Microtubule Acetylation Through HDAC6 Inhibition Results in Increased Transfection Efficiency

Erin E Vaughan1, R Christopher Geiger1-2, Aaron M Miller1, Phoebe L Loh-Marley3, Takayoshi Suzuki4, Naoki Miyata4 and David A Dean1,3

1Division of Pulmonary and Critical Care Medicine, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA; 2Department of Bioengineering, Florida Gulf Coast University, Ft. Myers, Florida, USA; 3Department of Pediatrics, University of Rochester, Rochester, New York, USA; 4Graduate School of Pharmaceutical Sciences, Nagoya City University, Aichi, Japan

The success of viral and nonviral gene delivery relies on the ability of DNA-based vectors to traverse the cytoplasm and reach the nucleus. We, as well as other researchers, have shown that plasmids utilize the microtubule network and its associated motor proteins to traffic toward the nucleus. While disruption of microtubules with nocodazole was shown to greatly inhibit cytoplasmic plasmid trafficking, it did not abolish it. It has been demonstrated that a pool of stabilized posttranslationally acetylated microtubules exists in cells, and that this acetylation may play a role in protein trafficking. In order to determine whether this modification could account for the residual DNA trafficking in nocodazole-treated cells, we inhibited or knocked down the levels of the tubulin deacetylase, histone deacetylase 6 (HDAC6), thereby generating higher levels of acetylated microtubules. Electroporation of plasmids into cells with inhibited or silenced HDAC6 resulted in increased gene transfer. This increased transfection efficiency was not because of increased transcriptional activity, but rather, because of increased cytoplasmic trafficking. When plasmids were cytoplasmically microinjected into HDAC6-deficient cells, they entered the nucleus within 5 minutes of injection, almost 10 times faster than in wild-type cells. Taken together, these results suggest that modulation of HDAC6 and the microtubule network can increase the efficiency of gene transfer.

Received 11 January 2008; accepted 8 August 2008; published online 9 September 2008. doi:10.1038/mt.2008.190

INTRODUCTION

A primary limitation of gene therapy, specifically nonviral gene therapy, is the inability to achieve high levels of expression. The cell and nuclear membranes as well as the dense meshwork of the cytoplasm all present obstacles to DNA transfer that must be overcome in order for the DNA to reach the nucleus and be transcribed. Much work has been devoted to overcoming the barriers presented by the cellular and nuclear membranes, while what occurs in the cytoplasm is only beginning to be discovered. Previous work has demonstrated that, after liposome mediated transfections, a significant amount of DNA remains free in the cytoplasm and does not reach the nucleus, and this may contribute to the low levels of expression. Further, the cytoplasmic environment is too densely populated by proteins and cytoskeletal elements for DNA to simply diffuse to the nucleus. It has been shown previously that DNA, like many nuclear localization sequence–containing proteins, utilizes the microtubule network and the molecular motor, dynein to navigate through the dense meshwork of the cytoplasm and reach the nucleus. However, we observed that even after pharmacological disruption of the microtubule network with the drug nocodazole, a low level of expression was still observed when plasmids were electroporated or cytoplasmically microinjected. Nocodazole treatment leaves the actin network primarily intact, thereby still providing a barrier to diffusion. Therefore, assuming that diffusion cannot occur, and that the microtubule network has been disrupted by drug treatment, we sought to determine how the plasmids were able to reach the nucleus. Interestingly, there is a subset of modified microtubules that remain intact even after nocodazole treatment. These stable microtubules are acetylated at lysine 40 (refs. 10,11). Intriguingly, it has been demonstrated recently that acetylated microtubules promote the binding of the molecular motors kinesin and dynein as well as their transport, suggesting a role for acetylated microtubules in trafficking. Further, we have previously observed that cyclic stretch increases gene transfer and alters the cytoskeleton. Therefore there may also be a role for acetylated microtubules in stretch-enhanced gene transfer. In view of these findings, we sought to determine whether acetylated microtubules play a role in the trafficking of plasmids to the nucleus, both in static and stretched cells.

In this study, after establishing that acetylated microtubules are increased in stretched cells and continue to be present in nocodazole-treated cells, we used drug treatments to inhibit the tubulin deacetylase, histone deacetylase 6 (HDAC6), and also used HDAC6 knockdown cells, so as to increase the acetylation...
of microtubules. We found that increased acetylation of microtubules increased gene transfer by increasing the trafficking of plasmids toward the nucleus. Finally, we looked at other targets of HDAC6 and determined that they are not responsible for the observed increase in expression. Taken together, these results suggest that modulation of HDAC6 and the microtubule network can increase the efficiency of gene transfer.

RESULTS

Acetylated microtubules remain intact after nocodazole treatment or cyclic stretch

In order to determine whether acetylated microtubules play a role in trafficking, we sought to first establish their presence. For this purpose, A549 cells were either stretched or treated with nocodazole. The cells were then harvested and lysed in either microtubule stabilization buffer (total tubulin) or microtubule stabilization buffer containing Triton X-100 (polymerized microtubules only). Equal amounts of sample were then analyzed for tubulin using western blot. We found that while the overall levels of tubulin remained constant, as expected, when cells were stretched or exposed to nocodazole, the numbers of microtubules was diminished. In the case of cells stretched for 3 hours, the polymerized microtubules decreased by ~40%, while treatment with nocodazole for the same period of time reduced microtubules by >80%, thereby confirming our previous findings. However, acetylated microtubules still remained, suggesting that, while there was an overall disruption of the microtubule network, there remained a stable subset of microtubules in the cells (Figure 1b). These results were confirmed by immunofluorescence of A549 cells for acetylated tubulin (Figure 1a). Again, after either cyclic stretch for 3 hours or exposure to nocodazole for 3 hours, a significant amount of acetylated microtubules remained present in the cells.

HDAC6 inhibition results in increased gene expression

In order to determine whether acetylated microtubules play a role in trafficking, we increased the amount of acetylated microtubules in A549 cells and assessed the transfection efficiency of electroporated plasmids. While acetylation of microtubules occurs through an as-yet-undefined mechanism, deacetylation occurs primarily through HDAC6. Therefore, in order to increase the amount of acetylated microtubules, we inhibited HDAC 6. Given that the most common inhibitors of HDACs inhibit various HDACs and not HDAC6 specifically, the use of these drugs can also inhibit nuclear HDACs, resulting in histone modifications that could ultimately affect the expression of our plasmids. In order to control for this, we took advantage of the various binding affinities of the different inhibitors. Sodium butyrate at 1 mmol/l inhibits all HDACs except HDAC6 (ref. 15). This then serves as a control for plasmid expression levels in the presence of nuclear histone deacetylase inhibition. Trichostatin A (TSA) at 1 μmol/l, however, inhibits all HDACs including HDAC6 (ref. 15). Therefore the difference between the two should be a reflection of the inhibition of HDAC6, that is, an increase in acetylated microtubules. Cells were electroporated with a luciferase-expressing plasmid, treated with the drugs at the given concentrations, and harvested at 3 hours for determination of luciferase expression (Figure 2). This early time point was chosen so that differences in gene expression would be primarily attributable to differences in trafficking as opposed to alteration in transcription which could accumulate over longer time periods. There was a slight but significant increase in the TSA-treated cells as compared to both vehicle-treated as well as sodium butyrate-treated cells, reflecting an increased expression when HDAC6 is inhibited (acetylated microtubules are increased).
Although suggestive of a role for HDAC6 inhibition in increasing the expression of an electroporated plasmid, we sought a more specific drug to determine the role of HDAC6 inhibition in trafficking. For this purpose we used the HDAC6-selective nonhydroxamate, aliphatic thiolate analog NCT-10b as an inhibitor. A549 cells were electroporated with a luciferase-expressing plasmid and treated with NCT-10b, and harvested after 3 hours to determine luciferase expression. Specific inhibition of HDAC6 again resulted in a significant increase in expression over dimethyl sulfoxide (vehicle) treatment (Figure 2).

**Inhibition of HDAC6 by NCT-10b does not alter transcriptional activity of the plasmid**

The nonhydroxamate, aliphatic thiolate analog NCT-10b has been shown to inhibit the tubulin deacetylase activity of HDAC6 specifically without inhibiting other HDACs. However, it is still possible that treatment with the drug may result in increased transcription, and that the increased expression is therefore not necessarily a reflection of increased or more efficient trafficking. In order to look at the effects of NCT-10b on transcription directly, we used a cell line stably transfected with a luciferase-expressing plasmid. These cells were then treated with TSA, sodium butyrate, or NCT-10b for 3 hours, the cells were harvested, and the expression was determined. Addition of NCT-10b for 3 hours did not show an increase in luciferase expression, thereby suggesting that the drug does not alter the transcription of the plasmid (Figure 3). Although addition of sodium butyrate and TSA increased the expression by ~25%, the increase did not reach statistical significance. These results suggest that when a plasmid is electroporated and the expression is increased, it is because of an increase in trafficking and is not attributable to events occurring once the plasmid is in the nucleus.

**Knockdown of HDAC6 results in increased trafficking**

In order to further address the role of HDAC6 inhibition, a luciferase-expressing plasmid was electroporated into stable cell lines, either overexpressing or knocking down HDAC6 and control cells. Cells were harvested after 3 hours or 24 hours, and expression was determined (Figure 4). In the control cell lines pSuper and pNeo, there was no increase in expression observed as compared to untransfected A549 cells at either of the time points. Further, overexpression of HDAC6 did not demonstrate a change in expression levels. Knockdown of HDAC6, however, resulted in a ~2.5-fold increase in expression at 3 hours after transfection, which was similar to the increase noted in cells in which HDAC6 had been knocked down with the chemical compound NCT-10b. However, when luciferase activity was measured in the cells 24 hours after transfection, the HDAC6 knockdown cells showed

**Figure 4** RNAi-induced silencing of histone deacetylase 6 (HDAC6) increases transfection efficiency. Wild-type A549 cells, or A549 cells stably transfected with control plasmids pSuper (pSup) or pNeo, HDAC6 overexpressing plasmid pHDAC6wt (OE), or an HDAC6 short-hairpin RNA–expressing plasmid to knockdown expression (KD) were electroporated with pCMV-Lux-DTS, and (a) 3 hours later or (b) 24 hours later luciferase activity was measured. Mean luciferase activities ±SD (relative luciferase unit (RLU)/mg cell protein) were normalized to values for transfected wild-type A549 cells. The experiments were performed in triplicate and repeated at least three times. Inset in panel a shows the western blot for HDAC6 in triplicate samples, indicating that >90% knockdown of HDAC6 was achieved. (c) TC7 cells from African Green Monkey kidney epithelium and human pulmonary artery smooth muscle cells were transfected with a control nontargeted small-interfering RNA (siRNA) or HDAC6-specific siRNA, and 48 hours later the cells were electroporated with pCMV-Lux-DTS. Three hours later, luciferase activity was measured. Mean luciferase activities ±SD (RLU/mg cell protein) were normalized to the values for their nontargeted siRNA controls. The experiments were performed in triplicate and repeated at least three times. *< 0.001 by paired student t-test.

© The American Society of Gene Therapy HDAC6 Regulates Cytoplasmic Plasmid Trafficking

**Molecular Therapy** vol. 16 no. 11 nov. 2008 1843
a >20-fold increase in expression. The increased transfection seen after HDAC6 knockdown is not restricted to lung epithelial cells but appears to extend to other cell types. While transfection of either a cell line of African Green Monkey kidney epithelial cells (TC7 cells) or primary human pulmonary artery smooth muscle cells with a nontargeted small-interfering RNA resulted in transfection efficiencies comparable to non-treated cells, transfection of these cells with HDAC6-specific small-interfering RNAs resulted in a two- to threefold increase in relative luciferase expression when HDAC6 was silenced by ∼90% (Figure 4c).

In order to demonstrate directly that silencing of HDAC6 affects DNA cytoplasmic trafficking, plasmids were fluorescently labeled with a Cy3-labeled PNA and microinjected into the cytoplasm of either wild-type A549 cells or A549 cells stably expressing short-hairpin RNA against HDAC6 (Figure 5). In wild-type cells, DNA began to localize to the nucleus within 30–50 minutes, as we have previously seen.16 However, even at 50 minutes after the cytoplasmic injection, not all of the DNA had moved to and into the nucleus. By contrast, almost all of the microinjected plasmid within the cell had localized to the nucleus of the HDAC6 knockdown cell within 5 minutes after microinjection. When quantified, almost 50% of the HDAC6 knockdown cells showed nuclear import at 10 minutes after microinjection, as compared to 2% of the control A549 cells (Figure 6), thereby confirming that the effects of HDAC6 inhibition are indeed caused by cytoplasmic trafficking.

Other targets of HDAC6

It has been suggested that HDAC6 may have roles other than acetylation of microtubules. Therefore it is possible that the increased expression observed when HDAC6 is inhibited is not mediated by increased trafficking on acetylated microtubules but through another pathway. In order to address this possibility we looked at various other targets of HDAC6. To begin with, HDAC6 has also been found to deacetylate heat shock protein-90 (Hsp90) and thereby alter its ability to chaperone proteins.18 Therefore when HDAC6 is inhibited Hsp90 becomes hyperacetylated and is unable to chaperone its client proteins. In order to determine whether this alteration in Hsp90 by HDAC6 inhibition is responsible for the increased expression observed, we chemically inhibited Hsp90 by treatment with 17-AAG. A549 cells were treated with 17-AAG immediately after electroporation of a luciferase-expressing plasmid, the cells were harvested 3 hours later, and luciferase expression was determined (Figure 7). There was no increase in expression observed when 17-AAG treatment was used. In fact, expression levels were dampened, thereby suggesting that the increase in expression observed with HDAC6 inhibition is not mediated by HSP90.

HDAC6 has also been shown to form a complex with protein phosphatase I,19 and inhibition of HDAC6 disrupts this complex, resulting in an inactive protein phosphatase I. In order to mimic this we inhibited protein phosphatase I with okadaic acid. A549 cells were treated with okadaic acid immediately after electroporation with a luciferase-expressing plasmid, and the cells were harvested 3 hours later to determine luciferase expression (Figure 7). Treatment with okadaic acid did not result in increased expression noted when HDAC6 was inhibited, thereby suggesting that the disruption of the protein phosphatase I–HDAC6 complex is not responsible for the increase in expression observed when HDAC6 is inhibited.

This article has been reviewed by the authors and is published as written.

Figure 5 Knockdown of histone deacetylase 6 (HDAC6) increases the rate of cytoplasmic trafficking and nuclear localization of plasmid DNA. Wild-type or stably transfected A549 cells expressing short-hairpin RNA for HDAC6 (HDAC6 KD) were cytoplasmically microinjected with Cy3-PNA-labeled plasmid (0.5 mg/ml). Immediately after injection, the cells were imaged for injected DNA (red) at the indicated times. Nuclear DNA was visualized by 4',6-diamidino-2-phenylindole staining (blue). At least 100 cells were injected for each condition and experiments were repeated three times. Representative cells are shown.

Figure 6 Quantitation of effect of histone deacetylase 6 (HDAC6) knockdown on cytoplasmic trafficking and nuclear localization of plasmid DNA. The number of cells showing nuclear plasmid at 10 minutes after cytoplasmic injection were counted for wild-type or stably transfected A549 cells expressing short-hairpin RNA for HDAC6 (HDAC6 KD) used in Figure 5. The mean percentages of cells showing nuclear localized plasmid ±SD are shown for >300 injected cells in three independent experiments for each cell type. *P < 0.001 by paired student t-test.

Figure 7 Inhibition of non-tubulin histone deacetylase 6 (HDAC6) targets does not alter DNA trafficking. A549 cells or stable A549 transfectants expressing short-hairpin RNA against hypoxia-inducible factor 1-α (HIF-1α) were transfected by electroporation with pCMV-Lux-DTS. Transfected wild-type A549 cells were immediately treated with vehicle [dimethyl sulfoxide (DMSO)], 10 nmol/l okadaic acid (OA), or 1 mmol/l 17-AAG, as indicated. Luciferase expression was determined 3 hours later and mean luciferase activities ±SD (relative luciferase unit/mg cell protein) were normalized to values for transfected wild-type A549 cells. The experiments were performed in triplicate and repeated at least three times.
Finally, HDAC6 inhibition has also been shown to inhibit the transcription factor hypoxia-inducible factor 1-α (HIF-1α). In order to determine whether the increase in expression could be attributed to HIF-1α suppression, we created a stable HIF-1α short-hairpin RNA–expressing knockdown cell line. We electroporated the knockdown cell line with luciferase-expressing plasmid, again harvested the cells at the 3-hour time point, and found that there was no change in expression between A549 cells and the HIF-1α knockdown cells (Figure 7).

**HDAC6 overexpression prevents stretch-enhanced gene transfer**

After electroporation, gene transfer is enhanced in cells that are exposed to cyclic stretch. We have shown that, after cyclic stretch, acetylated microtubules remain (Figure 1) and, further, that inhibition of HDAC6 results in an increase in gene transfer that is not attributable to any known interactions of HDAC6 other than an increase in acetylated microtubules. In this manner we sought to explore the role of acetylated microtubules in stretched cells. Stable A549 cell lines that overexpressed HDAC6 (few acetylated microtubules) or had knocked down HDAC6 levels (many acetylated microtubules) were exposed to cyclic stretch immediately after electroporation. After 3 hours the cells were harvested, and luciferase activity was determined. In the overexpressing cell line, where very few acetylated microtubules are present, cyclic stretch did not induce the enhancement normally found when cells are stretched (Figure 8). However, when HDAC6 was knocked down and consequently more acetylated microtubules were present, an increase (approximately twofold) in enhancement was seen in stretched cells.

**DISCUSSION**

A primary limitation of nonviral gene therapy is the low levels of expression achieved. One of the underlying reasons for this is that DNA is not free to move in the cytoplasm and therefore cannot simply diffuse to the nucleus. Therefore much of the DNA never even reaches the nucleus. This is true not only when the plasmid is microinjected but also in liposome-transfected cells after endosomal escape. Therefore, an understanding of the mechanism through which plasmids traverse the cytoplasm to reach the nucleus is imperative for improving nonviral gene therapy.

We have found earlier that when plasmids are electroporated into cells, they travel along the microtubule network to reach the nucleus. Yet, when cells were treated with nocodazole, a drug that disrupts the microtubule network, and should therefore prevent plasmids from reaching the nucleus, we still observed a low level of expression. Further, we established that when cells were stretched the microtubule network was also disrupted (Figure 1 and ref. 13). This finding was inconsistent with our data demonstrating that cyclic stretch actually enhances expression of an electroporated plasmid. One possible explanation for this apparent paradox would be the presence of acetylated microtubules. Here, we found that acetylated microtubules remain in A549 cells that have been either exposed to cyclic stretch or to the microtubule-depolymerizing drug, nocodazole, and that the acetylated microtubules may serve as a way for plasmids to reach the nucleus.

The function of acetylated microtubules is only beginning to be discerned. One hypothesis is that acetylation of microtubules confers stability on microtubules. If this were so, it would support our previous results showing that stabilization of the microtubule network with taxol increased the expression levels. Stabilization of the microtubule network either by taxol treatment or HDAC6 inhibition (and subsequent microtubule acetylation) show similar increases in expression. However, other work has suggested that acetylation of microtubules does not necessarily confer stability but only serves to make the microtubules more dynamic, i.e., demonstrating significantly higher growth and shorting rates. It is unclear, therefore, whether stability, or the lack thereof, plays a role in increasing trafficking on microtubules. Interestingly, an alternative explanation for increased trafficking has been proposed: that acetylation of microtubules may result in conformational changes in the microtubules, resulting in a higher affinity for molecular motors. In the light of this, and knowing that plasmids traffic through the molecular motor dynein and that HDAC inhibitors such as TSA increase acetylated tubulin, we sought to explore this relationship further.

In the past, TSA has been used for increasing expression in transfected cells, the general hypothesis being that inhibition of nuclear HDACs results in the subsequent acetylation of the DNA and increased expression. However, TSA inhibits all class I and class II HDACs, including not only nuclear HDACs but also the cytoplasmic HDAC6. It is therefore possible that there could be two distinct means by which broad HDAC inhibitors could be acting: on transcription in the nucleus and on trafficking in the cytoplasm. The idea that HDAC inhibition can play a role in

![Figure 8](image-url) Acetylated microtubules are required for stretch-enhanced gene expression. A549 cells were electroporated with pCMV-Lux-DTS and immediately exposed to cyclic stretch (10% change in basement membrane area at 15 cycles/min) or grown statically for 3 hours, after which the cells were harvested and luciferase activity was measured. Mean luciferase activities ±SD (relative luciferase unit/mg cell protein) were normalized to the values for unstretched, transfected wild-type A549 cells. The experiments were performed in triplicate and repeated at least three times. *P < 0.01 as compared to unstretched matched cells.
trafficking is supported by the finding by Dompiere et al. that TSA increases intracellular transport of brain-derived neurotropic factor. We found (Figure 3) that TSA actually has a limited effect on transcriptional activity of a chromosomally integrated plasmid at early time points in electroporated cells. We should point out that without directly studying transcription from these constructs by nuclear run-on assays or the like we cannot definitively rule out minor effects on transcription. However, our finding indicates that there may be an alternative explanation for the increase in the expression levels of transfected plasmids. Using both chemical and stable knockdown approaches, we demonstrate that inhibition of the cytoplasmically localized HDAC6 results in increased transgene expression (Figures 2 and 4) because of enhanced movement of the plasmid through the cytoplasm to the nucleus (Figure 5). Further, we have excluded several other major HDAC6 targets as potential causes for these increases (Figure 6). Taken together, these results suggest that the increase in acetylated tubulin, on account of HDAC6 inhibition, is responsible for the increase in trafficking to the nucleus, and consequently to the increase in expression.

This finding is not surprising in the light of data from recent work. It has been shown that, in primary hippocampal neurons, acetylated microtubules serve as “high occupancy vesicle” lanes for kinesin-mediated delivery of JIP-1-containing vesicles to the tips of neuritis. Further, Gardiner et al. suggest that a lack of tubulin acetylation, and the concomitant disruption in microtubule-associated protein trafficking, may be underlying causes of Familial Dysautonomia. In addition Dompiere et al. demonstrated, using biochemical assays, that TSA treatment enhances the association of kinesin heavy chain, p150 glued dynactin subunit, and dynein intermediate chain to microtubules. Further, they confirmed increased binding of kinesin and dynein to microtubules, using microscopy as well as in vitro assays. Indeed, based on our previous findings demonstrating that coinjection of an inhibitory antibody against dynein along with plasmids significantly inhibits cytoplasmic plasmid trafficking and subsequent gene expression, the increased transit time of DNA to the nucleus when HDAC6 is silenced (Figure 5), supports a model of increased motor loading and movement of plasmid–protein complexes along acetylated microtubules.

On the basis of the fact that acetylation of microtubules results in increased binding of molecular motors and inhibition of HDAC6 increases plasmid trafficking, we propose that the presence of increased acetylated microtubules in response to mild cyclic stretch and the persistence of acetylated microtubules after nocodazole treatment help explain the seeming paradox of plasmid trafficking increasing after stretch and remaining even after nocodazole treatment. As HDAC inhibitors continue to emerge as strong candidates in many drug trials, these results indicate a possible means to increase the efficiency of gene therapy.

**Materials and Methods**

**Cell culture, transfection, and microinjection.** Human adenoacarcinoma A549 cells (#CCL-185; American Type Culture Collection, Rockville, MD) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. A stable line of A549 cells expressing firefly luciferase was constructed by transfection of cells with plasmid pGL4.14[Luc2/Hygro] (Promega, Madison, WI) and selection with hygromycin. Colonies were pooled and passaged under selection. HIF-1α and HIF-2 knockdowns were created in A549 cells using retroviral methods, with the PT67 packaging cell line (Clontech, Mountain View, CA). PT67 cells were transfected with 10 to 15 μg of specific short-hairpin RNA-expressing or empty plasmids using lipofectin (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s protocol. Virus-containing medium was supplemented with 8 μg/ml of polybren (Sigma, St Louis, MO) for infection. Stable HDAC6 knockdown and stable HDAC6 overexpressing A549 cells were a generous gift from T.P. Yao (Duke University).

For electroporation, cells were grown to confluency in 6-well dishes, and rinsed twice in serum- and antibiotic-free media. Five micrograms of plasmids in 500 μl of serum- and antibiotic-free Dulbecco’s modified Eagle’s medium were added to each well, and one 125 mV square wave electric pulse was applied using a PetriPulser electrode (BTX, San Diego, CA). Immediately after electroporation (within 15 seconds), the indicated drug was added to the cells in 1 ml Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotic. Drugs were used at the following final concentrations: 1 μmol/l TSA, 1 mmol/l Sodium Butyrate, 20 μmol/l Nocodazole, 3 mmol/l NCT-10b, 1 mmol/l 17-AAG, and 10 mmol/l Okaida Acid. When indicated, 3 or 24 hours after electroporation, cells were lysed in Promega lysis buffer, and luciferase activity was measured using the Promega Luciferase Assay System as previously described. All luciferase measurements were normalized to total cell protein and reported as multiples of increase in expression relative to dimethyl sulfoxide-vehicle treated cells. All experiments were performed in triplicate wells and the experiments were repeated at least three times. For statistical analyses, student’s t-tests were used.

Cells grown on etched coverslips were microinjected as previously described. At least 100 cells were injected for each condition and the experiments were repeated three times.

**Plasmids.** Plasmid pCMV-lux-DTS expresses either luciferase from the cytomegalovirus immediate early promoter and contains the simian virus 40 DNA nuclear targeting sequence downstream of the reporter gene. All plasmids were purified from Escherichia coli using Qiagen Gigaprep kits as described by the manufacturer (Qiagen, Chatsworth, CA). Plasmid pDD306, expressing green fluorescent protein from the cytomegalovirus promoter and containing the GeneGrip1 PNA binding site (Gene Therapy Systems, San Diego, CA) was labeled with Cy3-labeled PNA as described. Briefly, plasmids were labeled with Cy3-PNA in the manufacturer’s labeling buffer at 37°C for 2 hours, followed by isopropanol precipitation to remove unbound Cy3-PNA. Plasmid labeling was verified by agarose gel electrophoresis and fluorescent detection in the absence of ethidium bromide.

**Equibiaxial cyclic stretch.** For all stretch-related experiments, A549 cells were plated on Pronectin-treated BioFlex culture plates. After allowing for attachment for 48–72 hours, the cells were stretched using 25 mm BioFlex loading stations with a 10% membrane surface area change at 15 cycles/min and a 50% duty cycle. These parameters are the same as previously reported and the 25 mm loading stations ensure an equibiaxial stretch in the radial and circumferential directions over the width of the loading post.

**Microtubule extraction.** Prior to collection, samples were washed once in phosphate-buffered saline (PBS) (37°C) and stabilized in two 15-minute washes in microtubule stabilization buffer (0.1 mol/l PIPES, pH 6.75, 1 mmol/l EGTA, 1 mmol/l MgSO4, 2 mol/l glycerol, protease inhibitors). Cells were then lysed in microtubule lysis buffer (25 mmol/l Tris–HCl, pH 7.4, 0.4 mol/l NaCl, and 0.5% sodium dodecyl sulfate).

**Western blots.** All extracted samples were mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis sample buffer (112.5 mmol/l Tris–HCl, pH 6.8, 10% β-mercaptoethanol, 3.6% sodium dodecyl sulfate, 1.8% glycerol, and 0.001% bromophenol blue) such that equal amounts of total protein were loaded into each lane and electrophoresed on a 10% gel and transferred to nitrocellulose. Antibodies were used against tubulin (Naviva, Inc.), acetylated tubulin (Novus Biologicals), HDAC6 (Novus Biologicals), KIF5A (Novus Biologicals), KIF5B (Novus Biologicals), and β-actin (Novus Biologicals). Western blots were scanned and quantified using the Odyssey Imaging System (LI-COR).
protein were loaded for each fraction. The samples were boiled for 3 min-
utes and separated using 12% sodium dodecyl sulfate polyacrylamide gel
electrophoresis gels in accordance with the methods previously described
by Lutengberg.21 Proteins were transferred to nitrocellulose and probed
using anti-acetylated (1:1,000, Sigma) or anti-tubulin (1:1,000, Sigma) in
PBS with 5% fat-free powdered milk. Blots were developed using a chemi-
luminescence detection kit, and autoradiograms were digitized before den-
sitometry using ImageJ (National Institutes of Health, Bethesda, MD). All
samples were run in duplicate and were from at least three independent
experiments.

**Immunofluorescence imaging.** After each treatment regime, the cells were
washed with PBS followed by a 10-minute incubation in fixation/permea-
bility buffer (60 mmol/l PIPES, 25 mmol/l HEPES, 10 mmol/l EGTA, 3 mmol/l MgCl2, 0.2% Triton X-100, and 3.7% paraformaldehyde). The
cells were then washed in PBS and blocked for 1 hour in PBS containing
1 mg/ml bovine serum albumin. After incubation, the cells were cyclized for 1 hour with the appropriate primary antibody in PBS with 0.1 mg/ml
bovine serum albumin, washed in PBS again, and reacted for 30 minutes
with Alexa 488– or 555–conjugated secondary antibody (1:200; Molecular
Probes, Eugene, OR) in PBS with 0.1% bovine serum albumin. Following
a second set of washes in PBS, the silastic membranes were excised from
the culture plates, placed face up on a microscope slide, and covered with
a coverslip. All images were acquired in OpenLab (Improvision, Lexington,
MA) using a Hamamatsu Orca II-ER camera attached to a Leica DRMX-2
upright fluorescent microscope with a 100× oil-immersion objective.

**Live cell imaging.** Cells were microinjected on an inverted Leica micro-
scope fitted with a 37 °C acrylic incubation chamber. Immediately after
microinjection, the cells were imaged over time using a Q Imaging Retiga
scope fitted with a 37 °C acrylic incubation chamber. Immediately after

ACKNOWLEDGMENTS

We thank T.P. Yao, Navdeep Chandel, Eric Bell, Brooke Emerling, Jacqueline Schriewer, and Rui Zhou for the generous gift of plasmids and/or cells, as well as for their excellent advice and help.

REFERENCES

codelivery of T7 RNA polymerase and T7 promoter sequence with cationic liposomes.
of size-dependent DNA mobility in cytoplasm: a new barrier for non-viral gene delivery.
mobility and nuclear accumulation of DNA plasmids associated with a karyophilic
Nuclear localization signal peptides induce molecular delivery along microtubules.
*Biophys J* **89**: 2134–2145.
alphatubulin in mammalian cells in culture: characterization, localization and function.
posttranslationally modified by acetylation on the epsilon-amino group of a lysine.
11. Gao, X and Huang, L (1987). Identification of an acetylation site of
12. Reed, NA, Cai, D, Blaisius, TL, Jih, CT, Myhoffer, E, Gaertig, J et al. (2006). Microtubule
reorganization of the cytoskeleton and its role in enhanced gene transfer. *Gene Ther*
**13**: 725–731.
17. Wilson, GL, Dean, BS, Wang, G and Dean, DA (1999). Nuclear import of plasmid DNA
in digitonin-permeabilized cells requires both cytoplasmic factors and specific DNA
regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid
inhibitors disrupt cellular complexes containing protein phosphatases and deacetylases.
inhibitors induce VHL- and ubiquitin-independent proteasomal degradation of
Class II histone deacetylases are associated with VHL-independent regulation of
23. Kim, SH, Kim, KW and Jeong, JW (2007). Inhibition of hypoxia-induced angiogenesis
by sodium butyrate, a histone deacetylase inhibitor, through hypoxia-inducible
In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation.
*EMBO J* **21**: 6820–6831.
dynamic instability to adenovirus nuclear localization efficiency. *Cell Mol Cell Biol*
**64**: 675–689.
Histone deacetylase 6 inhibition compensates for the transport defect in
acetylation and microtubule-based protein trafficking in familial dystonia. *Traffic*
**8**: 1145–1149.
decaylase HDAC6 regulates aegresome formation and cell viability in response to
29. Vack, J, Dean, BS, Zimmer, WE and Dean, DA (1999). Cell-specific nuclear import of
plasmid DNA. *Gene Ther* **6**: 1006–1014.
31. Dean, DA, Dean, BS, Muller, S and Smith, LC (1999). Sequence requirements for