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Inhibition of HDAC3 produces mitotic defects independent of alterations in histone H3 lysine 9 acetylation and methylation

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Running title: HDAC3 inhibition effect mitosis

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Abstract

The constitutive heterochromatin of the centromere is marked by high levels of tri-methylated histone H3 Lysine 9 (H3K9) and binding of the heterochromatin protein 1 (HP1), which are thought to also have an important role in mitosis. Histone deacetylase inhibitors (HDACi) are a class of anticancer agents which affect many cellular processes, including mitosis. Here we examine the mechanism by which these drugs disrupt mitosis. We have used *Drosophila* embryos to demonstrate that treatment with the HDACi 100 μg/ml suberic bishydroxamic acid (SBHA, IC50 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 tri-methylated 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.

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Introduction

Histone deacetylase inhibitors (HDACi) are a class of anti-tumour drugs that are proving to have clinical efficacy in a number of tumour types as either single agents or as part of a combination therapy. These drugs produce a range of effects on tumour cells including promoting the expression of differentiation markers, cell cycle effects and induction of apoptosis in tumour cells, but have little cytotoxic effect on normal cells (Bolden et al., 2006; Lindemann et al., 2004). 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 is been generally thought that the anti-tumour effects observed in response to HDACi are a consequence of transcriptional changes. However, histones deacetylases (HDACs) are associated with a range of other functions including the protein chaperone HSP90 (Aoyagi and Archer, 2005), and DNA damage recognition in association with Ku70 (Subramanian et al., 2005).

The anti-proliferative activity of HDACi result from a combination of inhibiting cell cycle progression and promoting cell death, the latter being more efficient in cycling cells (Bernhard et al., 2001; Burgess et al., 2004; Peart et al., 2003). HDACi-induced cell cycle effects include arrest at the G1/S transition, which is primarily a consequence of increased $p21^{CIP1}$ expression (Archer et al., 1998; Burgess et al., 2001), a G2 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 (Stevens et al., 2008; Warrener et al., 2003). Progression through the HDACi induced aberrant mitosis induces cell death (Warrener et al., 2003), thus understanding the mechanism(s) by which HDACi induce the aberrant mitosis is critical to understanding how these drugs promote cell death.

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HDACi induced mitotic defects are observed after relatively short term treatment with the drugs, and appear to be a consequence of exposure during the S phase prior to the defective mitosis (Blagosklonny et al., 2002; Dowling et al., 2005; Robbins et al., 2005; Warrener et al., 2003). HDACi treatment causes some disruption to the normal microtubule spindle architecture, failure of normal chromosome congression, extended duration of mitosis and mitotic slippage (Dowling et al., 2005; Qiu et al., 2000; Stevens et al., 2008; Taddei et al., 2001; Warrener et al., 2003). 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 (Robbins et al., 2005; Taddei et al., 2001).

Heterochromatin is specialised 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) (Bannister et al., 2001; Eissenberg and Elgin, 2000; Nakayama et al., 2001). Genetic knockout of the SUV39H1 and 2 methyltransferases responsible for H3K9 di-and trimethylation results in mitotic defects and genomic instability (Peters et al., 2001). Extended chronic treatment with low doses of HDACi also results in 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

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assembly checkpoint in response to HDACi. To investigate this possibility we have examined how S phase treatment with HDACi, under conditions which 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. SBHA was dissolved in dimethyl sulphoxide (DMSO).

Drosophila embryos

Drosophila strain w^{1118} were grown at 25°C on standard cornmeal-treacle media. Embryos were collected, dechorionated in 50% bleach for 2 min, then rinsed thoroughly in PBS. Following treatment in 100 μg/ml SBHA in PBS for 1h, embryos were fixed in 1:1 solution of 3.7% paraformaldehyde (in PBS): heptane for 30 min and devitenillised by the addition of an equal volume of methanol and shaken vigorously for 30 sec. 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 a Alexa Fluor 555 couple secondary antibody (Invitrogen).

Cell Culture, transfection and drug treatment

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All human cell lines used in this work, HeLa, HeLa Tet-On, Bcl2-HeLa and MCF7, were cultured in a humidified incubator at 37^{o}C with 5% CO_2. Cells were maintained in Dulbecco's modified Eagles medium supplemented with 10% (v/v) Serum Supreme (Biowhittaker). Cells were synchronised to the G1/S phase boundary by the addition of 2.5 mM thymidine to the culture media for 16h. Treatment with HDACi was performed at synchrony release and cells collected at times indicated. Knockdown of HP1α and/or HP1β was achieved by transfection with 50 μM of each siRNA using Dharmafect I reagent (Dharmacon), according to manufacturer's recommendations. The following siRNA were used; HP1α 1, HP1β1, HP1β2, (Dharmacon ON-TARGETplus), HP1α2 (5'-CCUGAGAAAAACUUGGAUUTT-3'), HDAC1, HDAC2, and HDAC3 (Dharmacon ON-TARGETplus SMARTpools). Cells transfected with siRNA were allowed to grow for 24h before being synchronized in G1/S phase with 2.5 mM thymidine for 16h. 100 μg/ml SBHA was added to the cells upon release and cells were harvested 9h after release in G2/M. Silencer Select Negative Control siRNA #1 (Ambion) 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) (Fodor et al., 2006). HeLa Tet-On cells were transfected using Lipofectamine 2000 (Invitrogen). After transfection, cells were synchronised overnight with 2.5 mM thymidine and plasmid expression 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) (Cloos et al., 2006). Bcl2-HeLa expressing cells were transfected using Lipofectamine 2000 (Invitrogen) and Jmjd2B expression confirmed by detection of the HA tagged protein.

Immunoblotting

Cell pellets were lysed in 1X SDS lysis buffer (0.4% SDS, 2% glycerol, 2.5mM Tris pH6.7 and 0.3M 2-mercaptoethanol) and cell lysates were quantified using Bio-Rad Protein Assay. 20-40 μg of samples were resolved by 12% SDS-PAGE, transferred electrophoretically to membrane. Membranes were immunoblotted with primary antibodies against HP1α, HP1β (Chemicon), HDAC1, HDAC2, HDAC3, acetyl lysine, di and tri-methylated H3K9, acetylated H3K9 and histone H3 antibodies (Cell Signaling), PCNA (DAKO) and α-tubulin (Sigma). These were detected with the appropriate HRP conjugated secondary antibodies (Zymed) and detected by chemiluminescence.

Immunofluorescent staining

Cells grown on poly-L-lysine coated cover-slips were fixed in ice cold methanol overnight at -20°C. Cover-slips 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 mins at room temperature before immunostaining with anti-bodies against α-tubulin, human ACA autoimmune serum, HP1α and HP1β for one hour at room temperature. For HP1 staining, cells were permeablised prior to fixing with 50 μg/ml digitonin, 130 mM sucrose, 50 mM KCl, 50 mM Na acetate, 20 mM Hepes pH7.5, 5 mM $MgCl_2$, 2 mM EGTA for 90 sec before washing twice with PBS and fixing with -20^{o}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 30min at room temperature. DNA was counterstained with 4'-6'Diamindino-2-phenylindole (DAPI; Sigma Aldrich). Fluorescent microscopy was carried out with Axioskop 2 plus (Carl Zeiss).

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Chromosome spreads

Nocodazole (0.5 μg/ml) was added to cell cultures 1h prior to harvest. Mitotic cells were mechanically dislodged then washed with PBS and resuspended in hypertonic buffer (10 mM Tris pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$) at $2x10^5$/ml for 10 min. 50 μl of cell suspension was spun onto microscope slides using a Shandon Cytospin at 5000 rpm for 2 min. Samples were fixed with 4% PFA diluted in PBS for 10 min and permeablised with 0.1% Nonidet-P40 in PBS for 10 min. Samples were blocked with 2% BSA/0.1% saponin for 30 min and antibodies applied in same for 16h at 4°C. Secondary antibodies and DAPI applied in 0.1% w/v skim milk powder in PBS for 2h. Samples were mounted under coverslips with ProLong® Gold anti-fade reagent (Invitrogen).

Fluorescence activated cell sorting (FACS)

Cells were fixed in ice-cold 70% ethanol in 1X PBS and stored at -20°C. Samples were washed in 1X PBS and resuspended in 0.5 mg/ml RNase A and 5 μg/ml propidium iodide in PBS. Cells were filtered through 37μm gauze and single cell suspensions analysed on a FACSCalibur system (BD Biosciences) using Cell Quest and Modfit data analysis software.

Results:

HDACi affect mitosis without transcription

To determine whether HDACi induced mitotic defects can occur in the absence of transcription, we turned to the *Drosophila* embryo. In *Drosophila*, the first 12 embryonic cell cycles post-fertilization consist of alternating S and M phases driven by maternally

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contributed mRNAs and protein. These cycles occur synchronously and do not require zygotic transcription, as *de novo* transcription does not commence until after cycle 13 (Foe, 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 (Stevens et al., 2008; Warrener et al., 2003). SBHA (IC50 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 taxol (Figure 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 (Figure 1a,b), a similar phenotype to that reported in human tumour cells (Stevens et al., 2008; Warrener et al., 2003). This indicated that the mitotic defects induced by HDACi treatment were unlikely to be due to transcriptional changes alone. As 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 tri-methylated 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

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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 synchronised 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 anti-acetylated lysine antibody, and both di and tri-methylated H3K9 were readily detected. There was an increase in the di-methylated H3K9 as cell progressed through S into G2 phase, although little obvious difference in the tri-methylated H3K9 was observed (Figure 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 synchronised HeLa cells in early S phase, the duration of G2/M phase was extended, due mainly 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 (Figure 2b and c). H3K9 tri-methylation was unaffected by HDACi treatment during progression into mitosis, marked by the increased level of MEK1 phosphorylated on Thr286 by cyclin B/cdk1 and a marker of mitosis (De Boer et al., 2008), but it blocked the increase of H3K9 tri-methylation observed in the following G1 phase, marked by decreased cyclin A and increased cyclin E (Figure 2c and Supplementary Figure 1). HDACi treatment blocked the increased H3K9 di-methylation during S phase, and the level of di-methylation was further reduced during the subsequent G1 phase, paralleling the decrease in the tri-methyl mark. The reduction in H3K9 methylation appeared to be independent on the level of H3K9 acetylation, as treatment with 100 μg/ml SBHA increased acetylation without reducing the methylation significantly more than observed with 10 μg/ml (Figure 2c). This suggests that the

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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. .

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

The lack of change in H3K9 tri-methylation and small decrease in di-methylation suggested that there was likely to be little change in HP1 binding following HDACi treatment. Examination of HP1α and β, the primary centromeric binding HP1 isoforms in G2/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 permeablised prior to fixation to remove HP1 not tightly associated with the chromatin (Schmiedeberg et al., 2004) (see Materials and Methods). A punctuate staining pattern was observed with HP1β after permeabilisation (Supplementary Figure 2). Little change was observed in HP1α or HP1β binding in G2/M phase cells with HDACi treatment (Figure 4a and b). The lack of effect on HP1 binding was also observed in normally fixed cells although the foci were less prominent (Figure 4c), and in MCF7 cells where the foci were very prominent without permeabilisation (Supplementary Figure 3). In cells that had

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progressed through mitosis and into the subsequent G1 phase in the presence of HDACi, there was the expected loss of HP1 foci corresponding to the reduction in H3K9 methylation at this time (Figure 2 and Supplementary Figure 4). Thus S phase treatment with HDACi did not appear 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 prior to entry into aberrant mitosis (Figure 2). To determine whether reduced H3K9 methylation was responsible for the aberrant mitosis observed, the H3K9 demethylase Jumonji (Jmjd) was transiently expressed and its effect on mitosis 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 tri-methyl H3K9 demethylase, and also had detectible activity against the di-methyl mark (Figure 5a and Supplementary Figure 5; (Cloos et al., 2006; Fodor et al., 2006)). Over expression 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 over expressed in HeLa cells stably over expressing the anti-apoptotic protein Bcl-2 (Burgess et al., 2004). Whereas this increased the numbers of cells over expressing Jmjd proteins, few Jmjd2B (Figure 5b) or Jmjd2C (not shown) over expressing cells were detected in mitosis. Those detected revealed the presence of normal metaphase and anaphase cells. This contrasted with HDACi treated cells where no metaphase or anaphase figures were observed (Stevens et al., 2008) (Figure 7C).

Although HDACi treatment had little effect on HP1α 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.

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Two independent siRNAs were used to knockdown each isoform and these reduced the protein levels efficiently by 24h post transfection. All four siRNAs were combined to efficiently deplete HP1α and β together (Figure 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 (Figure 6b and c). This contrasts to HDACi treatment where the majority of cells arrested in prometaphase (Figure 7c). In addition, over expression of a dominant negative mutant form of HP1β deleted for the chromoshadowdomain that reduced HP1 foci did not affect normal mitosis (data not shown). In addition, co-depletion of HP1α and β had no effect on the aberrant mitosis promoted by HDACi treatment (Figure 6d), indicating that HP1α and β are not required for the drug induced mitotic defects.

Depletion of HDAC3 mimics HDACi effects on mitosis

The only 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 localise to the mitotic spindle in association with the nuclear receptor corepressor N-Cor, and depletion of HDAC3 induced mitotic defects that appeared 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). Co-depletion 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 Figure 6a and b). By contrast, depletion of HDAC3

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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 tri-methylation. It did result in an accumulation of the mitotic marker phosphorylated B23 to a similar extent as HDACi treatment, indicating that it caused a delay in mitosis similar to HDACi treatment (Figure 7a and Supplementary Figure 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. Co-staining 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 (Figure 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 (Figure 7c).

The increased proportion of cell 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 ($p < 0.0001$; Figure 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 increase proportion of cells with multiple nuclei were observed in HDAC3 depleted cells to a level comparable with HDACi treatment Figure 8b and c). Thus, HDAC3 depletion induced aberrant mitosis and premature exit similar to the effect of HDACi.

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Discussion

HDACi have marked effects on mitosis, although the mechanism by which they produce these effects is at present unclear. We have demonstrated that HDACi can induce aberrant mitosis in *Drosophila* early embryos which run off maternal mRNA and protein stores, indicating that HDACi 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 (Dowling et al., 2005; Qiu et al., 2000; Robbins et al., 2005; Stevens et al., 2008; Taddei et al., 2001; Warrener et al., 2003). 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 (Dillon et al., 2002; Taddei et al., 1999; Tyler et al., 1999). 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 replicated DNA. Surprisingly, HDACi treatment had a stronger effect on the di-methyl

H3K9 mark than the tri-methyl mark, suggesting that H3K9 di-methylation is more dynamic than tri-methylation prior to 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 G1 phase as reported in *Xenopus* (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 occurs also occurs in G1 (Jansen et al., 2007).

The relative lack of effect of HDACi treatment on HP1 binding in G2/M phase cells is unsurprising given the very minor reduction in the tri-methylated H3K9, which appears to be the primary binding motif for HP1 given their co-localisation at particularly the centromeric regions. The reported loss of HP1 binding with HDACi treatment is a consequence of chronic 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; Taddei et al., 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 tri-methylation. The surprising lack of effect of the near complete loss of tri-methylated 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.

Our findings that alterations to H3K9 methylation or HP1 binding had little effect on mitosis suggested that the mitotic effects observed with HDACi are 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 as all of the HDACi used, representing

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examples from each class of HDACi, induce the same spectrum of mitotic defects (Cimini et al., 2003; Dowling et al., 2005; Robbins et al., 2005; Stevens et al., 2008; Warrener et al., 2003). The increased H3K9 acetylation is also unlikely to contribute to the aberrant mitosis observed as depletion of HDAC1 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, that latter appeared to be primarily the centromeric levels of this modification (Eot-Houllier et al., 2008). This was associated with loss of sister chromatid cohesion and loss of centromeric localisation of Shugoshin (Sgo1), a PP2A targeting protein which regulates sister chromatid separation (Wang and Dai, 2005). Extended treatment of mitotic arrested cells with HDACi produces similar loss of sister chromatid cohesion a 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 over expression 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 Figure 7). This latter had been reported previously with other HDACi, and it was suggested that increased acetylation of H3K9 was co-ordinated with increased H3K4 methylation (Nightingale et al., 2007).

Selective inhibition of HDAC3 appears 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 non histone 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 destabilisation 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 previously reported 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 (unpublished observations).

In summary, we have found that HDACi effects on mitosis appear 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 prior to mitosis, and depletion of tri-methylated 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 appears to be HDAC3, and selective depletion of this HDAC isoform mimicked the many of the effects of HDACi treatment on mitosis and promoted premature mitotic exit. These findings demonstrate that the mitotic effect 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 anti-cancer drugs.

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References

Aoyagi S and Archer TK (2005) Modulating molecular chaperone Hsp90 functions through reversible acetylation. *Trends Cell Biol* **15**:565-567.

Archer SY, Meng S, Shei A and Hodin RA (1998) p21(WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. *Proc Natl Acad Sci U S A* **95**:6791-6796.

Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC and Kouzarides T (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**:120-124.

Bernhard D, Skvortsov S, Tinhofer I, Hubl H, Greil R, Csordas A and Kofler R (2001) Inhibition of histone deacetylase activity enhances Fas receptor-mediated apoptosis in leukemic lymphoblasts. *Cell Death Differ* **8**:1014-1021.

Blagosklonny MV, Robey R, Sackett DL, Du L, Traganos F, Darzynkiewicz Z, Fojo T and Bates SE (2002) Histone deacetylase inhibitors all induce p21 but differentially cause tubulin acetylation, mitotic arrest, and cytotoxicity. *Mol Cancer Ther* **1**:937-941.

Bolden JE, Peart MJ and Johnstone RW (2006) Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* **5**:769-784.

Brinkmann H, Dahler AL, Popa C, Serewko MM, Parsons PG, Gabrielli BG, Burgess AJ and Saunders NA (2001) Histone hyperacetylation induced by histone deacetylase inhibitors is not sufficient to cause growth inhibition in human dermal fibroblasts. *J Biol Chem* **276**:22491-22499.

Burgess A, Ruefli A, Beamish H, Warrener R, Saunders N, Johnstone R and Gabrielli B (2004) Histone deacetylase inhibitors specifically kill nonproliferating tumour cells. *Oncogene* **23**:6693-6701.

Burgess AJ, Pavey S, Warrener R, Hunter LJ, Piva TJ, Musgrove EA, Saunders N, Parsons PG and Gabrielli BG (2001) Up-regulation of p21(WAF1/CIP1) by histone deacetylase inhibitors reduces their cytotoxicity. *Mol Pharmacol* **60**:828-837.

Cha TL, Chuang MJ, Wu ST, Sun GH, Chang SY, Yu DS, Huang SM, Huan SK, Cheng TC, Chen TT, Fan PL and Hsiao PW (2009) Dual degradation of aurora A and B kinases by the histone deacetylase inhibitor LBH589 induces G2-M arrest and apoptosis of renal cancer cells. *Clin Cancer Res* **15**:840-850.

Choi E, Choe H, Min J, Choi JY, Kim J and Lee H (2009) BubR1 acetylation at prometaphase is required for modulating APC/C activity and timing of mitosis. *Embo J* **28**:2077-2089.

Cimini D, Mattiuzzo M, Torosantucci L and Degrassi F (2003) Histone hyperacetylation in mitosis prevents sister chromatid separation and produces chromosome segregation defects. *Mol Biol Cell* **14**:3821-3833.

Cloos PA, Christensen J, Agger K, Maiolica A, Rappsilber J, Antal T, Hansen KH and Helin K (2006) The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. *Nature* **442**:307-311.

Dai J, Sullivan BA and Higgins JM (2006) Regulation of mitotic chromosome cohesion by Haspin and Aurora B. *Dev Cell* **11**:741-750.

De Boer L, Oakes V, Beamish H, Giles N, Stevens F, Somodevilla-Torres M, Desouza C and Gabrielli B (2008) Cyclin A/cdk2 coordinates centrosomal and nuclear mitotic events. *Oncogene* **27**:4261-4268.

Dillon N, Festenstein R, Cheung WL, Briggs SD and Allis CD (2002) Unravelling heterochromatin: competition between positive and negative factors regulates accessibility. Acetylation and chromosomal functions. *Trends Genet* **18**:252-258.

Dowling M, Voong KR, Kim M, Keutmann MK, Harris E and Kao GD (2005) Mitotic spindle checkpoint inactivation by trichostatin a defines a mechanism for increasing cancer cell killing by microtubule-disrupting agents. *Cancer Biol Ther* **4**:197-206.

Eissenberg JC and Elgin SC (2000) The HP1 protein family: getting a grip on chromatin. *Curr Opin Genet Dev* **10**:204-210.

Eot-Houllier G, Fulcrand G, Watanabe Y, Magnaghi-Jaulin L and Jaulin C (2008) Histone deacetylase 3 is required for centromeric H3K4 deacetylation and sister chromatid cohesion. *Genes Dev* **22**:2639-2644.

Fischle W, Tseng BS, Dormann HL, Ueberheide BM, Garcia BA, Shabanowitz J, Hunt DF, Funabiki H and Allis CD (2005) Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature* **438**:1116-1122.

Fodor BD, Kubicek S, Yonezawa M, O'Sullivan RJ, Sengupta R, Perez-Burgos L, Opravil S, Mechtler K, Schotta G and Jenuwein T (2006) Jmjd2b antagonizes H3K9 trimethylation at pericentric heterochromatin in mammalian cells. *Genes Dev* **20**:1557-1562.

Foe V, Odell, GM and Edgar, BA (ed) (1993) *Mitosis and morphogenesis in the Drosophila embryo: point and counterpoint.* . Cold Spring Harbor Laboratory Press, New York.

Gabrielli B, Warrener R, Burgess A and Beamish H (2004) Defining the chemotherapeutic targets of histone deacetylase inhibitors. *Ann N Y Acad Sci* **1030**:627-635.

Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG and Davidsen SK (2003) Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. *Mol Cancer Ther* **2**:151-163.

Hayakawa T, Haraguchi T, Masumoto H and Hiraoka Y (2003) Cell cycle behavior of human HP1 subtypes: distinct molecular domains of HP1 are required for their centromeric localization during interphase and metaphase. *J Cell Sci* **116**:3327-3338.

Hirota T, Lipp JJ, Toh BH and Peters JM (2005) Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin. *Nature* **438**:1176-1180.

Ishii S, Kurasawa Y, Wong J and Yu-Lee LY (2008) Histone deacetylase 3 localizes to the mitotic spindle and is required for kinetochore-microtubule attachment. *Proc Natl Acad Sci U S A* **105**:4179-4184.

Jansen LE, Black BE, Foltz DR and Cleveland DW (2007) Propagation of centromeric chromatin requires exit from mitosis. *J Cell Biol* **176**:795-805.

Lindemann RK, Gabrielli B and Johnstone RW (2004) Histone-Deacetylase Inhibitors for the Treatment of Cancer. *Cell Cycle* **3**:6.

Magnaghi-Jaulin L, Eot-Houllier G, Fulcrand G and Jaulin C (2007) Histone deacetylase inhibitors induce premature sister chromatid separation and override the mitotic spindle assembly checkpoint. *Cancer Res* **67**:6360-6367.

Nakayama J, Rice JC, Strahl BD, Allis CD and Grewal SI (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* **292**:110-113.

Nightingale KP, Gendreizig S, White DA, Bradbury C, Hollfelder F and Turner BM (2007) Cross-talk between histone modifications in response to histone deacetylase inhibitors: MLL4 links histone H3 acetylation and histone H3K4 methylation. *J Biol Chem* **282**:4408-4416.

Peart MJ, Smyth GK, van Laar RK, Bowtell DD, Richon VM, Marks PA, Holloway AJ and Johnstone RW (2005) Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. *Proc Natl Acad Sci U S A* **102**:3697-3702..

Peart MJ, Tainton KM, Ruefli AA, Dear AE, Sedelies KA, O'Reilly LA, Waterhouse NJ, Trapani JA and Johnstone RW (2003) Novel mechanisms of apoptosis induced by histone deacetylase inhibitors. *Cancer Res* **63**:4460-4471.

Peters AH, O'Carroll D, Scherthan H, Mechtler K, Sauer S, Schofer C, Weipoltshammer K, Pagani M, Lachner M, Kohlmaier A, Opravil S, Doyle M, Sibilia M and Jenuwein T (2001) Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* **107**:323-337.

Qiu L, Burgess A, Fairlie DP, Leonard H, Parsons PG and Gabrielli BG (2000) Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells. *Mol Biol Cell* **11**:2069-2083.

Robbins AR, Jablonski SA, Yen TJ, Yoda K, Robey R, Bates SE and Sackett DL (2005) Inhibitors of histone deacetylases alter kinetochore assembly by disrupting pericentromeric heterochromatin. *Cell Cycle* **4**:717-726.

Schmiedeberg L, Weisshart K, Diekmann S, Meyer Zu Hoerste G and Hemmerich P (2004) High- and low-mobility populations of HP1 in heterochromatin of mammalian cells. *Mol Biol Cell* **15**:2819-2833.

Stevens FE, Beamish H, Warrener R and Gabrielli B (2008) Histone deacetylase inhibitors induce mitotic slippage. *Oncogene* **27**:1345-1354.

Stewart MD, Sommerville J and Wong J (2006) Dynamic regulation of histone modifications in Xenopus oocytes through histone exchange. *Mol Cell Biol* **26**:6890-6901.

Subramanian C, Opipari AW, Jr., Bian X, Castle VP and Kwok RP (2005) Ku70 acetylation mediates neuroblastoma cell death induced by histone deacetylase inhibitors. *Proc Natl Acad Sci U S A* **102**:4842-4847.

Taddei A, Maison C, Roche D and Almouzni G (2001) Reversible disruption of pericentric heterochromatin and centromere function by inhibiting deacetylases. *Nat Cell Biol* **3**:114-120.

Taddei A, Roche D, Bickmore WA and Almouzni G (2005) The effects of histone deacetylase inhibitors on heterochromatin: implications for anticancer therapy? *EMBO Rep* **6**:520-524.

Taddei A, Roche D, Sibarita JB, Turner BM and Almouzni G (1999) Duplication and maintenance of heterochromatin domains. *J Cell Biol* **147**:1153-1166.

Tyler JK, Adams CR, Chen SR, Kobayashi R, Kamakaka RT and Kadonaga JT (1999) The RCAF complex mediates chromatin assembly during DNA replication and repair. *Nature* **402**:555-560.

Vader G and Lens SM (2008) The Aurora kinase family in cell division and cancer. *Biochim Biophys Acta* **1786**:60-72.

Wang X and Dai W (2005) Shugoshin, a guardian for sister chromatid segregation. *Exp Cell Res* **310**:1-9.

Warrener R, Beamish H, Burgess A, Waterhouse NJ, Giles N, Fairlie D and Gabrielli B (2003) Tumor cell-selective cytotoxicity by targeting cell cycle checkpoints. *Faseb J* **17**:1550-1552.

Footnotes

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Figure Legends

Figure 1: HDACi induce aberrant mitosis in Drosophila embryos independent of transcription. Drosophila embryos isolated immediately after fertilisation were either untreated or treated with 100 μg/ml SBHA or 10 ng/ml Taxol for 1 h, , then fixed and stained for either DNA (A), or DNA (blue) and α-tubulin (red). Scale bar in A represents 50 μM and in B represents 10 μM.

Figure 2: Cell cycle dependent effects of HDACi treatment on H3K9 modification. Synchronised 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 of the panel. Cell lysates were immunoblotted for H3K9 acetylation (Ac), di-methylation (2Me), tri-methylation (3Me), total acetylation with an acetyl lysine antibody (AcK), and total histone H3. α-tubulin was used as a loading control. (C) Synchronised HeLa cells 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 G1 phase, and lysates immunoblotted for the indicated proteins and modifications.

Figure 3: Chromosomal spreads show little change in tri-methyl 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 Figure 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 on the right. Bar represents 5 μM.

Figure 4: HDACi treatment has little effect on HP1 centromeric association. (A, B) Synchronised HeLa cells, either untreated (Con) or treated with 100 μg/ml SBHA (HDACi) in S phase were harvested in G2/M phase (7 h after synchrony release), permeablised then fixed and stained for HP1α or HP1β, ACA and DNA. (C) HeLa cells were treated as in B, harvested in G2 phase and fixed without permeablising first, and stained for HP1β and DNA. Scale bar = 10 μm.

Figure 5: Jmjd2C demethylation of H3K9 does not affect mitosis. (A) HeLa cells were transfected with either Jmjd2C or an inactive mutant (DD). 24h after transfection, cells were harvested and immunoblotted for GFP to define Jmjd2C over-expression, di-methyl (2Me) or tri-methyl (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.

Figure 6: HP1 depletion does not affect mitosis. (A) HeLa cells were transfected with either scrambled control siRNA (Scr), HP1 α, β or α and β siRNA. 48 h after transfection, cells were harvested and immunoblotted for HP1α, 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, synchronised and

harvested at G2/M (9 h after synchrony release). In parallel, synchronised HeLa cells were treated with 100 μg/ml SBHA in S phase then harvested in G2/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, b or α and β siRNA, synchronised then harvested in G2/M, fixed and stained as in B. 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. (D) HeLa cells were transfected with either scrambled (Scr) or HP1α and β siRNA, synchronised then treated with 100 μg/ml SBHA in S phase. Cells were harvested at G2/M (9 h after synchrony release) fixed and stained for α-tubulin (MT), DNA and ACA. Scale bar = 10 μm

Figure 7: Depletion of HDAC3 delays cells in prometaphase. HeLa cells were treated with scrambled (Scr) or HDAC3 siRNA, synchronised then harvested at G2/M (9 h after synchrony release). In parallel, synchronised HeLa cells were treated with 100 μg/ml SBHA in S phase and harvested at G2/M. Cells were analysed 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 to A were fixed at G2/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.

Figure 8: HDAC3 depletion promotes premature mitotic exit. (A) HeLa cells were transfected with either scrambled control (Scr) or HDAC3 siRNA, then synchronised 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, synchronised 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 over 100 cells in each experiment in three independent experiments.

Figure 1

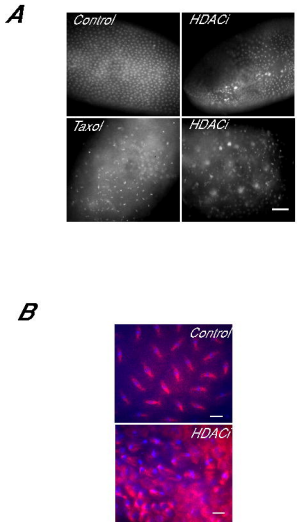
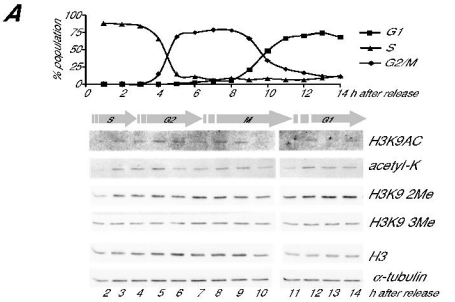
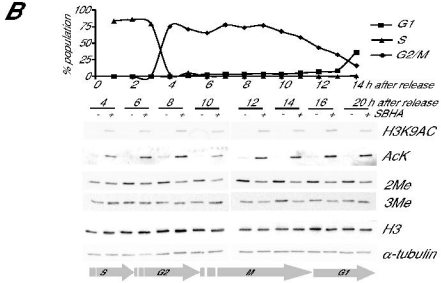


Figure 2


A
% population
100
75
50
25
0
0 2 4 6 8 10 12 14 h after release
G1
S
G2/M
S G2 M G1
H3K9AC
acetyl-K
H3K9 2Me
H3K9 3Me
H3
α-tubulin
2 3 4 5 6 7 8 9 10 11 12 13 14 h after release


B
% population
100
75
50
25
0
0 2 4 6 8 10 12 14 h after release
G1
S
G2/M
4 6 8 10 12 14 16 20 h after release
- + - + - + - + - + - + - + - + SBHA
H3K9AC
AcK
2Me
3Me
H3
α-tubulin
S G2 M G1

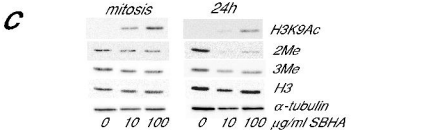

C
mitosis
24h
H3K9Ac
2Me
3Me
H3
α-tubulin
0 10 100 0 10 100 µg/ml SBHA

Figure 3

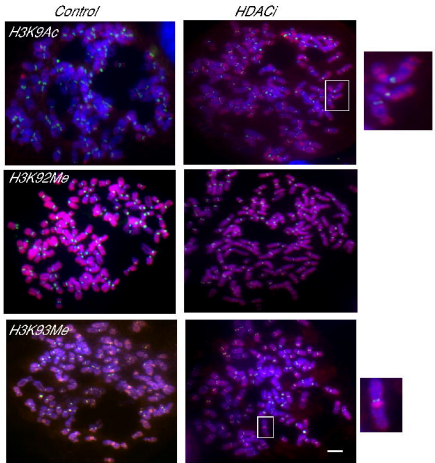


Figure 4

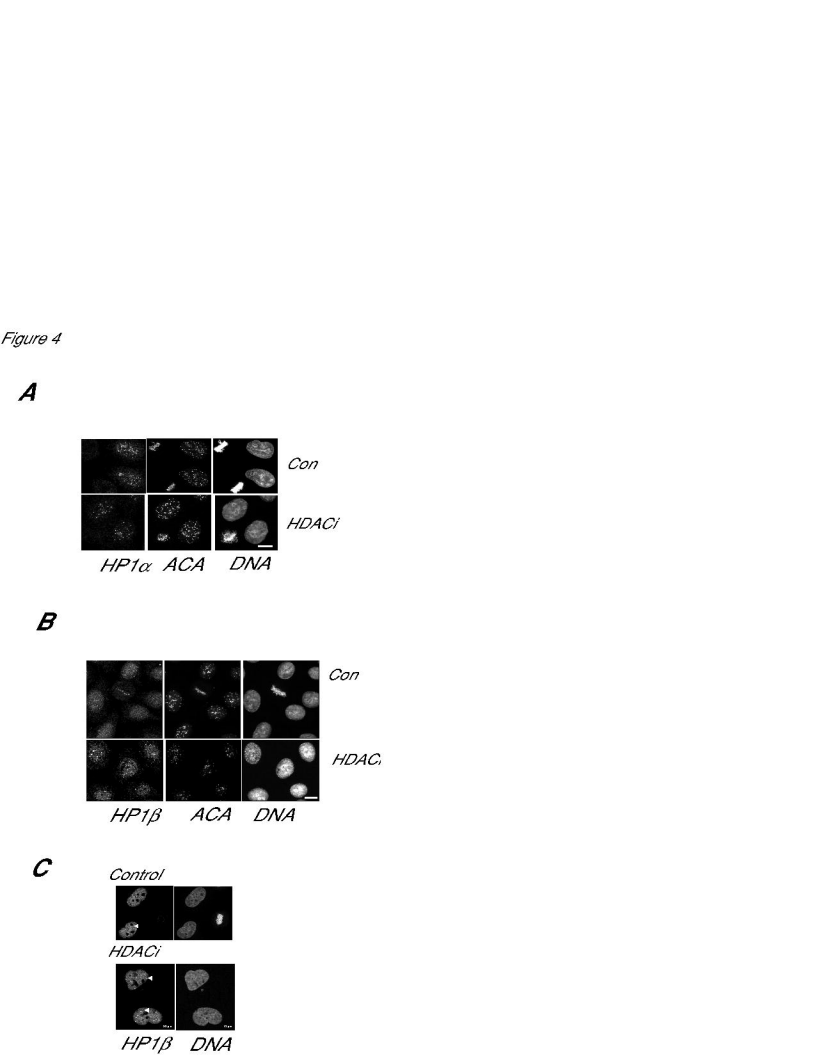
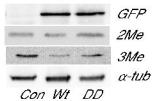

A
Con
HDACi
HP1α ACA DNA
B
Con
HDACi
HP1β ACA DNA
C
Control
HDACi
HP1β DNA

Figure 5

A



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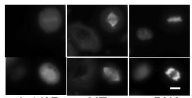
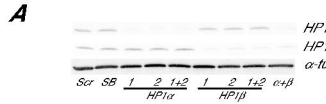


Figure 6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HP1α
HP1β
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Scr
SB
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2
1+2
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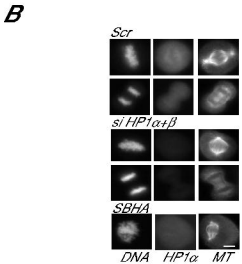
1+2
α+β
HP1α
HP1β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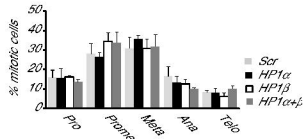
si HP1α+β
SBHA
DNA
HP1α
MT


C
% mitotic cells
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20
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Pro
Prometa
Meta
Ana
Telo
Scr
HP1α
HP1β
HP1α+β

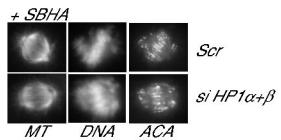
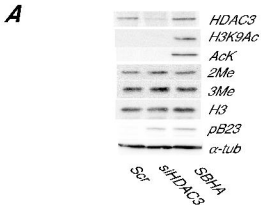

D
+ SBHA
Scr
si HP1α+β
MT
DNA
ACA

Figure 7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siHDAC3
SBHA
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MT
DNA
ACA
Scr
siHDAC3
SBHA
C
% mitotic cells
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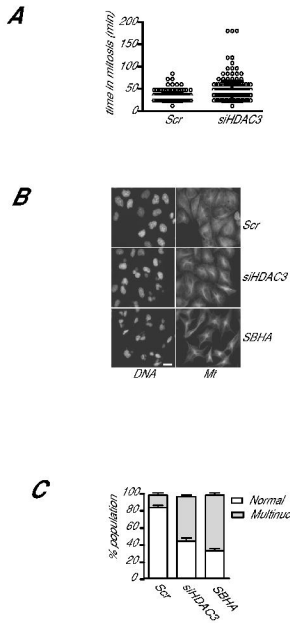
60
40
20
0
Scr
siHDAC3
SBHA
Pro
ProMeta
Meta
Ana
Telo

Figure 8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time in mitosis (min)
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150
100
50
0
Scr
siHDAC3
B
Scr
siHDAC3
SBHA
DNA
Mt
C
% population
100
80
60
40
20
0
Normal
Multinuc
Scr
siHDAC3
SBHA