

# HDAC6 deacetylation of tubulin modulates dynamics of cellular adhesions

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## Summary

Genetic or pharmacological alteration of the activity of the histone deacetylase 6 (HDAC6) induces a parallel alteration in cell migration. Using tubacin to block deacetylation of  $\alpha$ -tubulin, and not other HDAC6 substrates, yielded a motility reduction equivalent to agents that block all NAD-independent HDACs. Accordingly, we investigated how the failure to deacetylate tubulin contributes to decreased motility in HDAC6-inhibited cells. Testing the hypothesis that motility is reduced because cellular adhesion is altered, we found that inhibiting HDAC6 activity towards tubulin rapidly increased total adhesion area. Next, we investigated the mechanism of the adhesion area increase. Formation of adhesions proceeded normally and cell spreading was more rapid in the absence of active HDAC6; however, photobleaching assays and adhesion breakdown showed that adhesion turnover was slower. To test the role of

hyperacetylated tubulin in altering adhesion turnover, we measured microtubule dynamics in HDAC6-inhibited cells because dynamic microtubules are required to target adhesions for turnover. HDAC6 inhibition yielded a decrease in microtubule dynamics that was sufficient to decrease focal adhesion turnover. Thus, our results suggest a scenario in which the decreased dynamics of hyperacetylated microtubules in HDAC6-inhibited cells compromises their capacity to mediate the focal adhesion dynamics required for rapid cell migration.

Supplementary material available online at  
<http://jcs.biologists.org/cgi/content/full/120/8/1469/DC1>

Key words: HDAC6, Microtubule, Acetylation, Dynamics, Focal adhesion

## Introduction

Fibroblast motility in vitro constitutes a valuable model for understanding the mechanisms of tumor cell migration in vivo (e.g. Schlaepfer and Mitra, 2004). The finding that in an animal model nontransformed NIH-3T3 fibroblasts, normal mouse embryo fibroblasts (MEFs) and highly metastatic transformed NIH-3T3 cells all migrate equivalently from blood vessels into surrounding tissues (Koop et al., 1996) supports the notion that the characteristics of fibroblasts moving in vitro will inform us about mechanisms they share with tumor cells migrating in animals. Although actin is a key player in fibroblast crawling, microtubules have been shown to play at least two crucial roles. First, fibroblasts require microtubules (MTs) for persistent directional movement, perhaps because they polarize cell contents in the direction of the motility (Glasgow and Daniele, 1994). MT-dependent cell polarization has been studied most intensively in the wound-healing model of motility (reviewed in Noritake et al., 2005). Second, fibroblasts require dynamic MTs for the remodeling and eventual turnover of their focal adhesions (Kaverina et al., 2002). Adhesion turnover, which is rate-limiting for rapid migration of fibroblasts (Huttenlocher et al., 1997), has been shown to require MTs both for de-adhesion

events at the rear of moving cells (Kaverina et al., 1999) and also for remodeling of newly formed adhesions at the front of spreading cells (Wagner et al., 2002).

Motile fibroblasts possess two distinct MTs subsets. The majority of their MTs comprise a highly dynamic subset, whose distal plus ends are positioned randomly throughout the cell periphery (Salaycik et al., 2005). By contrast, a more stable (less dynamic) subset is typically arrayed between the fibroblast nucleus and its leading edge (Gundersen and Bulinski, 1988). The distal ends of these MTs appear to become stabilized by the binding of plus-end complexes of MT-associated proteins (MAPs) (Akhmanova et al., 2001; Lansbergen et al., 2006) and/or components of the forming adhesions (Palazzo et al., 2004), both of which are concentrated at the leading edge. Once stabilized, the  $\alpha$ -tubulin subunits within the non-dynamic MTs that face the leading edge become post-translationally modified by enzymes whose preferred substrate is MT polymer. For instance, tubulin subunits become acetylated along the entire length of the MT, and the acetylation state of the stable MTs may imbue them with special functional properties (reviewed in Westermann and Weber, 2003).

Although the enzyme that acetylates  $\alpha$ -tubulin has not been identified, histone deacetylase 6 (HDAC6) was shown to deacetylate tubulin (Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003). Although the NAD-requiring deacetylase SirT2 also deacetylates  $\alpha$ -tubulin (North et al., 2003), its activity appears to be confined to mitotic cells (Dryden et al., 2003; Vaquero et al., 2006), making HDAC6 the principal enzyme responsible for maintaining the observed low level of acetylated tubulin found in MTs in interphase fibroblasts and epithelial cells (e.g. Bulinski et al., 1988). Besides tubulin, histones (Zhang et al., 2006), and Hsp90 (Bali et al., 2005; Kovacs et al., 2005), are also deacetylation substrates of HDAC6.

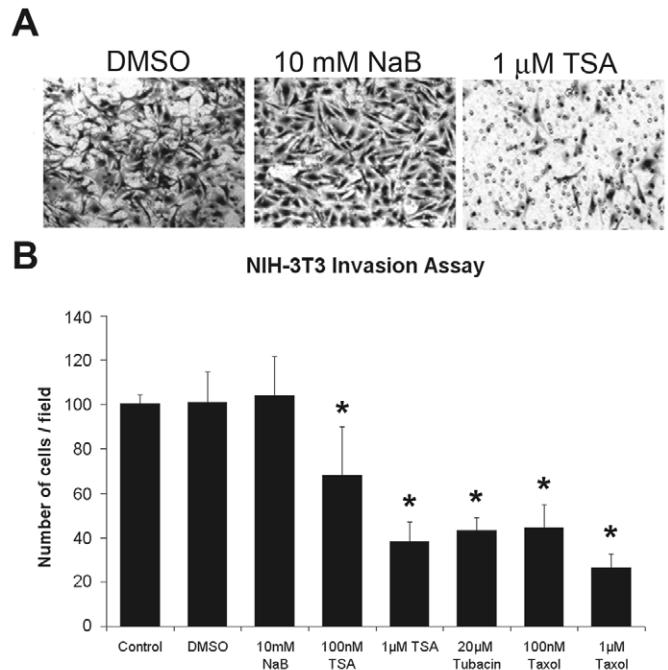
Overexpression of HDAC6 is sufficient to increase invasive motility of fibroblasts (Hubbert et al., 2002) and carcinoma cells (Saji et al., 2005) suggesting that deacetylation of at least one cytoplasmic HDAC6 substrate enhances invasive motility. The opposite may be true, too: trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), which inhibit all NAD-independent HDACs (i.e. HDAC1 to HDAC11) show promise as anti-cancer drugs (Drummond et al., 2005), partly because they block invasive motility (e.g. Eyupoglu et al., 2005; Liu et al., 2003). Although the anti-invasive activity of these compounds has been attributed to altered gene expression resulting from accumulation of hyperacetylated HDAC nuclear substrates, such as histones or transcription factors, hyperacetylated HDAC6 substrates not directly involved in gene expression may be involved. For example, the finding that tubacin also reduces invasion motility, even though it selectively inhibits deacetylation of tubulin but not histones (Haggarty et al., 2003), suggests that failure to deacetylate tubulin may contribute to the anti-invasive effects of broad-spectrum HDAC blockers.

In this paper, we test the hypothesis that preventing HDAC6 deacetylation of tubulin hinders invasive motility. We demonstrate that inhibiting HDAC6 deacetylation of tubulin blocks fibroblast invasion motility as efficiently as inhibiting all HDACs. We then show that the probable cause for the reduced cell migration in HDAC6-inhibited cells is an increase in steady-state level of focal adhesions, which in turn, is due to a decrease in the rate of adhesion turnover. Finally, probing the mechanism of decreased adhesion turnover, we show that MT dynamics are decreased in HDAC6-inhibited cells, and that this decrease is of sufficient magnitude to increase focal adhesion accumulation and thus decrease fibroblast motility.

## Results

### Inhibitors of tubulin deacetylation decrease cell motility

Previous studies demonstrated that treating cells with inhibitors of either all class I and class II HDACs (e.g. Eyupoglu et al., 2005; Liu et al., 2003) or only HDAC6 (Haggarty et al., 2003; Saji et al., 2005) reduced invasion motility by 50–90%. Because tubulin is an HDAC6 substrate (Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003), acetylated tubulin is one of the hyperacetylated proteins accumulated in HDAC inhibitor-treated cells. Thus, we assessed the contribution of hyperacetylated tubulin – rather than other HDAC substrates – in inhibiting cell migration, by comparing the effects of HDAC inhibitors. Fig. 1A shows that, as expected, the number of NIH-3T3 cells that migrated past the separating filter in an invasion assay was



**Fig. 1.** Inhibition of tubulin deacetylation decreases cell motility in Transwell chemotactic invasion assays of NIH-3T3 cells. (A) Micrographs of typical fields. (B) Quantification of invasion migration. Treatments were as indicated. Standard deviation is shown. Asterisks mark conditions statistically different from control ( $P < 0.05$ ).

significantly decreased by treatment with TSA, and Fig. 1B demonstrates that the extent of motility inhibition by TSA and by tubacin were indistinguishable, whereas sodium butyrate (NaB) showed no motility inhibition. As expected, treatment with TSA inhibited deacetylation of both nuclear substrates (i.e. histones and transcription factors) and cytoplasmic HDAC substrates, whereas NaB, an inhibitor of all class I and II HDACs except HDAC6 and HDAC10, inhibited deacetylation of histones but not tubulin (supplementary material Fig. S1A). Treatment with tubacin, a selective inhibitor of HDAC6 (Haggarty et al., 2003), yielded hyperacetylated tubulin, but did not affect the acetylation level of histones. Interestingly, within our treatment period, none of the three HDAC inhibitors used yielded a significant increase in acetylation of Hsp90 (supplementary material Fig. S1B), the only other HDAC6 substrate identified to date (Bali et al., 2005; Kovacs et al., 2005). Fig. 1B also shows motility assays of cells treated with the MT-stabilizing drug taxol, which we included as a positive control based upon its reported capacity to decrease invasion motility (Sgadari et al., 2000; Verschuere et al., 1994). Inhibition of tubulin deacetylation was sufficient to bring about a motility decrease indistinguishable from treatment with a low concentration of taxol (0.1  $\mu$ M; e.g. for 0.1  $\mu$ M TSA, 1  $\mu$ M TSA and 20  $\mu$ M tubacin,  $P$  values relative to 0.1  $\mu$ M taxol were  $P=0.09$ ,  $P=0.3$  and  $P=0.3$ , respectively). However, treatment with a high concentration of taxol (1  $\mu$ M) decreased invasion motility to a significantly greater degree than either 1  $\mu$ M TSA or 0.1  $\mu$ M taxol ( $P=0.01$  or  $P=0.004$ , respectively). These data

suggest that tubulin hyperacetylation contributes greatly to the robust decrease in invasion motility brought about by HDAC inhibitors.

Although these results indicate that tubulin is the only HDAC substrate whose increased acetylation is correlated with inhibited cell motility, we asked whether the presence of this HDAC itself, i.e. drug-inhibited HDAC6, is responsible for decreasing cell migration (Cabrero et al., 2006), perhaps acting as part of a complex bound to MTs (Kawaguchi et al., 2003). To assess this possibility, we assayed invasion motility of A549 knockdown (KD) lung carcinoma cells whose HDAC6 level had been decreased by small interfering RNA (siRNA) (A549 KD) (Kawaguchi et al., 2003). As shown in supplementary material Fig. S2, regardless of the fact that the HDAC6 KD cells are quite heterogeneous in siRNA expression, they showed a substantial decrease in motility, and they showed no statistically significant further motility decrease when treated with taxol (supplementary material Fig. S2D). This result corroborates the notion that cell motility inhibition results directly from increased acetylation of tubulin, due to loss of HDAC6 activity.

#### Decreased HDAC6 activity increases focal adhesion area

In fibroblasts, microtubules have been implicated in two steps in the motility process: reorientation of cellular organelles towards the direction of motility (e.g. Gundersen and Bulinski, 1988), and regulation of focal adhesion dynamics and turnover (e.g. Kaverina et al., 1999). Since detachment from the extracellular matrix, a limiting step in fibroblast migration (Huttenlocher et al., 1997), could slow invasion migration, we tested the hypothesis that altered HDAC6 activity somehow affects focal adhesion elaboration or turnover. In support of this hypothesis, TSA-treated cells appeared to possess more and/or larger adhesions, as visualized in micrographs of paxillin-immunostained cells (Fig. 2A). Quantification verified this visual impression; the percentage of total cell area covered by focal adhesions was significantly increased by TSA treatment (Fig. 2B). Both taxol (Kaverina et al., 1999; Zhou et al., 2001) and nocodazole (Bhatt et al., 2002; Ezratty et al., 2005; Kaverina et al., 1999) are known to increase size of adhesions. In our assay of adhesion area, nocodazole, taxol (either concentration) and TSA were all similarly efficacious in increasing adhesion area, as compared with control cells or those treated with the DMSO vehicle alone (Fig. 2B).

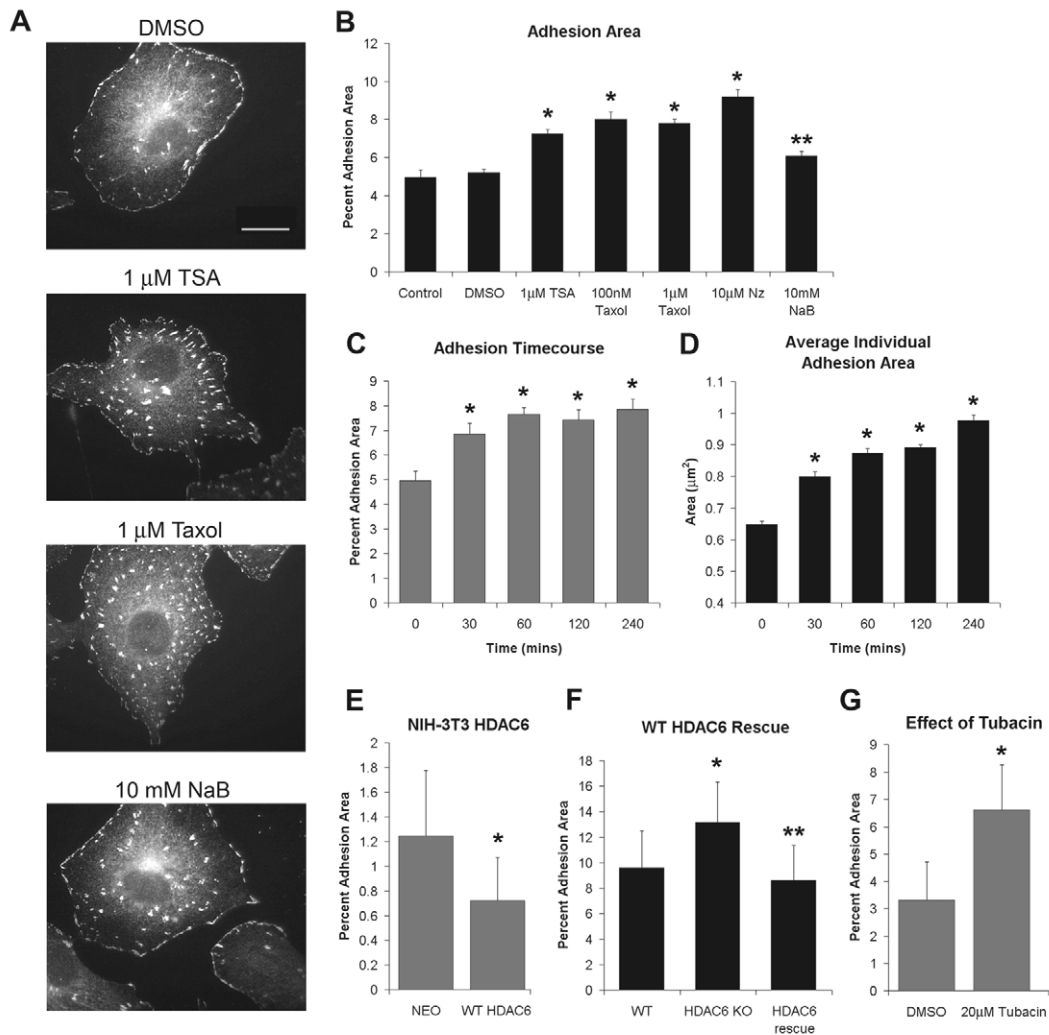
Although NaB treatment also increased adhesion area slightly relative to control (<20% increase due to NaB; Fig. 2B), the adhesion area in NaB-treated cells was significantly smaller ( $P < 0.05$ ) than that in TSA-treated cells (>50% increase due to TSA; Fig. 2B). This indicates that increasing adhesion to the same degree as MT-antagonistic drugs requires inhibition of the mostly cytoplasmic HDAC6 or partly cytoplasmic HDAC10, enzymes that are inhibited by TSA but unaffected by NaB (Matsuyama et al., 2002). Strengthening the assertion that increased adhesion area was largely attributable to cytoplasmic hyperacetylated proteins, we measured the time courses of the increase in adhesion area. After a 30-minute HDAC inhibitor treatment, no changes in mRNA transcripts are measurable (Peart et al., 2005) yet, in our experiments – after 30 minutes of TSA treatment – the adhesion area increase had already occurred (Fig. 2C).

An increase in cellular adhesion could occur either by formation of new adhesions or by growth of existing ones. Accordingly, we quantified the area of individual adhesions over a time course of TSA-treatment. The area of each individual adhesion was significantly increased as a result of a 30-minute TSA treatment (Fig. 2D). The striking similarity in the time course by which total cellular adhesion and individual adhesions increased (compare Fig. 2C with 2D) suggested that HDAC inhibition affects adhesion growth rate or turnover, rather than formation of new adhesions.

Based upon the imputed role of HDAC6 and/or HDAC10 activity in altering cell adhesion area, we tested the hypothesis that HDAC6 activity alone suffices to change adhesion area. Bolstering this notion, NIH-3T3 cells overexpressing wild-type (WT) HDAC6 showed decreased focal adhesion area (Fig. 2E), whereas HDAC6 knockout (KO) MEFs showed increased adhesion area (Fig. 2F). We also ascertained that increased adhesion in HDAC6 KO MEFs did not result from compensatory changes in other proteins that could have occurred during development of the mice lacking HDAC6, because A549-KD cells also showed increased focal adhesion area (supplementary material Fig. S2E). Also, exogenous expression of HDAC6 in the HDAC6 KO MEFs reversed the adhesion area increase (Fig. 2F), yielding adhesion areas that were slightly but significantly lower than those in WT MEFs. Finally, HDAC6 activity, rather than its physical presence, is required to change cell adhesion, because the HDAC6-specific inhibitor tubacin also increased focal adhesion area (Fig. 2G). Because tubacin does not alter the acetylation state of histones (Haggarty et al., 2003) or Hsp90 within the treatment period (supplementary material Fig. S1B), we conclude that preventing HDAC6 deacetylation of tubulin is sufficient to increase the size of individual focal adhesions and total focal adhesion area.

#### Loss of HDAC6 activity increases cell spreading

Cells require focal adhesion dynamics not only for dissociation of cell-substrate adhesions at the rear of motile cells, but also for remodeling of newly formed adhesions near the leading lamellae of moving or spreading cells (von Wichert et al., 2003). MTs are known to be required for the maintenance of adhesion dynamics during cell spreading, because nocodazole depolymerization of the MT network results in abnormal cell spreading and focal adhesions that are larger than usual (Wagner et al., 2002). Thus, we hypothesized that, if inhibition of tubulin deacetylation disrupts normal focal adhesion dynamics, quantitative and/or qualitative features of cell spreading will also be affected. To test this hypothesis, we compared the spreading of HDAC6 KO and WT MEFs, first by tracking the boundaries of the cell membrane with computer-assisted TIRF microscopy (Giannone et al., 2004). Fig. 3A shows that the lack of HDAC6 increased the rate of the initial phase of cell spreading on fibronectin-coated coverslips. Furthermore, HDAC6 KO MEFs showed a more sporadic spreading profile than HDAC6 WT MEFs. HDAC6 KO MEFs were deficient in the coordinated waves of contraction and retraction that normally typify spreading cells (in Fig. 3A, note in the lower graphs the many vertical stripes in the HDAC6 WT MEF, which appear only rarely in the HDAC6 KO MEF). Although automated analysis revealed differences in early events in cell spreading, this technique could not be applied to the analysis of a large number of cells or of spreading during longer intervals. Hence, we also quantified the adhesive area of cells



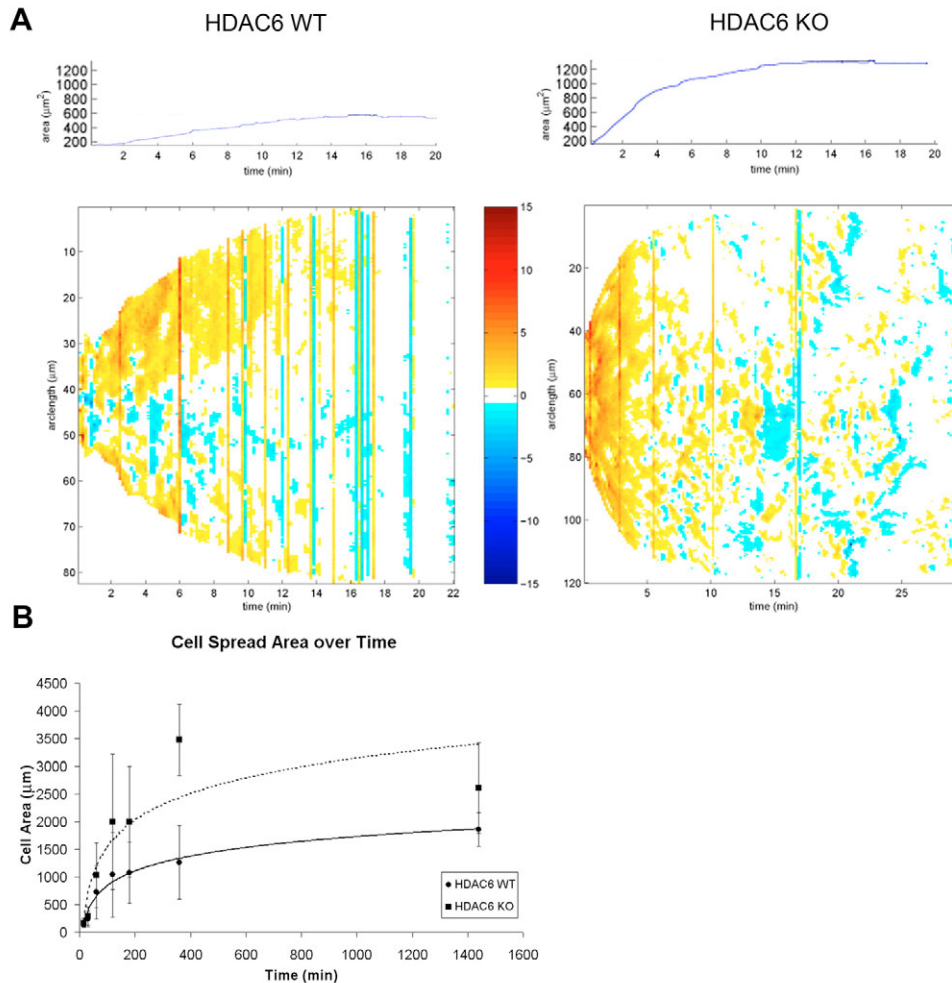
**Fig. 2.** Altered level or activity of HDAC6 alters focal adhesion area. (A) Paxillin immunostaining of typical TC-7 cells, following 2-hour treatments as indicated. Bar, 20 μm. (B) Quantification of adhesion area in TC-7 cells (shown in A) treated with TSA, taxol and Nz (nocodazole), and NaB; see Materials and Methods for details of measurements of paxillin immunofluorescence. (C,D) Time course of the increase in (C) total focal adhesion area, and (D) average area of each individual focal adhesion. (E) Quantification of total focal adhesion area in NIH-3T3 cells stably expressing neomycin plasmid alone (NEO) or WT HDAC6. (F) Quantification of total focal adhesion area in WT, HDAC6 KO and KO MEFs transiently transfected with HDAC6 (HDAC6 rescue MEFs). (G) Quantification of effects of tubacin on total focal adhesion area in TC-7 cells. \*, conditions statistically different from controls ( $P < 0.05$ ); \*\* in B, statistically significant difference both from control and from cells treated with TSA, taxol and Nz. \*\* in F, statistically significant difference both from WT control and from HDAC6 KO MEFs.

that had been allowed to spread for several hours on uncoated-glass coverslips before fixation and staining. Like the automated analysis of the first minutes of spreading, the long-term spreading of HDAC6 KO MEFs was also more rapid than that of HDAC6 WT control MEFs. As expected, the HDAC6 KO MEFs also displayed a greater final surface area (Fig. 3B).

#### Adhesion turnover rate is inhibited by decreased HDAC6 activity

Increased size of focal adhesions could result either from increased rate of adhesion growth, decreased rate of turnover or a combination of both. To our knowledge, adhesion growth rate in cells treated with nocodazole or taxol has not been investigated. Through fluorescence recovery after

photobleaching (FRAP) (supplementary material Movies 1, 2), we found that adhesions in HDAC6 KO cells showed a significantly increased half-time of recovery, indicating that increased tubulin acetylation perturbs focal adhesion dynamics (Fig. 4A,B). Next, we assayed the rate of adhesion disassembly in cells with and without HDAC6 activity, by quantifying the area of individual GFP-paxillin-labeled adhesions disassembling over time (Movies 3, 4). Fig. 4C shows that adhesion disassembly was significantly decreased in HDAC6 KO MEFs compared with HDAC6 WT MEFs. However, adhesion formation rates, quantified from the area of individual assembling GFP-paxillin-labeled adhesions at the front of the cell, were not significantly altered by the accumulation of hyperacetylated MTs (Fig. 4D). Thus, taken together, these



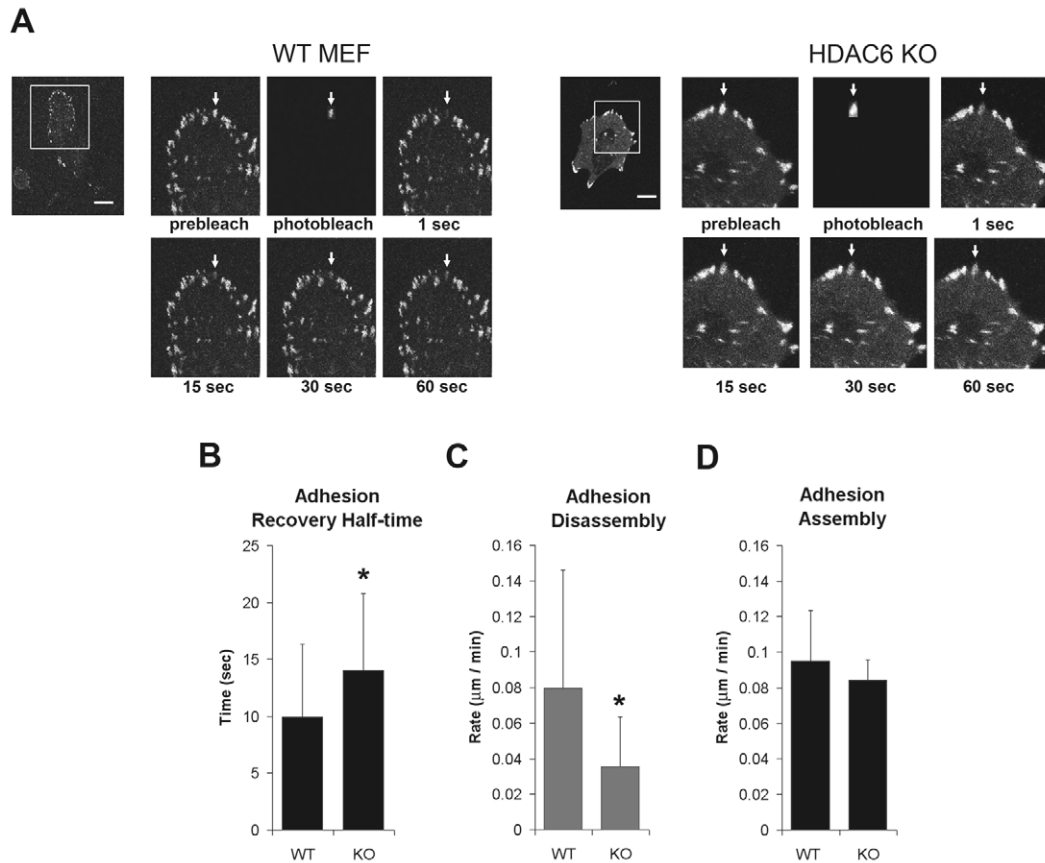
**Fig. 3.** Cell spreading is altered by HDAC6 level. (A) Early events in spreading of HDAC6 WT and HDAC6 KO MEFs, measured using computer-assisted TIRF microscopy to track the boundaries of the cell during spreading on fibronectin-coated coverslips. Top two panels show quantification of spread cell areas at each time interval. Bottom panel shows quantification of the percentage of membrane protrusions (red) and retractions (blue) in the two cell types, calculated as active length along the periphery vs total length along the periphery. The percentage of edge activity (protrusion in red, retraction in blue) was determined at each time by the quotient (active length of the cell periphery)  $\div$  (total length of the periphery). The warmth of color (green to blue; yellow to red) indicates greater velocity of retractions and protrusions, respectively. Note that the time course selected by the automated analysis software is slightly different for the two cell types in the lower graphs. (B) Long-term spreading of HDAC6 WT and HDAC6 KO MEFs that had been allowed to spread on uncoated-glass coverslips was quantified from the surface area drawn to include all adhesions.

results demonstrate that MT hyperacetylation in HDAC6-deficient cells causes larger adhesions and greater total adhesion area, because it interferes with focal adhesion turnover.

#### Increased acetylation inhibits MT dynamics

Dynamic MTs are involved in focal adhesion turnover, with a loss of MT dynamics resulting in stabilized, enlarged adhesions (Kaverina et al., 2000). In cells that lack HDAC6 activity, hyperacetylation could decrease the dynamics of MTs, and this could account for the decrease in adhesion turnover. To test this hypothesis, we quantified MT dynamic instability parameters from time-lapse images of TC-7 cells stably expressing 3xGFP-EMTB (Fig. 5A, supplementary material Movie 5), a MT-binding construct used because it very brightly labels MTs without perturbing their dynamics (Faire et al., 1999). MTs in

3xGFP-EMTB-TC-7 cells that had been TSA pre-treated for only 30 minutes showed decreased rates of depolymerization and polymerization as well as decreased dynamicity (Fig. 5B-D). Although HDAC6 inhibition significantly decreased MT dynamics, TSA was only about one-tenth as effective on a per-mole basis as taxol; that is, MT dynamic parameters in cells treated with 1  $\mu\text{M}$  TSA were roughly comparable to cells treated with 0.1  $\mu\text{M}$  taxol. Dampened MT dynamics were also evident from measured decreases in the percentage of time hyperacetylated MTs were polymerizing or depolymerizing (supplementary material Fig. S3A). Live-cell imaging of MTs in HDAC6 KO cells expressing 3xGFP-EMTB revealed increased MT stability, as measured by the percentage of time MTs were depolymerizing in cells lacking HDAC6 (Fig. 5E). Fig. 5E also documents that the normal level of stability could be rescued by expression of exogenous HDAC6, showing that



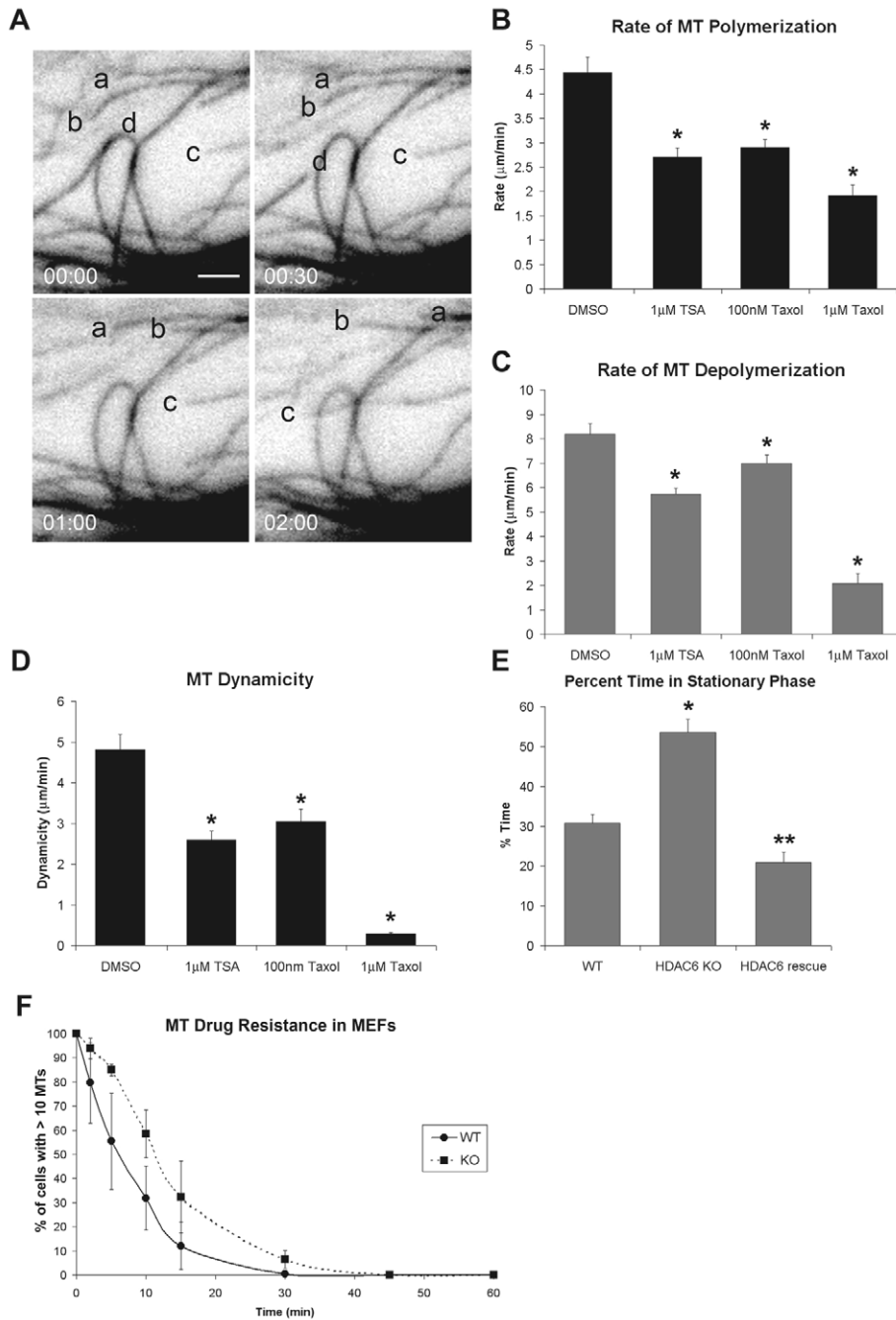
**Fig. 4.** Focal adhesion turnover and disassembly is affected by MT acetylation. (A) Focal adhesion turnover was calculated from FRAP images of WT and HDAC6 KO MEFs expressing paxillin-GFP. Bars, 20  $\mu\text{m}$ . (B) Average half-time ( $t_{1/2}$ ) of FRAP of adhesions in HDAC6 WT and HDAC6 KO MEFs expressing GFP-paxillin;  $n > 8$  adhesions in each type of MEF. (C,D) Rates of adhesion disassembly (C) and assembly (D) were quantified from the pixel-intensity of individual adhesions in time-lapse images of both HDAC6 WT and HDAC6 KO MEFs that were expressing GFP-paxillin;  $n > 12$  adhesions in each type of MEF. \*, conditions statistically different from control ( $P < 0.05$ ).

hyperacetylation of HDAC6 substrates indeed gave rise to the alteration in MT dynamics in HDAC6 KO vs WT MEFs. However, we noticed that neither the rate of depolymerization nor polymerization of HDAC6 KO cells was significantly altered compared with WT cells (data not shown). In addition, catastrophe and rescue frequencies were unaffected by either pharmacological inhibition or genetic knockdown of HDAC6, properties that HDAC6-inhibited cells shared with cells undergoing low-dose (0.1  $\mu\text{M}$ ) taxol treatment (supplementary material Fig. S3B-E). As a further assay of MT stability, we quantified the resistance of MTs against nocodazole-induced depolymerization in HDAC6 KO and WT MEFs. Fig. 5F shows that the MT arrays in HDAC6 KO MEFs were significantly more stable against drug depolymerization than those in HDAC6 WT MEFs, in agreement with our previous result. We repeated this assay with equivalent results in TSA-treated TC-7 and NIH-3T3 cells, as well as in A549 HDAC6 KD cells (supplementary material Fig. S4A,B, and C,D, respectively). Since nocodazole-resistance measures stability of MTs in a cytoplasmic context, we also tested the stability of MTs in cytoskeletons that had been detergent-extracted to remove soluble proteins that might be capable of altering the intrinsic stability of the MTs (Khawaja et al., 1988). In this assay of end-mediated depolymerization induced by ATP (Infante et al.,

2000), hyperacetylated MTs were also significantly stabilized; the rate and percentage of time MTs spent depolymerizing were both reduced and the MT array remained intact for a greater time period (supplementary material Fig. S5A-D). By contrast, MTs in cytoskeletons prepared from cells pre-treated with NaB showed no detectable increase in stability, that is, MTs were not protected against end-mediated depolymerization (supplementary material Fig. S5E,F). Taken together, these experiments clearly demonstrate that the dampened dynamics and increased stability of MTs in HDAC-inhibited cells are a function of the MTs themselves, rather than of extrinsic factors that would be removed during detergent extraction; the increased stability is brought about by hyperacetylation of tubulin, rather than substrates of HDACs whose deacetylation is also inhibited by NaB.

## Discussion

Dynamic MTs are crucial for modulating cell-substrate adhesions in migrating fibroblasts. Focal-adhesion breakdown at the rear of motile fibroblasts that is rate-limiting for fibroblast locomotion (Huttenlocher et al., 1997; Kaverina et al., 2000), requires MTs (Kaverina et al., 1999) and the MT motor protein, kinesin (Krylyshkina et al., 2002). MTs are thought to randomly approach, not accurately aim towards,



**Fig. 5.** MT acetylation dampens MT dynamics. (A) Typical micrographs used to measure MT dynamics in 3x GFP-EMTB-TC-7 cells by MT end-tracking; an edge of a cell is shown with elapsed time in minutes:seconds, and four sample MT ends are shown (a-d). Bar, 10 μm. (B-C) Rates of MT (B) polymerization, (C) depolymerization, and (D) dynamicity, following 30-minute treatment with TSA or taxol, as indicated (see Materials and Methods for details). (E) The percentage of time MTs spend in a stationary phase, i.e. pausing rather than growing or shrinking, in WT, HDAC6 KO MEFs, and KO MEFs transiently transfected with HDAC6 (HDAC6 rescue MEFs). (F) Nocodazole-resistance of MT arrays in HDAC6 WT and HDAC6 KO MEFs. Error bars represent + s.e.m.; \*, conditions statistically different from control ( $P < 0.05$ ); \*\*, statistically significant difference both from WT control and from HDAC6 KO MEFs.

adhesions because MT targeting to adhesions is excruciatingly sensitive to drug perturbation of MT dynamics. For example, treatment with 20 nM taxol, which decreases MT dynamics almost imperceptibly, increases the size of adhesions by limiting their dissolution (Kaverina et al., 1998).

The remodeling of newly formed adhesions also requires MTs. Wagner et al. showed that nocodazole treatment increased adhesion size and rate of cell spreading (Wagner et al., 2002), and Ballestrem et al. found that intact MTs were required to modulate adhesions ventral to the cell centroid as well as in the retracting tail of migrating cells (Ballestrem et al., 2000). Similarly, Kirchner et al. noted enlarged adhesions with significantly increased accumulation of paxillin and other

adhesion markers (Kirchner et al., 2003), in cells subjected to nocodazole-treatments that block adhesion turnover (Bhatt et al., 2002; Ezratty et al., 2005; Kaverina et al., 1999).

In our experiments, the size of adhesions was increased in cells in which tubulin was hyperacetylated, either due to genetic or pharmacological loss of HDAC6 activity. Treatment with TSA or tubacin, the HDAC inhibitors that yield hyperacetylated tubulin, equivalently increased cellular adhesions, whereas NaB, the HDAC inhibitor that induces hyperacetylation of only non-tubulin HDAC substrates, did not increase adhesions to a comparable extent. From these data, hyperacetylation of tubulin appears to be both necessary and sufficient to increase cellular adhesion.

In addition to MT acetylation changes, other proteins have been reported to be increased in acetylation or altered in activity when HDAC6 is inhibited or deleted from cells. For example, Hsp90, some of whose numerous client proteins are involved in adhesion and/or motility, is the only other known HDAC6 deacetylation substrate (Bali et al., 2005; Kovacs et al., 2005), although variations in Hsp90 acetylation level in cells treated with HDAC inhibitors suggest that Hsp90 is also a substrate of other HDACs (Bali et al., 2005; Kovacs et al., 2005; Nimmanapalli et al., 2003; Yu et al., 2002). We find it unlikely that Hsp90 is involved in affecting motility and adhesion dynamics, because we found that neither its acetylation nor protein level was markedly changed as a function of the HDAC inhibitor treatments we used (supplementary material Fig. S1B). However, we cannot discount involvement of protein phosphatase 1 (PP1), which has been shown to interact with HDAC1, HDAC6 and HDAC10 (Brush et al., 2004). Upon HDAC inhibition, release of PP1 from its complex with HDAC6 results in dephosphorylation of Akt (Chen et al., 2005) and this, in turn, might affect cell migration. However, the exact scenario by which this may occur is unclear, because different Akt isoforms have been shown to have opposite effects on cell motility (Zhou et al., 2006).

Besides the potential involvement of other HDAC6 substrates, could it be possible that HDAC6 itself alters invasion motility, cell adhesion or MT dynamics? All of our experiments gave indistinguishable results, whether we decreased HDAC6 action pharmacologically or genetically; thus, our results appear inconsistent with involvement of the HDAC6 protein in determining MT or motile properties of fibroblasts. In this way, our results contrast with the results of Cabrero et al. (Cabrero et al., 2006). This group found no inhibition of lymphocyte migration in the presence of tubacin or catalytically inactive HDAC6; migration was inhibited only by decreased levels of HDAC6. Thus, in lymphocytes, it was clearly the presence of HDAC6 and not its activity that cells required for maximal motility. However, one would expect discrepant results when comparing motility of spherical, highly contractile lymphocytes whose integrin-independent amoeboid motility is not limited by adhesion turnover (Carragher et al., 2006; Wolf et al., 2003), with 3T3 or mouse embryo fibroblasts, whose integrin-dependent mesenchymal motility is rate-limited by adhesion remodeling and/or turnover (Huttenlocher et al., 1997). Thus, only in fibroblasts would HDAC6 activity be needed to prevent MT hyperacetylation that would otherwise compromise adhesion dynamics. Since both amoeboid and mesenchymal strategies are involved at different stages of tumor cell motility, it is striking that HDAC6 is involved in both types of motility, either via its physical presence or its activity.

Experiments in which we used pharmacologically inhibited or genetically decreased HDAC6 to yield hyperacetylated tubulin resulted in decreased MT dynamics, similar to the observations of Matsuyama et al. (Matsuyama et al., 2002). At first glance, this result appears inconsistent with the work of two groups (Haggarty et al., 2003; Palazzo et al., 2003), wherein decreased HDAC6 activity was reported not to alter MT dynamics. In fact, it is probable that acetylation-induced changes in MT dynamics occurred in the experiments of these two groups as well, but that these modest changes in MT

dynamics went undetected simply because the assays they used were not sensitive enough. It is important to note that the decrease in MT dynamics induced by MT hyperacetylation is less marked than the decrease observed in a standard (e.g. 1  $\mu$ M) taxol treatment of cells. Our experiments confirm that neither the assay of increased Glu tubulin described by Palazzo et al. (Palazzo et al., 2003) (and data not shown), nor the test of resistance to a long-term nocodazole treatment carried out by Haggarty et al. (Haggarty et al., 2003) (and supplementary material Fig. S4C; NIH-3T3 cells 60 min nocodazole treatment) revealed a difference in MT dynamics in control cells and those with hyperacetylated MTs.

Our findings, that a global increase in cellular adhesion and a decrease in MT dynamics are both brought about by simply tweaking the post-translational modification state of a single amino acid on  $\alpha$ -tubulin subunits may seem surprising. However, acetylation of a single lysine has been shown to alter binding affinity of proteins; for example, binding of high-mobility-group 1 (HMG1) protein to DNA (Ugrinova et al., 2001) and binding of nuclear steroid hormone receptors to the ACTR coactivator (Chen et al., 1999). Analogously, monoacetylation of histone H4 is sufficient to alter recognition by transcriptional machinery (Girardot et al., 1994). Although acetylation exerts a milder effect on protein activity than phosphorylation (Polevoda and Sherman, 2002), modification of many subunits along a macromolecular structure such as a MT may propagate and, thus, enhance a modest effect.

Unlike histones, in which acetylation decreases the net basic charge of a sequence within the target proteins, tubulin has a net negative charge in the region surrounding the acetylated lysine. Nonetheless, because acetylation occurs exclusively on the MT polymer, alteration of hydrogen bonds may affect subunit conformation or subunit-subunit interactions sufficiently to decrease gain and loss of subunits. In addition, or instead, acetylation may change the conformation of the MT surface to which MAPs bind. Consistent with either scenario, MTs in cells with hyperacetylated tubulin were less dynamic and more stable. Structural studies (Nogales et al., 1999) and the failure of antibody against acetylated tubulin to label native (unfixed) MTs (Thompson et al., 1984) provide evidence that the acetylated lysine on  $\alpha$ -tubulin (K-40) faces the MT lumen. Thus, an allosteric change in MT structure may be required for tubulin acetylation to change surface properties such as binding of coflamentous MAPs.

By what mechanism(s) could MT hyperacetylation disrupt the normal dynamics of cellular adhesions? Four mechanisms seem most plausible, including alteration in Rac and Rho signaling, targeting of MTs to focal adhesions, activity of MT motors and localization of adhesion assembly and disassembly signals. The first possibility is that hyperacetylated MTs alter signaling by Rac and Rho. MT polymer level, stability and growth rate all modulate the activity of Rac and Rho (Gauthier-Rouviere et al., 1998; Waterman-Storer et al., 1999). However, although Rho family members are involved in early steps of adhesion (Wen et al., 2004) and growth (Clark et al., 1998), they are not involved in adhesion turnover (Ezratty et al., 2005). Since increased adhesion in HDAC6-inhibited cells results from decreased turnover rather than increased formation of adhesions, any modulation in Rac and Rho signaling that occurs is not likely to contribute to increasing the size of cellular adhesions.

Cell migration requires that MTs approach within a few MT diameters of focal adhesions, in order to trigger their remodeling (Kaverina et al., 1999). Cells use dynamic MTs to explore their environment (Gundersen, 2002). Extending MTs to randomly reach focal adhesions exemplifies this model of MT targeting. Hence, a second way in which cells with hyperacetylated MTs might decrease adhesion dynamics is that dampened MT dynamics could decrease the probability that MTs successfully target adhesions. Supporting this scenario is the demonstration that adhesions increase in size and decrease in turnover rate in cells subjected to low-dose (20 nM) taxol treatments (Kaverina et al., 1999). Since MT dynamics are more markedly compromised in HDAC6-inhibited cells, closely resembling cells subjected to 100 nM taxol treatment, HDAC6-inhibition is likely to decrease adhesion targeting. A full test of the hypothesis that MT hyperacetylation decreases MT-adhesion targeting frequency will require extensive TIRF imaging of live cells expressing both MT and adhesion labels. If true, though, this hypothesis would further predict that MT antagonistic drug therapies that only slightly perturb MT dynamics would interfere not only with cell division (e.g. Jordan et al., 1998), but also with cell adhesion dynamics and motility. In fact, we found that treatment of 3T3 fibroblasts with 100 nM taxol, which dampens MT dynamics to an extent similar to 1  $\mu$ M TSA treatment, also decreases cell motility (Fig. 1B).

A third possibility is that hyperacetylation of MTs changes the activity of one or more MT motors needed for adhesion remodeling. For example, antibodies against conventional kinesin (also known as KIF5 or kinesin-1) prevented adhesion turnover, whereas inhibition of dynein motors via dynamitin overexpression was ineffectual (Krylyshkina et al., 2002). This result suggests that signals directing adhesion breakdown travel on MTs toward MT plus ends that lie in close proximity to adhesions. Kinesin has been shown to interact preferentially with acetylated MTs in neuronal cells and in vitro (Reed et al., 2006), as well as with MTs modified by detyrosination in non-neuronal cells and in vitro (Kreitzer et al., 1999; Liao and Gundersen, 1998). It is, therefore, conceivable that in motile fibroblasts acetylation could positively or negatively influence MT motor activity; thus modulating adhesion breakdown. The mechanism by which kinesin alters transport along hyperacetylated MTs must involve a conformational change that is allosterically propagated within the tubulin molecule, because kinesin interacts with – and structurally changes – a region of  $\alpha$ -tubulin (H3 region) that is slightly removed from the acetylation site (H1-S2 loop) (Krebs et al., 2004).

Finally, a fourth mechanism by which hyperacetylation could slow adhesion turnover is by delocalizing signals normally provided by acetylated MTs. Since post-translational modifications are normally confined to the stable subset of MTs that faces the leading edge of a motile fibroblast (Gundersen and Bulinski, 1988), it has been proposed that covalent modifications along the length of these MTs signal to other cytoplasmic elements the intracellular position of the stable MTs (Bulinski and Gundersen, 1991). Hyperacetylation of the plus ends of all MTs, instead of only a small subset, could disrupt the localized recruitment of complexes comprised of CLIP-170 and CLASPs (Akhmanova et al., 2001) and/or components of forming adhesions (Palazzo et al., 2004) to the plus ends of dynamic (and therefore, non-acetylated)

MTs. In support of this mechanism, the distal ends of MTs in HDAC6-inhibited cells showed greatly decreased binding of the plus-end binding MAPs, p150glued (46% decrease; A.A.S., unpublished observations). Altered MT binding of plus-end MAPs could result either from structural changes that alter MAP-binding affinity, and/or as a secondary effect of decreased MT dynamics. There is precedent for the latter: binding of CLIP-170, whose binding specificity for plus-end tips of dynamic MTs mimics that of p150glued (Vaughan et al., 1999) is abolished in cells treated with a 200 nM taxol (Perez et al., 1999). Similarly, if all MTs within a cell are hyperacetylated, signals along the length of MTs will be delocalized. For example, if – analogous to the study by Reed et al. (Reed et al., 2006) – the kinesin motor selectively uses acetylated MTs as tracks, spatial information will be lost and the preference negated because only one variety of MTs – hyperacetylated ones – ramify throughout cells lacking functional HDAC6. Although this hypothesized mechanism for thwarting adhesion turnover might be the most plausible, it could be the most difficult to test. However, one prediction of the hypothesis has already been born out: in cells with hyperacetylated MTs, MT-dependent organelle polarization typically found in migrating cells, e.g. the Golgi complex, MT-organizing center and stable MTs (Gundersen and Bulinski, 1988), fails to occur (A.A.S., unpublished observations). Thus, each of the last three proposed mechanisms is likely to contribute to increasing cellular adhesions in HDAC6-inhibited cells. Even though the molecular details are still unknown, testing each of these hypotheses forms the basis for ongoing experiments.

## Materials and Methods

### Cell lines, treatments and antibodies

MT dynamics were measured in 3xGFP-EMTB-TC-7 cells (Faire et al., 1999); that is, cells from the TC-7 derivative of CV-1 African green monkey kidney epithelial cells (Kasamatsu et al., 1983) that stably express the 3xGFP-EMTB construct. Expression of this construct, comprised of three tandem GFP proteins linked to a small (28 kDa) MT-binding domain of ensconsin, has been shown not to alter the normal MT dynamics of the cells (Faire et al., 1999). Preparation of NIH-3T3 HDAC6 cells stably expressing wild-type (WT) HDAC6 and A549 HDAC6 knockdown (KD) cells stably expressing HDAC6-siRNA as well as their control isolates, were described by Hubbert et al. (Hubbert et al., 2002) and Kawaguchi et al. (Kawaguchi et al., 2003), respectively. MEFs derived from HDAC6-null mice have been described previously (Boyault et al., 2006). HDAC6 knockdown (KO) MEFs were rescued via transient transfection of HDAC6, with 3xGFP-EMTB co-transfected for visual selection. Moderate expressors were chosen for analysis. Anti-paxillin antibody was obtained from Sigma-Aldrich. Antibody against total  $\beta$ -tubulin has been described previously (Nguyen et al., 1997). All cells were grown and treated in DMEM supplemented with 10% calf serum (NIH-3T3 cells) or 10% fetal bovine serum (other cell lines) in a 37°C, 5% CO<sub>2</sub> incubator.

### Transwell chemotactic motility assay

Procedures were as described (Hubbert et al., 2002; Malinda et al., 1999), except that, following 18 hours in serum-free DMEM,  $2.5 \times 10^5$  cells were applied to each 12-well Transwell membrane insert or, in some experiments,  $0.65 \times 10^5$  cells per 24-well Transwell insert (both having 8- $\mu$ m pores; inserts from Corning Inc. were coated with 1% collagen for 1 hour and rinsed twice with serum-free medium before use). Inserts were placed in bottom chambers containing DMEM with 10% fetal bovine serum, and cells were permitted to migrate through the insert for 6 hours (time-course studies determined that this yielded half-maximal migration). Cells that had not migrated were removed from the upper side of the membrane insert with a cotton swab, the insert was stained (0.1% Crystal Violet or Coomassie Brilliant Blue in 5% acetic acid, 10% methanol), de-stained and for each condition ten to 12 randomly chosen fields from duplicate wells were photographed using a Cool-SNAP cooled-CCD camera (Photometrics, Tucson, AZ). At least three independent experiments were performed for each condition of drug treatment or for each cell line (i.e.  $n \geq 3$ ). Student's *t*-test was used to determine the statistical significance of differences between control and each experimental condition ( $P < 0.05$ ).

### Assay of adhesion and cell-spreading dynamics

Adhesion area was analyzed in cells, plated on acid-washed glass coverslips, that were allowed to spread overnight, fixed in 3.7% formaldehyde in PBS and permeabilized with 0.1% Triton X-100 in TBS containing 1% BSA. Immunofluorescence staining with anti-paxillin (Sigma-Aldrich) was performed and quantified by determining the threshold area of paxillin staining in percent as described by Finkelstein et al. (Finkelstein et al., 2004). Significance was assessed using Student's *t*-test ( $P < 0.05$ ). Surface area of spreading cells was quantified from the outline of the cell area, drawn to include all paxillin-stained adhesions. Although results with antibodies against other adhesion markers (e.g. anti-zyxin) gave qualitatively similar results, the superior signal-to-noise ratio obtained with the commercially available anti-paxillin antibody made it the most amenable for quantitative analysis.

For measurement of adhesion dynamics in live cells, WT and HDAC6 KO MEFs expressing a GFP-paxillin construct were prepared and individual adhesions were either quantified from time-lapse images or subjected to FRAP, as described by von Wichert et al. (von Wichert et al., 2003). Analysis of adhesion growth, shrinkage and turnover half-life ( $t_{1/2}$ ) was calculated as described previously (Bulinski et al., 2001).

Early events in cell spreading were quantitatively assayed, as described by Giannone et al. (Giannone et al., 2004). Briefly, trypsinized MEFs from non-confluent cultures were pre-loaded with Calcein AM Orange-Red (incubated for 30 minutes with 0.2  $\mu$ M dye from Molecular Probes), plated on fibronectin-coated coverslips and observed in a TIRF microscope, to generate maps of the velocity of membrane protrusions and the increase of cell surface area during spreading;  $n = 12$  cells of each type (HDAC6 WT and HDAC6 KO) analyzed over 20-minute to 30-minute intervals.

### Measurement of real-time MT dynamics

MT dynamics were measured in living cells exactly as described in Faire et al. (Faire et al., 1999). Briefly, 3xGFP-EMTB-TC-7 cells were pre-treated for 30 minutes with drug or vehicle, then placed in a perfusion chamber and imaged during the next 30 minutes with a Zeiss 63 $\times$  Planapochromat objective. MT images were captured with a shuttered Hamamatsu Orca cooled-CCD camera, controlled by MetaMorph software, at 5-second to 10-second intervals. Each 5-minute sequence was analyzed by tracking positions of MT ends using the 'TrackPoints' function of MetaMorph and MT dynamics were calculated for the time course. Dynamicity was calculated as [(total distance all MTs polymerized) + (total distance all MTs depolymerized)]  $\div$  (total elapsed time) (Toso et al., 1993). MEF WT and HDAC6 KO were transiently transfected with 3xGFP-EMTB prior to imaging.

### Nocodazole-resistance assay

To assay MT stability, cells were subjected to a time course of nocodazole-depolymerization as described in Nguyen et al. (Nguyen et al., 1997). Briefly, nocodazole-resistance was quantified as the proportion of cells in which >10 MTs remained after each time interval of treatment with 10  $\mu$ M nocodazole.

### Depolymerization assay of cytoskeletal MTs

To measure stability of MTs against dilution-induced depolymerization in live cells, we treated 3xGFP-EMTB-TC-7 cytoskeletons (prepared by extracting cells grown on coverslips with 0.2 mg/ml saponin at 37°C for 3 minutes) with 1  $\mu$ M ATP in 0.1 M PIPES pH 6.9, 1 mM EGTA, and 1 mM MgSO<sub>4</sub> to induce depolymerization, exactly as described by Infante et al. (Infante et al., 2000). The rate of depolymerization of individual GFP-labeled MTs was measured from time-lapse images and MT end-tracking, as described above. To determine the intrinsic stability of MTs, depolymerization of entire MT arrays was also quantified in cytoskeletons that had been incubated with 10  $\mu$ M ATP for 5-minute to 60-minute intervals, and then fixed. Following anti-tubulin immunofluorescence staining, arrays in which >10 MTs remained were quantified.

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