17

Analysis of Checkpoint Responses to Histone Deacetylase Inhibitors

Heather Beamish, Robyn Warrener, and Brian G. Gabrielli

Summary

Cell cycle checkpoints respond to a wide range of stresses to prevent compromise to the integrity of the cell. The best studied checkpoints are those induced by genotoxic agents that cause DNA damage. Histone deacetylase inhibitors not only increase the acetylation state of chromatin histones, but they also perturb the cell cycle, causing both G1 and G2 phase arrests, the latter by initiating a checkpoint response. In this chapter we will describe the analysis of the histone deacetylase inhibitor-sensitive G2 checkpoint using synchronized cell populations.

Key Words: Histone deacetylase inhibitor; G2 checkpoint; acetylation; histone; mitosis.

1. Introduction

Cell cycle checkpoints are mechanisms that respond to a diverse range of stresses that could ultimately compromise cell integrity. Histone deacetylase inhibitors (HDACIs) are a class of drugs that block the deacetylation of proteins, the best characterized being the chromatin histone proteins, although an increasing number of nonhistone proteins have also been shown to be modified by acetylation *(1)*. HDACI treatment of cells often results in perturbations of the cell cycle, notably cell cycle arrests in G1 and G2 phase. The G1 arrest resulting from HDACI treatment can be in part ascribed to transcriptional up-regulation of the Cdk2 inhibitor $p21^{Waf1/Cip1}$, a direct result of increased histone acetylation affecting transcription factor accessibility to the promoter of p21 *(2)*. On the other hand, the mechanism by which HDACI treatment imposes the G2 phase arrest is not clear, but a checkpoint response is initiated as opposed to the transcriptional up-regulation of an inhibitor of CDK activity *(3)*. Activation of the G2 checkpoint is dependent on the concentration of HDACI used *(2,4)*. Many immortalized and tumor cell lines are defective for this checkpoint, but are competent to impose a G2 checkpoint

From: Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

arrest in response to other agents, such as DNA-damaging drugs, indicating that this checkpoint is distinct from other G2 checkpoint mechanisms *(3)*. A consequence of failure of this checkpoint following HDACI treatment is that cells undergo an aberrant mitosis and often fail cytokinesis, resulting in cells with tetraploid DNA which have in fact exited mitosis and entered the subsequent cell cycle. In this chapter, we will outline methods for analyzing the effects of HDACI treatment on the cell cycle, primarily examining the G2 checkpoint response.

2. Materials

1. Dulbecco's modified Eagle's medium (DMEM; GIBCO), supplemented with 10% (v/v) donor calf serum.
2. Trichostatin A (TSA; Sigma-Aldrich).
3. Sodium butyrate (NaBu; Sigma-Aldrich): 20 m*M* stock solution in H_2O, make freshly before addition.
4. Azelaic bishydroxamic acid (ABHA) (generously synthesized by Mike West, Institute of Molecular Biosciences, University of Queensland), dissolved in DMSO at a concentration of 100mg/mL.
5. Thymidine: 100 m*M* stock solution in PBS. This will last for 1 wk at 4°C, stock solutions are maintained at –20°C.
6. 2-deoxycytidine: 10 m*M* stock solution in PBS, store at 4°C for 10 d.
7. Hydroxyurea: 400 m*M* stock solution in PBS, for long-term storage keep at –20°C.
8. Propidium iodide (PI): 1 mg/mL stock solution in H_2O, store at 4°C in the dark to prevent photo-bleaching.
9. RNase A: 10 mg/mL stock solution in H_2O, store at –20°C. To inactivate any contaminating DNase present, heat the stock solution to 95°C for 10 min.
10. 4',6-diamidino-2-phenylindole (DAPI): 1 mg/mL stock solution in H_2O, store at 4°C in the dark to prevent photo-bleaching.
11. Bromodeoxyuridine (BrdU): 5 m*M* stock solution in H_2O, store at 4°C.
12. Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody (Becton Dickson). Keep at 4°C in the dark to prevent photo-bleaching.
13. BrdU detection buffer: 0.25% (v/v) Tween-20, 2.5 mg/mL BSA, 0.1 mg anti-BrdU-FITC (Becton Dickson). This buffer should be made when required and be kept in the dark to prevent bleaching of the FITC-conjugated antibody.
14. Antibody to mitotic-specific phosphoproteins: MPM-2 antibody (DAKO Corporation, M3514, 0.3 mg/mL).
15. Flow cytometer.
16. 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich).
17. Microplate reader.
18. NETN buffer: 20 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA, 100 m*M* NaCl, 0.5% (v/v) Nonidet P-40, store at 4°C. Immediately before use add 5 µg/mL of each of

leupeptin, aprotinin, and pepstatin, 1 m*M* PMSF (phenylmethylsulfonyl fluoride; 100 m*M* stock in isopropanol; store at –20°C), 10 m*M* NaF (sodium fluoride, stock is 1 *M*, store at –20°C), and 0.1 m*M* Na_3V0_4 (sodium orthovanadate; stock is 20 m*M*, store at –20°C).

19. 5X SDS sample buffer: 0.313 *M* Tris-HCl (pH 6.8), 10% (w/v) SDS (sodium dodecyl sulfate), 50% (v/v) glycerol, 0.5 *M* DTT (dithiothreitol), 0.05% (w/v) bromophenol blue.
20. Polyacrylamide gel electrophoresis equipment.
21. Western blot transfer equipment.
22. 0.2 μ*M* PVDF membrane (NEN Life Science Products).
23. Ponceau S (Sigma).
24. PBS/Tween: 0.05% (v/v) Tween-20 in PBS. Store at room temperature.
25. Blotto: 5% (w/v) dried skimmed milk powder in PBS/Tween. Make fresh, store at 4°C.
26. Protein A–sepharose (Roche).
27. RC buffer: 20 m*M* Tris-HCl (pH 7.4), 15 m*M* $MgCl_2$, 1 m*M* DTT, 0.2 m*M* ATP, 5 μCi [γ^{32}P] ATP, 15 μg histone H1. This buffer contains the radioisotope γ^{32}P; therefore, all work with this buffer should be carried out in a suitably designated area. Strict guidelines for exposure, handling, and waste disposal should be outlined in the radiation safety plan for the laboratory in question.
28. Acrylamide gel staining buffer: 40% (v/v) methanol, 10% (v/v) acetic acid, 0.5 % (w/v) Comassie blue. Acetic acid is corrosive; aliquot the stock solution in a fume hood.
29. Acrylamide destaining buffer: 40% (v/v) methanol, 10% (v/v) acetic acid. Acetic acid is corrosive; aliquot the stock solution in a fume hood.
30. PhosphorImaging system (Molecular Dynamics).
31. Rehydration buffer: 0.1% (v/v) Tween-20 and 3% (w/v) BSA in PBS.
32. Vectorshield (Vector Laboratories).
33. Immunofluorescent microscope.

3. Methods

The methods below outline the analysis of the HDACI-initiated G2 checkpoint in both synchronized and asynchronously growing cells. Two cell lines will be described: the human cervical cancer cell line, HeLa, an example of a cell line with a defective HDACI-sensitive G2 checkpoint; and primary cultures of neonatal foreskin fibroblasts (NFF), which have a functional checkpoint arrest. Three different HDACIs will be mentioned: sodium butyrate (NaBu), trichostatin A (TSA), and azelaic bishydroxamic acid (ABHA).

3.1. Cell Lines and Culture Conditions

Mycoplasma-free HeLa and NFFs are cultured in complete DMEM (containing 10% [v/v] donor calf serum) at 37°C in the presence of 5% CO_2.

3.2. Cell Synchrony

Generally, drug treatment experiments are performed on asynchronously cycling cells, disregarding any cell cycle phase-specific drug effects. However, in the case of HDACIs, very significant differences are observed, depending on which phase of the cell cycle the drugs are administered in (*see* **Note 1**). The use of synchronized cells helps to overcome this problem. It also permits more refined analysis of the cell cycle phase progression, particularly progression through G2/M, than can be obtained using asynchronously growing cultures, which are normally composed of 60% G1-phase cells.

3.2.1. HeLa Cell Synchrony

HeLa cells are synchronized using a double thymidine block release protocol as described below.

1. To block cells in late G1/early S phase, trypsinize cells and replate at 50% confluence. Twenty-four hours later, remove medium and replace with prewarmed complete DMEM supplemented with 2.5 m*M* thymidine for 16 h.
2. Release cells from the block with three washes of prewarmed DMEM (without donor calf serum).
3. Trypsinize and reseed the cells into 10-cm-diameter dishes at a density of 1×10^6 cells per dish, followed by the addition of prewarmed complete medium supplemented with 24 μ*M* thymidine and 24 μ*M* 2-deoxycytidine (*see* **Note 2**).
4. After 8 h, block the cells again by the addition of 2.5 m*M* thymidine directly into the medium. Incubate for a further 16 h.
5. Release the cells from the block with three washes of prewarmed DMEM (without donor calf serum), followed by the addition of prewarmed complete medium supplemented with 24 μ*M* thymidine and 24 μ*M* 2-deoxycytidine.

   HeLa cells normally enter mitosis within 10 h after release from the second thymidine block with 60–80% synchrony using this protocol (*see* **Note 3**).

3.2.2. Fibroblast Culture Synchrony

One to two million NFF cells are seeded into 10-cm-diameter dishes, and then synchronized using a single hydroxyurea block. This synchronizes cells in late G1/early S phase.

1. Treat exponentially growing NFFs with 2 m*M* hydroxyurea for 24 h; the hydroxyurea can be added directly into the medium on the plate.
2. Release the cells from the hydroxyurea arrest with three washes of prewarmed DMEM (without donor calf serum), followed by the addition of prewarmed complete media.

This normally results in up to 50% of cells cycling synchronously through S phase and into mitosis by 12–14 h after release (*see* **Note 4**).

3.3. HDACI Treatment of Cells

1. Treat cells with varying concentrations of HDACIs (*see* **Note 5**) (*see* **Subheading 3.11.**), either immediately after release from the second thymidine or single hydroxyurea block (G1 phase) or within 4–8 h post-release (S-G2 phase).
2. Harvest floating and attached treated and untreated cells by trypsinization at various times after release.
3. Pellet the cells by centrifugation at 5000*g* in a microfuge. Wash the cell pellet twice with PBS. At this stage, the cell pellet can be divided, and one-third fixed for FACS analysis (*see* **Subheading 3.4.**) and two-thirds set aside for biochemical analysis (*see* **Subheadings 3.8.** and **3.9.**).

3.4. Flow Cytometric Analysis of the DNA Content of Cells

One-dimensional flow cytometric analysis is used to examine the cell cycle phase distribution of cell populations. It is particularly useful in analyzing cell cycle arrest in synchronized cell populations, where the transit of cells through cell cycle phases can be followed by analyzing DNA content at successive time points after release from the synchrony arrest.

1. Treat cells (normally 0.5–1 $\times$ 10^6), harvest and pellet as in **Subheading 3.3.**, wash twice with PBS, and fix in 70% ethanol at –20°C. Fixed cells may be stored for up to 2 wk at –20°C before analysis.
2. Wash fixed cells twice with PBS. Care should be taken when aspirating the PBS, as the cell pellet is fragile at this stage, and can easily be lost.
3. Stain nuclear DNA with a solution of PI (4 µg/mL) and RNase A (400 µg/mL) in 500 µL PBS for 15 min at room temperature in the dark.
4. Wash the cells three times in PBS, and then filter them through fine silk gauze (35 µm) to remove cell clumps.
5. Analyze the single cell suspension by flow cytometry, exciting the cells at 488 nm, and measuring the PI intensity through a 600 nm wavelength filter on a FACScalibur (Becton Dickinson) using CellQuest and ModFit data analysis software. Analyze at least 10^5 cells for each DNA distribution.

ModFit allows the analysis of the diploid and subdiploid populations, the former representing the viable population and the latter the nonviable cells. Nonviable cells are normally reported as the debris in the ModFit analysis. A G2 checkpoint arrest will be detected as a stable accumulation of cells in a G2/M peak at a time when the untreated control cells have progressed through mitosis into the subsequent G1 phase. This will be apparent as a decrease in the G2/M population and an increase in the G1 peak.

3.5. Bromodeoxyuridine (BrdU) Labeling

This method selectively labels S phase cells, allowing their passage through mitosis to be monitored without the need for drug arrest synchronization, avoid-

ing any potential artifacts caused by synchronization. Cells are pulse-labeled with the thymidine analog BrdU, which is incorporated into the newly synthesized DNA during S phase. DNA containing BrdU can be identified by using a fluorescently tagged antibody specifically against BrdU.

1. Pulse-label exponentially growing cells (1×10^7) with 10 μ*M* BrdU for 2 h. Wash the cells on the dish twice with pre-warmed PBS to remove the unincorporated BrdU, followed by addition of prewarmed complete media.
2. Immediately treat the cells with HDACIs for up to 24 h. Harvest all cells both floating and attached, wash with PBS, and resuspend the cell pellet in 300 μL PBS. Fix the cells with dropwise addition of 700 μL 100% ethanol at –20°C to a final concentration of 1×10^6/100 μL.
3. Following fixation, resuspend the cells in 2 *M* HCl in 0.5% (v/v) Triton X-100. Incubate for 30 min at room temperature. Pellet cells gently, by centrifugation at 3300*g* for 5 min.
4. Neutralize the cells by resuspending the cell pellet in 0.1 *M* sodium tetraborate (pH 8.5) for 5 min at room temperature.
5. Detect BrdU incorporation by staining with FITC-conjugated anti-BrdU monoclonal antibody in 400 μL BrdU detection buffer supplemented with PI (2 μg/μm) to counterstain the DNA. Incubate in the dark for 30 min at room temperature.
6. Wash the cells three times with PBS and filter them through fine silk gauze (35 μm) to produce single-cell suspensions.
7. Analyze the stained cells by two-dimensional flow cytometry. Excite cells at 488 nm and collect green fluorescence from FITC through a 514 nm filter, indicating the amount of incorporated BrdU. Measure PI fluorescence though a 600 nm wavelength filter. Use electronic compensation to prevent contamination of the PI channel by the FITC fluorescence. Plot FITC fluorescence intensity (FL-1) on the Y axis and PI staining intensity (FL-3) on the X axis.

   In untreated cells, the S phase BrdU-stained population will have progressed to the G2/M peak by 6 hr post-labeling. By 24 h there should be a population of BrdU-stained cells in the G1 and S phases, representing cells that have undergone mitosis (**Fig. 1**, upper panels). If HDACI treatment results in a G2/M arrest, then the BrdU-positive cells (those cells in S phase at the time of treatment) will remain in the G2/M peak at 24 h, while in the case of a G1 arrest, the BrdU positive cells will appear only in the G1 peak (**Fig. 1**, lower panels). These cells will have been in S phase when treated with HDACI, passed through G2/M, and arrest in the subsequent G1 phase.

3.6. MPM-2 Staining FACS Analysis

To determine the percentage of cells in M phase by FACS analysis, the specific mitotic phospho-epitope MPM-2 can be used. This will identify the proportion of mitotic cells in a PI-stained G2/M peak.

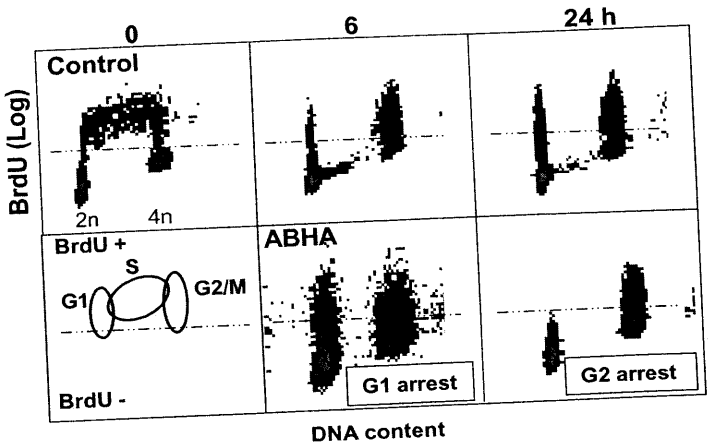


Fig. 1. FACS analysis of BrdU staining. Fibroblasts were pulse labeled with BrdU for 2 h, then either harvested or chased with fresh medium for 6 or 24 h. For the ABHA treated cells, HeLa cells (G1 arrest, *see* **Note 12**) or NFF (G2 arrest) were labeled with BrdU as above, and then treated with 100 µg/mL ABHA for 24 h. The mask shows the positions of the G1, S, and G2/M phase BrdU-labeled populations. Cells not labeled with BrdU would appear below the dotted line.

1. Treat either exponentially growing cells or synchronized cells with HDACIs and harvest at various times after treatment.
2. Fix cells in –20°C 70% ethanol for 2 h. Wash with PBS and incubate with anti-MPM-2 antibody diluted 1/200 in PBS for 1 h at 4°C. Wash cells twice with PBS and incubate with species-specific FITC- labeled secondary antibody and PI (2 µg/mL) to counter-stain the DNA, for 30 min at 4°C in the dark.
3. Wash cells twice with PBS and counterstain the DNA with PI (2 µg/mL) for 30 min at 4°C in the dark. Filter the cells through a fine silk gauze (35 µm).
4. Analyze MPM-2-positive cells using bivariate flow cytometry with Cell Quest and Modfit software (Becton Dickinson).

Mitotic cells have a 4N DNA content by PI staining and are positive for MPM-2 staining, whereas G2-phase cells have a 4N DNA content by PI staining and no MPM-2 staining.

3.7. MTT Cell Proliferation Assay

MTT (3-[4, 5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) measures mitochondria activity, which is a good marker of cell viability and measures proliferation.

1. Seed cells into 96-well plates at a density of $2–5 \times 10^3$ per well, 24 h prior to the addition of HDACIs.

2. On day 0 (the day HDACIs are added) and for the following 3–4 d, measure the cell viability and proliferation in untreated control and HDACI-treated cells. Add MTT to the culture media at a final concentration of 0.5 mg/mL and incubate the plates for 4 h at 37°C.
3. Precipitate the insoluble formazan product by centrifugation of the plate, followed by aspiration of the supernatant (*see* **Note 6**).
4. Dissolve the formazan crystals in 100 µL DMSO with gentle shaking at room temperature. This routinely takes 5 min, but if a large amount of formazan crystals form, then a longer time is required to dissolve all the crystals.
5. Measure absorbance at 570 nm using a microplate reader.

3.8. Immunoblotting for Acetylated Histones

As a measure of inhibition of histone deacetylases, the level of histone acetylation can be determined by immunoblotting, using either a generic anti-acetyl lysine antibody or acetylation site-specific antibodies.

1. Pellet and lyse synchronized and treated cells in 500 µL of NETN buffer on ice for 30 min.
2. Determine the protein levels using bicinchoninic acid according to the manufacturer's instructions (BCA; Pierce Chemical Co.) using bovine serum albumin (BSA) as a standard.
3. To 20 µg of protein lysate diluted to 20 µL in NETN, add 5 µL 5X SDS sample buffer and boil for 5 min.
4. Resolve samples on a 12.5% SDS-PAGE, and transfer to a PVDF membrane using semi-dry Western blotting equipment according to manufacturer's instructions. Assess sample loading by brief Ponceau S (Sigma) staining of the membranes. The histone proteins stain as prominent bands between 15 and 25 kDa. Remove Ponceau S staining by washing membrane three times for 5 min in PBS/Tween.
5. Block the membrane in Blotto for 1 h. Detect acetylated histone levels with either generic or acetylation site-specific antibodies, and the appropriate horseradish-peroxidase-conjugated secondary antibody using enhanced chemiluminescent (ECL; NEN) detection.

This method analyzes the whole cell lysate, including the chromatin histones which are not extracted using normal detergent and low-salt buffers. With the generic anti-acetyl lysine antibodies, other acetylated nonhistone proteins are also detected. **Fig. 2** shows acetylation of α-tubulin in response to ABHA and TSA, but not NaBu treatment.

3.9. Immunoprecipitation and Cyclin B/Cdc2 Kinase Assay

Cyclin B/Cdc2 is activated at the G2/M transition. Its activity is essential for entry into mitosis, and the inactivation and destruction of cyclin B is required for exit from mitosis *(5)*. Thus, measurement of the activity of cyclin B/Cdc2

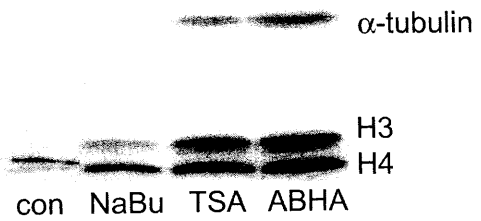


Fig. 2. Whole-cell lysates of HeLa cells treated for 24 h with either 10 m*M* sodium butyrate (NaBu), 100 ng/mL TSA, or 100 µg/mL ABHA. Lysates were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane, immunoblotted with an anti-acetyl lysine antibody (Cell Signalling Ac-K-103), and then developed with enhanced chemiluminescence staining. The blot shows acetylated histone H3 and H4 and acetylated α-tubulin (*see* **Note 13**).

and the abundance of the cyclin B protein by immunoblotting are excellent indicators of entry into and exit from mitosis. The measurement of cyclin B/Cdc2 activity is particularly useful when used in conjunction with synchronized cell cultures, as it provides a biochemical measure of transit through mitosis. Cyclin B/Cdc2 activity is determined by the immunoprecipitation of the complex followed by an in vitro kinase assay performed on histone H1, which is its in vivo substrate. Flow cytometry can provide only an indirect measure of G2/M transition, and may be inaccurate, especially if a high proportion of cells fail cytokinesis. The latter may result in cells with 4N DNA content, which will be detected in a G2/M peak, although these cells are actually in G1 phase. The failure of cytokinesis is common in HDACI treated cells.

1. Resuspend and lyse treated cells ($1–5 \times 10^6$) on ice for 30 min in 1 mL NETN buffer supplemented with 0.15 *M* NaCl.
2. Remove cellular debris by centrifugation at 16,000*g* for 10 min at 4°C.
3. Determine the protein concentration of the lysates (*see* **Subheading 3.8.**) transfer 1 mg to a fresh microfuge tube.
4. Incubate the precleared cell lysate (*see* **Note 7**) with anti-cyclin B antibody (0.5 µg) prebound to 30 µL 50% suspension of protein A–sepharose (Roche) (*see* **Note 8**), for 3 h at 4°C with constant mixing. Wash precipitates three times with NETN, once with 20 m*M* Tris-HCl (pH 7.4), 1 m*M* DTT.
5. In an approved area for radioactive work, resuspend the precipitates in 30µL RC buffer. Incubate at 30°C for 15 min. Add 10 µL 5X SDS sample buffer and boil for 5 min. Run samples on a 10% PAG, stain with acrylamide staining buffer for 30 min with rocking, and destain with acrylamide destain buffer to determine the even loading of histone H1.
6. Quantitate histone phosphorylation using a PhosphorImaging system (Molecular Dynamics).

7. In addition to the immunoprecipitation of the cyclin B complex and kinase assay as performed previously, a 20µg sample of each lysate should be prepared for immuno-blotting. Add 5X SDS sample buffer to the lysate and boil (*see* **Subheading 3.8.**). These samples can be kept at –20°C for 2 wk before running on a PAG to determine the levels of cell cycle regulatory proteins cyclin B1 and Cdc2. The level of cyclin B1 should increase as cells enter mitosis, and decline on exit. The level of Cdc2 remains constant through the cell cycle and acts as a convenient loading control.

3.10. Immunofluorescent Staining

One of most dramatic consequences of a defect in the HDACI-sensitive G2 checkpoint is the appearance of cells undergoing an aberrant mitosis following HDACI treatment (*see* **Note 9**). To examine this phenotype, immunostaining of fixed cells is performed.

1. Seed exponentially growing cells onto coverslips (*see* **Note 10**). To obtain a higher proportion of cells undergoing mitosis, these cells can be synchronized as described in **Subheading 3.2.**
2. Treat cells with HDACI, and harvest the cells on the coverslips at various times after treatment. Wash cells with PBS, fix with –20°C 100% methanol, and store at –20°C in methanol until required. The optimal time for harvesting the synchronized cultures is when the control and HDACI-treated samples both display a high proportion of the rounded mitotic phenotype, which normally occurs 8–12 h after synchrony release. With asynchronous cultures, a high proportion of mitotic cells are evident 16–24 h after drug treatment.
3. Air-dry coverslips, and rehydrate with a drop of rehydration buffer for 1 h at room temperature.
4. Stain the cells with anti-α-tubulin (1:1000 in rehydration buffer; Amersham), for 1 h at room temperature in a humidified chamber (*see* **Note 11**).
5. Wash coverslips three times for 15 min with PBS containing 0.1% dried skimmed milk powder, then incubate with an appropriate fluorochrome-conjugated secondary antibody and DAPI (5 µg/mL), to stain the DNA, for 20 min in the dark in a humidified chamber.
6. Wash coverslips three times with PBS containing 0.1% dried skimmed milk powder, once with PBS, and then mount by inverting onto a glass slide on a drop of Vectorshield mounting solution to preserve the fluorescence.
7. Store coverslips in the dark at 4°C, or analyze immediately with a immunofluorescence microscope. Determine the number of aberrant mitotic cells with each treatment (*see* **Note 9**).

3.11. Determining the Effective Dose of HDACI

We have used a range of different HDACIs to examine the cell cycle effects of these drugs. In each case, it is important to define the concentration of drug required to produce a G2/M effect—either G2 arrest in cells with an intact

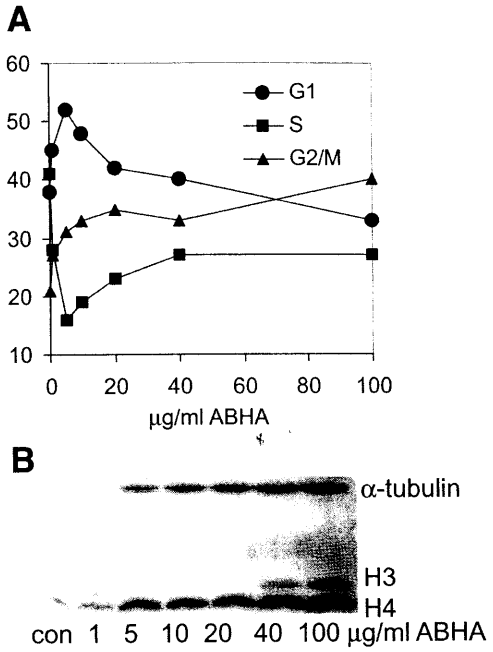


Fig. 3. Titration of ABHA with DG75 Burkitts lymphoma cells._ Asynchronously growing cells were treated with the indicated concentrations of ABHA for 24 h and then harvested. A portion of each sample was fixed for FACS analysis of the DNA content (**A**), and the remainder was lysed and immunoblotted with anti-acetyl lysine antibody (**B**). The levels of acetylated histone H3 and H4, and acetylated tubulin are shown.

HDACI-sensitive checkpoint or aberrant mitosis in cells defective for this checkpoint control. For example, a low dose of ABHA, which produces only a relatively small increase in histone acetylation in DG75 Burkitts lymphoma cells, produces a very strong accumulation of these cells in G1 phase and loss of the S phase population. In contrast, at higher doses, which produce substantial histone acetylation, the accumulation of these cells in G2/M is more prominent (**Fig. 3**). To examine the effective dose of HDACI, the following protocol should be used.

1. Treat asynchronous cell cultures with a range of doses of HDACI (*see* **Note 5**).
2. Harvest at 4, 8, and 24 h after treatment. Determine histone and nonhistone protein acetylation by immunoblotting (*see* **Subheading 3.8.**).
3. In addition, determine the effect on the cell cycle by analyzing cells harvested at 24 h by flow cytometry (*see* **Subheading 3.4.**).

At low doses of HDACIs, a G1 arrest is often observed without any G2/M effects. This is in part a consequence of the transcriptional up-regulation of the CDK inhibitor $p21^{WAF1/CIP1}$ *(2)*. Higher doses are required to observe G2/M effects.

4. Notes

1. When 100 µg/mL ABHA is added to cells in the S phase, a large proportion of the cells will undergo an aberrant mitosis, and subsequently within 24 h a large proportion are dead. If the same amount of ABHA is added to cells in the S phase of the cell cycle and removed in the G2 phase, then cell death does not occur. In addition, 100 µg/mL ABHA added in G2 phase does not produce an aberrant mitosis and causes little cell death *(6)*. Similar results have been observed for additional HDACIs tested.
2. Thymidine depletes the deoxycytidine triphosphate pool, and in this way induces a G1 block in the cell cycle. The inhibitory action of thymidine is alleviated by removal of the high concentration of thymidine and the addition of 2-deocytidine to the medium, which appears to be the rate limiting factor.
3. HeLa cells are the best characterized cell line for use with a double block release protocol. Many cell lines will not successfully exit the second arrest, or if they do, they will have a reduced level of synchrony. For most cell lines, an overnight block with 2 m*M* hydroxurea produces a robust G1/S arrest that is readily reversed upon removal of the drug, resulting in populations which progress into mitosis with 50% synchrony. Synchrony with all these procedures is improved if the tissue-culture hood or room in which the release is performed is also prewarmed to 37°C.
4. Primary fibroblast cultures are difficult to synchronize, and after release a variable proportion of the population often remains arrested in G1. This does not provide any difficulties in interpreting data, as it is effectively a noncycling population that remains constant throughout the experiment. A higher degree of synchrony can be obtained with NFF if they are first synchronized by serum starvation with 0.5% serum for 48 h, then stimulated to enter the cell cycle with 10% serum readdition, then blocked with 2 m*M* hydroxyurea for 24 h, and then released from this arrest by removal of the drug as described. The drawback with this method is that the proportion of noncycling cells is often higher than after the single hydroxyurea block.
5. All HDACIs examined appear to have similar effects on the cell cycle. Stability is a problem for both TSA, with a half-life of 8 h, and NaBu. In addition, NaBu has a number of additional effects on the cell; therefore other HDACIs are used in preference. Typical concentration ranges for HDACIs used on Hela and NFF cells are shown in **Table 1.**
6. To aspirate the supernatant from the plate, a multi-channel pipet is used to remove approx 90% of the total volume in each well.
7. Preclearing a lysate removes all proteins present which will bind nonspecifically to the protein A or G sepharose used to purify the antibody of interest. This is

Table 1
Typical Concentration Ranges for HDACIs Used on Hela and NFF Cells

ABHA	10–200 µg/mL
TSA	100–500 ng/mL
NaB	10–20 m*M*
SBHA (Suberohydroxamic Acid)	50–200 µg/mL
Oxamflatin	5–20µ*M*
Depsipeptide	0.5–2.0 µ*M*

done by first incubating the lysate with 30 µL of protein A/G–sepharose for 30 min with rocking at 4°C. The protein A/G is removed by centrifugation of the lysate at 8000*g* for 1 min in a microfuge, and transfer the supernatant to a fresh microfuge tube for the subsequent immunoprecipitation.

8. To prebind the antibody to the protein A–sepharose, take 0.5 µg anti-cyclin B antibody and mix it with 30 µL 50% protein A–sepharose. Incubate with rocking for 20 min at 4°C, pellet the complex by centrifugation at 5000*g* in a microfuge, wash with an equal volume of PBS, and remove the supernatant. Repeat the washing step three times.

The decision to use protein A or protein G to pull down the antibody of interest will depend on the species of animal in which the antibody was raised and which subclass of immunoglobulin it is. Protein A binds well to antibodies raised in humans, pigs, rabbits, guinea pigs, and mice, while protein G binds antibodies generated in humans, horses, cows, pigs, sheep, goats, rabbits, hamsters, guinea pigs, rats, and mice.

Protein A/G–sepharose is typically stored in 20% ethanol; the ethanol will need to be removed before addition into a cellular lysate. This is done by washing the protein A/G–sepharose five times in a large volume of PBS. The washed protein A/G–sepharose can be stored at 4°C for 10 d.

9. Aberrant mitosis refers to a mitotic cell which is abnormal in appearance (**Fig. 4** and **ref. *6***). Normally, chromosomes congress to the metaphase plate, with a very distinctive spindle cap on either side of the midline. HDACI-treated mitotic cells contained well-formed spindles, but DNA staining shows chromosomes failing to migrate to the midline of the cell. Staining of the kinetochores can also be carried out to demonstrate the failure of the chromosomes to migrate to the midline.
10. Owing to the variation in quality of coverslips, all coverslips are precleaned before use, to ensure that cells will adhere to them. Coverslips (10 mm) are first boiled in water for 20 min, then rinsed in 100% ethanol, and allowed to air dry. When dry, they are packaged into tubes and autoclaved before use.
11. As the volumes involved in the immunofluorescent staining of coverslips are low, a humidified chamber is used. This should be a dark box with a lid. Line the

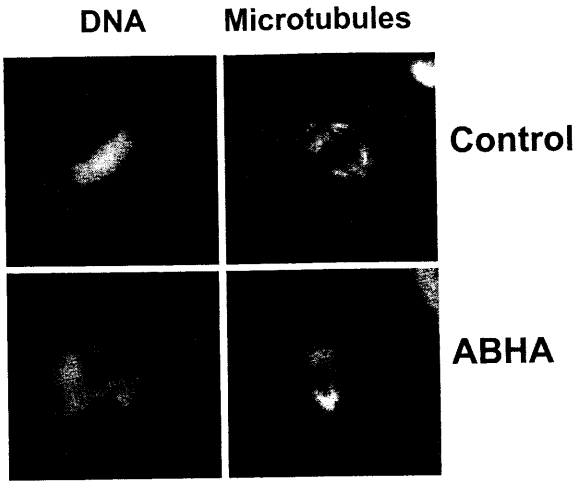


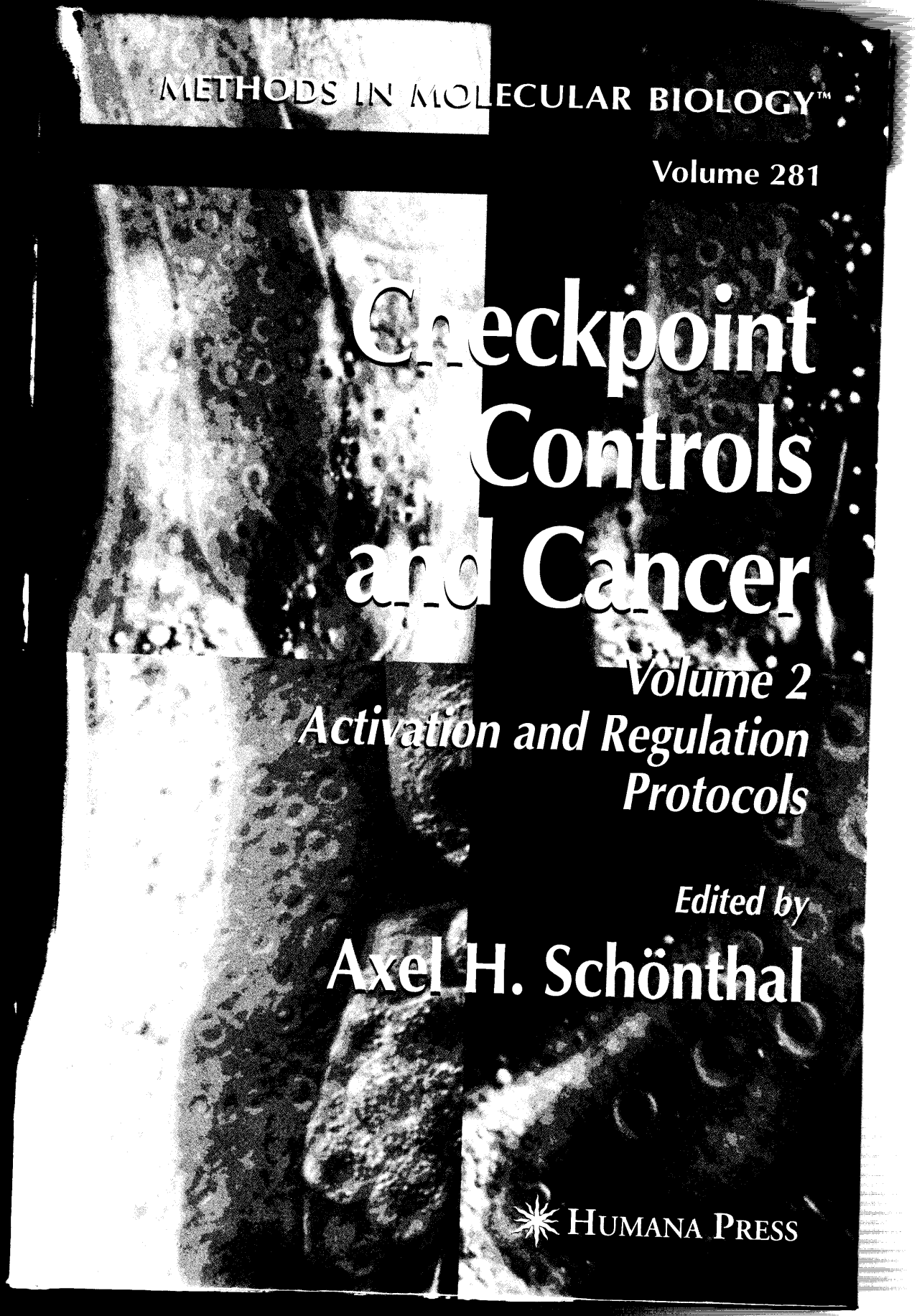
Fig. 4. Aberrant mitosis phenotype. Cells attempting mitosis in the presence of ABHA undergo aberrant mitosis. HeLa cells synchronized by a double thymidine block and then released and treated without (upper panels) or with 100 µg/mL ABHA (lower panels), cells were fixed as the cultures entered mitosis. Cells were stained for DNA and microtubules. The normal congression of condensed chromosomes at metaphase is clearly observed with the DNA staining. The ABHA-treated mitotic cells contained well formed spindles, but DNA staining shows chromosomes failing to migrate to the midline of the cell.

bottom of the box with blotting paper, and wet it with PBS. Take a length of parafilm large enough to place all the coverslips on, and place on the blotting paper. Mark the parafilm with the identification of each coverslip, to prevent a mix-up of the samples. Use this chamber for the rehydration and the incubations with the primary and secondary antibodies. Place the coverslips cell-side down on a drop of the buffer/solution in question for each step. For washing, transfer the coverslips to a 24-well plate cell-side up.

12. In NFF cells, a G2/M checkpoint is activated in the presence of the HDACI, ABHA. In HeLa cells this checkpoint is not activated, and the cells progress through the cell cycle into G1 phase. In the figure of the BrdU-Hela cells, there are a significant proportion of cells still in the G2/M or 4N peak after 24 h of treatment. This peak corresponds to cells which have gone through mitosis but have failed cytokinesis; therefore, they are in fact G1-phase cells.
13. Differential acetylation of the H3 and H4 histone can be seen when cells are treated with different HDACIs. At present it is unclear whether this is a result of the inhibitors having differential effects, or whether it is a result of the relative instability of TSA and NaBu compared to ABHA.

References

1. Kouzarides, T. (2000) Acetylation: a regulatory modification to rival phosphorylation? *EMBO J.* **19,** 1176–1179.
2. Richon, V. M., Sandhoff, T. W., Rifkind, R. A., and Marks, P. A. (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. *Proc. Natl. Acad. Sci. USA* **97,** 10,014–10,019.
3. Qiu, L., Burgess, A., Fairlie, D. P., Leonard, H., Parsons, P. G., and Gabrielli, B. G. (2000) Histone deacetylase inhibitors trigger a G2 checkpoint in normal cells that is defective in tumor cells. *Mol. Biol. Cell* **11,** 2069–2083.
4. Burgess, A. J., Pavey, S., Warrener, R., et al. (2001) Up-regulation of p21(WAF1/CIP1) by histone deacetylase inhibitors reduces their cytotoxicity. *Mol. Pharmacol.* **60,** 828–837.
5. Morgan, D. O. (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu. Rev. Cell. Dev. Biol.* **13,** 261–291.
6. Warrener, R., Beamish, H., Burgess, A., et al. (2003) Tumor cell–specific cytotoxicity by targeting cell cycle checkpoints. *FASEB J.* **17,** 1550–1552.


METHODS IN MOLECULAR BIOLOGY™
Volume 281
Checkpoint Controls and Cancer
Volume 2
Activation and Regulation Protocols
Edited by
Axel H. Schönthal
HUMANA PRESS