HDAC6 activity is required for efficient polarization and intracellular transport of organelles in directionally migrating cells

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ABSTRACT

HDAC6 activity is required for efficient polarization and intracellular transport of organelles in directionally migrating fibroblasts

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Deacetylation of non-histone substrates by histone deacetylase 6 (HDAC6) has been shown to affect chemotactic invasion (e.g., Haggarty et al., 2003; Hubbert et al., 2002; Tran et al., 2007) or wound healing motility (Lafarga et al., 2012) of fibroblasts. Chemotactic invasion and wound-healing motility are both examples of directional migration, a process which requires polarization of cytoskeletal and cellular components to facilitate directed organelle and vesicle transport. This ultimately establishes a persistent leading edge and represses random migration. Given that tubulin is a known substrate of HDAC6 and since microtubules (MTs) are known to play an important role in polarization of directionally migrating cells, we hypothesized that HDAC6 activity regulates important MT dependent aspects of directional cell motility.

We were able to test this hypothesis by observing cellular polarization and organelle and vesicle transport in the presence or absence of HDAC6 activity using a wound-healing directional motility model. Experiments were carried out in HDAC6 null and wild type fibroblasts, and results were corroborated by pharmacological inhibition of HDAC6 activity. Loss of HDAC6 activity resulted in hyper-acetylation of tubulin, but not of Hsp90 or cortactin, two other HDAC6 substrates known to be involved in actin-mediated regulation of cell motility. Cells lacking HDAC6 activity showed significantly reduced wound-healing velocity but increased lamellipodial protrusions and random migration. These cells also failed to polarize

their array of posttranslationally modified MTs as well as organelles such as their MT-organizing center (MTOC), Golgi apparatus, mitochondria, and vimentin filament array. Localizations of cortical dynein and its MT plus-end binding partner and processivity factor, p150Glued, known to be involved in MTOC reorientation via a MT capture mechanism, were also compromised in the absence of HDAC6 activity. In addition, polarized bi-directional transport of Golgi vesicles in motile cells was decreased. Retrograde and anterograde organelle transport were independently affected: Golgi reclustering during recovery from a Golgi inhibitor and mitochondrial trafficking in spreading cells, two canonical dynein- and kinesin- mediated events, were inhibited. Our results were specific to HDAC6 as re-expression in HDAC6 null cells rescued bi-directional Golgi vesicle transport. Testing the specific contribution of the acetylation state of lysine 40 of α-tubulin however, proved inconclusive as both re-expression of wild type α-tubulin and a non-acetylatable mutant equivalently rescued the Golgi vesicle motility defect.

We also report initial data from our investigation into two parallel cell polarization pathways reported to orient a stable, modified MT array towards the direction of migration. Localizations of components of a MT-capture complex involved in stable MT array reorientation, cortical LL5β, and its MT plus-end tracking partner, CLASP2, were inhibited by loss of HDAC6, reminiscent of our results with cortical dynein and p150Glued. Paradoxically, localization of EB1, a MT plus-end binding protein involved in an mDia-mediated MT stabilization pathway was not affected. The localization of CLIP170, another MT plus-end binding factor related to p150Glued and known to be important for the recruitment of CLASPs to MT ends, was also not affected.

These data provide novel insight into the role of HDAC6 in fibroblast motility showing that its activity is required for regulating MT-based cellular polarization and dynein- and kinesin-

mediated vesicular and organellar transport in directionally migrating cells. Our data also point to possible mechanisms by which deregulation of MT acetylation, by abrogating or inhibiting HDAC6 activity, inhibits directional motility and underscore the importance of this MT subset in establishing cellular polarization. We propose that HDAC6 activity modulates bidirectional transport in motile cells by regulating levels of MTs enriched in Ac subunits in response to external cues.

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ABBREVIATIONS

MT	Microtubule
MTOC	Microtubule organizing center
MAP	Microtubule associated protein
Glu	Detyrosinated
Tyr	Tyrosinated
Ac	Acetylated
+TIP	MT plus-end binding protein
TCP	Tubulin carboxy peptidase
TTL	Tubulin tyrosine ligase
TAT	Tubulin acetyl transferase
WT	Wild type
KO	Knockout
MEF	Mouse embryonic fibroblast
HDAC	Histone deacetylase
TDAC	Tubulin deacetylase
HAT	Histone acetyltransferase
SIRT	Sirtuin
TSA	Trichostatin A
NaB	Sodium butyrate
GSK3β	Glycogen synthase kinase
GRK2	G protein coupled receptor 2
ER	Endoplasmic reticulum

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In lumine Tuo videbimus lumen

Dedicated to my family,

Iffat, Asghar and Omar Salam and

my monster, Aaisha

Chapter 1

Introduction

THE MICROTUBULE CYTOSKELETON

MTs are dynamic cytoskeletal polymers present in all eukaryotes that are important in diverse cellular processes, including cell motility, axonemal-based ciliary and flagellar motility, intracellular transport, mitosis, and morphogenesis. In these roles MTs provide structural integrity to cells, allow for the separation of sister chromatids in mitosis, establish polarity in morphogenetic events like motility, form specialized structures like the spindle, axonemes, and neuronal processes, and function as tracks for intracellular transport. Moreover, these ubiquitous polymers are found throughout the entire cell, connecting all cellular proteins and organelles in signaling pathways. Their ability to polymerize and depolymerize quickly, and undergo rapid reorganization in response to various stimuli and cell cycle requirements, is critical to their function and requires precise temporal and spatial regulation. How MTs are regulated in order to perform the vast repertoire of functions associated with them is vital to understanding their distinct role in each function.

MT Structure

MTs are helical polymers composed of α and β tubulin heterodimers, the head-to-tail arrangement of which forms polar linear protofilaments (Fig. 1). Parallel protofilaments associate laterally to form curved sheets that close to create hollow MT cylinders with a standard outer diameter of approximately 25 nM. Lateral interactions between protofilaments in the resulting lattice are homologous (α-α or β-β), except at the MT 'seam' where tubulin dimers make heterologous (α-β) contacts (Krebs et al., 2005). This architecture forces the MT lattice into a conformation in which the protofilaments run straight, without winding, thereby facilitating processive movements of MT-associated motors on continuous protofilament tracks.

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α and β- tubulin monomers exhibit high sequence homology (~ 40%) (Luduena, 1998), and apart

from slight differences in curvature, appear to be identical in high-resolution structural models

(Krebs et al., 2005; Li et al., 2002; Nogales et al., 1998).
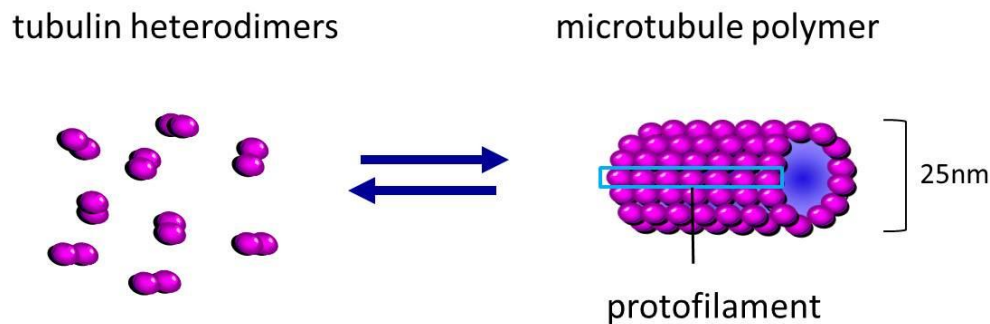
Figure 1. Microtubules are composed of α and β tubulin heterodimers. Head to tail

assembly leads to the formation of linear protofilaments, which laterally associate to form

polar, hollow polymers with a diameter of 25 nm.

The amino-terminal domain of each monomer binds GTP at a nucleotide-binding pocket,

termed the N (non-exchangeable) site in α-tubulin that is buried at the intradimer surface, and the

E (exchangeable) site in β-tubulin that is partially exposed to allow for nucleotide exchange

(Nogales et al., 1998). In polymerized MTs, the amino-terminal domains face the MT lumen and

form a bumpy inner surface. The intermediate domain is proposed to mediate interactions with

MT drugs, like the taxol-binding site in β-tubulin, while the carboxyl terminal domain forms a

crest on the outside surface of the MT polymer. The highly acidic, carboxyl terminal tails, whose

structure is divergent among the various α- and β-tubulin isoforms, protrude from the outside

surface of MTs providing the main sites of interaction for a wide variety of MT-associated proteins (MAPs), including the MT motors, dyneins and kinesins.

During protofilament polymerization, which is driven by a gain of entropy, the α-tubulin in the heterodimer being added contacts the exposed β-tubulin E-site in the previous dimer for GTP hydrolysis. This head-to-tail arrangement of the heterodimers in protofilaments imparts polarity to MTs. Although MTs from tissue culture cells can self-assemble in vitro (Bulinski and Borisy, 1979), in vivo MTs are nucleated in the cytoplasm from MT-organizing centers (MTOCs) (Mitchison and Kirschner, 1984b) or the Golgi apparatus (Efimov et al., 2007). The slow-growing or minus ends of each MT are affixed to the MTOCs or the Golgi apparatus, which protects them from depolymerization, while the fast- growing, more dynamic plus ends are free to polymerize and depolymerize in the cytosol.

MT Dynamics

In the test tube and in the cellular context, MTs alternate between phases of stochastic polymerization and depolymerization, known as dynamic instability (Mitchison and Kirschner, 1984a). This rapid lengthening and shortening of MTs is important for many functions, as it allows the plus ends to explore the intracellular space and to increase contacts with cytoplasmic structures and proteins. This MT 'search and capture' mechanism enables the attachment and separation of chromosomes by kinetochores and the cortical capture of MTs leading to local MT stabilization and cell polarization (Gundersen et al., 2004) (Fig. 2). MT dynamics are also important in protrusive activity at the leading edge, focal adhesion turnover, and delivery of cargo to organelles and cell structures (Kaverina et al., 1999; Krylyshkina et al., 2002; Wittmann and Waterman-Storer, 2001).
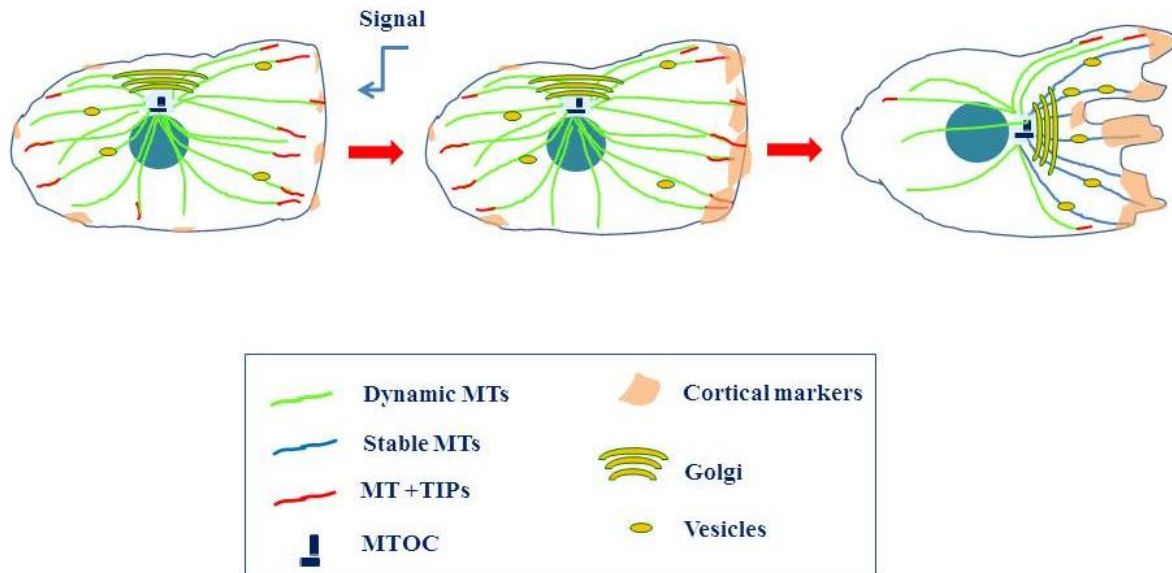
Figure 2. Cortical capture of MTs leads to selective MT stabilization and cellular polarization. In unpolarized cells (left), dynamic MTs bound by +TIPS, the MT plus-end binding proteins, search the extracellular space. Upon an external signal, for example, the accumulation of a chemo attractant or wounding which trigger cell motility, cortical factors redistribute and initiate MT capture by interacting with +TIPs (middle). This localized MT capture leads to selective MT stabilization and MTOC reorientation which in turn cause global cellular polarization represented here by Golgi reorientation and polarized vesicle transport (right). Figure adapted from Gundersen et al. (2004).

Dynamic instability is a property inherent to MT structure, resulting from the capacity of α and β tubulin to bind and hydrolyze GTP. Subunits that add to an existing protofilament necessarily possess GTP in their β-tubulin E-site; the slow and constant rate of hydrolysis of E-

site GTP ensures that the plus end of a growing MT possesses a GTP cap. The length of the GTP cap varies according to the relative rates of polymerization and GTP hydrolysis; MTs with slow polymerization rates lose their GTP caps. The GTP cap is thought to stabilize MTs by imposing structural constraints on the GDP-bound heterodimers in the body of the MTs, since GTP protofilaments appear to have a straight conformation and intrinsic stability, while GDP protofilaments, are curved, and thus subject to depolymerization (Nogales, 2001). Loss of the GTP cap is responsible for dynamic instability; it induces rapid depolymerization by exposing the less stably-bound GDP-tubulin subunits. MT dynamics are also regulated by interaction by several cofilamentous MAPs that stabilize subunit-subunit contacts within the protofilament (Konno et al., 2012).

Studies of the structure of tubulin and MTs have provided vital information on binding mechanisms of drugs that affect MT dynamics. For example, Taxol, which binds to a site near the M-loop of β-tubulin, may stabilize MTs by altering lateral contacts that occur between the M-, H1-S2 and H3 loops of adjacent protofilaments, which face the MT lumen (Nogales, 2001). Taxol may also stabilize MTs by influencing interdimer longitudinal contacts within a protofilament. The drugs colchicine and nocodazole, which inhibit MT polymerization, bind the intradimer interface on the lumenal side, thus destabilizing longitudinal contacts. Vinblastine, another polymerization inhibitor, binds a region on β-tubulin exposed at the surface of MT ends, thus preventing further MT polymerization. Vinblastine and Taxol are widely used in chemotherapy because their capacity to inhibit MT dynamics impairs mitosis and motility.

Microtubule associated proteins: cofilamentous MAPs, +TIPs and motors

MAPs comprise a diverse group of proteins that regulate functional properties of MTs including MT dynamics and stabilization, cellular polarization and MT-dependent transport. The so-called cofilamentous MAPs, including MAP1, MAP2, MAP4 and Tau, were originally identified by in vitro polymerization/depolymerization purification schemes. These large, positively charged proteins ionically bind the carboxyl terminal domains of α and β tubulin and stabilize MT polymer (Nogales, 2001). Except for the ubiquitously expressed MAP4, these MAPs are primarily expressed in differentiated cells in neurons and glia, so they are excluded from our discussion here.

Other MAPs serve as MT destabilizers. Op18/stathmin binds to MT plus ends and increases GTP hydrolysis; free Op18/stathmin dimers bind and sequester tubulin subunits, further destabilizing MTs. In addition, katanin and spastin enzymatically sever MTs, generating more free ends with exposed GDP-tubulin lattices that rapidly depolymerize. Finally, the Kin1 kinesins potently depolymerize MTs by inducing a destabilizing conformational change in the tubulin lattice.

Yet another group of MAPs, the +TIPs, interact preferentially with the growing, plus ends of MTs. +TIPs include EB1, APC, and CLASPs, which are involved in MT stabilization, as well as the CAP-GLY domain-containing proteins, p150Glued, CLIP170, and Arp1, which are involved in recruiting dynein for intracellular transport (Akhmanova and Steinmetz, 2010). p150Glued also plays a well characterized role in MTOC reorientation (Dujardin et al., 2003). +TIPs interact with each other to form functionally different dynamic complexes at the plus ends of MTs, but there exists a hierarchy of binding wherein some +TIPs bind MTs directly to recruit others. +TIPs regulate MT dynamics and can act as intermediary factors in MT capture by

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various organelles and cellular structures (Fig. 2), or in MT interactions with other cytoskeletal structures, notably microfilaments.

Finally, MT-activated ATPases of the kinesin and dynein families, serve as motor proteins that actively transport cargo bidirectionally along MTs (Mallik and Gross, 2004). Both types of motors transit along the outside surface of MT protofilaments; kinesins interact ionically with the carboxyl terminal tails of tubulin there. Transport of numerous organelles is important for their steady-state positioning as well as for their intracellular movement during cell migration.

MT Diversity

Functional diversity of MTs can be generated in the cell through differential expression of tubulin isotypes, through changes in the complement of MAPs that may alter MT dynamics and stabilization, and through post-translational modifications of tubulin (reviewed in Nogales, 2001). The existence of several α- and β-tubulin isotypes expressed in eukaryotes led to the 'multitubulin hypothesis' for creating functional diversity of MTs (Fulton and Simpson, 1976). To date, however, experimental evidence suggests that different isotypes are mostly interchangeable, with only modest functional differences that help in long-term differences that are implemented over several cell cycles, and usually irreversibly (Luduena, 1998). Hence, MT diversification via isoform-switching usually contributes to organogenesis or other differentiative events. All isotypes are highly homologous, with the carboxyl terminal acidic tails of α and β tubulin being the most divergent among isoforms. In contrast, altered complements of MAPs and posttranslational modifications both of which primarily involve the carboxyl terminal acidic tails

of α and β tubulin, contribute more significantly to the cells' regulation of MT dynamics and function.

MT stabilization and posttranslational modifications

In undifferentiated vertebrate cells, stable MTs comprise a small subset of the total MT array, but form a polarized array early in the polarization process, for example, in differentiating neuronal and myogenic cells and in directionally migrating fibroblasts (Bulinski and Gundersen, 1991) (see Fig. 2 for migrating cells). Temporal and spatial regulation of MT stabilization is thought to be important for directional migration and differentiation, perhaps because stable MT arrays serve as long-lived tracks for iterative intracellular transport events. Stable MTs also exist in specialized structures like cilia and flagella, where their stability may be important for intraflagellar transport.

MT stabilization may be instigated via the persistence of a GTP cap, capture by a +TIP complex, or binding of cofilamentous MAPs. Stable MTs are thought to acquire different functions due to the posttranslational modifications they accumulate. Tubulin subunits within the MT polymer are subject to the action of post-translational modification enzymes that carry out lysine-40 acetylation, detyrosination, polyglutamylation, polyglycylation and Δ2- tubulin generation (Janke and Bulinski, 2011) (Fig. 3). All modifications except for Δ2-tubulin, which is formed by the additional cleavage of the glutamic acid residue exposed by detyrosination on α-tubulin, are reversible, and all except for α-tubulin lysine-40 acetylation, occur on the carboxyl terminus of α- and/or β-tubulin. Thus, carboxyl terminal modifications alter the surface of the MT, where they are then poised to modulate interactions with a diverse group of MAPs. Despite the identification of these modifications over the past several decades, until recently, insufficient

knowledge concerning the modifying/demodifying enzymes and their inhibitors has thwarted the testing of each modification's role(s).
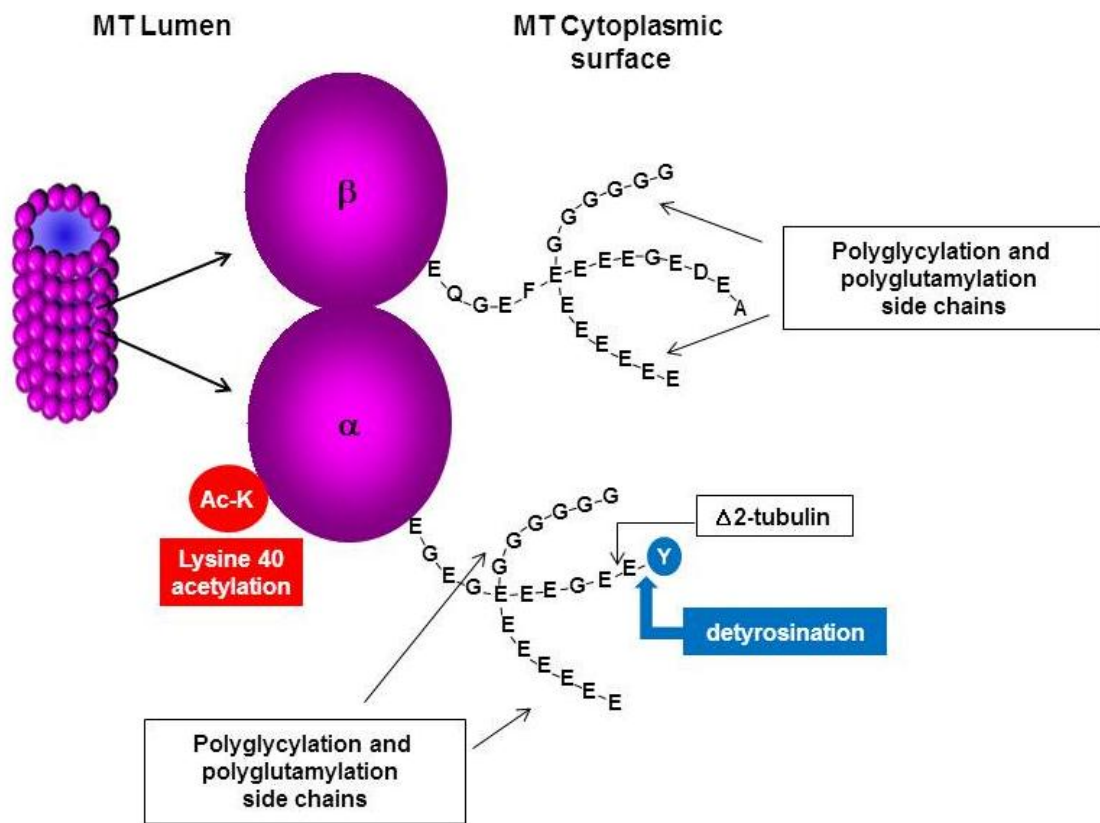
Figure 3. Microtubule posttranslational modifications. Figure shows lysine-40 acetylation, detyrosination, Δ2- tubulin, and polyglutamylation and polyglycylation side chains on an α-β tubulin heterodimer in MT polymer. All modifications, except lysine-40 acetylation, occur on the cytoplasmic carboxyl-terminal tails of α or β tubulin where they affect interactions with a wide variety of MAPs. Lysine 40 acetylation occurs on the amino-terminal of α-tubulin which faces the MT lumen. The amino acid sequences of the

carboxyl terminal tails from representative mouse tubulin isotypes are shown to indicate

positions of modifications. Figure adapted from Janke and Bulinski (2011).

Detyrosination and acetylation predominate in the oriented stable MTs of directionally

migrating cells, e.g., in fibroblasts and epithelial cells. Polarized acetylated and detyrosinated

arrays (Fig. 4) are postulated to play important roles in signaling pathways during cell migration

(Bulinski and Gundersen, 1991). The early elaboration of a stable, modified MT array oriented

towards the direction of migration is thought to form a template for further polarization of cell

contents and intracellular transport (Bulinski and Gundersen, 1991; Kirschner and Mitchison,

1986) (also see Fig. 2). Detyrosination and acetylation are postulated to mark preferred MT

tracks that polarize intracellular transport. Polarized transport to the plasma membrane

contributes to membrane turnover during directional migration (Bergmann et al., 1983).

Although detyrosination and acetylation demarcate the same population of stable MTs

(Bulinski et al., 1988), the elaboration of the two modifications according to different timing and

regulation (Chang et al., 2002; Gundersen et al., 1989; Quinones et al., 2011) argues that

detyrosination and acetylation serve different functions during differentiation or motility.

Moreover, detyrosination is an effect and not a cause of MT stability (Skoufias and Wilson,

1998), unlike MT acetylation, in which a relationship between stability and modification is still

not well understood. The roles of both detyrosination and acetylation in modulating membrane

trafficking have been explored (Gurland and Gundersen, 1995; Hammond et al., 2010; Lin et al.,

2002; Reed et al., 2006).

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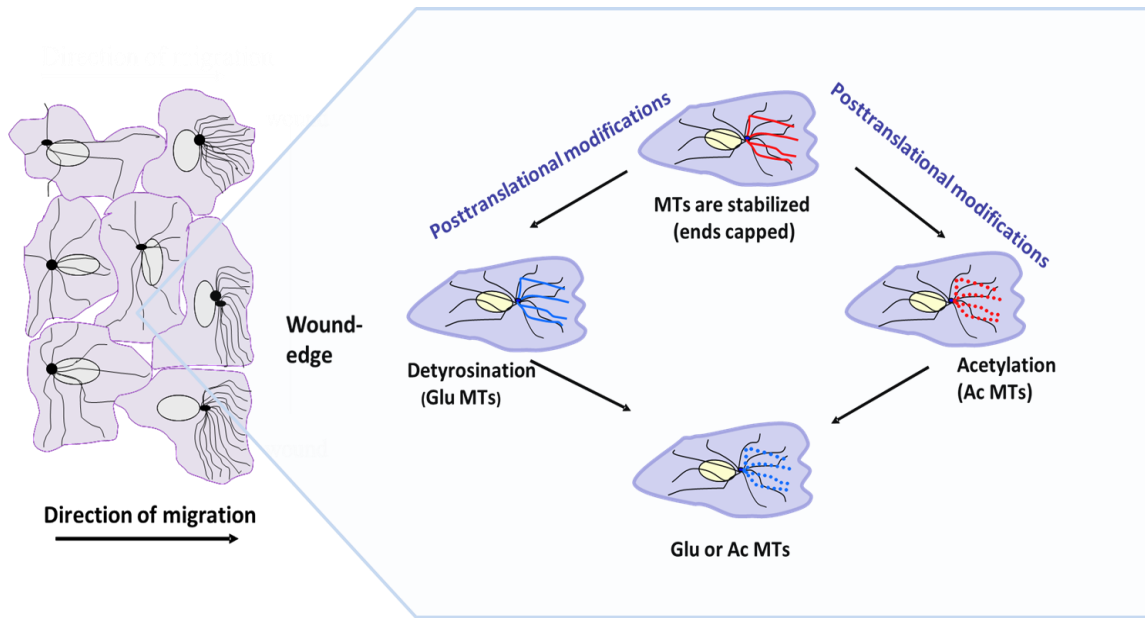
Figure 4. MTs are stabilized and then modified at the leading edge in directionally migrating cells. Figure shows a representative motile cell from a fibroblast monolayer undergoing wound healing, in which MT stabilization, via an end-capping mechanism, for e.g., the MT capture of dynamic MTs by the leading edge (see Fig. 2), is followed by detyrosination and acetylation resulting in a polarized and modified stable MT array facing the leading edge. The early elaboration of a polarized, modified MT array is thought to form a template for further polarization of cell contents and intracellular transport. Note that while detyrosination and acetylation are independent modifications (Chang et al., 2002), they occur on the same subset of stabilized MTs. Dynamic MTs are depicted in black, while polarized, stable MTs are shown in red, detyrosinated MTs in blue, acetylated MTs in stippled red and a stable, detyrosinated and/or acetylated MT array in stippled blue.

Detyrosination

Detyrosination is a reversible posttranslational modification unique to tubulin, in which an unidentified tubulin carboxypeptidase (TCP) removes the genetically encoded carboxyl terminal tyrosines from polymerized α-tubulin subunits, thus exposing glutamic acid residues on the surface of MT polymers (reviewed in Janke and Bulinski, 2011) (see Fig. 3). As with all reversible post-translational modifications of tubulin, the modification cycle is completed by the reversal of the modification, in this case the tyrosination of depolymerized tubulin subunits by tubulin tyrosine ligase. Due to the high efficiency of this ligase, all newly polymerized MTs are composed only of tyrosine residues, such that presence of detyrosinated subunits 'marks' those MTs that have been stabilized.

Despite the fact that the tyrosination enzyme, tubulin tyrosine ligase, was the first MT post-translational modification enzyme to be cloned and sequenced (Ersfeld et al., 1993), reagents to study the detyrosination reaction have been lacking. Only a partial purification of the tubulin-specific tyrosine carboxypeptidase (TCP) (Argarana et al., 1980), has been reported to date. Thus, experiments to test the role of Glu MTs have been largely designed to use indirect methods. For example, inhibition of Glu MT formation by incorporation of nitrotyrosine, a non-cleavable tyrosine analog, blocked early differentiation events in myogenesis (Chang et al., 2002). Similarly, microinjection of Glu tubulin antibody blocked kinesin-mediated vimentin and transferrin receptor transport towards the leading edge of migrating cells, respectively (Gurland and Gundersen, 1995; Lin et al., 2002). The interpretation of these results was that kinesin preferentially binds to Glu MTs. Subsequent studies demonstrated a modest preference of

kinesin-1 for Glu MTs through in vitro (Liao and Gundersen, 1998) and in vivo (Konishi and Setou, 2009, and less directly, Hammond et al., 2010) studies.

The CAP-GLY domains of MT +TIPs show decreased binding to Glu MTs (Akhmanova and Steinmetz, 2010; Badin-Larcon et al., 2004; Erck et al., 2005). Studies in yeast that express an α-tubulin mutant unable to be tyrosinated (Badin-Larcon et al., 2004), and mice lacking tubulin tyrosine ligase (Erck et al., 2005) showed that increased Glu MTs (and in mice, Δ-2-tubulin enriched MTs) mislocalize CLIP-170, but not EB1, from MT plus ends. Budding defects in the mutated yeast cells, which require dynein for budding, suggested that dynein motility is impaired in these cells. Since mice null for tubulin tyrosine ligase die shortly after birth and exhibit major neuronal defects (Erck et al., 2005) and since CLIP170 is known to link dynein to its cargo for retrograde transport (Perez et al., 1999), these data may also imply a dynein-mediated transport defect in animals with increased Glu and/or Δ-2-tubulin modified MTs. Thus, Glu MTs may enhance kinesin-based but decrease dynein-based MT transport. These data nonetheless provide evidence for the hypothesis that detyrosinated tubulin functions as a marker for altered intracellular transport along detyrosinated MTs.

Acetylation

Reversible acetylation of α-tubulin (LeDizet and Piperno, 1987) was first discovered in Chlamydomonas ciliary axonemes (L'Hernault and Rosenbaum, 1985), and on a few cytoplasmic MTs in Chlamydomonas, Drosophila, and mammalian cells (Bulinski et al., 1988; Piperno and Fuller, 1985b; Piperno et al., 1987). Unlike all other tubulin modifications, which occur at the carboxyl terminus of tubulin, acetylation occurs on the highly conserved amino terminal lysine 40 (LeDizet and Piperno, 1987), more specifically, within the H1-S2 loop that faces the lumen of

the MT (Nogales et al., 1999). Despite extensive studies showing that increased acetylation of stable MTs correlates with many differentiation and polarization events (Bulinski and Gundersen, 1991), the role(s) of this evolutionarily conserved modification have not been determined.

Despite tubulin's abundance in the cytoplasm, and the fact that it was the first cytoplasmic protein that was shown to be reversibly acetylated (Piperno et al., 1987), it has not been straightforward to decipher the role of acetylated MTs in any organism. Neither mutation of lysine 40 to a non-acetylatable residue in protists (Gaertig et al., 1995; Kozminski et al., 1993), nor overexpression of a non-acetylatable tubulin in C. Elegans (Fukushige et al., 1999) yielded obvious phenotypes in those organisms. Moreover, mice genetically null for the tubulin deacetylation enzyme, HDAC6, showed hyper-acetylated tubulin in most tissues, but they developed into adults and exhibited only subtle phenotypes (Lee et al., 2008; Zhang et al., 2008) that have yet to be fully characterized.

Although tubulin acetylation appears to be unnecessary for cell and organism survival, the identification of the enzymes involved in reversible tubulin acetylation is beginning to uncover its functions and regulation in cellular processes. Confirmed α-tubulin lysine acetyltransferases are αTAT1 in mammalian cells, and MEC17 and αTAT2 in C.Elegans (Akella et al., 2010; Shida et al., 2010). αTAT1 was first identified by its interaction with the Bardet-Biedl complex of proteins (the BBSome) required for genesis of the primary cilium; this implicated MT acetylation in development and/or function of cilia (Shida et al., 2010). Moreover, disruption of αTAT1 in Tetrahymena and zebrafish decreased MT stability and led to neuromuscular defects, respectively, while disruption of MEC17 disregulated touch sensitivity in C. Elegans (Akella et al., 2010). Other candidate lysine 40 tubulin acetyltransferases have been

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reported as well, such as ARD1-NAT1 (Ohkawa et al., 2008), the elongator complex protein, ELP3 (Creppe et al., 2009; Solinger et al., 2010), and GCN5 (Conacci-Sorrell et al., 2010). However, the identification and verification of tubulin acetyltransferases is still underway, as the correlation between the activity of αTAT and MT acetylation is imperfect, and in vivo activity has thus far been rigorously demonstrated only for αTAT/MEC17 enzymes, not other putative acetyltransferases.

Tubulin deacetylation was definitively shown to be carried out by the cytoplasmic histone deacetylase family member, HDAC6 (Hubbert et al., 2002). Sirtuin 2 (SIRT2) was also shown to deacetylate tubulin (North et al., 2003), but is present in small amounts and is most likely inactive in interphase cells (Hubbert et al., 2002; North et al., 2003). Accumulation of hyper-acetylated tubulin in HDAC6 null cells and tissues, further suggests that SIRT2 does not deacetylate tubulin in vivo (Zhang et al., 2008).

Pharmacological manipulation of HDAC6 activity, and consequently of MT acetylation level, also suggested a number of cellular functions of acetylated MTs (Haggarty et al., 2003; Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003). For example, increased MT acetylation levels were shown to impact MT-dependent functions such as MTOC reorientation and immune synapse formation in lymphocytes (Serrador et al., 2004), osteoclast maturation (Destaing et al., 2005), viral infection (Huo et al., 2011), cell cycle and cytokinesis (Wickstrom et al., 2010), ciliary assembly and function (Loktev et al., 2008), kinesin function (Hammond et al., 2010; Reed et al., 2006) and cell motility (Haggarty et al., 2003; Hubbert et al., 2002; Lafarga et al., 2012). However, definitive proof for these putative functions of MT acetylation has not yet emerged.

We and others previously reported that MT hyper-acetylation modestly dampens MT dynamics (Matsuyama et al., 2002; Tran et al., 2007). The role of acetylation in MT stabilization has been controversial, though. Some studies assayed for a major shift in sensitivity to MT destabilizing drugs as a result of their increased acetylation; none was found (Haggarty et al., 2003; Palazzo et al., 2003). Another study showed that protein-protein interactions, rather than catalytic activity, of HDAC6 results in decreased MT growth parameters (Zilberman et al., 2009).

We also determined that MT hyper-acetylation reduces focal adhesion turnover during cell motility (Tran et al., 2007); this turnover might be expected to result from altered MT dynamics, as highly dynamic MTs are necessary to mediate adhesion breakdown (Kaverina et al., 1999). Furthermore, increased MT acetylation was reported to destabilize MTs by recruiting the destabilizing MT severing protein katanin (Sudo and Baas, 2010). However, interpretation of this study was confounded by increased glutamylation within the same population of MTs, as post-translational glutamylation, not acetylation has been shown to increases katanin's severing activity in vitro (Lacroix et al., 2010).

Perhaps the most striking function proposed for MT acetylation is augmenting motor binding and increasing intracellular trafficking, such that acetylation, like detyrosination, could serve as a marker for preferential intracellular transport. Kinesin-1 was reported to bind acetylated MTs with higher in vitro binding affinities, while in vitro kinesin-1 binding to, and velocity of transport on MTs polymerized from a non-acetylatable form of tubulin was dramatically reduced (Reed et al., 2006). This study also reported that an increased level of MT acetylation, and not other posttranslational modifications, paralleled an increase in polarized kinesin-1-based transport of its in vivo cargo, JIP1. However, a later study from the same

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researchers reported conflicting data, demonstrating that acetylated MTs do not serve as preferential tracks for kinesin-1 transport in neurons, and increased MT acetylation is not sufficient to increase transport (Hammond et al., 2010). Hammond et al. (2010) concluded that a combination of MT modifications may enhance kinesin-1 transport, with the most critical modification being detyrosination; that detyrosination marks MTs for preferential kinesin transport in neurons was also shown by Konishi and Setou (2009).

Parallel studies with BDNF, a dynein cargo, showed increased bidirectional transport of BDNF vesicles on acetylated MTs in neuronal cells and on MTs that were chemically acetylated at a variety of lysine residues in vitro (Dompierre et al., 2007). Thus, some studies suggest that lysine-40 acetylation of MTs affects anterograde and/or retrograde transport via kinesin or dynein in neurons, but the methods, data, and interpretations of these studies remain to be sorted out.

MTs and directional migration

Directional migration of cells is critical during embryonic development, wound healing, immune function, and invasiveness of metastatic cells (Ananthakrishnan and Ehrlicher, 2007). Directional migration requires the polarization of cytoskeletal and cellular contents so as to scaffold intracellular transport towards the leading edge of the motile cell (see Fig. 5 for a polarized migrating cell). Polarized trafficking enables transport of cytoskeletal, membrane and signaling components to the leading edge, facilitating proper assembly and turnover of proteins involved in the motility machinery (Wakida et al., 2010). In migrating cells, both actin and MT networks polarize and contribute to the polarization of other cellular components (Wittmann and Waterman-Storer, 2001). The regulation of actin filaments is critical for the mechanics of cell

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movement. MTs, on the other hand, are required for polarization of the actin network and for

persistent directionality of movement. Therefore, tight coordination of the actin and microtubule

cytoskeletons, as well as membrane and adhesion dynamics, is critical for directional motility

(Ananthakrishnan and Ehrlicher, 2007; Mitchison and Cramer, 1996).
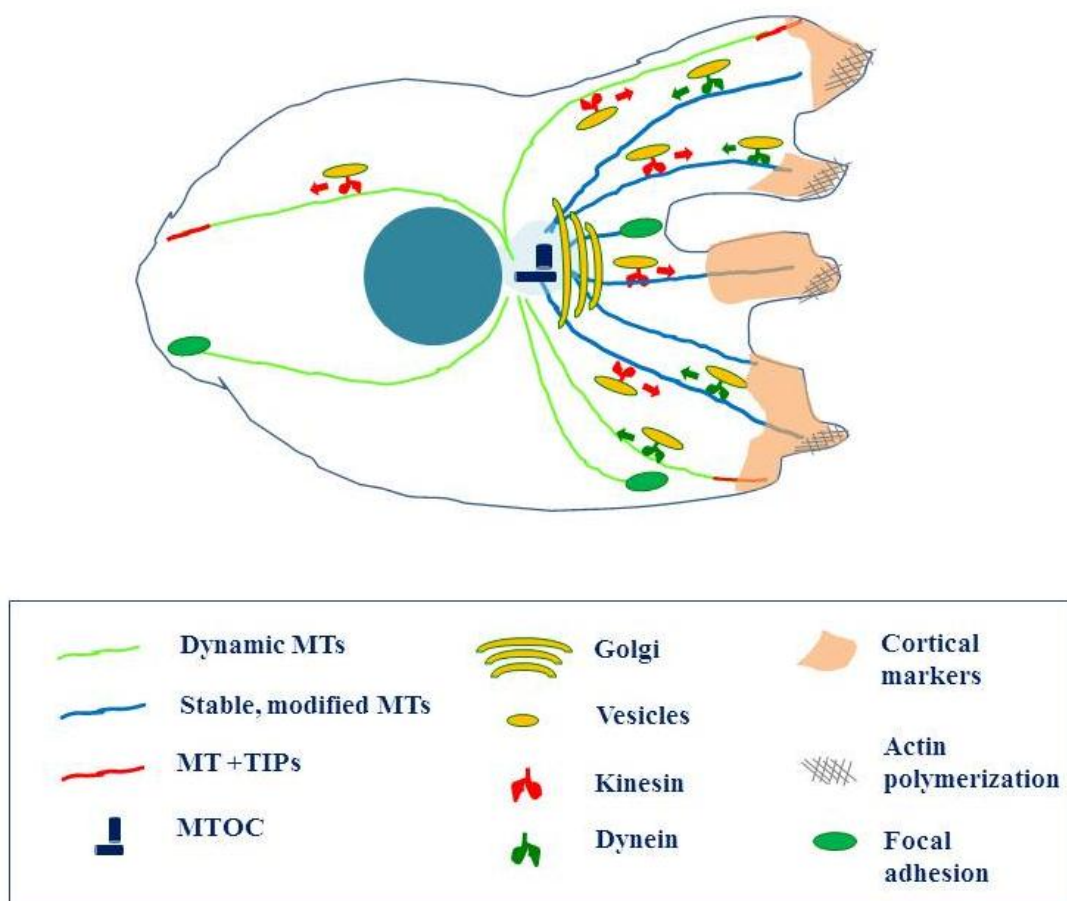
Figure 5. A polarized motile cell. Migrating cells polarize actin and stable, modified MT

networks towards the leading edge. While actin polymerization is important for

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generating forces for forward movement, MTs are important for establishing cellular

polarization. The redistribution of cortical factors to the leading edge causes MT capture

via the +TIPs leading to selective MT stabilization and posttranslational modification.

Stable, modified MTs instigate cellular polarization of organelles such as the MTOC and

Golgi, as well as intracellular vesicular transport which is mediated by MT motors

kinesins and dyneins in anterograde and retrograde directions, respectively. Focal

adhesions form at the front of the cell and are turned over by dynamic MTs at the rear of

the cell.

Actin polymerization at the leading edge creates protrusive forces that generate

lamellipodial protrusions (Small et al., 1978). This protrusive activity is coupled with retrograde

actin flow; together these generate forces sufficient to push the cell forward (Waterman-Storer

and Salmon, 1999). Adhesion formation at the leading edge of the cell and turnover at its trailing

edge allow for cell-substrate adhesion de-adhesion cycles during movement. The actin

cytoskeleton is regulated by activities of the Rho GTPases, RhoA, Rac1, and Cdc42, which each

redistribute to specific compartments during migration: Rac1 and Cdc42 localize to lamellipodia,

while RhoA is maintained in the cell body (Nobes and Hall, 1999). Thus Rac1 and Cdc42 are

appropriately positioned to induce lamellipodial protrusions and cell polarization, respectively,

whereas RhoA activity is in the correct location to regulate contractility and adhesion formation

during motility.

MTs play critical roles in the polarization of motile cells; they stabilize lamellipodia and

repress random protrusions, thus establishing directional persistence (Petrie et al., 2009). Cells

whose MTs are disrupted lose lamellipodial polarity and ruffling, and increase random migration (Bershadsky et al., 1991; Glasgow and Daniele, 1994). MTs are hypothesized to play a role in polarizing cellular contents, as well as intracellular trafficking of vesicles and organelles during cellular motility, thereby further enhancing directional persistence.

HDAC6, A MAJOR CYTOPLASMIC PLAYER

The discovery that HDAC6 is the tubulin deacetylase (Hubbert et al., 2002) led to a number of initial reports linking MT acetylation to cell motility (Haggarty et al., 2003; Hubbert et al., 2002) immune cell function (Serrador et al., 2004), and kinesin-based transport (Reed et al., 2006). While the acetylation of tubulin, despite being the first cytoplasmic protein shown to be reversibly acetylated (Piperno et al., 1987), still does not have well characterized functional roles, HDAC6 has since emerged as a key cytoplasmic regulator of many important biological processes including cell migration, immune function, degradation and transport of ubiquitinated and misfolded proteins in response to cellular stress, as well as cancer and neurodegeneration (Aldana-Masangkay and Sakamoto, 2011; Valenzuela-Fernandez et al., 2008).

Although tubulin is an abundant substrate whose acetylation/deacetylation is a potential regulator of the cytoskeletal function, the diverse processes regulated by HDAC6 are mediated by an impressive range of mechanisms that involve a growing list of substrates and interacting proteins (see Tables 1 and 2). The distinction of substrates vs. interacting proteins is important because HDAC6 can act in both deacetylase-dependent and -independent modes in cell motility of fibroblasts and lymphocytes, respectively, and in its role in the binding and clearing of ubiquitinated proteins (Cabrero et al., 2006; Hubbert et al., 2002; Kawaguchi et al., 2003). This functional complexity necessitates that studies investigating the role of HDAC6 in diverse

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cellular processes and in different cell types address multiple mechanisms and substrates via which HDAC6 may mediate its effects.

Lysine acetylation and the acetylome

Posttranslational modification of proteins is a major regulatory mechanism that increases the overall coding potential of the genome by generating functional diversity in cellular proteins (Walsh et al., 2005). Multiple aspects of a protein's function during its cellular lifetime, including its folding, activity, stability, intracellular localization, and interactions with other proteins and nucleic acids, can all be modulated by posttranslational modifications (Soppa, 2010). The most common modifications are phosphorylation, acetylation, ubiquitination, sumoylation, methylation, glycosylation, and proline isomerization (Yang and Seto, 2008). The potential for regulatory crosstalk between modifications is becoming more obvious; for example, ubiquitination and acetylation both occur on lysine residues and each can thus 'lock' the site from the other modification.

Acetylation of proteins at the amino terminus or on the ε-amino group of an internal lysine, is thought to be the most common posttranslational modification in eukaryotes (Soppa, 2010). N-terminal acetylation, which occurs on the α-amino group of methionine or the penultimate residue exposed after cleavage of methionine, is irreversible, well conserved, and widespread, occurring on as many as 90% of proteins (Helbig et al., 2010), yet its functional significance is not well understood. Acetylation on internal lysines, on the other hand, is often reversible and occurs on numerous cellular proteins (the so-called 'acetylome'). Reversible acetylation has been shown to be involved in regulation of numerous pathways and processes. The regulatory potential of the acetylome is turning out to be very broad and its functional

significance in cells has been proposed to rival that of protein phosphorylation (Choudhary et al., 2009; Kouzarides, 2000). Accordingly, cells precisely regulate lysine acetylation of proteins.

Reversible acetylation on internal lysine residues occurs on both nuclear and cytoplasmic proteins. Tubulin was the first cytoplasmic protein found to be acetylated (L'Hernault and Rosenbaum, 1985; Piperno and Fuller, 1985), followed by p53 (Gu and Roeder, 1997). The addition of an acetyl moiety to a lysine side chain neutralizes the cationic charge on lysine to alter interactions with other proteins and with DNA (Soppa, 2010). Acetylation of histones, therefore, induces chromatin remodeling so that gene transcription can occur. However, acetylation can also cause either gene activation or inhibition, by acetylating transcriptional coactivators or repressors, respectively.

Whether acetylation's changing of the charge on α-tubulin lysine 40 impacts protein-protein binding is not known. This is further confounded by the fact that the site for acetylation on lysine 40 of α-tubulin, the acetylation site studied here, faces the MT lumen (Nogales, 2001). The discovery of novel acetylation sites on both α- and β-tubulin may be functionally relevant; these are expected to have different roles in MT function (Choudhary et al., 2009; Chu et al., 2011). However, most of these sites have not yet been verified to exist in vivo, and thus will not be considered here. The exception is acetylation on lysine 252 of β-tubulin, which does occur in vivo (Chu et al., 2011), but whose physiological significance still remains to be determined. Moreover, acetylation of lysine 252 in unpolymerized β-tubulin is carried out by a novel tubulin acetyltransferase, SAN, and appears to block MT polymerization. Given how different the properties of acetylation of lysines 252 and 40 are, we will confine our discussion here to the modulation of MTs by acetylation of lysine 40.

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Histone deacetylases and HDAC6

Histone deacetylases (HDACs) and acetyltransferases (HATs), enzymes first shown to modify histones, are now known to regulate reversible lysine acetylation of several nuclear and cytoplasmic proteins (Sadoul et al., 2011). As the number of non-histone and cytoplasmic proteins known to be acetylated continues to grow these enzymes are also referred to as lysine deacetylases (KDACs) and acetyltransferases (KATs). Lysine acetylation occurs on the epsilon-amino group of internal lysine residues in proteins and serves to neutralize the inherent positive charge of this amino acid.

There are 18 known members of the mammalian HDAC family; these are classified into four groups based on their homology with yeast orthologues (Witt et al., 2009) (Fig. 6). Despite high homology, HDACs are generally not considered to be functionally redundant (Dokmanovic et al., 2007). Moreover, they co-exist and interact with other HDACs within multi-subunit complexes (Zhang et al., 2003b). Class I, II, and IV encompass classical HDACs dependent on the zinc ion, which is essential for the removal of the acetyl moiety via a mechanism of charge transfer within the HDAC molecule (de Ruijter et al., 2003). Class I HDACs, homologs of yeast Rpd3, a global gene regulator and transcriptional repressor, include HDACs 1, 2, 3 and 8. These ubiquitously expressed HDACs are active in nuclear transcriptional complexes, in which they regulate gene repression. Phenotypes of HDAC-null mice show that HDAC1- and 3- nulls are embryonic lethals, while HDAC2-null mice die perinatally of severe cardiac defects (Witt et al., 2009).

The Class II HDACs related to yeast Hda1 are further subdivided into Class IIa, which includes HDACs 4, 5, 7 and 9, and Class II b, which includes HDACs 6 and 10 (Sadoul et al., 2011). The expression profile of these transcriptional repressors is usually tissue-specific. HDAC

Class IIa-null mice are viable, with the exception of HDAC7, which is embryonic lethal due to defects in endothelial cell-cell adhesions (Witt et al., 2009). HDAC4 null mice show developmental bone ossification defects and exhibit early onset chondrocyte hypertrophy, while HDACs 5 and 9 null mice display major cardiac defects.
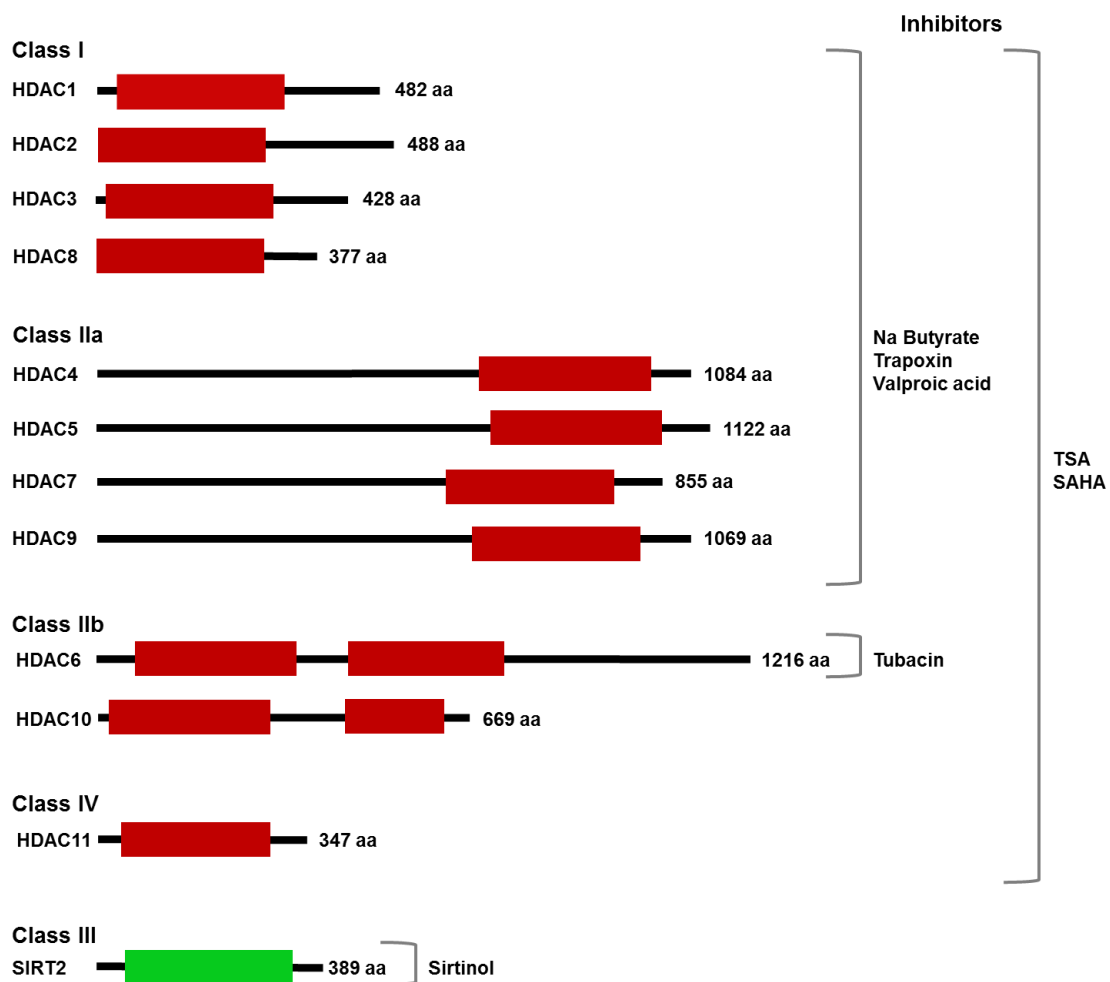
Figure 6. The histone deacetylase (HDAC) family. The figure shows the catalytic domains of the classical HDACs belonging to Classes I (HDACs 1, 2, 3, and 8), IIa

(HDACs 4, 5, 7 and 9), IIb (HDACs 6 and 10) and IV (HDAC11), and of SIRT2, which

is shown as a representative member of the Class III sirtuins (SIRTS 1-7). The

structurally distinct deacetylase domains of the zinc-dependent classical HDACs and the

NAD-dependent SIRT2 are depicted with red and green boxes, respectively.

Representations reflect the relative sizes of these HDACs. The number of amino acid (aa)

residues and the inhibitory profiles of standard HDAC inhibitors are shown to the right.

Figure adapted from Bolden et al. (2006).

All Class II HDACs, except HDAC6, which is predominantly cytoplasmic (Hubbert et

al., 2002), are able to shuttle between the nuclear and cytoplasmic compartments (Sadoul et al.,

2011). HDACs 4 and 6, however, are the sole HDACs so far known to have cytoplasmic

substrates. HDAC4 has four known substrates, MLP (Gupta et al., 2008), DNAJB8 (Hageman et

al., 2010), HIF1α (Geng et al., 2011), and connexin 43 (Colussi et al., 2011), which regulate

muscle contraction, suppression of cytotoxic protein aggregate formation, cancer cell hypoxia-

induced angiogenesis, and GAP junction formation, respectively.

HDAC6 is a unique member of the HDAC family and belongs to a subgroup sometimes

referred to as Class IIb. HDAC6 is structurally and functionally divergent from other HDACs

and resides almost exclusively in the cytoplasm (de Ruijter et al., 2003). It is the only HDAC

with two functional catalytic domains, a nuclear exit signal and an ubiquitin binding domain.

Surprisingly, despite the loss of HDAC6 function in several important biological processes and

high levels of hyper-acetylated MTs, HDAC6 null mice are viable with only minor behavioral

and immune-response defects, and faster bone growth (Fukada et al., 2012; Zhang et al., 2008)

being noted thus far in comparison with WT littermates. HDAC6-specific substrates include α-tubulin and other cytoplasmic substrates and will be discussed in more detail below.

HDAC10, also in Class IIb, is the HDAC structurally most related to HDAC6. However, the second catalytic domain in HDAC10 is not functional and it is not clear whether HDAC10 works as a deacetylase in vivo (de Ruijter et al., 2003). HDAC10 interacts with deacetylated proteins including cytoplasmic Hsp70 (Park et al., 2008; Yao and Yang, 2011). Whether these are substrates of HDAC10 remains to be determined. HDAC 11, with structural features unique to HDACs, is assigned to its own class, Class IV (Witt et al., 2009). HDAC6 has been shown to interact with both HDAC10 and 11 (Gao et al., 2002), but functions of HDACs 10 and 11 are largely unknown and null mice have yet to be generated and analyzed.

Finally, the Class III HDACs, also known as sirtuins, are related to yeast Sir2 and form a functionally and mechanistically distinct class (Michan and Sinclair, 2007). The dependence of this class on NAD for catalysis links these enzymes to the cell's energy stores and sirtuins are known to regulate metabolic pathways (Yao and Yang, 2011). Sir2 in yeast is a transcriptional repressor and regulates longevity, chromosomal stability and DNA repair and recombination. The seven mammalian homologs, SIRTs 1-7, also regulate longevity, caloric restriction, DNA repair, and tumorigenesis (Michan and Sinclair, 2007).

SIRT2 is a Class III HDAC relevant to studies of HDAC6 since SIRT2 is also predominantly localized to the cytoplasm, is capable of deacetylating α-tubulin and is shown to interact with HDAC6 (North et al., 2003). SIRT2 can however, translocate to the nucleus where it deacetylates p53, histones and p300, an acetyltransferase, among others (Michan and Sinclair, 2007). SIRT2 is abundant in the brain where it may impinge upon MT-dependent processes (Yao and Yang, 2011). It is also implicated in regulating centrosome cohesion. However, SIRT2 is not

thought to be active in interphase cells as it is present at a low level upregulated during mitosis and functions as a checkpoint protein to regulate proper mitotic exit (Dryden et al., 2003; Vaquero et al., 2006).

The role of HDACs as global transcriptional modulators of cell differentiation, cell cycle and apoptotic events associates their aberrant regulation to several pathologies, especially cancers. HDAC inhibitors, which impact cell growth, induce terminal differentiation, and show greater cytotoxicity towards tumor cells, are promising therapeutic candidates; in fact, numerous clinical trials are in process for cancer (Ververis and Karagiannis, 2011). In addition, HDAC inhibitors are being investigated for use in neurodegenerative, infectious and cardiac diseases. Inhibitors of the classical HDACs typically work by chelating the zinc ion bound at the base of the catalytic pocket thereby disrupting the charge-relay system within the molecule. There are five classes of inhibitors for the classical HDACs. Short-chain fatty acids like butyrate and valproic acid, hydroxamic acids like TSA and SAHA, benzamides like MS-275, cyclic tetrapeptides like trapoxin and FK228, and electrophilic ketones (de Ruijter et al., 2003; Gallinari et al., 2007) (see Fig. 6 for the specificity of the widely-used inhibitors). Sirtuins, on the other hand, can be non-competitively inhibited by nicotinamide, a by-product of the deacetylation reaction, or by NAD analogs, including specific small molecule inhibitors like sirtinol that competitively inhibit NAD-requiring processes (Spange et al., 2009).

HDAC6 Domains

HDAC6 is the only member of the HDAC family with two functional catalytic domains (Fig. 7), making it the largest HDAC with 1216 amino acids in humans (Grozinger et al., 1999; Verdel and Khochbin, 1999). The second catalytic domain is thought to have arisen from a gene

duplication event during evolution as the two domains are highly homologous and conserved

across species (Boyault et al., 2007). Whether one or both domains contribute to the

deacetylation functions in HDAC6 has been controversial. Using catalytic point or deletion

mutants, one group reported that both domains possess HDAC activity, but only the second

domain possesses tubulin deacetylase (TDAC) activity (Grozinger et al., 1999; Haggarty et al.,

2003). Others reported that the second domain alone is responsible for all HDAC and TDAC

activities, with the first domain being non-functional (Zou et al., 2006). Finally, a third group

used deletion and multi-site catalytic core mutants to show that the presence and integrity of the

catalytic cores of both domains are critical to all deacetylase activities of HDAC6 (Zhang et al.,

2006; Zhang et al., 2003b).
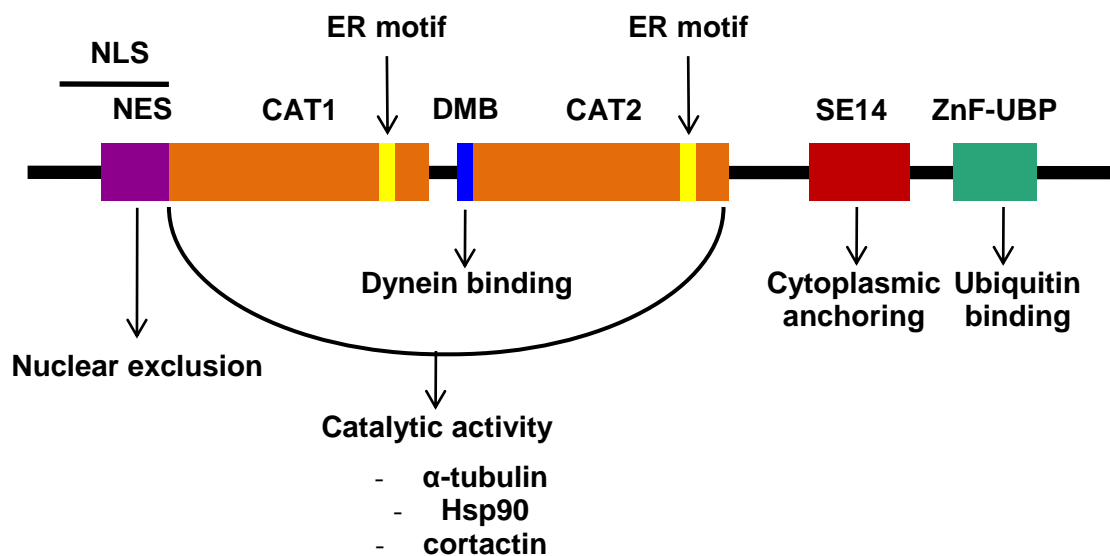
Figure 7. HDAC6 domain map. The figure shows the organization of HDAC6 domains as

marked: NLS, nuclear localization signal, that is masked under most conditions; NES,

nuclear exclusion signal, a unique, potent conserved nuclear exit signal; CAT, catalytic

sites, the two fully functional HDAC6 catalytic domains that deacetylate α-tubulin,

Hsp90 and cortactin among other identified substrates; ER, Esa1-Rpd3 motifs, conserved

substrate recognition regions present in both catalytic domains; DMB, dynein motor

binding domain; SE14, serine glutamate tetradecapeptide repeat motif, a novel repeat

motif which ensures cytoplasmic anchoring; and ZnF-UBP, zinc finger ubiquitin binding

protein or BUZ domain, that mediates binding to ubiquitinated proteins

Additional features also affect the deacetylase properties of HDAC6. Mutations in

conserved substrate recognition regions, the Esa1-Rpd3 (ER) motifs present in both catalytic

domains, showed that the ER motif in the second domain is critical for HDAC and TDAC

activity (Zhang et al., 2006). Mutations in the ER motif of the first domain only affected HDAC

and TDAC activities modestly, although greater decrements were observed in tubulin

deacetylation. The precise length of the linker region between the two catalytic domains is also

critical for HDAC6 activity as reducing this linker by even 5 amino acids was sufficient to

dramatically reduce activity. Again, TDAC activity was more sensitive to alterations in the

length of the linker region. This linker region's sensitivity may be one reason for the conflicting

results when others tested each catalytic domain separately. Furthermore, experiments with

artificial chimeric proteins with different HDAC domains showed that the second domain of

HDAC6 is indispensable for its activity. TSA, a Class I and II HDAC inhibitor, as well as

tubacin, an HDAC6-specific inhibitor, have also been shown to bind to the second domain of

HDAC6 (Haggarty et al., 2003).

Given these data and the fact that no activity of HDAC6 towards histones has been shown *in vivo*, it may be that the second domain is critical for cytoplasmic substrate recognition and catalytic activity. Regardless of the mechanism, the presence of two catalytic domains no doubt confers HDAC6 with its unique biological properties and expands the diversity of its substrates in the cytoplasm. In support of this, binding of HDAC6 with different interacting proteins and substrates can either occur through a single domain, with different proteins preferring one domain over the other, or through both deacetylase domains (Azuma et al., 2009; Jiang et al., 2008; Lafarga et al., 2012; Wickstrom et al., 2010; Wu et al., 2010; Zhang et al., 2007; Zhang et al., 2003b). The presence of two domains may be important for histone deacetylation *in vivo* as well as many HDACs co-exist in active complexes and experiments with artificial chimeric proteins with two domains lead to de novo activity (Zhang et al., 2006).

HDAC6 has other features important for its function as well. What is called a cytoplasmic retention signal is actually mediated by a potent, conserved nuclear exit signal (NES) on the HDAC6 amino-terminus, a signal unique to HDAC6. In humans, stable cytoplasmic localization of HDAC6 is ensured by an additional SE14 domain, which is a novel tetradecapeptide repeat motif, and that provides cytoplasmic anchoring (Bertos et al., 2004). HDAC6 also contains an intrinsic amino-terminal nuclear localization signal (NLS) (Verdel et al., 2000). This NLS appears to be masked under most conditions; nuclear localization is rare occurring only under certain conditions like cell cycle arrest or in certain cancers (Riolo et al., 2012; Verdel et al., 2000; Westendorf et al., 2002; Zhang and Kone, 2002). NLS and nuclear exit signals have been shown not to be required for HDAC6 activity.

HDAC6 has been mapped to show a small domain responsible for interacting directly with the dynein motor (Kawaguchi et al., 2003). The dynein motor binding domain, or DMB, is

adjacent to the second deacetylase domain of HDAC6 (see Figure 7). Finally there is a cysteine

and histidine rich, ubiquitin-binding zinc finger domain, ZnF-UBP, also known as the BUZ

domain, on the carboxyl-terminal end of HDAC6 (Grozinger et al., 1999; Verdel and Khochbin,

1999). The BUZ domain is highly homologous to those found in several ubiquitin binding

proteases (Seigneurin-Berny et al., 2001). However, it is unique to HDAC6 and it mediates the

novel HDAC6 response to cellular stress and injury; i.e., clearing of ubiquitinated and misfolded

proteins. The BUZ domain of HDAC6 possesses the highest known affinity for ubiquitinated

proteins (Boyault et al., 2006) and can bind both mono and poly ubiquitinated proteins (Boyault

et al., 2006; Hook et al., 2002; Seigneurin-Berny et al., 2001). This high-affinity binding is

thought to be important in stabilizing poly-ubiquitinated proteins. Importantly, the binding of

ubiquitinated proteins does not affect the concurrent deacetylase activities of HDAC6

(Seigneurin-Berny et al., 2001). The BUZ domain is also implicated in the MT-dependent

transport of misfolded and ubiquitinated proteins to lysosomal and aggresomal compartments, as

it has been shown to link cargo to dynein motors (Boyault et al., 2007; Kawaguchi et al., 2003).

Inhibitors

     HDAC6 shows selective sensitivity to classical HDAC inhibitors (see Fig. 6). It is

inhibited by the hydroxamic acids TSA and SAHA, but shows no response to treatment with the

short chain fatty acid butyrate or the cyclic tetrapeptides trapoxin and FK228 (Furumai et al.,

2001; Guardiola and Yao, 2002; Hubbert et al., 2002; Matsuyama et al., 2002). This selective

drug resistance is due to the presence of the duplicated catalytic domain as HDAC10 displays a

similar resistance (Guardiola and Yao, 2002) and provides an indirect way to assay the activity

of HDAC6, and HDAC10, distinct from other HDACs.

The development of a specific small molecule inhibitor of HDAC6, tubacin (Haggarty et al., 2003), which is as potent as TSA in inhibiting chemotactic invasion motility of fibroblasts (Tran et al., 2007), and its congenor, tubastatin (Butler et al., 2010), have been critical in investigating HDAC6-specific functions. Crucially, tubacin inhibition may be specific for α-tubulin as Hsp90, cortactin and the in vitro substrate, histones, were not affected by treatment with this inhibitor (Lafarga et al., 2012; Tran et al., 2007). Two additional types of HDAC6-specific inhibitors have recently been reported, mercaptoacetamides, and thiolate analogs (Li et al., 2011b). Inhibition with mercaptoacetamides and thiolate analogs has been shown to confer neuroprotective and anticancer properties, respectively.

Substrates

HDAC6 has been shown to deacetylate the following cytoplasmic substrates to date: α-tubulin (Hubbert et al., 2002), Hsp90 (Kovacs et al., 2005), cortactin (Zhang et al., 2007), β-catenin (Li et al., 2008), peroxiredoxins I and II (Parmigiani et al., 2008), and survivin (Riolo et al., 2012) (see Table 1). The deacetylation of α-tubulin, Hsp90 and cortactin are involved in regulating invasion motility (Hubbert et al., 2002; Kovacs et al., 2005; Zhang et al., 2007). The deacetylation of Hsp90, a vital cellular chaperone, is also involved in the induction of heat shock transcription factors in response to cellular injury and stress. β-catenin deacetylation by HDAC6 is required for its nuclear localization and activation of target genes involved in cell proliferation in response to EGF signaling (Li et al., 2008). The deacetylation of the peroxiredoxins, PrxI and II, which can induce apoptosis, is important in the regulation and balancing of their redox activities and in decreasing hydrogen peroxide cytotoxicity (Parmigiani et al., 2008). Survivin is a recently identified HDAC6 substrate important in apoptosis and cell proliferation (Riolo et al.,

2012). The nuclear deacetylation of survivin by HDAC6 leads to its cytoplasmic shuttling where it induces cell death in breast cancer cells.

Additionally, HDAC6 has also been shown to deacetylate the endoplasmic reticulum (ER) protein GRP78 (Rao et al., 2010), which dissociates from the ER upon cellular stress, and Ku70 (Subramanian et al., 2011), a factor that regulates apoptosis, in breast cancer and neuroblastoma cells, respectively. HDAC6 also deacetylates the HIV viral Tat protein important in HIV infection (Huo et al., 2011).

Table 1. Cytoplasmic HDAC6 substrates and their related biological functions.
Adapted from Valenzuela-Fernandez et al., 2008

Substrates	Related biological functions
Motility-related	
α-tubulin	MT-dependent cell migration; intracellular transport; immune synapse formation; viral infection; cell cycle and cytokinesis; myogenesis
Cortactin	actin-dependent cell migration
Hsp90	actin-dependent cell migration; chaperone; heat shock response
Non-motility related	
β-catenin	activation of target genes in cell proliferation
PrxI/II	regulation of redox activities; hydrogen peroxide cytotoxicity; apoptosis
Survivin	apoptosis; cell proliferation
GRP78	ER misfolded-protein stress response
Ku70	apotosis in cancer and neuroblastoma cells
HIV Tat	HIV infection

Interacting partners

In addition to having many deacetylation substrates, HDAC6 interacts with several cellular proteins. Many of these co-exist in multi-subunit complexes with HDAC6 (see Table 2). It is striking that a significant proportion of these interacting proteins are regulators or components of the MT cytoskeleton and/or are involved in the regulation of cell migration. HDAC6 interacts with β-tubulin through either of its deacetylase domains (Zhang et al., 2003b). This interaction is independent of its catalytic activity or its inhibition and is unaffected by treatment with TSA. Moreover, HDAC6 associates with both the tubulin dimer and polymerized MTs (Zhang et al., 2003b) and colocalizes with the Ac MT subset (Hubbert et al., 2002). The interaction of HDAC6 with its substrate, α-tubulin, to which it can independently bind via its catalytic domains, is thought to be mediated through the HDAC6-β-tubulin interaction (Zhang et al., 2003b).

HDAC6 also interacts with MT +TIPs, which are known modulators of MT dynamics and function. Immunoprecipitation experiments have thus far identified p150glued (Hubbert et al., 2002; Kawaguchi et al., 2003), Arp1, and EB1 (Zilberman et al., 2009) as HDAC6-interacting proteins. The EB1 interaction was further determined to occur through the N- and C-terminal domains of HDAC6 that are not involved in deacetylation. HDAC6 also directly associates with the MT motor dynein (Kawaguchi et al., 2003). This interaction is mediated through a specific dynein-binding domain that is adjacent to the second deacetylase domain of HDAC6 (see Figure 7). The CAP-GLY domain proteins, p150glued and Arp1, are both subunits of dynactin, a regulatory complex of dynein (Vaughan, 2005), and HDAC6 interactions with p150glued and dynein are enhanced upon proteasome inhibition (Kawaguchi et al., 2003). The

interactions of HDAC6 with dynactin and dynein have been shown to mediate the MT-based

transport of ubiquitinated and misfolded proteins to aggresomes.

Table 2. Cytoplasmic HDAC6 interacting proteins and their related biological functions.

Adapted from Valenzuela-Fernandez et al., 2008

Proteins	Related biological functions
Tubulin and MAPs	
β-tubulin	α-tubulin deacetylation; binding to MTs; MT dynamics
p150glued	intracellular transport; aggresome formation; dynein adaptor
EB1	regulation of MT dynamics; stabilization
Arp1	regulation of MT dynamics; intracellular transport; dynein adaptor
Dynein	intracellular transport; aggresome formation
Inhibitory upstream regulators	
CYLD	cell migration; MT polymerization; cell cycle and cytokinesis; tumor invasiveness
IIp45	cell migration; HDAC6 stability; mitosis; tumor suppressor
TPPP/p25	cell migration; MT bundling and dynamics; aggresome formation; neurodegenerative disease
Tau	MT stabilization; aggresome formation; neurodegenerative disease
EGFR	EGF-dependent signaling; EGFR degradation and recycling
p300	cell migration
Dysferlin	myogenesis
Activating upstream regulators	
mDia2	MT and actin dynamics; cell migration; osteoclast maturation
GSK3β	MT and actin dynamics; kinesin-based MT transport
GRK2	cell migration; cell spreading

Table 2 continued

Proteins	Related biological functions
Cellular stress response proteins	
Parkin	ubiquitin-aggresome pathway
p97/VCP	ubiquitin-aggresome pathway
PLAP	ubiquitin-aggresome pathway
Ubiquitin	ubiquitin-aggresome pathway; proteasomal degradation
Cancer-related	
Erα	breast cancer tumor invasiveness and proliferation
BRMS1	breast cancer tumor invasiveness and proliferation
HIF1α	tumor invasiveness; angiogenesis; hypoxia
Miscellaneous	
Bcl-3	CYLD-mediated cell cycle delay
BBIP10	ciliogenesis; MT acetylation, polymerization and stability; centrosome cohesion
PP1	regulation of Akt phosphorylation; cell migration and apoptosis
HDACs	
SIRT2	deacetylation of α-tubulin; mitosis; cytokinesis
HDAC10	PP1 regulation
HDAC11	PP1 regulation

Another major group of HDAC6-interacting proteins has been identified to serve as inhibitory upstream regulators of its activity. Of these, CYLD, a deubiquitinase and tumor suppressor (Gao et al., 2008; Wickstrom et al., 2010), IIp45, invasion inhibitory protein 45

(Wang et al., 2011; Wu et al., 2010), TPPP/p25, tubulin polymerization promoting protein (Tokesi et al., 2010), EGFR (Deribe et al., 2009; Han et al., 2009) and p300, a histone acetyltransferase (Han et al., 2009) are all known cell migration regulators that are likely to regulate the role of HDAC6 in cell motility. Moreover, interactions of HDAC6 with TPPP/p25 and tau are shown to be involved in aggresome formation in response to cellular stress (Guthrie and Kraemer, 2011; Perez et al., 2009; Tokesi et al., 2010), while dysferlin, a transmembrane protein that binds HDAC6 and α-tubulin and regulates the deacetylation reaction, links HDAC6 function to myogenesis (Di Fulvio et al., 2011).

Only a few interacting proteins thus far have been shown to stimulate HDAC6 activity. These are also implicated in regulating cell migration via their effects on the MT cytoskeleton and include the Rho effector, mDia2, glycogen synthase kinase β (GSK3β), and the G protein coupled receptor kinase 2 (GRK2) (Chen et al., 2010; Destaing et al., 2005; Lafarga et al., 2012). mDia1 and mDia2 are formin homology proteins that are involved in distinct actin nucleation and MT stabilization pathways (Bartolini et al., 2008; Eng et al., 2006; Palazzo et al., 2001a; Wen et al., 2004). Thus, through both its enzymatic activity and its binding partners, HDAC6 has been hypothesized to coordinate the MT and the actin cytoskeletons, respectively. For example, mDia2 binds to and stabilizes MTs directly (Bartolini et al., 2008) and can also bind either of the two deacetylase domains of HDAC6 (Destaing et al., 2005). It is thought that mDia1 also interacts with HDAC6 (Bershadsky et al., 2006). In migrating fibroblasts, mDia1 regulates MT stabilization towards the leading edge by the plus end binding proteins, EB1 and APC (Wen et al., 2004).

Like mDia, regulation of GSK3β affects many MT-dependent processes. GSK3β is involved in distinct MT-stabilizing pathways, one of which involves EB1 and APC, in which

GSK3β is negatively regulated by mDia. It is also involved in the CLASP2- mediated MT

stabilization pathway (Eng et al., 2006; Lansbergen et al., 2006; Wen et al., 2004). A recent

paper has implicated GSK3β regulation of HDAC6 in kinesin-1 mediated mitochondrial

transport in neurons (Chen et al., 2010). Furthermore, GSK3β may also coordinate the actin and

MT networks required for efficient cell migration via GSK3β's regulation of the interaction

between CLASP2 and IQGAP1, which can bind both actin and MTs (Watanabe et al., 2009).

Finally, GRK2 has recently been shown to be a major stimulator of HDAC6 tubulin deacetylase

activity that is important in cell migration (Lafarga et al., 2012).

HDACs are known to interact with protein phosphatases that regulate cell proliferation

and transformation. The interaction between HDAC6 and protein phosphatase 1 (PP1) is of

interest as TSA has been shown to physically disrupt this interaction (Brush et al., 2004). The

only other instance in which an HDAC inhibitor physically disrupts binding is the HDAC6

inhibitor, tubacin, which disrupts dynein-HDAC6 binding (Hideshima et al., 2005). HDAC6

activity is required in order for HDAC6 to complex with PP1, which is also active in the

complex (Brush et al., 2004). The release of PP1 upon TSA treatment has been shown to cause

dephosphorylation of Akt, which also plays a role in cell migration (Brush et al., 2004; Chen et

al., 2005; Zhou et al., 2006).

HDAC6 interacts with several factors involved in cancer metastasis and invasiveness,

including the estrogen receptor alpha (ERα) (Azuma et al., 2009), the breast cancer metastasis

suppressor 1, BRMS1 (Hurst et al., 2006), and the Hypoxia-Inducible Factor 1α, HIF1α (Qian et

al., 2006). HDAC6 also interacts with ubiquitin, parkin, the chaperone p97/VCP and the

phospholipase PLAP2 all of which are involved in the ubiquitin-aggresome pathway triggered by

cellular stress, as well as the BBSome interacting protein BBIP10 (Loktev et al., 2008), which is

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involved in ciliogenesis. The HDAC6-mediated roles of these interacting proteins are discussed in more detail below.

As outlined here, almost all known HDAC6-interacting proteins are functionally linked to the MT cytoskeleton and effect HDAC6-mediated regulation of MTs in cell migration. It is significant that HDAC6 interacts with several CAP-GLY proteins; these are a subset of MT plus-end binding proteins (+TIPs) that possess CAP-GLY domains (Kawaguchi et al., 2003; Wickstrom et al., 2010; Zilberman et al., 2009). HDAC6 also interacts with transcriptional activators like NF-κB and Runx2 to modulate target gene repression (Westendorf et al., 2002; Zhang and Kone, 2002). Moreover, farnesyltransferase, a prenylating enzyme, is found in a complex with HDAC6 on MTs and though this enzyme does not interact directly with HDAC6, its activity modulates HDAC6 activity (Zhou et al., 2009). Finally, HDAC6 also interacts with SIRT2 (North et al., 2003), and HDACs 10 and 11(Gao et al., 2002), although the functional consequences of these interactions are less well understood.

Role in cell motility

The localization of HDAC6 to the leading edge lamellipodia of fibroblasts that have been stimulated with growth factors provided the first clue that HDAC6 is involved in the regulation of cell motility (Gao et al., 2007; Hubbert et al., 2002). Overexpression of HDAC6, but not of a catalytically inactive mutant, increased while HDAC6 pharmacological or genetic inhibition significantly decreased chemotactic motility (Gao et al., 2007; Haggarty et al., 2003; Hubbert et al., 2002; Tran et al., 2007). These results demonstrate that HDAC6 catalytic activity is required for the regulation of fibroblast cell motility. Moreover, tubacin, which increases levels of Ac MTs without affecting the acetylation of Hsp90 (Tran et al., 2007) or cortactin (Lafarga et al.,

2012), was shown to equivalently inhibit fibroblast chemotactic motility as compared with TSA (Tran et al., 2007), suggesting that tubulin may be the major hyper-acetylated protein underlying these effects. In support of this, we have previously shown that the hyperacetylation of MTs in pharmacologically inhibited or genetically null HDAC6 fibroblasts imparts a moderate stabilization to cellular MTs (Tran et al., 2007). Since HDAC6-inhibited or -null cells also show increased adhesions and decreased adhesion turnover, and since MT dynamics have been shown to be critical for regulating adhesion turnover (Kaverina et al., 1999), these results provide the first mechanism for a MT-dependent role of HDAC6 activity in chemotactic invasion of cells.

GRK2, which also binds β-tubulin (Pitcher et al., 1998), has recently been identified as a major player in HDAC6-mediated modulation of directional migration of fibroblasts and epithelial cells (Lafarga et al., 2012). GRK2 and HDAC6 were shown to colocalize in the lamellipodia at the leading edge of migrating cells, and HDAC6 activity was shown to be stimulated by GRK2 phosphorylation, thus regulating MT acetylation levels in these regions. Inhibition of HDAC6 by GRK2 mutation impaired cell migration and protrusive activity. Furthermore, a tight regulation of Ac MT levels by the GRK2-HDAC6 interaction was found to be important for both early and late phases in spreading cells. Cortactin acetylation was unaltered by HDAC6 activity, however, a role of the actin network cannot be ruled out as Hsp90 acetylation was not investigated in this study. GRK2 has also been shown to regulate cell motility via integrin activation in the Rac/PAK/MEK/ERK1/2 signaling pathway that affects downstream actin and focal adhesion remodeling (Penela et al., 2008).

However, Zilberman et al. reported that it was HDAC6 level and not activity that suppresses MT dynamics, suggesting that MT acetylation does not affect stabilization of MTs and cell migration (2009). Other studies have disputed a role for tubulin acetylation in cell

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motility also explicating mechanisms involving a role of the actin cytoskeleton instead (Gao et al., 2007; Zhang et al., 2007). Cortactin and Hsp90, like HDAC6, localize to F-actin enriched ruffles and lamellipodia upon growth factor stimulation. HDAC6-mediated deacetylation of Hsp90 is critical for cell motility and ruffle formation; only a 'non acetylatable', and not an acetylation mimetic mutant of a critical lysine residue in Hsp90 rescued ruffle formation in HDAC6 null cells (Gao et al., 2007). Hsp90 deacetylation was also shown to be important in macropinocytosis, a process important for ruffle formation. Moreover, HDAC6 and Hsp90 were shown to stimulate ruffle formation by activating Rac1, a major actin effector of cell migration.

The fact that overexpression of WT Hsp90 in HDAC6 -null cells only partially rescued ruffle formation indicated that other factors are involved in this process. Cortactin is a Rac1-regulated, F-actin binding protein that stimulates actin polymerization and branching by interacting with the actin-nucleating complex, Arp2/3 (Zhang et al., 2007). F-actin polymerization leads to membrane ruffling and leading edge protrusions that are intrinsic to cell migration (Wittmann and Waterman-Storer, 2001). In an elegant study using non acetylatable and acetylation mimetic mutants, Zhang et al. showed that only deacetylated cortactin could bind and localize to the F-actin-rich membrane ruffles to stimulate migration (2007). Cortactin binds HDAC6 and F-actin through its repeat region that contains 8 out of the 11 lysines acetylated in cortactin. These residues represent a positive 'charged patch' region in deacetylated cortactin, which is required for F-actin binding. Accordingly, the charge-neutral hyperacetylated cortactin cannot bind F-actin. Only deacetylated cortactin can translocate to the cell periphery upon growth factor binding, while an acetylation mimetic mutant that can bind HDAC6, is defective in its ability to localize to the leading edge membrane. This mutant was also shown to reduce the rate of migration. In conflict with the Hsp90 activation of Rac1, cortactin and HDAC6 were

shown to be downstream of Rac1 activation. These results clearly show that HDAC6 catalytic activity is required for cell migration but induces effects on both actin and MT networks.

In direct contrast the migration of lymphocytes was shown to be independent of HDAC6 deacetylase activity, requiring only the physical presence of the enzyme (Cabrero et al., 2006; Hubbert et al., 2002). This is not surprising when one considers that in vivo motility of cells employs vastly different modes of motility for single cells (e.g., fibroblasts and other mesenchymal cells) than cells that move in sheets or layers (e.g., epithelial derived cells). Fibroblast or epithelial cells moving as part of a monolayer require MT-dependent polarization to generate a persistent direction of motility. However, single cells that tend to move more rapidly, e.g., leukocytes, lymphocytes or keratinocytes do not depend on intact MTs for their motility (Cabrero et al., 2006; Waterman-Storer and Salmon, 1999) and thus, clearly exhibit a mode of motility in which cellular polarization is not rate-limiting. It is of interest that in fibroblasts it was later shown that the catalytic and the BUZ domain deletion mutants were impaired in their ability to bind Hsp90, although they could localize to membrane ruffles (Kovacs et al., 2005). Intriguingly, the BUZ domain deletion mutant inhibited cell motility and ruffling to a similar extent as the catalytic inactive mutant indicating that deacetylase independent functions are also at play in fibroblast motility (Gao et al., 2007). Whether the BUZ domain regulates fibroblast motility in a MT-dependent or independent manner is however not known.

HDAC6 also plays an important role in the metastatic potential of many cancers. HDAC6, the product of an estrogen-regulated gene (Saji et al., 2005), was shown to directly interact with the estrogen receptor alpha (ERα) (Azuma et al., 2009). A membrane-targeted ERα protein was shown to associate with HDAC6 and MTs at the plasma membrane, thus inducing

local tubulin deacetylation. This in turn increased migration and proliferation of MCF-7 cells, a breast cancer cell line. This result is significant as HDAC6 upregulation in breast cancer is known to be an indicator of poor patient prognosis. HDAC6 activity is also required for the TGFβ-1 dependent epithelial-mesenchymal transition in many invasive tumors (Shan et al., 2008). Furthermore, HDAC6 interacts with the breast cancer metastasis suppressor 1, BRMS1, the destabilization of which by deacetylated Hsp90 was shown to decrease its tumor suppressor functions (Hurst et al., 2006).

HDAC6 activity may also play a role in hypoxia-induced angiogenesis, as HDAC6 interacts with and stabilizes Hypoxia-Inducible Factor 1α, HIF1α, which mediates transcription of genes needed for angiogenesis, in response to hypoxic conditions (Qian et al., 2006). In support of this idea, recent studies showed that HDAC6 activity promotes MT-dependent cell migration and angiogenesis in endothelial cells (Birdsey et al., 2012; Li et al., 2011a), and that this occurs through the MT plus-end binding protein, EB1 (Li et al., 2011a). On the other hand, deacetylation of cortactin by HDAC6 has also been reported to contribute to angiogenesis and cancer cell migration, thus implying a role for actin in these processes (Kaluza et al., 2011; Tsunoda et al., 2011).

Thus, multiple mechanisms govern the complex role of HDAC6 in modulating cell migration. As outlined above, a majority of substrates and interacting proteins are directly involved in regulating motility. Through the specific interactions derived here, HDAC6 not only alters directly the properties of both MTs and actin networks, but it also functions through deacetylase-dependent and -independent means of regulating cell motility.

Role in diverse biological processes

The ubiquitin-aggresome pathway, a hallmark of neurodegenerative diseases, is triggered by the aggregation of ubiquitinated proteins in excess of the cell's degradation capacity upon cellular stress (Boyault et al., 2006; Kawaguchi et al., 2003). HDAC6 is a required component of the ubiquitin-aggresome pathway. For example, Kawaguchi et al. showed that HDAC6 binds both dynein and polyubiquitinated proteins to mediate the MT-dependent transport of cytotoxic ubiquitinated aggregates to the aggresome (2003). The linking of polyubiquitinated cargo to the dynein motor by HDAC6 is essential for cell survival, as HDAC6 knockdown cells with increased accumulations of polyubiquitinated and misfolded proteins are prone to apoptosis. Moreover, both the deacetylase-independent ubiquitin-binding BUZ domain and the HDAC6 deacetylase activity are required for this dynein-mediated clearing of cytotoxic proteins.

HDAC6 function in the ubiquitin-aggresome pathway can be negatively regulated by its interactions with several proteins. In normal, unstressed cells, the chaperone protein, p97/VCP, and the phospholipase PLAP2 bind and sequester HDAC6 and mediate release of ubiquitinated proteins from the complex (Boyault et al., 2006; Seigneurin-Berny et al., 2001). The subsequent proteasomal degradation of these ubiquitinated proteins prevents aggresome formation. In fact, HDAC6, in complex with Hsp90 can also sequester the transcription factor HSF1 (Whitesell and Lindquist, 2005) which is required to induce a heat shock response in stressed cells (Boyault et al., 2006). Ubiquitin binding disrupts this complex, releasing HDAC6 to its aggresome function, and allowing HSF1 to translocate to the nucleus to induce transcription of heat shock genes. p97/VCP, however, may also disrupt aggresome formation by disorganizing the MT network.

HDAC6 has not been shown to deacetylate Hsp90 within the Hsp90/HSF1 complex. However, HDAC6 deacetylation of Hsp90 was previously reported to regulate its chaperone activity (Bali et al., 2005; Kovacs et al., 2005). Hyperacetylation of Hsp90 leads to its

dissociation from its co-chaperone, p23, thus impairing its ability to chaperone and cause

maturation of glucocorticoid receptor, ultimately decreasing glucocorticoid receptor function

(Kovacs et al., 2005). Via a similar mechanism, HDAC6 inhibition also affected Hsp90

chaperone function on several oncogenic client proteins, such as Bcr-Abl and Akt (Bali et al.,

2005).

TPPP/p25 and tau are two negative neuronal regulators of HDAC6 that work by directly

inhibiting HDAC6 deacetylase activity and thus allowing increased aggresome formation

(Guthrie and Kraemer, 2011; Perez et al., 2009; Tokesi et al., 2010). The TPPP/p25 interaction

with HDAC6 could be prevented by tubulin thus it is unlikely that TPPP/p25 interacts with

HDAC6 in the cytoplasm, where tubulin is abundant. However, TPPP/p25 has been shown to be

involved in Parkinson's disease, in which it accumulates in Lewy bodies, where HDAC6

inhibition could occur. Though the pharmacological inhibition of HDAC6 by TSA or tubacin did

not disrupt the tau-HDAC6 interaction it was shown to modify tau phosphorylation (Ding et al.,

2008). The hyper-phosphorylation of tau disrupts its MT binding and promotes its own

aggregation.

The interaction of parkin with HDAC6 and MTs also regulates the ubiquitin-aggresome

pathway. Parkin is an E3 ubiquitin ligase that is involved in Lewy body formation, an aggresome

like structure, in Parkinson's disease (Jiang et al., 2008). The translocation of parkin to the

centrosome upon proteasome inhibition (Kawaguchi et al., 2003; Kopito, 2000), was shown to be

HDAC6- and dynein-dependent (Jiang et al., 2008). The transport of parkin was found to be

important in its function for further ubiquitination and processing of the proteins undergoing

trafficking to the aggresome. The localization of parkin was found to be dependent on both MT

motors as the peripheral steady-state localization of parkin was mediated by kinesin-1.

Consistent with the findings of Kawaguchi et al. (2003), the MT-dependent bidirectional movements of parkin also require HDAC6 deacetylase activity (Jiang et al., 2008). Cortactin has been shown to be involved in HDAC6- and parkin-dependent clearing of ubiquitinated mitochondria (Lee et al., 2010) indicating that aggresome formation is also dependent on actin.

HDAC6 deacetylase activity and its ubiquitin-binding property regulate another important function in response to cellular stress. Stress granules are involved in the reversible suppression of protein translation to help avoid stress-induced cell death. HDAC6 was shown to bind ubiquitinated G3BP, a stress granule component, to mediate the dynein-dependent translocation of these proteins to stress granules (Kwon et al., 2007). Pharmacological inhibition or genetic ablation of HDAC6 abrogated the formation of stress granules, as did MT and dynein inhibitors. Thus, this process, which is directly parallel to aggresome formation, also requires both the deacetylase activity and ubiquitin-binding functions of HDAC6.

In addition, in response to DNA damage, HDAC6 blocks cell cycle progression and cytokinesis via a mechanism dependent on MTs and CYLD (Wickstrom et al., 2010). Interestingly, HDAC6 has been shown to localize to mitotic MT structures including the spindle, centrosomes and midbody (Zhang et al., 2003b). Since SIRT2 deacetylates tubulin during mitosis, it regulates the levels of Ac MTs even in HDAC6 null cells resulting in normal spindle formation and dynamics.

HDAC6 is also involved in the regulation of immune function in cells (Serrador et al., 2004). Formation of a proper immune synapse requires transient deacetylation of MTs upon initial contact with the target cell, as well as an increase in Ac MTs at later stages of synapse formation. HDAC6 overexpression, but not that of a catalytically inactive mutant, disorganized the immune synapse, while HDAC6 inhibition reversed this defect. Moreover, HDAC6

overexpression disrupted MTOC reorientation and IL-2 production. These data show a role for Ac MTs in immune synapse formation and MTOC reorientation, though the mechanism for this is not yet understood. Several viruses have also been shown to manipulate HDAC6 activity during infection (Valenzuela-Fernandez et al., 2008). Induction of MT acetylation near the cell cortex at the viral contact site seems to be a paradigm for viral infection in HIV and Herpes viruses. Ac MTs at cortical sites may stabilize viral contacts. Alternatively, decreased HDAC6 activity/increased acetylation may enhance MT-based translocation to the nucleus, as some viruses seem to prefer transport on Ac MTs. However, other viruses appear to require deacetylated MTs. HDAC6 has recently been shown to deacetylate the HIV transcriptional activator, Tat (Huo et al., 2011).

HDAC6 has been implicated in several other biological processes, including myogenesis, osteoclast maturation, and ciliogenesis. During myogenesis, dysferlin, a transmembrane protein that binds HDAC6 and α-tubulin and regulates the deacetylation reaction, links HDAC6 function to a progression of myogenesis (Di Fulvio et al., 2011). Elaboration of an oriented, acetylated array of MTs occurs before myoblast fusion (Gundersen et al., 1989). Generation of a stable, posttranslationally modified microtubule array is an early event in myogenic differentiation; these results argue that this array may be necessary for myogenic differentiation. Inhibition of HDAC6 by tubastatinA, a tubacin congener, early in myogenic differentiation impaired myotube fusion and concomitantly increased nocodazole and cold-resistant Ac MTs that were not oriented intracellularly (Di Fulvio et al., 2011). We previously showed that precluding the elaboration of detyrosinated (Glu) MTs abrogated myotube formation and blocked the synthesis and/or activation of key myogenic factors (Chang et al., 2002). Thus, it is not surprising that

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dysregulation of HDAC6 and MT acetylation early in the myogenic program is sufficient to disrupt morphological muscle differentiation.

HDAC6 is involved in bone development; stimulation of HDAC6 by mDia2 prevents osteoclast maturation, by disrupting formation of the podosome belt that is dependent on Ac MTs, thus leading to bone defects (Destaing et al., 2005). In contrast, HDAC6 null mice showed slightly increased bone density (Zhang et al., 2008). Finally, HDAC6 is also involved in ciliogenesis through BBSome function. BBSomes are protein complexes whose loss or malfunction causes Bardet-Biedl syndrome; the BBSome interacting protein, BBIP10, also interacts with HDAC6 and regulates MT acetylation. BBIP10's role is unique, as other subunits of the BBSome are principally involved in the generation and function of the primary cilium (Loktev et al., 2008). Independent of its HDAC6 interaction, BBIP10 also binds directly to MTs, enhancing their stability and therefore further enhancing MT acetylation. BBIP10 is also thought to play a role in centrosome cohesion, though whether this is a result of binding to centrosomal MTs is unknown. The significance of these results to ciliogenesis or other functions remains to be determined, since HDAC6-null animals do not show defects in ciliogenesis (Zhang et al., 2008).

Concluding remarks

Though a role of MT acetylation is implicated in cell migration (Haggarty et al., 2003; Hubbert et al., 2002; Lafarga et al., 2012; Tran et al., 2007), the underlying mechanisms by which HDAC6-mediated deacetylation of tubulin modulates directional cell motility have not been shown. Here we show our novel results that HDAC6 activity on its substrate tubulin

regulates polarization of subcellular organelles and intracellular transport towards the leading

edge required for persistent directional migration.

Chapter 2

HDAC6 activity is required for polarization of cells and

intracellular trafficking during directional migration

INTRODUCTION

The deacetylase HDAC6 is involved in chemotactic or wound-healing motility of fibroblastic, epithelial, and endothelial cells (Birdsey et al., 2012; Hubbert et al., 2002; Lafarga et al., 2012), all of which require an intact MT cytoskeleton for cell migration and polarization (Aldana-Masangkay and Sakamoto, 2011; Gotlieb et al., 1983; Valenzuela-Fernandez et al., 2008). Furthermore, HDAC6, which is upregulated in many cancers, has been linked to the metastatic potential of tumors (Aldana-Masangkay and Sakamoto, 2011; Saji et al., 2005). In previous studies, fibroblasts subjected to pharmacological or genetic treatments that either increase (Hubbert et al., 2002) or decrease (Haggarty et al., 2003; Tran et al., 2007) the activity of HDAC6 showed a corresponding increase or decrease in cell motility in a chemotactic invasion assay, thus establishing the importance of HDAC6 deacetylase activity in motile cells. Strikingly, unlike for other HDACs, alpha-tubulin was found to be the most abundant HDAC6 substrate (Hubbert et al., 2002; Matsuyama et al., 2002; Zhang et al., 2003b). However, despite an evident functional link between HDAC6, MTs and cell motility, the mechanism(s) by which α-tubulin deacetylation by HDAC6 impacts on directional migration remain unclear.

MTs are not required for the mechanics of cell crawling, which is dependent upon the actin microfilament system. Instead, ablation experiments have shown that MTs are critical for polarizing cellular contents and intracellular trafficking, thereby generating and maintaining a leading edge lamellipodia to provide directional persistence of motility (Bershadsky et al., 1991; Glasgow and Daniele, 1994; Wittmann and Waterman-Storer, 2001). MT-dependent cell polarization has been studied most intensively in the wound-healing model of motility (reviewed by Noritake et al., 2005), in which cells migrate to close a wound that has been scratched into a monolayer of fibroblasts.

An initial step in polarizing fibroblasts for wound-healing motility is the selective stabilization and subsequent posttranslational modification by detyrosination (Gundersen and Bulinski, 1988) and acetylation (Akhmanova et al., 2001) of a subset of their MTs whose distal (plus) ends are closest to the wound edge. Reorientation of the MT-organizing center (MTOC) and Golgi apparatus towards the wound-edge (Kupfer et al., 1982) is known to be important for polarized membrane trafficking towards the leading edge (Bergmann et al., 1983) and the stable, modified MT array was proposed to provide a template for polarization of these organelles (Bulinski and Gundersen, 1991; Kirschner and Mitchison, 1986). These classic experiments raised two questions about how MT arrays function to polarize wound-edge cell components: First, how does a subset of MTs that faces the wound-edge become stabilized? Second, how does the post-translational modification of the newly stabilized MT subset alter its functions, e.g., what role do posttranslationally modified MTs play in polarizing MT-dependent transport of organelles and plasma membrane vesicles?

A wealth of information now effectively answers the first question: Stabilization of MTs facing the wound-edge is mediated by capping of their distal (plus) ends (Infante et al., 2000), via one of two distinct molecular mechanisms. Akhmanova et al. (2001) showed that two +TIPs, that is, proteins that bind to the plus ends of growing MTs, attach as a CLIP170-CLASP complex that then docks in the cell cortex with an LL5β and ELKS receptor complex (Lansbergen et al., 2006). The second mechanism, discovered concurrently, consists of RhoA stabilization through mDia (Palazzo et al., 2001a), with binding and stabilization via the additional +TIPs, EB1 and APC (Wen et al., 2004). Since both pathways are regulated analogously by the kinase, GSK3β (Eng et al., 2006; Lansbergen et al., 2006), and since HDAC6 interacts with both GSK3β (Chen

et al., 2010) and mDia (Bershadsky et al., 2006; Destaing et al., 2005) it is possible that HDAC6 protein and perhaps its deacetylation activity could modulate wound-edge MT stabilization.

The second question, that is, how the post-translational modification state of the wound-edge MT subset alters its functions, has not been as well-explored. One means by which detyrosinated and/or acetylated tubulin subunits along the length of stable MTs could signal the stability of that MT or the location of its plus end is for these modified subunits to modulate MT-dependent polarization and transport. For example, MTOC reorientation, independent of MT stabilization (Palazzo et al., 2001b), occurs by a capture of MT plus ends by p150glued, which is a +TIP and a dynein receptor (Vaughan, 2005). p150glued then docks to dynein anchored in the cortex at the leading edge of motile cells (Dujardin et al., 2003; Gomes et al., 2005). Moreover, Golgi reorientation occurs via one or a combination of several mechanisms. Golgi polarization could occur either through its close association with the MTOC (Kupfer et al., 1982), its preferential recognition of posttranslationally modified MT arrays (Skoufias et al., 1990; Thyberg and Moskalewski, 1993) or its nucleation of MTs (Efimov et al., 2007), especially stable MTs destined for acetylation or detyrosination (Chabin-Brion et al., 2001). Thus, differential recognition of posttranslationally modified MTs by MT-associated proteins (MAPs) and MT motor proteins might be sufficient to polarize cellular contents during directional migration. There is suggestive evidence for this hypothesis. For example, intracellular transport was shown to be inhibited in Taxol-treated cells (Hamm-Alvarez et al., 1994; Hamm-Alvarez et al., 1993). Taxol increases both the cellular density of MT polymers and the content of posttranslationally modified acetylated and detyrosinated subunits within MTs (Gundersen et al., 1987). In vitro biochemical studies of the kinesin-MT interaction showed modestly increased affinity and MT-activated ATPase activity for MTs polymerized with pure detyrosinated tubulin,

as compared to tyrosinated tubulin (Liao and Gundersen, 1998). Similarly, microinjection of IgGs specific for detyrosinated tubulin inhibit kinesin-mediated anterograde transport of kinesin cargoes, suggesting a preference of kinesin for the population of MTs that is enhanced in detyrosinated tubulin (Gurland and Gundersen, 1995; Kreitzer et al., 1999; Lin et al., 2002).

MT acetylation, like detyrosination, has also been reported to increase intracellular trafficking. Using MTs from ciliary axonemes or chemically acetylated mammalian brain MTs, kinesin-1 and dynein were found to bind acetylated MTs with higher in vitro binding affinities (Dompierre et al., 2007; Reed et al., 2006). In vivo, hyperacetylation of MTs in HDAC6-inhibited cells increased polarized kinesin-1-based transport of JIP1 and increased bidirectional transport of BDNF vesicles in neuronal cells (Dompierre et al., 2007; Reed et al., 2006). However, since both of these studies used heterologous MT preparations and HDAC6 inhibitors, they are hard to extrapolate to the situation in motile, genetically null fibroblasts. In that system, altered MT function by HDAC6 inhibition or deletion was linked to decreased focal adhesion turnover; an effect mediated by moderately stabilized MTs (Tran et al., 2007).

Recently, GRK2, a G-protein coupled receptor, was shown to contribute to regulation of HDAC6-mediated modulation of MT acetylation, in turn enhancing directional migration of fibroblasts and epithelial cells (Lafarga et al., 2012). GRK2 and HDAC6 were colocalized at the leading edge of migrating cells, and inhibition of HDAC6 by GRK2 mutants impaired cell migration and protrusive activity. Furthermore, a tight regulation of acetylated MT levels by the GRK2-HDAC6 interaction was found to be important for both early and late phases in spreading cells. Cortactin acetylation was unaltered by HDAC6 activity in these cells, but a scenario in which altered HDAC6 deacetylation of Hsp90 regulated motility through regulation of the actin network was not ruled out.

Thus, based upon the fact that both MT-dependent cellular polarization and HDAC6 deacetylase activity are important for directionally migrating cells, and that tubulin is a major substrate of HDAC6, we hypothesized that HDAC6 activity towards tubulin/MTs is required for efficient polarization of motile cells. However, although hyper-acetylated MTs have been implicated in decreasing directional motility of HDAC6-inhibited fibroblasts, other in vivo deacetylation substrates of HDAC6 may also function in cell migration; for example, Hsp90 (Bali et al., 2005; Kovacs et al., 2005) and/or cortactin (Zhang et al., 2007). Indeed, both cortactin (Zhang et al., 2007) and Hsp90 (Gao et al., 2007) were shown to affect cell motility via regulation of actin-dependent processes. Therefore, delineating the role of HDAC6 in directional motility warrants consideration of the acetylation state and role(s) in motility of all of HDAC6 substrates and dissection of the mechanism(s) by which HDAC6 activity acts in the motility process.

RESULTS

Loss of HDAC6 and its activity decrease directional migration.

To test whether the loss of HDAC6 impacts directional migration of fibroblasts, we compared the wound-healing motility of mouse embryo fibroblasts (MEFs) genetically null for HDAC6 (HDAC6 KO MEFs) prepared from viable HDAC6 (-/y) mice (Zhang et al., 2008), to wild-type fibroblasts (WT MEFs) prepared from HDAC6 (+/+) littermates. HDAC6 KO MEFs showed greatly decreased ability to migrate into an experimental wound; Fig. 1A shows that speed of wound closure was reduced by more than 60%, compared to WT MEFs. We corroborated these results with live cells; we examined motility of HDAC6 WT and KO MEFs by live imaging using phase microscopy (see supplementary movies). These wound-healing movies were consistent with results from fixed-cell assays; both clearly show that HDAC6 KO MEFs are impaired in their directional migration into the wound, showing instead more random migration than did WT MEFs. However, membrane ruffling at the leading edge appeared similar in HDAC6 WT and KO MEFs, suggesting that actin-dependent processes in migrating cells are not altered by the loss of HDAC6.

As predicted, HDAC6 KO MEFs showed a level of acetylated (Ac) tubulin much increased over WT MEFs, an even higher level than that observed in HDAC6 WT MEFs treated with TSA, a general Class I and II HDAC inhibitor, for 4 hours (Fig. 1B). Importantly, the level of detyrosinated or Glu tubulin, a reporter of MT stabilization (Gundersen et al., 1987) was unaffected by loss of HDAC6 activity in both HDAC6 KO and TSA-treated WT MEFs. However, both HDAC6 KO and WT MEFs showed a similar capacity to generate Glu tubulin in response to Taxol treatment. In contrast, Taxol treatment did not detectably alter the level of Ac tubulin in WT MEFs. These results confirm our finding that, at commonly applied

concentrations (1 µM for both) Taxol stabilizes MTs more potently than TSA (Tran et al., 2007), and that TSA treatment is amenable to a dissection of the role of microtubule acetylation in wound-healing motility, independent of any role of detyrosination.

Chemotactic invasion motility of a different cell type, lymphocytes, which like other fast moving cells do not require intact MTs or polarization of their contents for their motility (Cabrero et al., 2006; Waterman-Storer and Salmon, 1999) is dependent on level, not deacetylase activity, of HDAC6 (Cabrero et al., 2006). Accordingly, we compared the wound-healing migration of HDAC6 inhibited WT MEFs, which have normal level but reduced activity of HDAC6, to KO MEFs which have no HDAC6 protein or activity. As shown in Fig. 1C, treating WT MEFs with trichostatin A (TSA), to inhibit all class I and II HDACs, including HDAC6 (Hubbert et al., 2002; Matsuyama et al., 2002), significantly reduced the rate of wound closure. TSA effects were dose-dependent (data not shown), but we used 1 µM TSA in all experiments, to maximize the level of hyper-Ac tubulin. In contrast, treatment with sodium butyrate (NaB), to inhibit all class I and II HDACs except HDAC6 and HDAC10 (HDAC10 has no known substrates) (Guardiola and Yao, 2002), did not alter wound-healing rate, relative to the control (Fig.1C).

Notably, neither TSA nor NaB changed wound-healing migration rate of HDAC6 KO MEFs (Fig. 1C). These data indicate that activities of all other HDACs, except HDAC6, that would be inhibited by these drugs are not important for wound-healing migration. Furthermore, since enzymatically inactivated HDAC6 can associate with MTs (Haggarty et al., 2003), or modulate cellular functions (Cabrero et al., 2006; Zilberman et al., 2009), the fact that results from TSA-treated WT and HDAC6 KO MEFs are indistinguishable suggests that it is the

deacetylase activity of HDAC6, rather than a structural role of inactive HDAC6, which mediates

the inhibition of directional motility.

Loss of HDAC6 increases the acetylation of tubulin, but not Hsp90 and cortactin.

To understand the role of HDAC6 deacetylation activity, it is necessary to determine

which hyper-Ac substrate proteins might accumulate and affect cell migration. HDAC6 has

several known substrates; although the best-characterized of these is tubulin (Hubbert et al.,

2002; Matsuyama et al., 2002; Zhang et al., 2003b), other well-known substrates are histones

(Zhang et al., 2003b), the chaperone protein, Hsp90 (Bali et al., 2005; Kovacs et al., 2005), and

the actin-binding protein, cortactin (Zhang et al., 2007). In fact, the latter two, Hsp90 (Gao et al.,

2007) and cortactin (Zhang et al., 2007), have both been shown to enhance cell motility by

modulating actin. We therefore analyzed which HDAC6 substrates, besides tubulin, became

hyper-acetylated, both under the drug regimens we used to reduce HDAC6 activity in motile

MEFs and in HDAC6-null fibroblasts. In previous work, we showed that TSA treatment

substantially increased acetylation of tubulin, as well as histones, but not Hsp90 (Tran et al.,

2007). Here, we used the same Keyhole limpet Ac protein (KLAC) antibody, designed to react

with all Ac-lysines irrespective of the sequence of the flanking amino acids, to determine which

HDAC substrate proteins, in addition to tubulin, become hyper-acetylated in MEFs lacking

HDAC6.

As shown in Fig. 2, Hsp90 acetylation was not detectably greater in HDAC6 KO than in

WT MEFs, even when the former were treated with TSA suggesting that Hsp90 is not

deacetylated exclusively by HDAC6 or other classical HDACs (Fig. 2A). In contrast, tubulin was

more Ac in HDAC6 KO than in WT MEFs but was not further increased in acetylation level in

TSA-treated HDAC6 KO MEFs (Fig. 2A), corroborating the identification of HDAC6 as the only HDAC that deacetylates tubulin (Matsuyama et al., 2002). Figure 2A also documents that hyper-acetylation of histones did not occur in HDAC6 KO cells except when they had been treated with TSA. This corroborates results of Zhang et al. (2008); although HDAC6 has deacetylation activity towards histones (Zhang et al., 2003b) many other HDACs continue to deacetylate histones in the absence of HDAC6.

Similar to Hsp90 and histones, immune-precipitated cortactin also showed the same degree of acetylation in HDAC6 KO MEFs compared to WT MEFs (Fig. 2B). We tested the specificity of the KLAC antibody for lysine ε-amino group acetylation by preabsorbing KLAC with N-epsilon-acetyl-L-lysine. Cortactin, is a deacetylation substrate of either HDAC6 or SIRT2 (Zhang et al., 2007), so it is important to evaluate its acetylation state in HDAC6 KO cells. We therefore performed KLAC antibody-labeled western blots of cortactin IPs, following treatment with inhibitors of other deacetylases (Figure 2C). Inhibition of HDAC6 KO MEFs with TSA, to block HDACs 1-11, did not change cortactin acetylation level, but inhibition of all the sirtuin family of NAD-dependent deacetylases (SIRTs) with sirtinol yielded increased acetylated cortactin (Fig. 2C). This result agrees with the finding that cortactin is a deacetylation substrate of either HDAC6 or SIRT2 (Zhang et al., 2007), and further suggests that SIRT2 compensates for increased acetylation of at least one HDAC6 substrate in the absence of HDAC6 background. Taken together, these results show that tubulin, but not Hsp90, histones, or cortactin, is exclusively dependent upon HDAC6 activity for its deacetylation in MEFs. Importantly, these data also suggest that in wound-healing of MEFs that lack HDAC6 activity, MTs are hyper-Ac and could impact motility, while the other HDAC6 substrate proteins, Hsp90 and cortactin are unchanged in acetylation level and thus unlikely to affect motility of HDAC6 KO MEFs.

Loss of HDAC6 activity yields abnormal acetylated MT arrays.

We hypothesized that hyper-acetylation of all cellular MTs precludes formation of a polarized array of Ac MTs during directional migration. To test this hypothesis we treated wound-edge WT and HDAC6 KO MEFs with vehicle alone (DMSO), with TSA, and with tubacin. We examined the distribution of Ac MTs by immunofluorescence with Ac tubulin antibody. Tubacin, a small molecule that selectively inhibits HDAC6 increases Ac-tubulin without altering Ac-histones (Haggarty et al., 2003), Ac-Hsp90 (Tran et al., 2007) or Ac-cortactin (Lafarga et al., 2012). Further, tubacin has been shown to be as effective as TSA, in blocking chemotactic invasion motility of fibroblasts (Tran et al., 2007). Consistent with our prediction, complete ablation of HDAC6 expression through gene knock out, inhibition of all HDACs via TSA-treatment, or specific inhibition of tubulin deacetylation by blocking HDAC6 with tubacin (Figs. 3A and B, Supplementary Fig. 1A, and Supplementary Fig. 2A, respectively), all yielded wound-edge cells with hyper-Ac MTs that lacked a polarized array of Ac MTs.

However, while levels of tubulin hyper-acetylation were greatly increased in TSA-treated WT or HDAC6 KO MEFs (Fig. 1B), tubacin treatment, though equivalent in the extent of mislocalization of oriented Ac MT arrays (compare Fig. 3A and Supplementary Fig. 1A to Supplementary Fig. 2A), was less effective in inducing hyper-acetylation of tubulin (Ac-tubulin levels were increased by as much as 67% in TSA treatment; Supplementary Fig. 2B). Treatment with Taxol to stabilize all MTs instead of only a polarized subset also mislocalized the Ac MT array in a majority of cells (data not shown), even though Taxol treatment did not increase MT acetylation level, as detected by western blot (Fig. 1B) or immunofluorescence (data not shown). This result shows that, irrespective of the magnitude of increase in MT acetylation, either

HDAC6 inhibition or depletion disrupts the selective stabilization of the Ac MT subset towards the wound-edge. Moreover, Taxol, whose potent stabilization of MTs causes a significant increase in Glu tubulin (Gundersen et al., 1987), also mislocalizes the Ac MT subset. However, HDAC6 inhibition prevents the polarization of the Ac MT array independent of any major effects on MT stabilization (Tran et al., 2007) or detyrosination.

Loss of HDAC6 activity prevents polarization of subcellular organelles.

We originally proposed that post-translational modification of a subset of MTs functions to scaffold reorientation of other organelles (Bulinski and Gundersen, 1991). We next tested this hypothesis, asking whether the failure to polarize an Ac MT array affects polarization of each of four other subcellular components: 1) the stable MT subset, which is identified by its heightened content of Glu tubulin (Gundersen et al., 1987); 2) the MTOC, which rapidly reorients during wound-healing motility by a mechanism independent of the reorientation of the stable MT array (Palazzo et al., 2001b); 3) the Golgi complex, whose polarization appears to polarize vesicular transport and secretion to the leading edge (Bergmann et al., 1983); and 4) the vimentin intermediate filament array, whose reorientation towards the leading edge of motile cells requires kinesin and a polarized Glu MT subset (Gurland and Gundersen, 1995; Kreitzer et al., 1999). As shown in Figs. 4A and 4B, all four organelles examined were mislocalized in HDAC6 KO, as compared to WT, MEFs. HDAC6 KO MEFs were affected similarly in organelle reorientation (Fig. 4B) and wound-healing motility speed (compare Fig. 4B to Fig. 1A). For example, ablation of HDAC6 expression decreased polarization of the Golgi by ~60%; similar to the decrement in wound-closure speed. Each of the four organelles we monitored also failed to orient towards the leading edge in A549 HDAC6 siRNA (HDAC6 KD) cells (data not shown) and in TSA-treated

WT and KO MEFs (Supplementary Fig. 1B). These data suggest that either inhibition or loss of

HDAC6 activity inhibits organelle polarization, suggesting that these MT-dependent events may

result from MT hyperacetylation or failure to orient an Ac MT array towards the leading edge.

Moreover, failure to polarize cellular contents may be coupled to a decrease in directional

migration.

We also tested the effects of treating WT MEFs with the HDAC6-selective inhibitor,

tubacin (Haggarty et al., 2003). Tubacin treatment yielded a significant disruption in polarization

of all four organelles examined (Supplementary Fig. 2C). However, the extent of inhibition was

less than that seen in HDAC6 KO MEFs, or in TSA-treated WT MEFs (compare Figs. 4B, Supp.

Fig. 1B, and Supp. Fig. 2C). These results are consistent with the fact that, in our hands, tubacin

yields a lower level of MT hyper-acetylation than does TSA treatment or complete ablation of

HDAC6, as in HDAC6 KO MEFs (Supplementary Fig. 2B). This and other results show that the

degree to which organelles fail to reorient in wound-edge cells is always commensurate with

their degree of MT hyper-acetylation.

Loss of HDAC6 disrupts dynein-mediated cortical attachment of MTs via the +TIP

p150glued

Redistribution and accumulation of cortical proteins at the leading edge is an early step in

the polarization process that reorients the MTOC in response to the monolayer wounding

stimulus (Dujardin et al., 2003; Lansbergen et al., 2006). The MT +TIP, p150glued, a subunit of

the dynactin complex, mediates selective capture of MT plus ends by dynein localized in the cell

cortex at the leading edge of migrating cells (Dujardin et al., 2003). The p150glued-dynactin

interaction has been implicated in MTOC reorientation, (Etienne-Manneville and Hall, 2001;

Gomes et al., 2005; Palazzo et al., 2001b), and persistent directional migration (Dujardin et al., 2003), independent of selective MT stabilization. Moreover, HDAC6 has been shown to directly bind the retrograde MT motor dynein (Kawaguchi et al., 2003) and interact with p150glued (Hubbert et al., 2002; Kawaguchi et al., 2003). To address the mechanism underlying the defect we observed in MTOC positioning in directionally migrating cells, we localized both p150glued (Figs. 5A and B) and its cortical docking receptor, dynein (Figs. 5C and D) in WT and HDAC6 KO MEFs. HDAC6 KO MEFs showed greatly reduced p150glued localization on MT plus ends (~ 45%, Figs. 5A and B), while they showed similarly reduced cortical dynein at the leading edge cell cortex (also ~ 45%, Figs. 5C and D). While the few small and dim cortical patches of dynein in KO cells (Fig 5C) almost entirely coincided with MTs converged at the leading edge (data not shown), the converse was not true; sites at which MT distal ends converged in the lamellipodia showed markedly reduced cortical dynein (Fig. 5C and D) in HDAC6 KO MEFs as compared to their WT counterparts. These data suggest that loss of HDAC6 decreases MT-cortex interactions interfering with the p150glued-dynein pathway. Not only do these results provide a plausible mechanism for the MTOC reorientation defect, they also suggest that loss of HDAC6 might impact on other dynein pathways.

Loss of HDAC6 activity decreases bidirectional Golgi vesicle transport.

Polarization of membrane trafficking towards the leading edge mediates polarized membrane renewal during directional migration (Bergmann et al., 1983). Moreover, bidirectional transport mediated by kinesin-1 and dynein was reported to occur preferentially on Ac MTs and to increase on hyper-acetylated MTs (Dompierre et al., 2007; Reed et al., 2006). The MT receptor of dynein, p150glued, that is part of the dynactin complex, helps load dynein onto MTs

(Vaughan, 2005) and also serves as a processivity factor for dynein motility (King and Schroer, 2000). From our result that p150glued was decreased in binding to MT ends in HDAC6 KO, compared to WT MEFs, and that HDAC6 directly binds dynein (Kawaguchi et al., 2003) we hypothesized that MT-dependent dynein transport is impaired by loss of HDAC6. To test this hypothesis, we analyzed Golgi vesicle trafficking, known to be mediated by dynein retrograde motility (Vaughan, 2005), by live-imaging of Golgi vesicles fluorescently-labeled with BODIPY ceramide, a trans-Golgi lipid marker that labels Golgi vesicles that undergo bidirectional transport (Klausner et al., 1992).

While steady-state Golgi structure did not appear to be greatly altered (see Fig. 7A), a marked change was observed in the proportion of the total vesicles that showed motility during the 5-minute recordings: the percentage of motile Golgi vesicles was reduced by ~50% in HDAC6 KO, compared to WT MEFs (Fig. 6A). Quantification of vesicle motility from time-lapse images revealed significant but modest inhibition of vesicle trafficking, also in agreement with our hypothesis. In fact, quantification of vesicle motility parameters in HDAC6 KO MEFs showed that all vesicle motility parameters measured, except for run time, were significantly decreased in retrograde or dynein-mediated transport (Fig. 6B). Surprisingly, a similar decrement in all vesicle motility parameters was also measured in anterograde or kinesin-based MT transport of Golgi vesicles (Fig. 6B).

Quantification of Golgi vesicle transport in wound-edge migrating HDAC6 KO MEFs gave parallel reductions (see supplementary movies, data not shown). Moreover, TSA-treated L6 myoblast cells we examined previously showed a similar inhibition in bidirectional Golgi vesicle transport as compared to the DMSO treated and NaB controls (data not shown). These data suggest that HDAC6 deacetylase activity perturbs MT-dependent dynein-based vesicle motility

in vivo. However, from these experiments we cannot determine whether parameters of MT-dependent kinesin-based transport are inhibited directly by interference with kinesin or indirectly by decreased dynein-mediated transport or stalling of dynein-cargo complexes on MTs.

Loss of HDAC6 inhibits both dynein- and kinesin-mediated transport processes.

From the bidirectional inhibition of MT-based Golgi vesicle transport in HDAC KO cells, we hypothesized that loss of HDAC6 activity manifests direct effects on dynein- and/or kinesin-based transport along MTs. To test whether one or both motors is directly inhibited we investigated two canonical dynein- and kinesin-specific intracellular transport events.

We first analyzed dynein-mediated retrograde movement of Golgi elements as they clustered and reformed during recovery from treatment with brefeldin A (BFA) (Bulinski et al., 1997; Burkhardt et al., 1997). BFA induces a MT- and kinesin-dependent (Feiguin et al., 1994; Lippincott-Schwartz et al., 1995) redistribution of the Golgi into the peripheral ER, completely dispersing the Golgi apparatus into distinct peripheral puncta (Klausner et al., 1992; Lippincott-Schwartz et al., 1989).

Analysis of Golgi clustering, performed by immunofluorescence with Golgi antibody, at steady-state in WT and HDAC6 KO MEFs (Fig. 7A) showed that although most cells of both types clustered around the periphery of the nucleus, a significant proportion of HDAC6 KO MEFs exhibited Golgi staining along a greater circumference of the nucleus, rather than a tight clustering along one side of the nuclear periphery (Fig. 7A). A brief treatment with BFA dispersed the Golgi in both WT and HDAC6 KO and MEFs; the less pronounced dispersal in HDAC6 KO MEFs suggested an inhibition of the kinesin motor responsible for transporting the Golgi vesicles into the ER (Fig. 7A) consistent with data from Lippincott et al. (1995).

66

Immunofluorescence and quantification during recovery from BFA treatment showed inhibited Golgi re-clustering in the HDAC6 KO MEFs (Figs. 7A, B). For example, clustered Golgi were again evident in many WT MEFs 15 minutes after BFA release and in a significant majority of cells at 45 minutes (Fig. 7B). In contrast some Golgi had not yet re-clustered even at 3 hours after BFA release in HDAC6 KO MEFs (Fig. 7B). These data confirm and extend Golgi vesicle motility data (Fig. 6), showing that MT-dependent dynein-based vesicular transport is inhibited by genetic ablation of HDAC6, while suggesting that kinesin-dependent vesicular transport may also be affected in our system.

To determine whether kinesin-mediated transport is altered, we next assayed transport of canonical kinesin cargoes, the mitochondria. Mitochondrial distribution and movement in axons has been shown to be dependent on kinesin-1 (Chen et al., 2010; Hollenbeck and Saxton, 2005). We tested kinesin-based transport directly in two ways: Initially we examined distribution of mitochondria at steady-state in sparse and wound -edge WT and HDAC6 KO MEFs. No difference in mitochondrial distribution was measured when we calculated the degree to which mitochondria spread towards cell edges (mitochondrial spread area was calculated as a proportion of total spread area of MEFs; see Materials & Methods) in sparse cells at steady-state (data not shown). However, the quantification of both the mitochondrial area spread towards the leading edge (Supplementary Fig. 3) and the extent of the polarization of this organelle towards the leading edge which was similar to the extent polarization of other organelles examined in Fig. 4B (data not shown) showed a significant decrease in wound-edge HDAC6 KO MEFs.

We also hypothesized that mitochondrial kinesin-based anterograde trafficking is directly inhibited by loss of HDAC6. To test this, we quantified mitochondrial spread area over time in spreading MEFs. Compared to WT MEFs, HDAC6 KO MEFs spread more rapidly, as they

accumulate focal adhesions that turn over slowly (Tran et al., 2007, also see Supp. Fig. 4).

Accordingly, we quantified mitochondrial area as a proportion of total cell area, to compensate

for inherent cell size differences during spreading. We also confined our measurements to early

times during spreading (0-20 minutes) because at these times the HDAC6 KO and WT MEFs are

similar in size, and also, mitochondria show net movement only in the anterograde direction that

would be attributable to kinesin. Fig. 7C shows that consistent with our hypothesis, we observed

a significant inhibition of mitochondrial transport at early time points in the cell spreading

process. Kinesin-based mitochondrial transport may also be altered when cells are more

completely spread, but the interpretation of these data is confounded by the increased surface

area of HDAC6 KO MEFs. Thus, we confined our analysis to the initial 20 minutes of spreading

which show that anterograde mitochondrial movement, a canonical kinesin cargo, was

specifically and independently inhibited in the absence of HDAC6 protein.

Re-expression of HDAC6 or non-acetylatable tubulin rescues Golgi vesicle motility.

Re-expression of full-length HDAC6 in HDAC6 KO MEFs was technically challenging

in wound-edge cells; the low efficiency of transfection precluded qualitative and quantitative

analysis of directional migration. Therefore, we used the single-cell assay of Golgi-vesicle

transport, as it is amenable to testing the effects of HDAC6 re-expression. We quantified

bidirectional vesicle motility in HDAC6 KO MEFs transfected with HDAC6 plus GFP,

transfecting GFP alone as a control. HDAC6 KO cells re-expressing HDAC6 showed rescued

Golgi vesicle motility parameters in both directions (compare Fig. 8A to Fig. 6B) showing

irrefutably that the alteration of MT-dependent bidirectional vesicular transport in HDAC6 KO

MEFs is HDAC6-specific.

Finally, since a loss in HDAC6 activity results in hyper-Ac MTs, we tested whether a prevention of MT hyper-acetylation prevents the MT-dependent defects in intracellular transport caused by HDAC6 inhibition or loss. To create HDAC6 KO MEFs in which MTs are not highly acetylated, we expressed GFP plus K40R-FLAG-α-tubulin into sparse HDAC6 KO MEFs; we also expressed GFP plus WT-FLAG-α-tubulin and GFP alone, as controls. K40R is a non-acetylatable tubulin mutant, whose arginine mutation prevents the acetylation that normally occurs at lysine 40 of α-tubulin. We cloned the K40R into a FLAG-tagged-α-tubulin rather than into GFP-α-tubulin as is commonly used (Creppe et al., 2009; Gao et al., 2010) as we previously determined that GFP-α-tubulin is poorly acetylated, if at all, in vivo, possibly because of the bulky, N-terminal GFP tag (Weil, Marmo, and Bulinski, data not shown).

We subjected HDAC6 KO MEFs expressing each of these constructs to fluorescent labeling to examine effects on vesicle motility. Because these experiments present significant technical obstacles, we used only the most robust assay of Golgi vesicle motility, that is, a quantification of the proportion of motile vesicles in HDAC6 KO MEFs (an ~50% inhibition as seen in Fig. 6A). Quantification of the proportion of motile vesicles in cells transfected with each of the tubulin constructs showed that both WT and K40R tubulins equivalently rescued the defect in Golgi vesicle motility (Fig 8B). This result clearly demonstrates that a MT-based mechanism is responsible for inhibited bidirectional intracellular trafficking in HDAC6 KO cells. However, it is somewhat surprising that the wild-type α-tubulin achieved as effective a rescue as did the K40R mutant. We cannot conclude from these data whether some overall increase in α-tubulin, rather than a modulation of the acetylation state of lysine 40 is responsible for impacting motor-based vesicular transport in the absence of HDAC6.

Chapter 2 - Figures

Figure 1

A
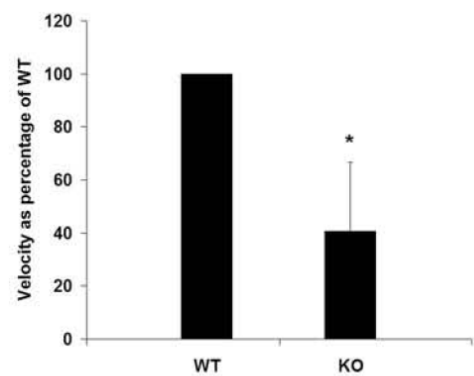
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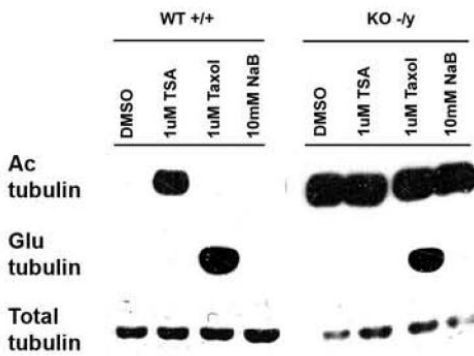
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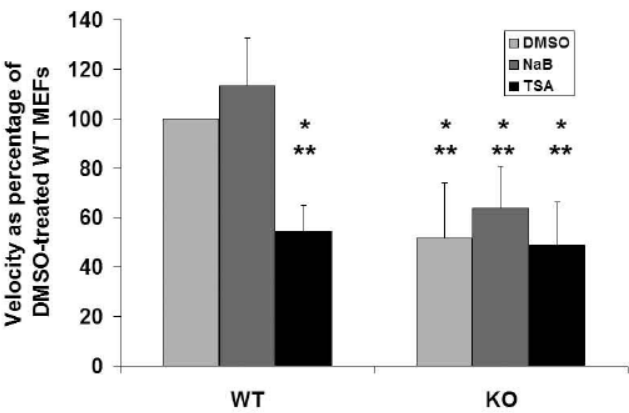
Figure 1. Loss of HDAC6 activity decreases directional wound-healing migration.

Quantification of wound-healing velocities of **A)** DMEM and **C)** drug-treated WT and HDAC6 KO MEFs. Scratch wounds were made in fibroblast monolayers and the rate of wound closure was quantified from coverslips fixed at regular time intervals between 0 and 10 hours (see Materials and Methods for details). The indicated drugs, DMSO, 1 μM TSA and 10mM NaB were added to the cultures at the time of wounding and maintained for the duration of wound-healing migration, i.e., for a total of 10 hours. In A) and C), migration velocities are represented as percent of control, i.e., WT MEFS and DMSO-treated WT MEFs, respectively. Asterisks indicate significant differences ($p < 0.05$) from controls. **B)** Western blot analysis of drug-treated WT and HDAC6 KO MEFs, blots were probed with antibodies against **Ac**, **Glu** and **total tubulin** (β-tubulin used to label total tubulin as a loading control). Extracts were made after **6 hour** treatments to ensure that cell polarization after the onset of migration had occurred.

Figure 2

A

| | WT +/+ | KO -/y | |
| | DMSO | DMSO | 1uM TSA |

Ac Hsp90

Ac tubulin

Ac histone

Total tubulin

B

IP anti-cortactin

WT +/+   KO -/y

IB anti-KLAC

IB anti-cortactin

WT +/+   KO -/y

IB anti-KLAC
(preabsorbed with
N-ε-Ac-L-K)

IB anti-cortactin

C

IP anti-cortactin

| WT +/+ | KO -/y | | |
| DMSO | DMSO | 1uM TSA | 50 uM Sirtinol |

IB anti-KLAC

IB anti-cortactin
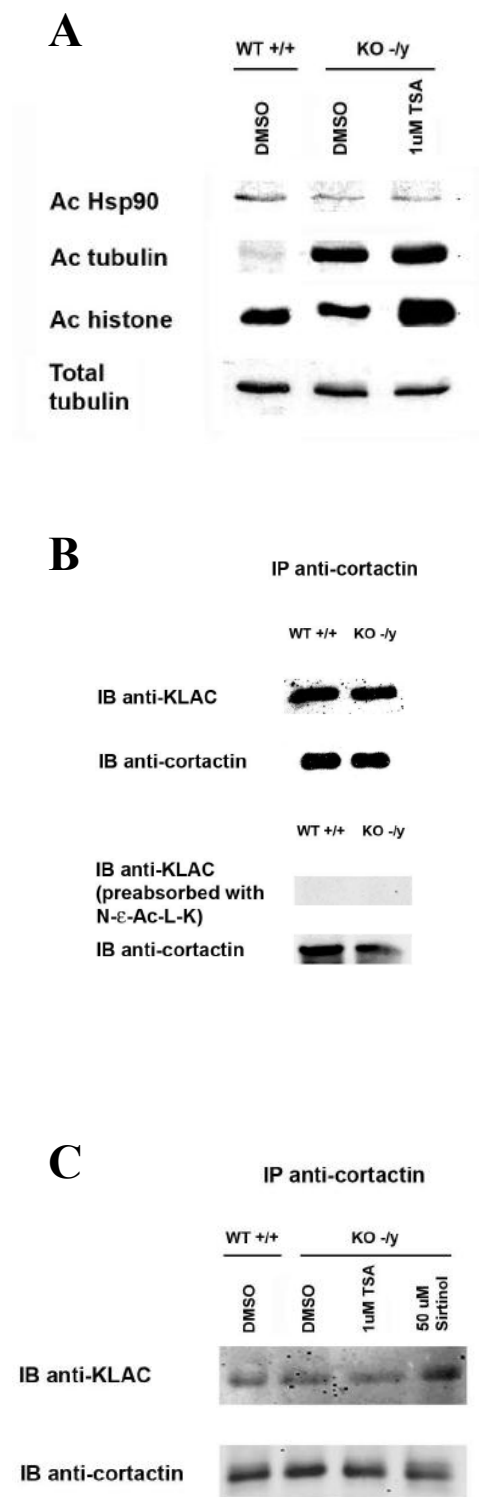
Figure 2. HDAC6 deacetylates tubulin, but not Hsp90 or cortactin.

Acetylation level of HDAC6 motility-related substrates from WT and HDAC6 KO MEFs. **A)** Lysates of WT and HDAC6 KO MEFs, or HDAC6 KO MEFs treated with TSA (1 µM, 4 hours) were probed for Ac proteins, using KLAC antibody (see Materials & Methods for details). **Ac-Hsp90**, **-tubulin**, and **–histones** are indicated. **Total tubulin** (stained with β-tubulin antibody) was used as a loading control. **B)**, **C)** Lysates from MEFs treated as indicated for 5 hours were immunoprecipitated with **anti-cortactin** antibody, and Ac-cortactin level was detected in each, via **KLAC** antibody. Blots were also probed with KLAC antibody that had been pre-incubated with **N-epsilon-acetyl-L-lysine** (N-e-Ac-L-K). **Anti-cortactin** antibody was used as loading control.

Figure 3

A
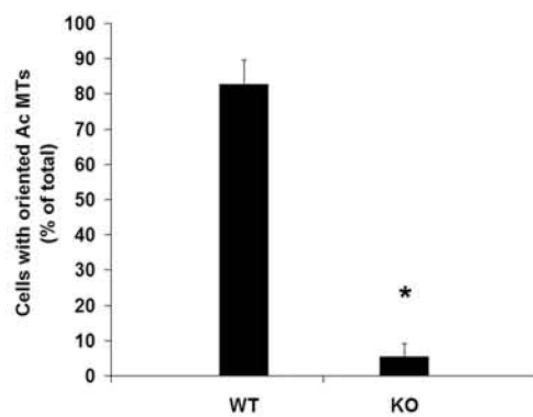
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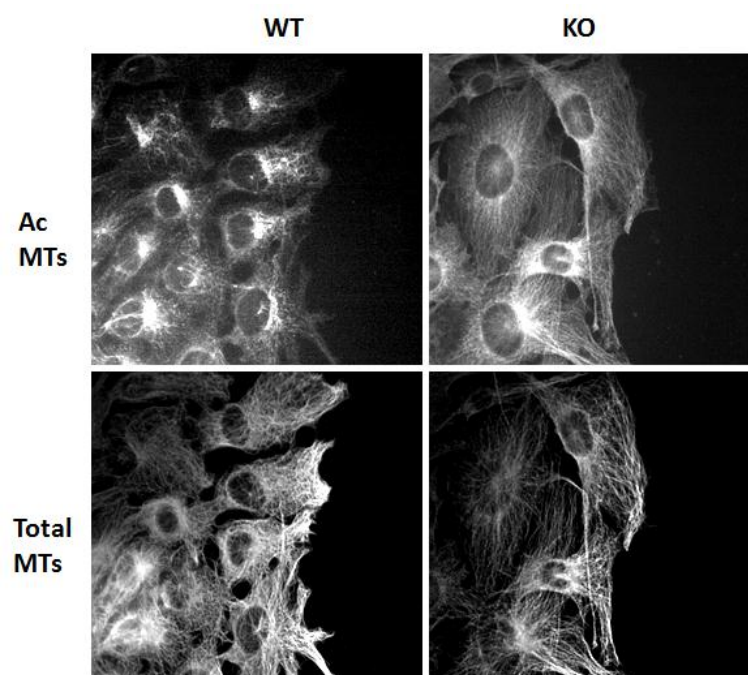
Figure 3. Loss of HDAC6 shows hyper-acetylation of all cellular MTs and loss of a polarized array of Ac MT array.

A) Immunofluorescence and **B)** Quantification of polarized Ac MT arrays in wound-edge WT and HDAC6 KO MEFs. Cells were wounded, treated as indicated and allowed to polarize for 5 hours before they were fixed and immunolabeled with antibodies against **Ac** and **total tubulin**. Asterisks indicate significance (p<0.05) as compared to WT MEFs.

Figure 4

A

B
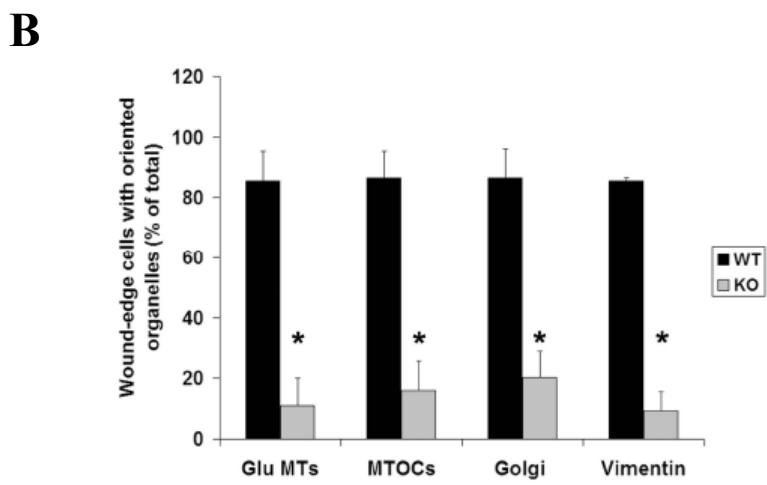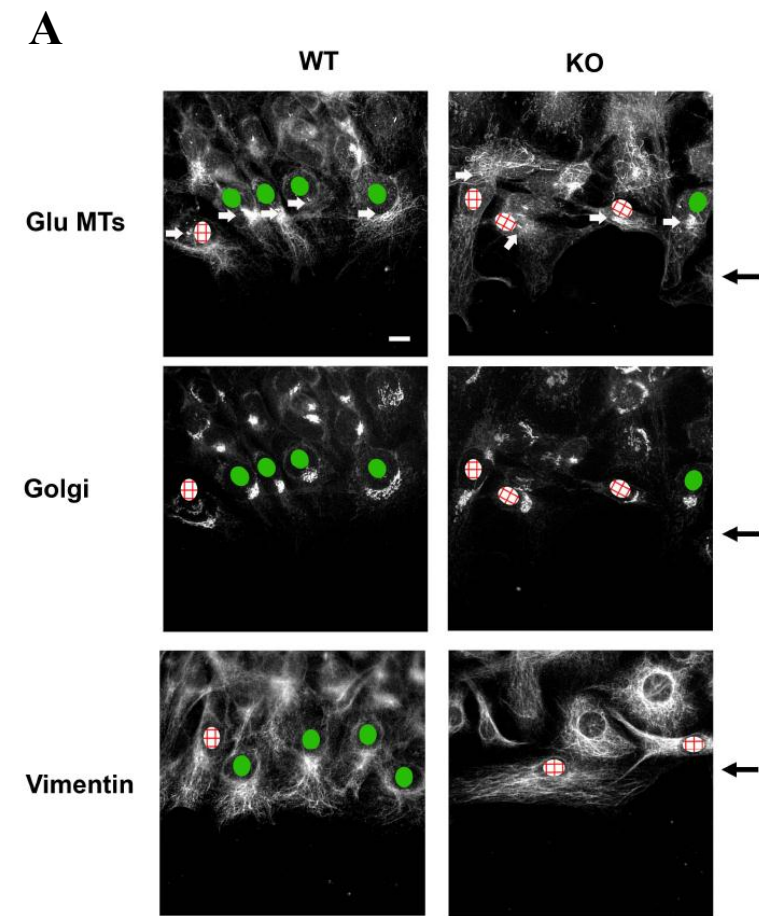
Figure 4. Loss of HDAC6 mislocalizes cellular organelles.

 A) Micrographs and **B)** quantification of the polarization of cellular organelles in WT and HDAC6 KO MEFs at the wound edge. Cells were wounded and allowed to polarize for 5 hours before fixation and immunolabeling. In A) antibody against Glu tubulin was used to detect stable, **Glu MTs** and **MTOCs**, and **Golgi** and **vimentin** antibodies were used to localize these cellular organelles. Quantification was performed by dividing cells into 4 quadrants and scoring the number of cells with oriented organelles, i.e., organelles found in the leading edge quadrant. Green circles (●) indicate polarized cells, red cross-hatched circles (⊞) indicate cells with mislocalized organelles (see Materials and Methods for details). MTOCs are indicated by white arrows. Wound edges are indicated by black arrows. Bar, 10 µm. In B) at least 200 cells were scored per experimental condition in each of three experiments. Asterisks indicate significance ($p<0.05$) as compared to WT MEF controls.

Figure 5

A
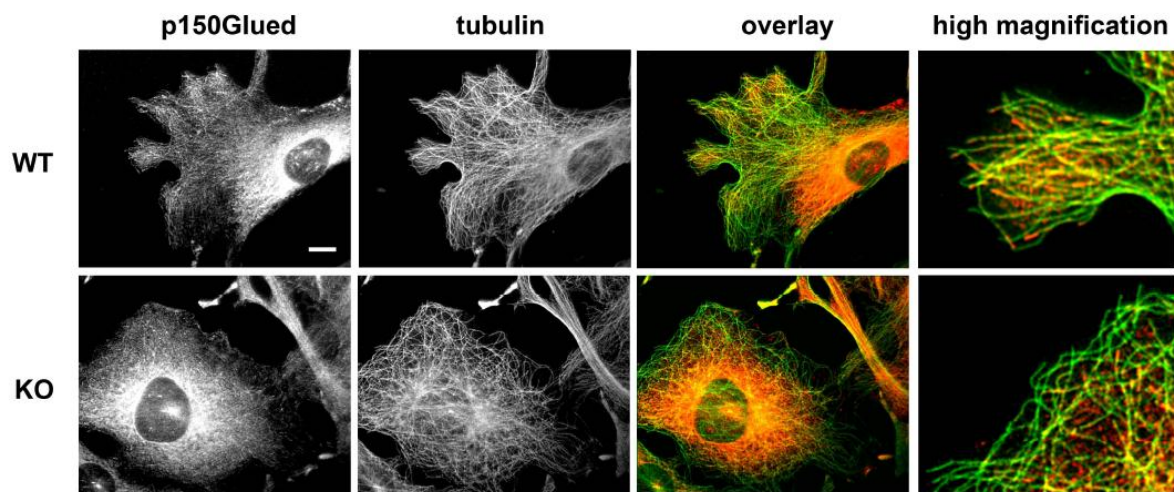
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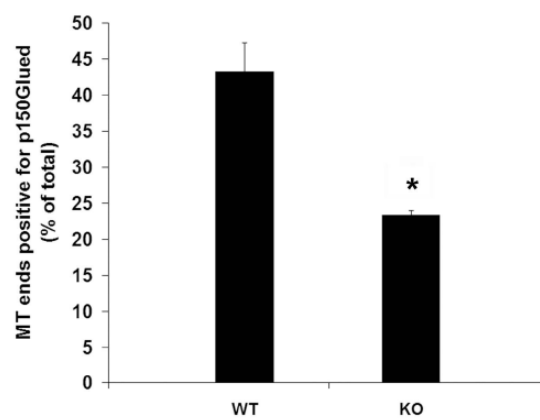
Figure 5 continued

C
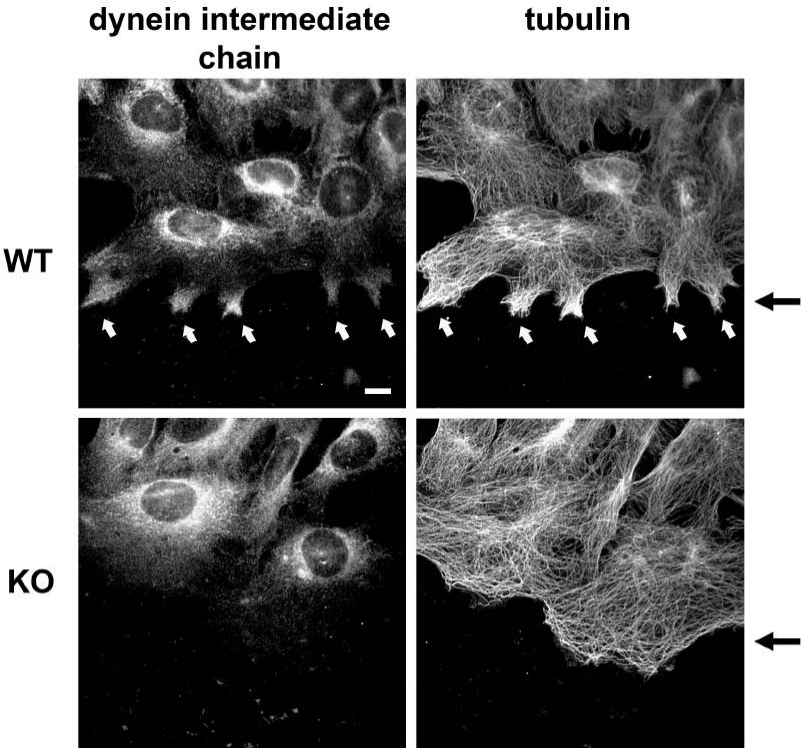
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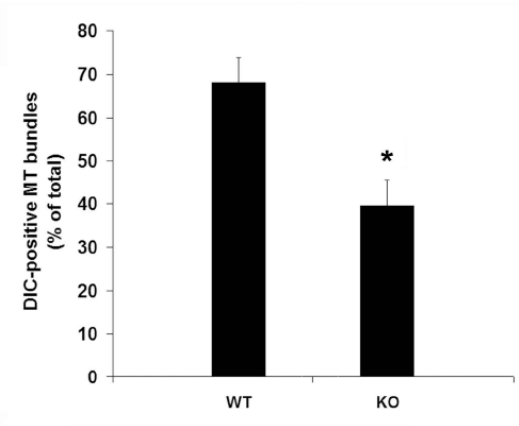
Figure 5. Loss of HDAC6 inhibits localization of p150glued and dynein to MT plus-ends and the leading edge cortex.

A) Micrographs and **B)** quantification (see Materials and Methods) of p150glued localization on MT plus ends in sparse WT and HDAC6 KO MEFs labeled with **p150glued** and **α-tubulin** antibodies. Bar, 10 µm. **C)** Micrographs and **D)** quantification of leading-edge localization of cortical dynein in WT and HDAC6 KO MEFs. **C)** MEF monolayers were wounded and allowed to polarize for 5 hours before they were fixed and immunolabeled with antibodies against **dynein intermediate chain (DIC)** and **α-tubulin**. White arrows indicate cortical DIC-positive leading edge patches in WT MEFs where MTs were observed to converge. Wound edges are indicated by black arrows. Bar, 10 µm. **D)** Asterisks indicate significant differences, compared to WT controls, (p<0.05).

Figure 6

A

B
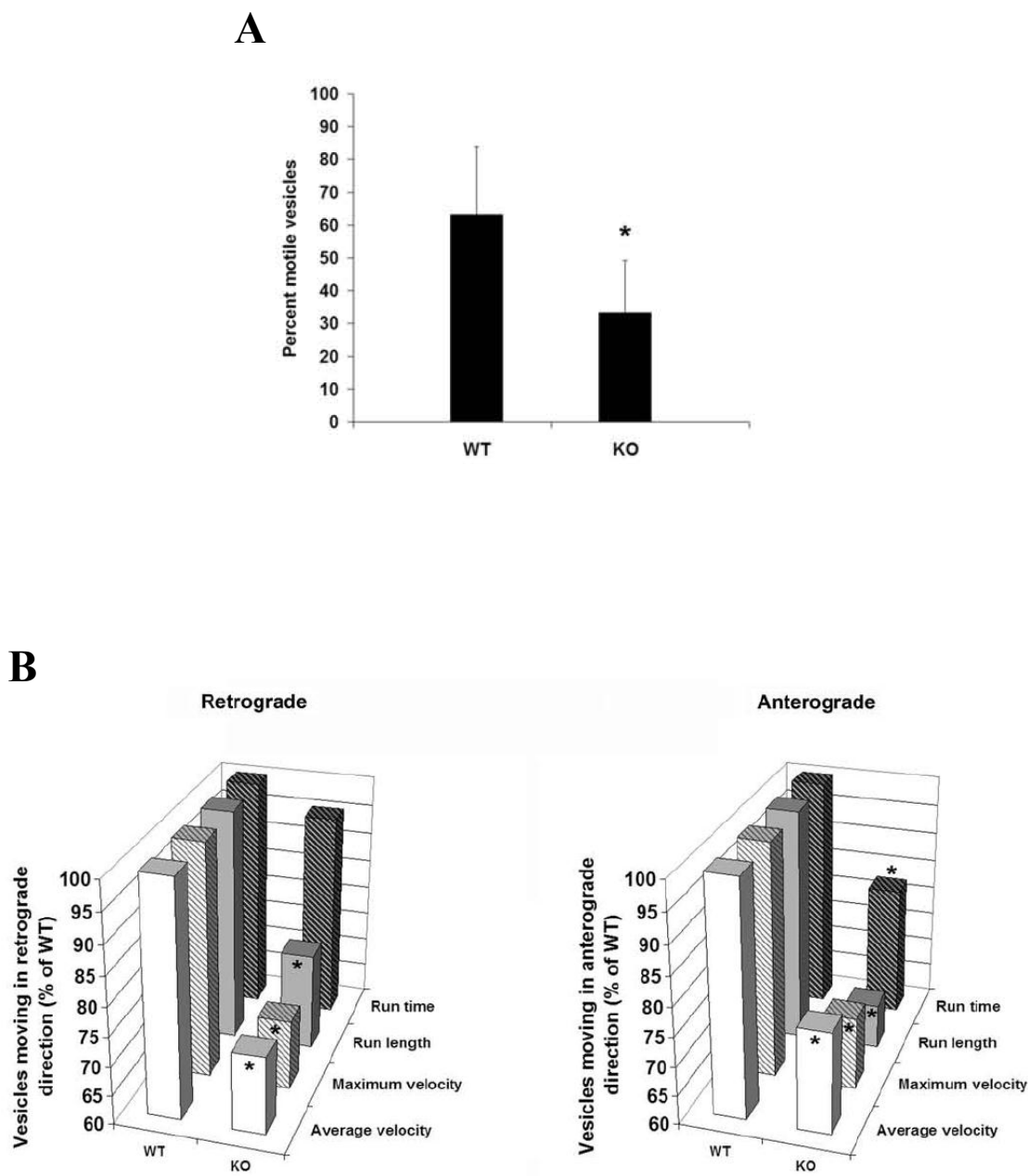
Figure 6. Loss of HDAC6 inhibits bidirectional transport of Golgi vesicles.

Quantification of **A)** proportion of motile Golgi vesicles and **B)** Golgi vesicle motility parameters in **retrograde** and **anterograde** directions in WT and HDAC6 KO MEFs, (see Materials and Methods for details). Only vesicles moving at speeds > 0.1 µm/second were considered motile. Asterisks indicate significant differences (p<0.05) as compared to WT controls.

Figure 7

A
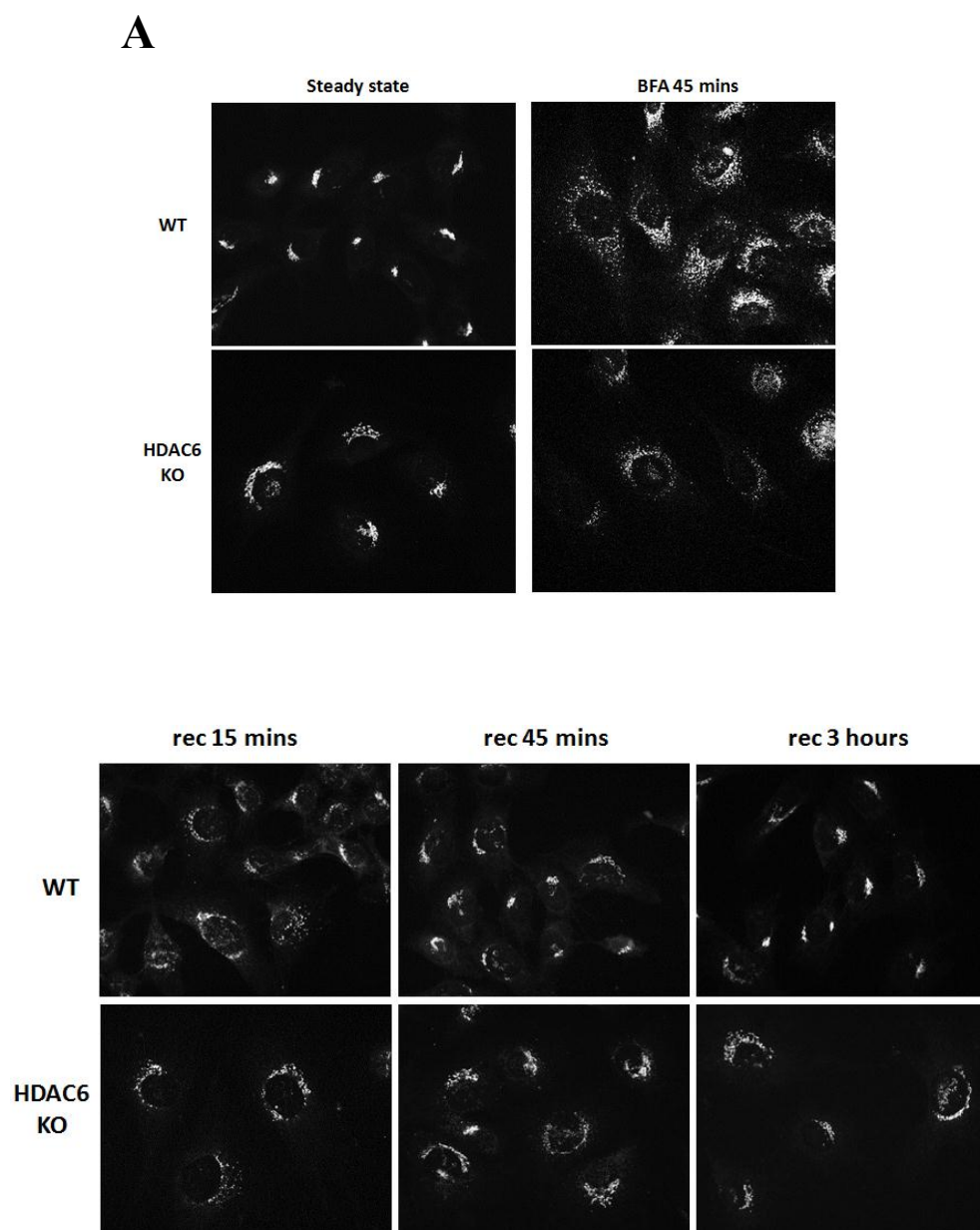
Figure 7 continued

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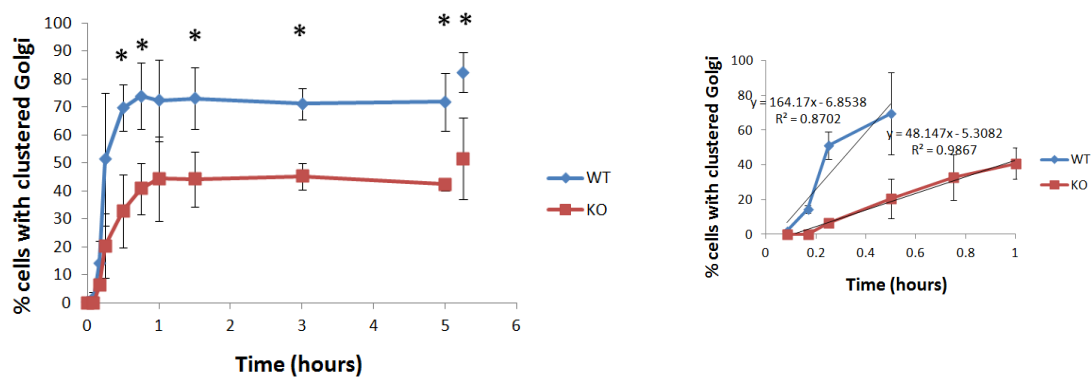
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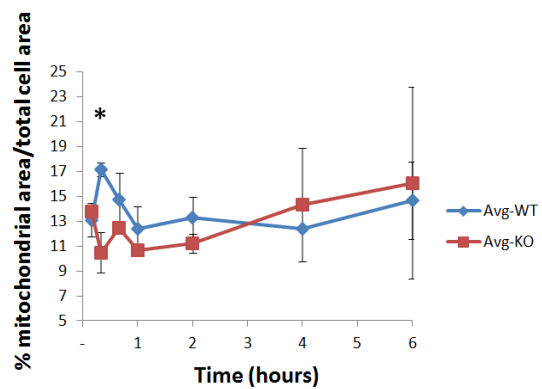
Figure 7. Loss of HDAC6 inhibits dynein and kinesin-mediated microtubule-based transport.

A) Micrographs and **B)** quantification (see Materials and Methods) of treatment and recovery from BFA treated WT and HDAC6 KO MEFs. A) Cells were treated with BFA for 45 minutes (top panel) and allowed to recover (bottom panel) as indicated, fixed and immunolabeled with **Golgi** antibody. B) BFA Recovery curve (left panel); linear portion of recovery curve (right panel) displays rate of recoveries from BFA release. C) Quantification (see Materials and Methods) of mitochondrial area in spreading cells as relative to total cell area in WT and KO MEFs. Asterisks indicate significant differences ($p<0.05$) as compared to WT controls.

Figure 8

A
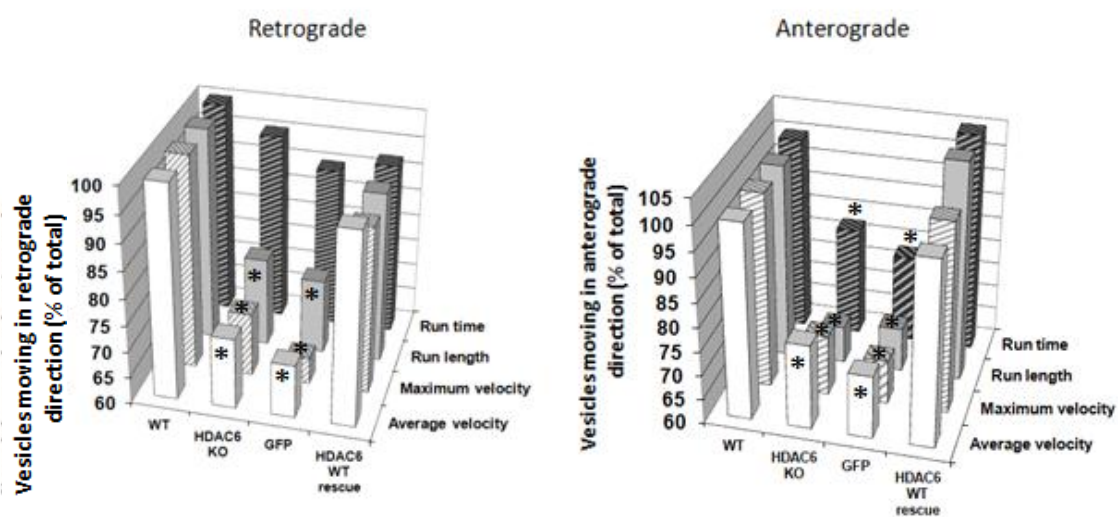
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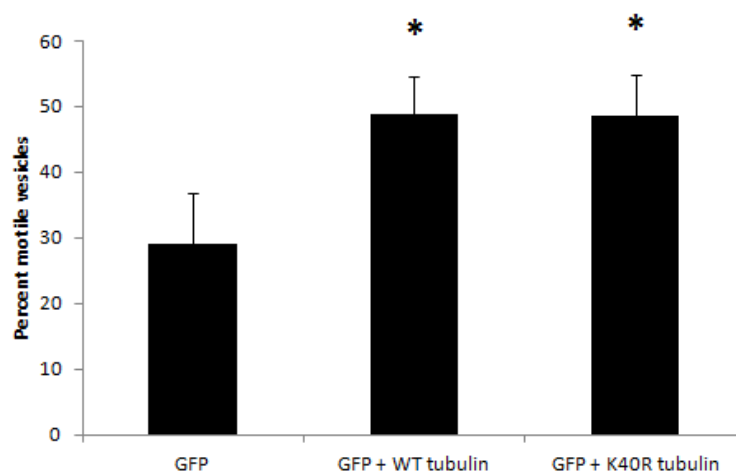
Figure 8. Re-expression of HDAC6 or non-acetylatable or WT tubulin rescues Golgi vesicle transport.

Quantification of **A)** Golgi vesicle motility parameters in **retrograde** and **anterograde** directions in WT and HDAC6 KO, and GFP- or GFP- and HDAC6- transfected HDAC6 KO MEFs, and **B)** proportion of motile Golgi vesicles in HDAC6 KO MEFs that were either transfected with GFP alone or GFP and 3X-FLAG-WT α-tubulin or GFP and 3X-FLAG-K40R. Only vesicles moving at speeds > 0.1 μm/second were considered motile. Asterisks indicate significant differences (p<0.05) as compared to WT controls.

Chapter 2 - Supplementary Figures

Supplementary Figure 1

A

B
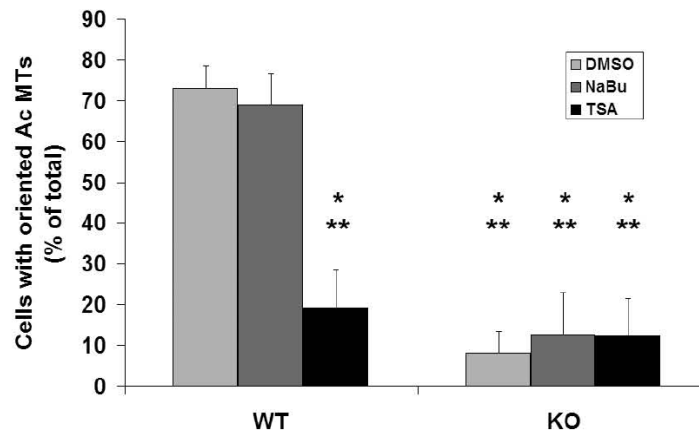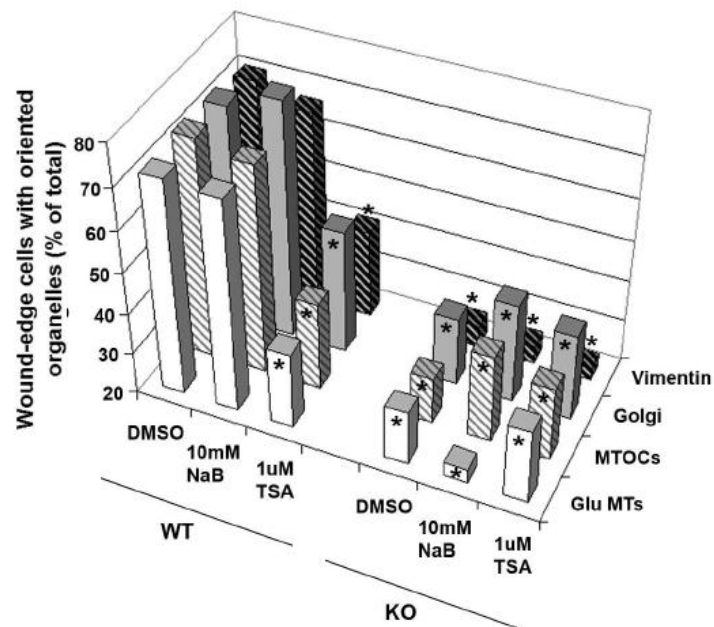
Supplementary Figure 1. TSA-treated WT and HDAC6 KO MEFs exhibit similarly inhibited polarization of Ac MT arrays and cellular organelles.

        A) Polarization of Ac MT arrays and **B)** organelle polarization were quantified in both drug-treated WT and HDAC6 KO MEFs. The drugs indicated were added to the cultures at the time of wounding. In A) and B), cells were fixed 5 hours after wounding and immunolabeled with Ac tubulin antibody in A) and Glu tubulin antibody (to detect stable, **Glu MTs** and **MTOCs**), and **Golgi** and **vimentin** antibodies in B). In A) and B) at least 200 cells were scored per experimental condition in each of three experiments. Note from panels A) and B) that TSA inhibited WT MEFs to an extent similar to HDAC6 KO, and TSA produced no discernible further inhibition of KO MEFs, compared to DMSO treatment of HDAC6 KO MEFs. Asterisks indicate significant differences ($p<0.05$) from the control, DMSO-treated WT MEFs.

Supplementary Figure 2

A
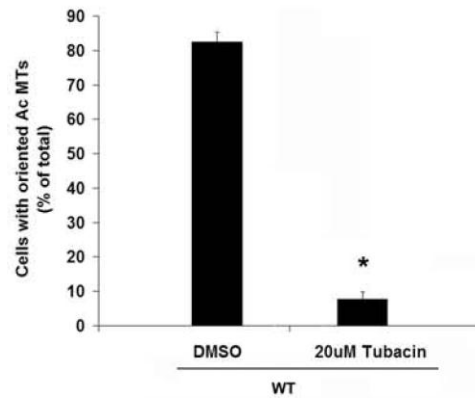
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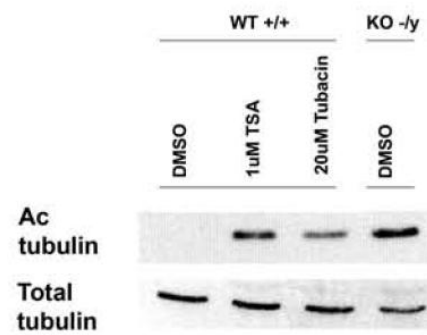
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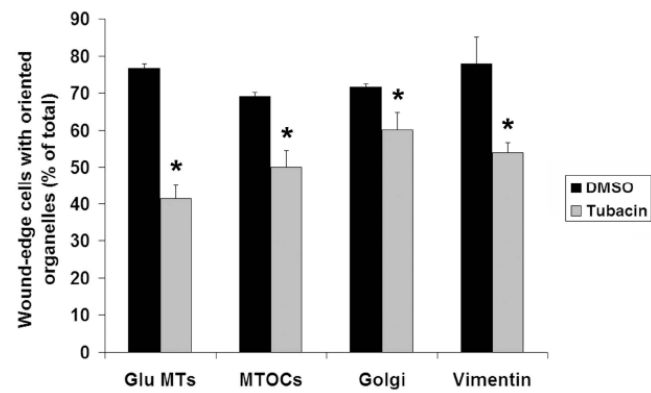
Supplementary Figure 2. Tubacin treatment of WT MEFs results in loss of polarization of the Ac MT array and cellular organelles.

Quantification of polarized A) Ac MT arrays and B) cellular organelles in wound-edge, tubacin-treated WT MEFs. Cells were wounded, treated as indicated and allowed to polarize for 5 hours before they were fixed and immunolabeled with antibodies against **Ac** and **total tubulin** in A), and Glu tubulin antibody (to detect stable, **Glu MTs** and **MTOCs**), and **Golgi** and **vimentin** antibodies in B). In A) and B) at least 200 cells were scored per experimental condition in each of three experiments. Asterisks indicate significance ($p < 0.05$) as compared to DMSO-treated WT MEFs. **C)** Western blots were performed on WT and HDAC6 KO MEFs in order to compare the effects of TSA and HDAC6 KO versus tubacin on the level of hyper-Ac tubulin. Cells were treated as indicated for 4 hours before harvesting. Blots were labeled with antibodies against **Ac** and **total** (β) **tubulin**, a loading control.

Supplementary Figure 3

A

Mitochondria

WT

KO

B
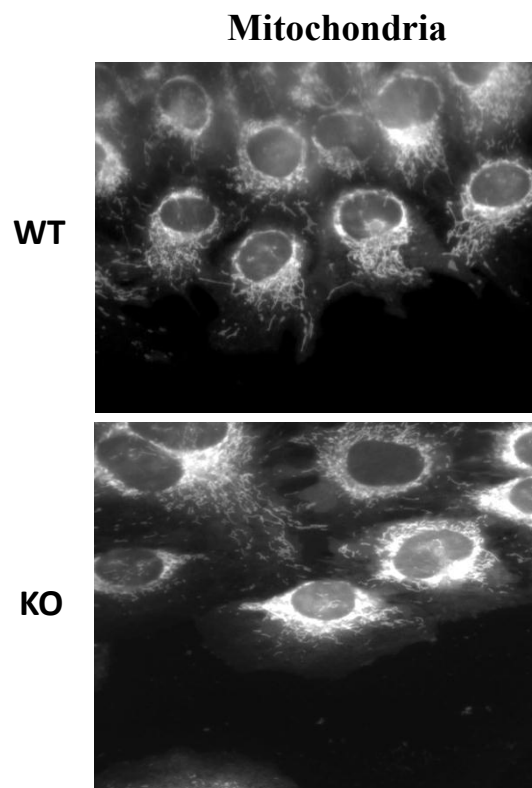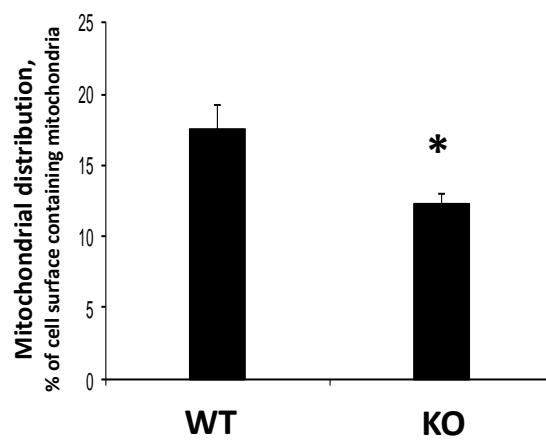
Supplementary Figure 3. Loss of HDAC6 inhibits polarization of mitochondria towards the leading edge.

A) Micrographs and **B)** quantification of mitochondrial polarization in wound-edge WT and HDAC6 KO MEFs. **A)** MEF monolayers were wounded and allowed to polarize for 5 hours and treated with a mitochondrial fluorescent dye before fixation. Bar, 10 µm. **B)** At least 200 cells were scored per experimental condition in each of three experiments. Asterisks indicate significant differences, compared to WT controls, ($p<0.05$).

Supplementary Figure 4
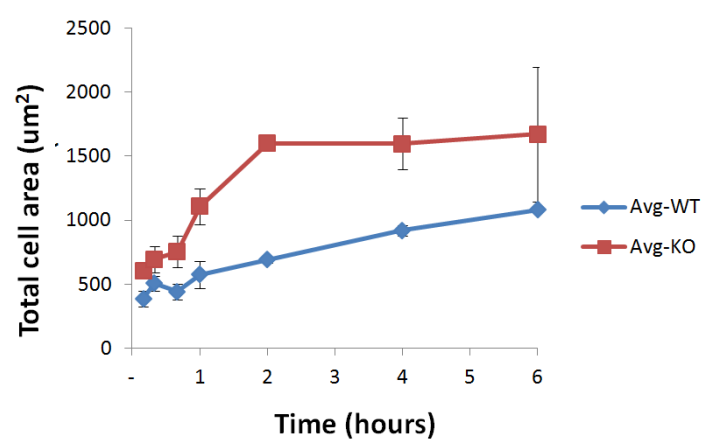
Supplementary Figure 4. Loss of HDAC6 increases cell spreading.

Quantification of spreading in WT and HDAC6 KO MEFs. Total cell area at different times after cell plating on glass coverslips was measured and plotted. At least 25 cells per time point and condition were measured. Asterisks indicate significant differences ($p<0.05$) from WT MEFs.

DISCUSSION

Our results provide strong support for our original hypothesis, that HDAC6 activity is required for MT-dependent cellular polarization and polarized intracellular transport in directional migration of fibroblasts. Below we discuss how our data, taken together with other findings, also contribute to answering three questions that extend from our hypothesis: First, in the absence of HDAC6 activity, what proteins become hyperacetylated, and thus potentially reduce motility? Second, what is/are the mechanism(s) by which HDAC6 activity impinges upon MT-dependent fibroblast polarization and directed motility? Third, are TSA's anti-motility effects on wound-healing likely to be replicated in clinical regimens using TSA or other hydroxamic acid HDAC inhibitors (Drummond et al., 2005; Minucci and Pelicci, 2006)?

Addressing the first question, of all the known in vivo HDAC6 substrates only three, i.e., tubulin (e.g., Hubbert et al., 2002); Hsp90 (Bali et al., 2005; Kovacs et al., 2005); and cortactin (Zhang et al., 2007) are known to be directly involved in cell motility. Using our KLAC antibody (Tran et al., 2007) that was designed to react with all Ac-lysines irrespective of the surrounding sequence, we show here that tubulin was the only one whose acetylation level detectably increased in HDAC6 KO and HDAC6-inhibited WT fibroblasts; an important result as no other study has simultaneously investigated the acetylation states of all three of the HDAC6 motility-related substrates to our knowledge. Presumably, like cortactin (Zhang et al., 2007), but unlike tubulin, Hsp90 is not deacetylated exclusively by HDAC6 in motile fibroblasts and may be deacetylated by SIRT2. Pharmacological experiments first suggested that more than one HDAC class deacetylates Hsp90 (Blagosklonny, 2002), which possesses multiple acetylation sites (Scroggins et al., 2007). Our observation that Hsp90 acetylation level was unchanged by HDAC6 inhibition is consistent with some studies (Carta et al., 2006; Tran et al., 2007), but contradictory

to others (Bali et al., 2005; Gao et al., 2007; Zhang et al., 2007). Plausible explanations for these discrepant results are that the levels of several HDACs vary with cell type or growth conditions (Dangond et al., 2001; Dryden et al., 2003), or that detection of Hsp90 acetylation varies with different antibodies or methods of assay. Thus, although cortactin and Hsp90 affect cell motility (Gao et al., 2007; Zhang et al., 2007), the HDAC6 substrate and interacting protein, tubulin and its polymer, are the most likely contributors to the motility inhibition we measured here.

However, the overexpression of both WT and a non acetylatable tubulin mutant sufficiently and equivalently rescued the Golgi vesicle motility defect in HDAC6 KO MEFs. While this result unequivocally shows a MT-dependent effect for HDAC6 activity and points to the relevance of the acetylated MT subset to the effects we have reported here, we cannot as of yet conclusively conclude that hyperacetylated MTs are the sole cause underlying these effects as the effects could be mediated by some other property of MTs, by an overall increase in total MT polymer level, or a failure to sufficiently acetylate tubulin residues overexpressed by the WT mutant. It is thus possible that one or more of the other identified substrates of HDAC6 (see non-motility related substrates in Table 1, Chapter 1) also modulate their effects on MT-based transport and polarization in migrating cells. Of these, β-catenin seems the most likely candidate to influence directional migration due to its functions in the classical Wnt signaling pathway and in cell-cell adherens junctions which are deregulated in many cancers (Li et al., 2008). However, none of these other substrates are known to be direct cell motility modifiers and whether altering the acetylation state of β-catenin or any of the other substrates contributes to altering directional migration indirectly or by as yet undiscovered function(s) remains to be determined.

It is also likely that HDAC6 has substrates involved in the regulation of cell motility that have not yet been identified. For example, presence of HDAC6 in protein complexes containing

the formin, mDia (Destaing et al., 2005), GSK3β (Chen et al., 2010), PP1 (Brush et al., 2004), or

SIRT2 (North et al., 2003), suggests that HDAC6 may deacetylate these or other substituents of

these protein complexes, many of which are known to interact with and impact the function of

MTs. Any putative HDAC6 substrate dependent exclusively upon HDAC6 or other NAD-

independent HDACs for its deacetylation would become hyper-Ac in HDAC6 KO or TSA-

treated fibroblasts, respectively, thus possibly contributing to decreased cell polarization and/or

motility. To have escaped detection thus far, such a hyper-Ac protein would likely be present in

small amount; nevertheless, it could contribute an important signaling function in motile

fibroblasts. In addition, we predict that motility defects would be markedly enhanced in

fibroblasts lacking both HDAC6 and SIRT2, i.e., cells with hyper-Ac tubulin, cortactin, and

possibly Hsp90 or other HDAC6 substrates. This prediction is amenable to test, using mice null

for HDAC6 (Gao et al., 2007; Zhang et al., 2008) and SIRT2 (Vaquero et al., 2006).

      Turning to our second question, there are several possible mechanisms of action of

HDAC6 activity that might explain the MT-based defect in cellular polarization and intracellular

transport. Migrating cells polarize stable MTs and other organelles (Bulinski and Gundersen,

1991), and Golgi-derived vesicle trafficking towards the leading edge, possibly to optimize the

directional membrane turnover required for persistent direction of migration (Bergmann et al.,

1983; Prigozhina and Waterman-Storer, 2004; Schmoranzer et al., 2003). Loss of HDAC6

activity abrogated polarization of organelles, i.e. the stable MT subset, the MTOC, the Golgi

complex, the vimentin intermediate filament array and mitochondria, towards the leading edge.

Testing components of the MTOC reorientation pathway revealed that loss of HDAC6 activity

inhibited localizations of the MT plus-end binding protein p150glued and cortical dynein which

are required for MT capture and subsequent polarization. Dynein- and kinesin-mediated

bidirectional transport of Golgi vesicles was also decreased suggesting an inhibition in the binding and/or activities of these motors on MTs.

Loss of HDAC6 activity results in a global acetylation of tubulin such that virtually every MT appears completely modified. One possible ramification of hyperacetylation of almost all tubulin subunits is that it could likely alter polymerization or surface structure of MTs; either would be expected to decrease the binding of MTs to +TIPs and motors thereby decreasing local MT capture and stabilization, and motor transport. Our data showing the decreased localization of the +TIP p150glued and decreased bidirectional motor-based transport are consistent with this premise. p150glued is proposed to mediate docking of dynein to both MTs and cargoes via a search-capture mechanism (Vaughan, 2005), and to enhance dynein processivity (Culver-Hanlon et al., 2006). Moreover, p150glued has been shown to activate kinesin-based transport as well as dynein (Berezuk and Schroer, 2004; Haghnia et al., 2007). Thus, loss of its localization on hyperacetylated MT plus-ends would attenuate the activities of both dynein and kinesin on MTs leading to decreased bidirectional transport. Our results are consistent with both dynein and kinesin loading and processivity defects in HDAC6-inhibited fibroblasts, since many vesicles did not move at all and those that did showed decreased run time of retrograde and anterograde motility. Alternatively, decreased motor binding to hyperacetylated MTs, independent of any role of p150glued, would also decrease bidirectional transport of vesicles thereby contributing to decreased directional migration. The decreased Golgi vesicle motility in HDAC6-inhibited cells could in turn impact migration through the failure of polarization of the Golgi from which stable MTs often emanate (Efimov et al., 2007).

Hyperacetylation of virtually all cellular MTs could also delocalize signaling pathways activated by the formation of an oriented array of acetylated MTs in motile cells. The formation

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of a stable, posttranslationally modified array is thought to be important for directional migration perhaps because stable MT arrays serve as long-lived tracks for reiterative intracellular transport events. Additionally, temporal and spatial regulation of MT stabilization and modification early in the polarization process in directionally migrating fibroblasts (Bulinski and Gundersen, 1991) is thought to be important for signaling downstream pathways that trigger global organelle polarization towards the leading edge. The loss of spatial information by hyperacetylation of all cytoplasmic MTs could hamper the localized recruitment of proteins at the leading edge preventing polarization of organelles and transport along oriented MT tracks marked specifically by acetylation. This premise is supported by our findings that HDAC6-inhibited fibroblasts did generate stabilized MT arrays, even though they failed to reorient these or other organelles towards the leading edge. In addition to the loss of HDAC6 activity substantially reducing the plus-end localization of p150glued, HDAC6-inhibited fibroblasts also failed to localize cortical dynein involved in MTOC reorientation. Naturally, mislocalization of dynein at the leading edge would significantly lower the likelihood of leading edge MT capture and MTOC reorientation.

Mechanisms for localizing dynein at the leading edge have not been described; thus, we can only speculate on the impact of HDAC6 activity and hyperacetylated MTs on this process. Lipid raft redistribution to the leading edge of migrating cells, activating PI3K signaling pathways, and recruiting proteins with plekstrin homology (PH) domains to the cortex (reviewed in Manes et al., 2003), may be involved in leading edge localization of dynein. While no dynein-interacting PH domain protein has been described in higher eukaryotes, at least in yeast, a PH-domain containing protein, Num1p, has been proposed as the cortical anchor for dynein (Yamashita and Yamamoto, 2006). Moreover, evidence of crosstalk among lipid rafts, Rho family members, and PI3K is proposed to establish and reinforce cellular polarization (Manes et

al., 2003). Although these processes occur downstream of actin reorganization (Manes et al., 2003), feedback loops between MTs and small GTPases have been reported in which MTs also interact with regulatory subunits of PI3K (reviewed in Wittmann and Waterman-Storer, 2001). Thus, it is possible that a feedback loop exists in which MTs or Ac MTs regulate upstream signaling pathways that then activate MT capture. Another possibility is that accumulation of dynein at the cortex may also require its loading onto MTs (Sheeman et al., 2003; Xiang, 2003) via the MT-loading factor, p150glued (Vaughan, 2005). Alternatively, dynein may undergo kinesin-based transport to the leading edge, as this occurs in Aspergillus (Zhang et al., 2003a). Thus, defects in bi-directional MT-based transport in HDAC6-inhibited cells may abrogate redistribution of dynein to the leading-edge cortex.

Altered MT dynamics of hyperacetylated MTs in HDAC6-inhibited fibroblasts (Tran et al., 2007) may provide yet another mechanism for the defect in MTOC reorientation which involves interaction of p150glued on dynamic MTs with cortical dynein (reviewed in Gundersen et al., 2004). Dampening of MT dynamics by a low dose of Taxol has been shown to inhibit the binding of a p150glued-related +TIP, CLIP170, to MT plus ends (Perez et al., 1999) The reduced dynamics of MTs in HDAC6-drug inhibited (Tran et al., 2007) fibroblasts may suffice to reduce the MT-binding affinity of p150glued which would inhibit cortical docking. The result that both MT dynamics and p150glued binding to MT ends are reduced in HDAC6 KO fibroblasts corroborates the findings that MTOC reorientation requires both dynein function and dynamic MTs (Yvon et al., 2002; Gomes et al., 2005). Reduced dynamics of MTs would also decrease the probability of MT targeting to leading edge docking sites thereby impacting cell motility directly by altering polarized transport key to membrane turnover in directed cell migration.

Finally, decreased or disrupted binding of inhibited HDAC6 with its interacting partners, p150glued and dynein, can also contribute to the decreased bidirectional motor-based transport we observe. Dynein directly binds HDAC6 (Kawaguchi et al., 2003), and tubacin has been shown to disrupt this binding (Hideshima et al., 2005). Thus, the loss of HDAC6 or its activity could either directly decrease dynein loading onto MTs, or indirectly by inhibiting p150glued plus-end binding, causing the decreased dynein-based motility. This can also explain the inhibition in dynein's cortical leading edge localization as HDAC6 itself is specifically localized to the leading edge cortex (Gao et al., 2007; Hubbert et al., 2002). While it is not known whether HDAC6 directly interacts with kinesin, kinesin-based transport of parkin was inhibited in tubacin and TSA treated cells (Jiang et al., 2008). Moreover, HDAC1 has recently been discovered to be present in the cytoplasm in neurodegenerative disease, where it has been shown to bind kinesin-1 thereby decreasing kinesin-cargo interactions and detrimentally inhibiting axonal transport (Kim et al., 2010). These results suggest that HDAC6 also regulates kinesin-1 activity. Interestingly, the MT motor kinesin-1 has been shown to regulate focal adhesion turnover (Krylyshkina et al., 2002), which we have previously shown is inhibited in HDAC6 KO MEFs by dampened MT dynamics (Tran et al., 2007). The inhibition of kinesin loading onto MTs by loss of HDAC6 or its activity may thus alternatively explain the decreased kinesin-based motility.

However, the decrease in MT-based dynein and kinesin vesicle transport we observed in HDAC6 null and inhibited fibroblasts appears inconsistent with the increased bidirectional dynein and kinesin-based MT transport observed in neuronal cells (Dompierre et al., 2007; Reed et al., 2006). However, those data are hard to interpret as they could be due to the effects of increased Ac MTs or inhibition of HDAC6 activity. Moreover, a later study has reported

conflicting data showing that MT acetylation is not sufficient for preferential transport in neurons and requires a combination of MT modifications including acetylation, detyrosination and polyglutamylation (Hammond et al., 2010). Our data on the other hand are consistent with a role reported for HDAC6 deacetylase activity being required for the anterograde and retrograde transport of parkin by kinesin and dynein, respectively (Jiang et al., 2008). Thus, deacetylation of acetylated residues along the MT track in normal cells could be important for the regulation of transport of organelles and vesicles required for steady-state homeostasis or other cellular activities; increased MT hyperacetylation could inhibit bidirectional motor-based transport as an effect of inhibition or loss of HDAC6 activity. Alternatively, it is possible that transport of different cargoes or by different kinesin family members are selectively regulated by MT acetylation (Carta et al., 2006; e.g., Hirokawa and Takemura, 2005; Ikegami et al., 2007; Naranatt et al., 2005).

At first glance, one would expect inhibition of bidirectional trafficking to be compounded in axonal transport, in which cells use numerous reiterative transport events to generate movement over long distances. However, HDAC6 KO mice are viable (Gao et al., 2007; Zhang et al., 2008), without gross brain defects (Zhang et al., 2008). Perhaps, the fact that HDAC6 expression is low and the tubulin is heavily Ac in brains of WT mice (Zhang et al., 2008) predicts that a small further increase in tubulin acetylation in HDAC6 KO mice would have no effect, or would yield only subtle behavioral effects (as reported by Fukada et al., 2012). In other tissues, hyper-acetylation of MTs could compromise wound healing or alter tumorigenicity or metastatic potential. The immune system defects of HDAC6 KO mice (Zhang et al., 2008) are consistent with the previous demonstration that MTOC reorientation in immune cell function appears to be sensitive to MT acetylation level (Serrador et al., 2004).

Concerning the last question, the extraordinary promise of HDAC inhibitors as anti-tumor agents, (Drummond et al., 2005; Minucci and Pelicci, 2006) makes it useful to assess the potential relevance of our results to the action of these drugs in people. Most current studies of the anti-oncogenic potential of HDAC inhibitors addresses their effects on gene expression pathways involved in cell proliferation, differentiation, apoptosis, and angiogenesis (reviewed in Drummond et al., 2005). It is difficult to extrapolate from in vitro assays of mouse fibroblast motility in culture to motility effects of tumor cells in vivo in humans. We do note, however, that we measured motility deficits in HDAC-inhibited fibroblasts both in 3D (Tran et al., 2007), and 2D-motility, which we explored in depth here. Similar motility defects are likely to be replicated in vivo, especially when hydroxamic acid-type HDAC inhibitors are used to treat mouse models of sarcomas, which maintain numerous motility and morphology characteristics of the fibroblasts from which they arise (Johnson et al., 1971; Paku et al., 2003; Sroka et al., 2002). The HDAC6-selective drug, tubacin, inhibits 3D invasion motility (Haggarty et al., 2003), and its inhibition is as potent as that of TSA (Tran et al., 2007). However, tubacin also greatly enhances cytotoxicity in multiple myeloma cells, when used in conjunction with a known anti-tumor drug (Hideshima et al., 2005). Thus, the degree to which HDAC inhibitor effects on cell motility prove to be beneficial contributors to the overall anti-tumor effects of these drugs remains to be seen.

In summary, our study provides plausible mechanisms for the roles of HDAC6 and MTs in directionally migrating fibroblasts. These results may have long term implications in the drug-targeting of diseases that involve aberrant cell migration, and/or in understanding of off-target anti-motility effects of some HDAC inhibitors currently in use.

MATERIALS AND METHODS

Cell culture and treatments

Experiments were performed in wild type (WT) and HDAC6 knockout (KO) MEFs (Boyault et al., 2008) and corroborated in A549 and HDAC6 knockdown (KD) A549 cells (Kawaguchi et al., 2003). All cells were cultured in DMEM supplemented with 10% fetal bovine serum, while all drugs except water-soluble sodium butyrate (NaB) were dissolved in DMSO, whose final concentration in media of treated cells was $\leq 0.1\%$. Rescue experiments were performed in HDAC6 KO MEFs by transient transfections of HDAC6 WT, WT-FLAG-α-tubulin, and K40R-FLAG-α-tubulin constructs co-transfected with GFP for visualization. GFP constructs alone were expressed as controls. Low to moderate expressors were chosen for data analysis.

Antibodies and reagents

All antibodies and chemicals were purchased from Sigma-Aldrich unless indicated. Antibodies specific to detyrosinated (Glu) and tyrosinated tubulin were described in Gundersen at al. (1984). Antibodies to β-tubulin (3F3); p150glued; and dynein intermediate chain (DIC) were generous gifts from Drs. J. Lessard (University of Cincinnati), K. Vaughan (University of Notre Dame) and Vallee (Columbia University), respectively. Anti-Golgi (GM130), and anti-EB1 antibodies were from BD Transduction Laboratories (Lexington, KY). Monoclonal cortactin antibody (clone 4F11) was purchased from Upstate (Millipore) (see Zhang et al., 2007). Anti-acetylated lysine antibody (Keyhole Limpet Ac antibody, or KLAC) (Tran et al., 2007) was raised against Keyhole Limpet Hemocyanin that was chemically acetylated by reaction with acetic anhydride (Piperno and Fuller, 1985). KLAC antibody was affinity-purified before use by

binding to and low pH elution from chemically acetylated Keyhole Limpet Hemocyanin-Agarose beads. Specificity of KLAC antibody for the ε-amino acetylation of lysines was determined by preabsorption of the antibody with 1mM N-epsilon-acetyl-L-lysine (N-e-Ac-L-K) for 1 hour at room temperature. Sirtinol was purchased from Calbiochem. Mitotracker Red CMXRos was purchased from Invitrogen.

Immunoprecipitations, immunoblotting and immunofluorescence

Cortactin immunoprecipitates (IPs) were prepared from MEF lysates under identical high stringency conditions and using the same source of cortactin antibody as described in Zhang et al. (2007). All western blots of whole cell extracts and immunofluorescence of fixed cells were performed as described in Chang et al. (2002).

Wound healing migration assays

Cells were seeded overnight at a density of 1 x 10^6 per acid-washed glass coverslips to form confluent monolayers; after 16 hours they were wounded as described (Gundersen and Bulinski, 1988). For velocity measurements, coverslips were fixed at 0-10 hour time intervals in 3.7% formaldehyde. Phase micrographs (> 15) of wound margins were obtained for each time point with a CoolSNAP-fx cooled-CCD camera (Photometrix, Roper, NJ) and wound-closure rate was measured as described by Yvon et al. (2002). Average velocities (reported in μm/hour) were divided by two to obtain rates of movement in a single direction.

Quantification of cellular polarization and MT end-binding proteins

Polarization of cellular organelles and cortical leading edge markers was quantified in fixed and immunolabeled wound-edge cells as described (Finkelstein et al., 2004). The localization of cortical dynein at the leading edge was analyzed in parallel with analysis of MT ends that converged at these lamellipodial regions (Dujardin et al., 2003). MT plus end localization of +TIP p150glued was quantified in sparse cells from immunofluorescence images double-labeled for p150Glued and β tubulin; pixel intensities, for a total of ≥ 1000 MTs were quantified from 15 cells per cell type with MetaMorph software (Molecular Devices, Downingtown, Pennsylvania).

Time-lapse Imaging of Golgi vesicles and cell migration

Imaging of both live and fixed cells was performed as described by Tran et al. (2007), unless indicated. Golgi vesicle motility in sparse or wound-edge cells was visualized using BODIPY TR ceramide [*N*-((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)phenoxy)acetyl] sphingosine (Invitrogen, Carlsbad, CA). Vesicles were analyzed in areas of whole cells according to the criteria specified by Hamm-Alvarez et al. (1993). The following vesicle motility parameters were quantified using the 'Track Points' function in MetaMorph: average velocity, maximum velocity, run length, run time, and percentage of motile vesicles; ≥ 1000 vesicles total in 15 cells per cell type were analyzed.

Brefeldin recovery assay

WT and HDAC6 KO MEFs were sparsely seeded on coverslips and allowed to grow overnight. Cells were treated with 1 µg/ml Brefeldin A (BFA) for 45 minutes and allowed to recover in culture medium after washing 5 times in serum-free medium. Recovery was monitored at

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intervals of 0, 5, 15 and 45 minutes and at 1, 1.5 3, and 5 hours during 0 to 5 hours by methanol fixation and immunofluorescence with Golgi GM130 antibody. Golgi clustering was scored as described in Bulinski et al. (1997) and data plotted to quantify rates of Golgi recovery from BFA treatment.

Assay of mitochondrial area in spreading cells

WT and HDAC6 KO cells pretreated with 100nM MitoTracker Red CMXRos for 15 minutes were rinsed, trypsinized and re-plated on coverslips to analyze and quantify mitochondrial area in spreading cells. Cells were fixed in 3.7% formaldehyde at regular intervals during 0 to 6 hours of plating, and then permeabilized in 0.1% Triton X-100. Images were taken and processed with the threshold function in Image J software to calculate mitochondrial area. Data were plotted as a percentage of total cell area to compensate for inherent cell spreading differences in WT and HDAC6 KO MEFs. At least 20 cells per time point and condition were used for analysis.

Data Analysis

Data from all cellular motility, immunofluorescence, western blot, and IP experiments were quantified from at least three independent experiments. For microscopic scoring, at least 200 cells or fields of view were scored for each experimental condition. Statistical significance was evaluated using a student's t-test (significance required p-value < 0.05).

Chapter 3

HDAC6 regulates cellular polarization by mediating cortical-microtubule anchoring via a subset of plus-end binding proteins

INTRODUCTION

HDAC6 activity regulates directional migration of fibroblasts through MT-dependent effects on polarization of cellular organelles and directed organelle and vesicular intracellular transport (Salam et al., manuscript in preparation, see Chapter 2). Loss or inhibition of HDAC6 activity resulted in a global defect in polarization of organelles including inability of HDAC6 null fibroblasts to reorient their MTOCs. Experiments to elucidate a mechanism for defects in MTOC reorientation in HDAC6 null fibroblasts revealed decreased localizations of two factors involved in the MTOC reorientation pathway: p150glued, a MT +TIP protein that binds to growing or dynamic MT ends was inhibited in its localization to MT plus tips, while its receptor, cortical dynein, was inhibited in its leading edge localization in migrating cells (Dujardin et al., 2003; Gomes et al., 2005). Furthermore, both dynein-mediated and kinesin-mediated MT organelle and vesicle transport was inhibited in motile cells. We also observed defects in the elaboration of a stable, modified MT array oriented towards the leading edge (Chapter 2) suggesting that loss of HDAC6 activity may impact MT stabilization pathways in migrating cells.

MTs play essential roles in polarizing cellular organelles in persistent directional migration (Glasgow and Daniele, 1994; Wittmann and Waterman-Storer, 2001). One of the earliest detectable steps in the polarization of fibroblasts towards an experimental wound is the selective stabilization of a subset of MTs whose distal (i.e., plus) ends are situated in the cortical region of the cell, closest to the wound edge (Gundersen and Bulinski, 1988). While most MTs in fibroblasts are highly dynamic, exhibiting a turnover half-time of 6-10 min (Schulze et al., 1987), the plus ends of MTs that face the wound edge appear to be capped (Infante et al., 2000), such that they persist for many hours.

Two distinct molecular mechanisms have been elucidated by which wound-edge MTs become selectively stabilized: Akhmanova et al. (2001) found that CLIP-170, called a +TIP because it is normally bound to the plus ends of dynamic MTs, recruits CLIP-170-associated proteins (CLASPs) to MT tips; this complex then stabilizes MTs by attaching their plus ends to the cell cortex through association of CLASPs with the cell cortex components, LL5β and ELKS (Lansbergen et al., 2006). Concurrently, Palazzo et al. (2001a) found that RhoA stabilizes MTs through its downstream mediator, mDia; mDia binds additional proteins, APC and EB1, and these then bind to and stabilize MT plus ends (Wen et al., 2004).

Interestingly, GSK3β is involved in the regulation of both MT stabilization pathways. GSK3β is negatively regulated by mDia1 in the MT stabilization pathway involving EB1 and APC (Eng et al., 2006; Wen et al., 2004). The inhibition of GSK3β, which inhibits HDAC6 (Chen et al., 2010) is also involved in the regulation of CLASP2 (Akhmanova et al., 2001; Kumar et al., 2009; Lansbergen et al., 2006; Watanabe et al., 2009; Wittmann and Waterman-Storer, 2005). Phosphorylation of CLASP2 by active GSK3β disrupts the localization of CLASP2 and EB1 from MT plus ends (Watanabe et al., 2009). Furthermore, GSK3β may coordinate the actin and the MT networks required for efficient cell migration via regulating the interaction between CLASP2 and IQGAP1 which can bind both actin and MTs. Although the interface between the two MT stabilization mechanisms is not understood, a link between mDia and HDAC6 (Bershadsky et al., 2006) and GSK3β and HDAC6 (Chen et al., 2010) may implicate HDAC6 in wound-edge MT stabilization.

Based on that MT +TIPs are involved in conserved MT capture mechanisms involved in cellular polarization (Gundersen et al., 2004), that p150glued and dynein localizations were affected in HDAC6 KO MEFs resulting in a defect in MTOC reorientation, and that stable,

modified MT arrays were not oriented towards the leading edge, we hypothesized that loss of HDAC6 activity in HDAC6 null fibroblasts regulates MT stabilization pathways. Here we report our data on MT +TIP localization involved in the two other, independent MT stabilization pathways showing that only the pathway involving CLASP2 and its cortical receptor LL5β is affected in HDAC6 null fibroblasts.

RESULTS

Loss of HDAC6 inhibits localization of CLASP2, and its cortical receptor LL5β

Accumulation of cortical proteins at the leading edge is an early step in the polarization response to monolayer wounding (Dujardin et al., 2003; Lansbergen et al., 2006). The plus ends of MTs that approach the wound edge of cells have been shown to be stabilized through the binding of +TIP family proteins (Carvalho et al., 2003). Previously, we showed that a +TIP-cortex interaction mediated by p150glued, a +TIP and dynein adaptor, by docking with dynein anchored in the leading-edge cortex of motile cells (Etienne-Manneville and Hall, 2001; Gomes et al., 2005; Palazzo et al., 2001b) was altered by loss of HDAC6 in HDAC6 KO MEFs leading to MTOC reorientation defects. We investigated a parallel pathway in cells which is involved in MT stabilization (Lansbergen et al., 2006), involving the MT + TIP CLASPs (Akhmanova et al., 2001) which dock MT plus ends to leading edge plasma membrane receptors such as LL5β (Lansbergen et al., 2006).

CLASPs and LL5β specifically redistribute to the leading edge lamellipodia at the onset of cellular migration (Akhmanova et al., 2001; Lansbergen et al., 2006). To address the mechanism(s) underlying defects in the formation of properly oriented stable MTs we localized CLASP2 (Figs. 1A and 1B) and its docking receptor, LL5β at the leading-edge cortex in wound-edge cells (Figs. 1C and 1D) in WT and HDAC6 KO MEFs. Analogous to the factors involved in the MTOC reorientation pathway (Chapter 2), both markers were markedly reduced in their localization at the cell cortex in HDAC6 KO MEFs and few MT ends converged at the cell cortex facing the wound edge were CLASP2 or LL5β positive (Fig 1 A-D). Like dynein, while the noticeably fewer cortical CLASP2 and LL5β patches in HDAC6 KO cells almost entirely coincided with converged MTs (data not shown), the converse was not true. Lamellipodia

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enriched in converged MT distal end networks were markedly reduced for cortical CLASP2 and

LL5β localization (Fig. 1 A-D) in HDAC6 KO MEFs as compared to their WT counterparts.

These data indicate that like factors involved in MTOC reorientation, CLASP2 and LL5β are

inhibited in their localizations to the cortex providing a mechanism for the defect in polarization

of a stable, modified MT array oriented towards the leading edge in directionally migrating cells

(Chapter 2).

Loss of HDAC6 inhibits localization of CLASP2 at MT plus ends but not of EB1 or

CLIP170

Since CLASP2 cortical localization was inhibited we wanted to test whether CLASP2

MT plus tip localization is also altered by loss of HDAC6 in HDAC6 KO MEFs. To assay this

we immunolabeled and quantified the number of MT plus ends positive for CLASP2

localization, as described for p150glued (see Materials and Methods). HDAC6 KO MEFs

showed greatly reduced CLASP2 localization on MT plus ends (~ 60%, Figs. 2A and 2B),

reminiscent of our previous results with p150glued. Thus, CLASP2 localization is inhibited both

at the leading edge cortex and on MT plus ends by loss of HDAC6 and provides a mechanism for

the defect in orientation of a stabilized, oriented MT array at the leading edge in migrating cells.

Since CLASPs are thought to be recruited by CLIP170 onto MT plus ends (Akhmanova

et al., 2001), we assayed whether CLIP170 localization is affected in HDAC6 KO MEFs to begin

to elucidate mechanisms affecting CLASP2-MT plus end localization. Moreover since CLIP170

is known to provide the link between dynein and its cargo, and dynein-mediated MT based

transport and dynein's cortical localization was affected by loss of activity of HDAC6, we

predicted that CLIP170 would also exhibit altered localization in HDAC6 KO MEFs. In

addition, we also determined whether EB1, which is a downstream factor for mDia in the

alternate MT stabilization pathway (Eng et al., 2006; Palazzo et al., 2001a; Wen et al., 2004),

and is thought to stabilize MTs by specifically binding to the MT GTP cap, is inhibited in its

ability to interact with MT plus ends in HDAC6 KO MEFs.

To test these possibilities, we quantified the localizations of CLIP170/CLIP115 and EB1,

which are implicated in dynein function (Pierre et al., 1992) and MT stabilization (Akhmanova et

al., 2001; Wen et al., 2004), respectively. However, no reduction in the distributions of CLIP170

(data not shown) or EB1 (Fig. 2C and D) on MT distal ends was observed in the absence of

HDAC6 protein. These data suggest that CLIP170 is not involved in the CLASP2 MT

stabilization or the dynein MTOC reorientation pathways. Although, we did not assay APC, the

other +TIP involved in the EB1 MT stabilization pathway, the localization with EB1 suggests

that perhaps this MT stabilization pathway is not regulated by HDAC6 activity. These data,

together with our data on p150glued and dynein, suggest that loss of HDAC6 activity decreases

MT-cortex interactions by inhibiting localizations of cortical membrane proteins and their +TIP

MT-linking partners in both MTOC reorientation and MT stabilization pathways, thus providing

a plausible mechanism for the associated polarity defects in migrating cells.

Chapter 3 - Figures

Figure 1

A
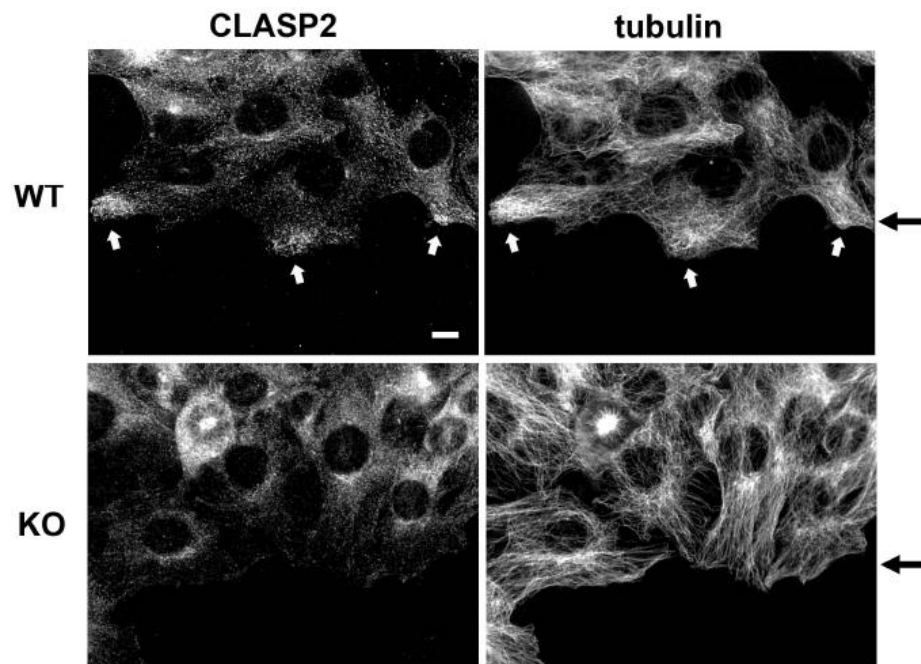
B
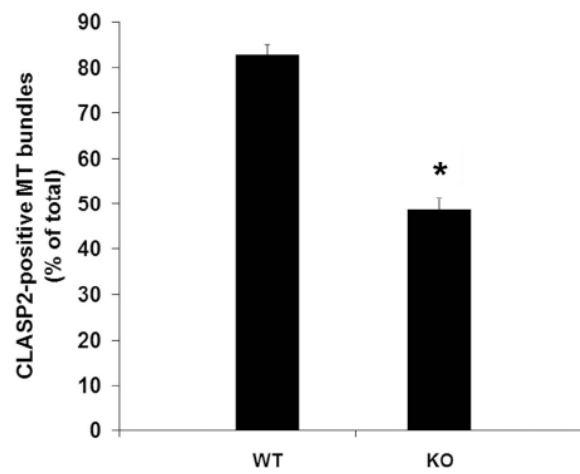
Figure 1 continued

C

D
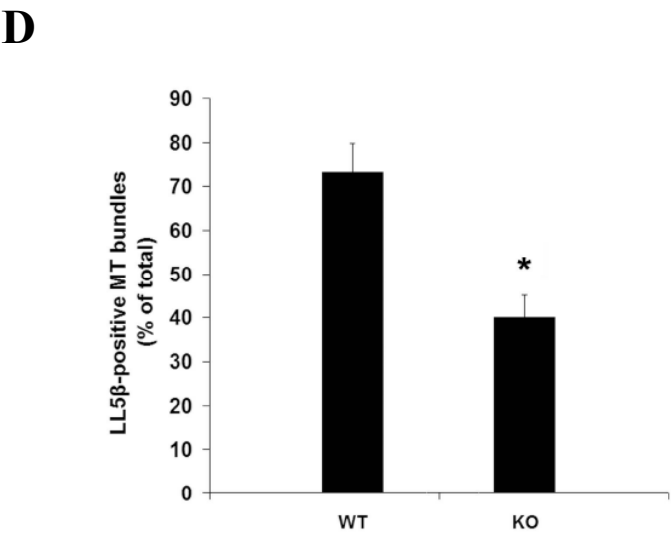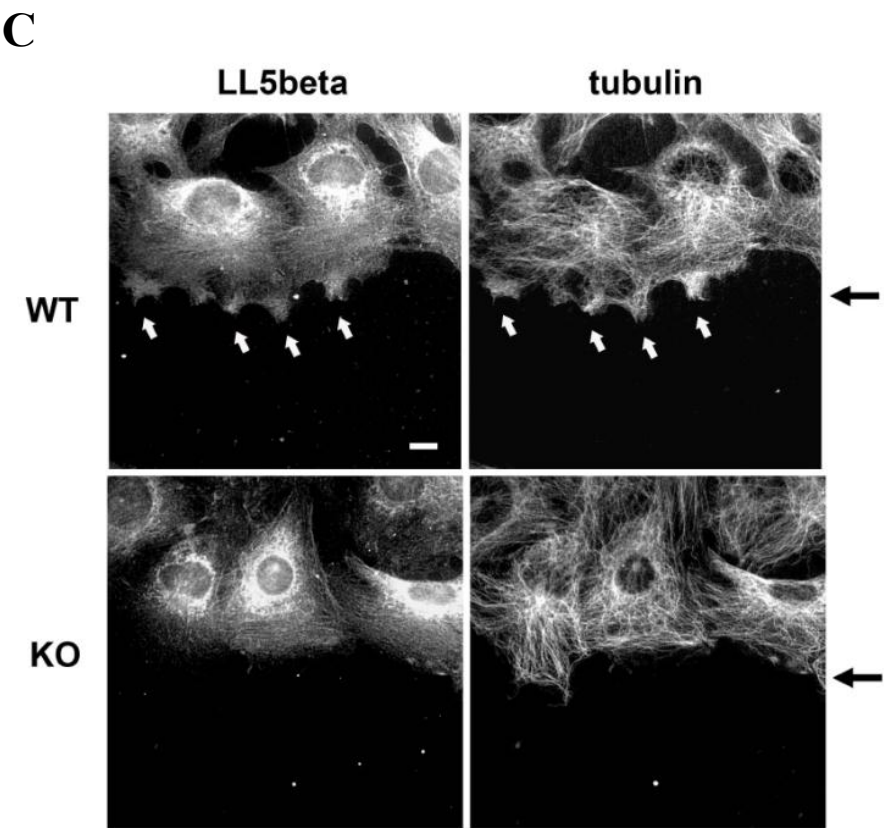
Figure 1. Loss of HDAC6 inhibits localization of CLASPs and their cortical docking protein, LL5β, to MT plus-ends and the leading edge.

A) Micrographs and **B)** quantification (see Materials and Methods) of leading edge localization of CLASP2. **C)** Micrographs and **D)** quantification of leading edge localization of LL5β, as in B). **A)** and **C)** WT and HDAC6 KO MEF monolayers were wounded and allowed to polarize for 5 hours before they were fixed and immunolabeled with antibodies against **CLASP2**, **LL5β** and **total (β) tubulin**. White arrows indicate CLASP2- or LL5β-positive leading edge regions in WT MEFs where MTs were observed to converge. Wound edges are indicated by black arrows. Bar, 10 µm. **B)** and **D)** Asterisks indicate significant differences, compared to WT controls, (p<0.05).

Figure 2

A
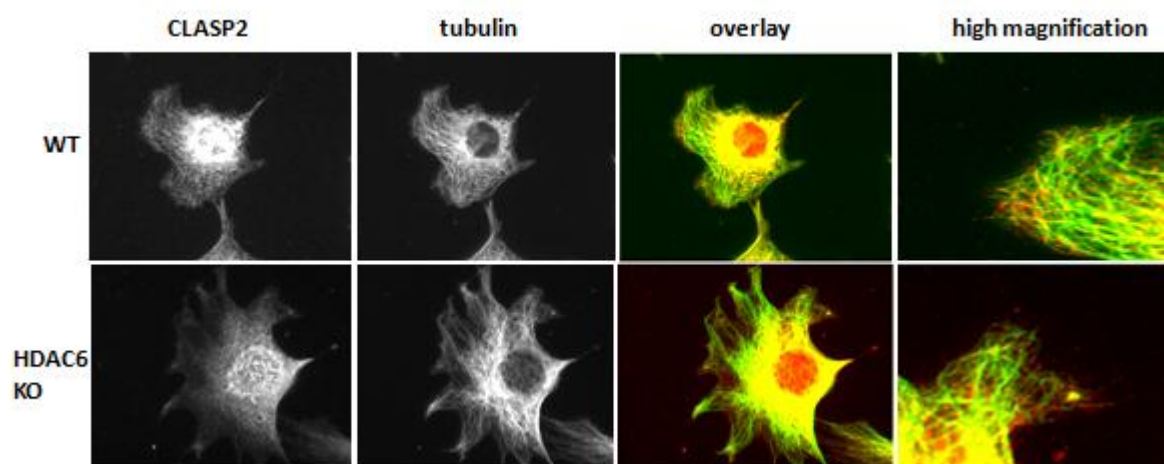
B
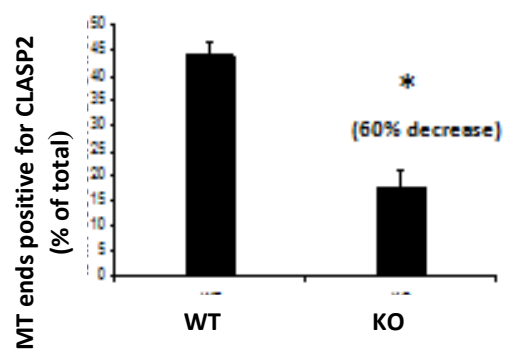
Figure 2 continued

C
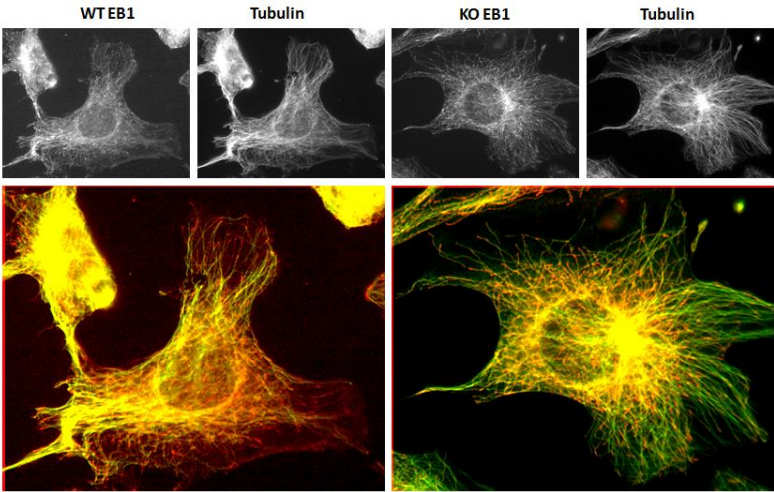
D
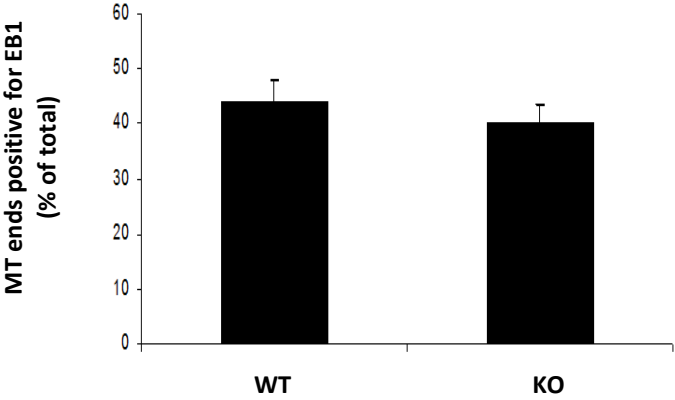
Figure 2. Loss of HDAC6 inhibits localization of CLASP2, but not EB1, to MT plus-ends.

A, and C) Micrographs and **B, and D)** quantification (see Materials and Methods) of CLASP2, and EB1 localization on MT plus ends in sparse WT and HDAC6 KO MEFs, labeled with **CLASP2** and **total (β) tubulin** antibodies in A), and **EB1** and **total tubulin** antibodies in C). Bar, 10 µm. B, D, F) Asterisks indicate significant differences, compared to WT controls, (p<0.05).

DISCUSSION

Taken with our previous results, our data demonstrate HDAC6 enzymatic function in two non-overlapping pathways, polarization of the stable MT array, and reorientation of the MTOC (e.g., Drabek et al., 2006; Dujardin et al., 2003; Palazzo et al., 2001a; Palazzo et al., 2001b). The MT stabilization and MTOC reorientation pathways are independent, but nonetheless parallel, involving interaction of a +TIP on a dynamic MT, (CLASP or p150glued, respectively) with a cortical receptor concentrated at the leading edge (LL5β or dynein) (reviewed in Gundersen et al., 2004). The fact that loss of HDAC6 yields a more pronounced reduction in fibroblast migration than either CLASP2-depletion (Drabek et al., 2006) or dynein-inhibition (Dujardin et al., 2003) corroborates the notion that HDAC6-inhibition impacts on both stable MTs and MTOC polarization pathways.

Loss of HDAC6 substantially reduced the localizations of the +TIP CLASP2 on MT ends and in the cortex, as well as its cortical receptor, LL5β, thereby inhibiting the formation of a stable MT array oriented towards the direction of migration in motile fibroblasts. Conversely, EB1 localization was not altered in HDAC6 null fibroblasts suggesting that the EB1 MT stabilization pathway is active and functional in these cells. In support of this, MT stabilization of a select subset of MTs was not found to be inhibited in wound-edge cells, its orientation was. Also unlike CLASPs, which are selectively localized to the leading edge, EB1 does not redistribute to the leading edge upon the onset of directional migration (Lansbergen et al., 2006). This implies that while both the CLASP2 and the EB1 pathways can stabilize MTs, the regulation of the LL5β-CLASP2 pathway by HDAC6 is critical for the formation of oriented stable MT arrays towards the direction of migration.

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On analysis, the MT +TIP binding profile in HDAC6 null fibroblasts is perplexing, making it hard to pinpoint a common mechanism of altered localization of these proteins. HDAC6 has been reported to interact with several MT + TIPs including p150glued (Hubbert et al., 2002; Kawaguchi et al., 2003), Arp1 and EB1 (Zilberman et al., 2009). While some of these are CAP-GLY domain containing proteins like p150glued, Arp1, and CYLD (Kawaguchi et al., 2003; Wickstrom et al., 2010; Zilberman et al., 2009), others like EB1 (Zilberman et al., 2009) and CLASP2 are not. Thus, while it is not surprising that loss of HDAC6 inhibited the MT plus end localization of p150glued, it is surprising that the localization of CLIP170 was not inhibited, as both are CAP-GLY +TIPs. Paradoxically, the MT plus end localization of EB1, which also binds HDAC6 (Zilberman et al., 2009), was not affected in HDAC6 null fibroblasts, consistent with the observation that in experiments with HDAC6 KD cells only Arp1, a CAP-GLY protein, but not EB1, was affected in its pattern of localization on MT plus ends (Zilberman et al., 2009). It is equally intriguing that CLASP2 localization was inhibited by loss of HDAC6, while the localization of the CAP-GLY containing, p150glued-related CLIP170 which helps to recruit CLASPs on MT plus ends (Akhmanova et al., 2001) was not. It remains to be shown whether CLASPs and CLIP170 bind HDAC6.

EB1 is an interesting and unique MT +TIP protein as it can bind MTs independently of other MT +TIP and binding partners (Dixit et al., 2009). EB1 is postulated to be the key recruiting factor that establishes a hierarchy of different complements of +TIPs at the MT plus ends, as it can bind almost all other MT +TIPs (Akhmanova and Steinmetz, 2010). EB1's well-studied C-terminal EEY/F motif, which is also present in tubulin, mediates binding to CAP-GLY domain containing proteins like p150glued and CLIP170 recruiting them to MT plus ends. EB1 also binds CLASP2, a member of the CLASP family which do not contain CAP-GLY domains

but instead mediate binding to MTs through their TOG domains and additional SxIP motifs (Al-Bassam and Chang, 2011; Mimori-Kiyosue et al., 2005; Slep and Vale, 2007). The N-terminal EB homology (EBH) domains of EB1 interact with the SxIP motifs of CLASPs thereby recruiting them to MT plus ends. Due to the important role of EB1 in establishing different networks of MT +TIPs bound to the growing, plus ends of MTs, it is not surprising that its localization was not altered by loss of HDAC6. Similarly, CLIP170 also plays an important role in hierarchical recruitment of a subset of +TIPs as it recruits CLASPs (Akhmanova et al., 2001) and p150glued (Lansbergen et al., 2004) to MT ends. Moreover, CLIP170 plays an essential role in dynein function providing a link between dynein and many of its cargoes (Pierre et al., 1992). The MT localization of EB1 and CLIP170 is thus probably under tight regulatory control since inhibition of these two +TIPs would lead to global cellular malfunction. This might explain why in HDAC6 null fibroblasts only the localizations of p150glued and CLASP2 are affected.

Hyperacetylation of all MTs might affect the binding of a subset of the MT +TIPs. However, we cannot as yet distinguish whether it is the specific loss of HDAC6 activity or the physical protein that alters the +TIP protein localizations on MT plus ends as we did not investigate the localizations of these proteins in HDAC6 drug-inhibited cells. The moderately reduced dynamics of MTs in HDAC6-inhibited (Tran et al., 2007) and HDAC6-null fibroblasts (Tran and Bulinski, unpublished data) might not be sufficient for inhibiting MT plus end localization for the +TIPs we have investigated and do not provide a satisfactory explanation unless different MT + TIPs exhibit different sensitivities to reduction in MT dynamics. Dampened MT dynamics may, however, contribute indirectly to decreasing at least CLASP2-MT binding: MT dynamics is a local regulator of Rac activity (Waterman-Storer et al., 1999), which, in turn, regulates CLASP-MT affinity (Wittmann and Waterman-Storer, 2005). Doing

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more detailed investigation of these possibilities will shed more light on HDAC6-mediated mechanisms controlling MT +TIP binding patterns in HDAC6 null fibroblasts and are the focus of future efforts.

Finally, loss of HDAC6 in fibroblasts also failed to localize cortical docking proteins involved in leading edge orientation of the stable MT array, i.e., LL5β and CLASP2, parallel to cortical dynein in the MTOC reorientation pathway. Mislocalization of these cortical receptors further lowers the likelihood of leading edge MT capture inhibiting the formation of an oriented stable MT array in migrating cells. Mechanisms for localizing cortical MT receptors have not been well described; thus, we can only speculate on the impact of loss of HDAC6 and/or MT hyperacetylation. In contrast to accumulation of dynein at the cortex which may be dependent on its loading onto MTs (Sheeman et al., 2003; Xiang, 2003), perhaps via the MT-loading factor, p150glued (Vaughan, 2005) or kinesin-based MT transport (Zhang et al., 2003a), cortical accumulations of LL5β and CLASP2, are MT-independent (Drabek et al., 2006; Lansbergen et al., 2006). Interestingly, CLASP2 cortical localization, though MT-independent, appears to be targeted via a Golgi-based mechanism (Mimori-Kiyosue et al., 2005), thus indirectly mediated by MT-based transport. Thus, defects in bi-directional MT-based transport in HDAC6 null cells (Chapter 2) may abrogate redistribution of both dynein and CLASP2 to the leading-edge cortex.

While it is not known whether LL5β or CLASP2 interact with HDAC6, HDAC6 itself could be responsible for both LL5β's and CLASP2's leading edge localization as it is specifically localized to the leading edge at the onset of fibroblast migration (Kawaguchi et al., 2003). Lipid raft redistribution to the leading edge of migrating cells, activating PI3K signaling pathways, and recruiting proteins with plekstrin homology (PH) domains to the cortex (reviewed in Manes et al., 2003), may also be responsible for leading edge localization of LL5β as it is

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PI3K-regulated and has a PH domain (Paranavitane et al., 2003). Lipid rafts have been implicated in stabilizing MTs, by stimulating Rho GTPase signaling (Palazzo et al., 2004). Evidence of crosstalk among lipid rafts, Rho family members, and PI3K is proposed to establish and reinforce cellular polarization (Manes et al., 2003). Although these processes occur downstream of actin reorganization (Manes et al., 2003), feedback loops between MTs and small GTPases have been reported in which MTs also interact with regulatory subunits of PI3K (reviewed in Wittmann and Waterman-Storer, 2001). Thus, it is possible that a feedback loop exists in which HDAC6, MTs or acetylated, but not hyperacetylated MTs regulate upstream signaling pathways that then activate MT capture. Another possibility is that HDAC6 activity, MTs or MT acetylation influences cellular polarization via actin regulation. Regulation of cortical accumulation of CLASP2, in addition to PI3K signaling (Akhmanova et al., 2001), has been shown to be mediated by ACF7 (Drabek et al., 2006), a MT-actin cytoskeletal linker protein whose loss also disrupts polarization in migrating cells (Kodama et al., 2003).

These results, though preliminary, reveal the importance of HDAC6 in the CLASP2-LL5β MT stabilization pathway. Elucidating the specific mechanisms that affect MT plus end localization of CLASP2, and not EB1 or CLIP170, and cortical accumulations of CLASP2 and LL5β in HDAC6 null and drug-inhibited fibroblasts are the focus of ongoing studies.

MATERIALS AND METHODS

Cell culture and treatments

All cells were cultured in DMEM: Media of mouse embryonic fibroblasts (MEFs), generated from wild-type (WT) and HDAC6 knockout (KO, genotype, HDAC6 -/y) mice (Zhang et al., 2008) was supplemented with 10% fetal bovine serum.

Antibodies and reagents

All antibodies and chemicals were purchased from Sigma-Aldrich unless indicated. Antibodies to β-tubulin (3F3); CLASP2 and CLIP170/115; and LL5β were generous gifts from Drs. J. Lessard (University of Cincinnati), N. Galjart (Erasmus University), A. Akhmanova (Erasmus University), respectively. Anti-EB1 antibodies were from BD Transduction Laboratories (Lexington, KY).

Quantification of cellular polarization and MT end-binding proteins

Polarization of cellular organelles and cortical leading edge markers was quantified in fixed and immunolabeled wound-edge cells as described (Finkelstein et al., 2004). The occurrence of +TIP CLASP2 and cortical LL5β at the leading edge were analyzed in parallel with analysis of MT ends consolidated at these lamellipodial regions (Dujardin et al., 2003). MT plus end localization of +TIP CLASP2 was quantified in sparse cells from immunofluorescence images double-labeled for CLASP2 and β tubulin; pixel intensities, for a total of ≥ 200 MTs were quantified from 15 cells per cell type, with MetaMorph software (Molecular Devices, Downingtown, Pennsylvania).

Data Analysis

Data were quantified from at least three independent experiments. For microscopic scoring, at least 200 cells or fields of view were scored for each experimental condition. Statistical significance was evaluated using a student's t-test (significance required p-value < 0.05).

CONCLUSIONS AND FUTURE PERSPECTIVES

Consistent with our hypothesis that HDAC6 activity regulates important MT-dependent aspects of directionally migrating cells, we report here that HDAC6 enzymatic activity, and not level, is required for MT-dependent organelle polarization and polarized intracellular transport in motile fibroblasts. Of the three HDAC6 substrates directly involved in cell motility, we find that only tubulin's acetylation state, and not Hsp90's or cortactin's, which are involved in actin-mediated motility, was altered by abrogating or inhibiting HDAC6 activity.

In motile cells, the early elaboration of a stable, modified MT array oriented towards the direction of migration is thought to template further polarization of cellular contents and intracellular transport (Bulinski and Gundersen, 1991; Kirschner and Mitchison, 1986). Our data show HDAC6 function in two independent but parallel, MT-dependent cellular polarization pathways: the formation of an oriented stable MT array, and reorientation of the MTOC (e.g., Drabek et al., 2006; Dujardin et al., 2003; Palazzo et al., 2001a; Palazzo et al., 2001b). Investigation into the mechanism(s) by which HDAC6 activity and its substrate tubulin or its acetylation/deacetylation state impacts directional migration revealed that loss of HDAC6 activity altered localizations of particular MT plus-end binding and cortical protein partners, i.e. p150glued and dynein, and CLASP2 and LL5β, and/or activities of the MT motors, dynein and kinesin, thereby inhibiting polarization of organelles and intracellular transport towards the leading edge.

By what underlying mechanism(s) does HDAC6-mediated deacetylation of tubulin modulate these effects in cell motility? It is possible that the binding of a subset of the MT +TIP or motor proteins is specifically altered by MT hyperacetylation. Chemical modification of tubulin subunits via acetylation could alter polymerization or surface structure of MTs. Either of these would be expected to decrease MT +TIP and motor binding as well as the probability of

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MT targeting to leading edge docking sites. It, however, remains to be determined how MT acetylation may be playing a role in either signaling as the lysine 40 site of α-tubulin faces the MT lumen. A MT 'breathing' mechanism has been proposed involving the weaker heterologous contacts between tubulin dimers at the MT seam (Nogales, 2001); this may explain how enzymes and interacting proteins have quick access to this site, as the kinetics of enzymatic activity which are quite rapid are not consistent with slow diffusion down the inside of the bumpy MT lumen or through the smaller MT fenestrations inherent in the MT structure. However, as lysine 40 occurs on the H1-S2 loop, a region in proximity to the corresponding Taxol binding site on β-tubulin which stabilizes lateral protofilament contacts (Nogales, 2001), it is possible that the acetylation of lysine 40 in this region imparts moderate stabilization to MTs as we previously determined (Tran et al., 2007) and/or induces a conformational change in MT structure that alters protein-MT interactions. Elucidating the mechanism(s) by which hyper-acetylation decreases binding of the +TIPs p150glued and CLASP2 and the motors dynein and kinesin to MTs will require in vitro binding experiments with purified Ac MTs and MAP components and is the subject of current efforts.

Interestingly, only the localizations of a subset of +TIPs, namely p150glued and CLASP2, were inhibited in HDAC6 null fibroblasts. The reduced dynamics of hyperacetylated MTs in HDAC6-inhibited or knockout fibroblasts (Tran et al., 2007), may suffice to reduce the MT-binding affinity of these TIPs, and not others, even though the MT binding domains of some of these +TIPs are similar (Carvalho et al., 2003; Perez et al., 1999; Slep and Vale, 2007). For example, while CLIP-170 and CLIP115 (data not shown) were not reduced on MT tips, p150glued was (Chapter 2). CLIP170, CLIP115 and p150glued are related MT +TIPs and contain CAP-GLY domains that interact with both HDAC6 and MTs through their C-terminal

EEE/Y motifs (Akhmanova and Steinmetz, 2010; Wickstrom et al., 2010). The localization of EB1, which is a different subclass of + TIPs, was also not affected. However, the localization of CLASP2, a distinct subclass family member that contain TOG domains (Al-Bassam and Chang, 2011; Mimori-Kiyosue et al., 2005), which is thought to be recruited to the MT plus ends via its interaction with CLIP170 (Akhmanova et al., 2001), was inhibited.

However, we previously found that the effect on MT dynamics is moderate and comparable to the low dose of 100nm Taxol treatment (Tran et al., 2007). Perez et al. have previously reported that for the related MT +TIP, CLIP170, which shares the CAP-GLY domain homology with p150glued, a concentration of 200nM Taxol is sufficient to inhibit its binding to MT plus ends (1999). As we did not see any effect of loss of HDAC6 on CLIP170 in our cells, the dampening of MT dynamics we see in cells with hyperacetylated MTs may not be sufficient to inhibit +TIP binding. However, it is possible that different MT +TIPs exhibit different sensitivities to reduction in MT dynamics. In the case of CLASP2, a member of the CLASP family which do not contain CAP-GLY domains and mediate binding to MTs through their TOG domains and additional SxIP motifs (Al-Bassam and Chang, 2011; Mimori-Kiyosue et al., 2005; Slep and Vale, 2007), dampened MT dynamics may contribute indirectly to decreasing CLASP2-MT binding, instead: MT dynamics is a local regulator of Rac activity (Waterman-Storer et al., 1999), which, in turn, regulates CLASP-MT affinity (Wittmann and Waterman-Storer, 2005). Additionally, a feedback loop exists between Rac1 and MTs; Rac1, a Rho GTPase involved in cell migration and ruffling, specifically localizes to the leading edge of migrating cells where it in turn could influence MT dynamics to regulate MT-+TIP interactions at the plus ends of growing MTs (Wittmann and Waterman-Storer, 2001).

The most plausible mechanism explaining our results may be that HDAC6 itself is directly involved in the recruitment of a subset of MT +TIPs to MT plus ends. Since HDAC6 interacts with p150glued and other CAP-GLY containing proteins like CYLD (Kawaguchi et al., 2003; Wickstrom et al., 2010) it may be involved in the recruitment of these proteins to MT +TIPs. This can explain why in HDAC6 KO cells, the loss of HDAC6 protein or activity inhibited p150glued localization at MT plus ends. Consistent with our model, in HDAC6 KD experiments, it was reported that only Arp1, a p150glued related protein and dynactin component, but not EB1, was slightly altered in its pattern of localization (p150glued was not tested in this analysis) (Zilberman et al., 2009). However, since CLIP170 was not affected, and EB1 binds to HDAC6, yet is present on MT plus ends in HDAC6 KO cells, there must be other levels of regulation determining the +TIP MT networks.

However, we cannot as yet distinguish whether it is the specific loss of HDAC6 activity or the loss of its physical presence that alters the +TIP binding of p150glued and CLASPs as we did not investigate localization of these proteins in HDAC6 drug-inhibited cells. There is some precedence for altered binding of MT +TIPs in the presence of HDAC6 inhibitor as Zilberman et al., reported some changes in localization patterns of Arp1, another related p150glued and dynactin component, as well as EB1 (2009), although the proteins remained localized to MT +TIPs. Perhaps this can be explained by the fact that the EB1 interaction has been determined to occur through the N- and C-terminal domains of HDAC6 which are not involved in deacetylation activity. There is a possibility that the binding of p150glued, and also CLASP2, to HDAC6 is inhibited in the presence of HDAC6 inhibitors, as reported for the disruption of the dynein-HDAC6 interaction in the presence of tubacin (Hideshima et al., 2005). It is interesting that the TOG domain containing CLASP2 is also affected by loss of HDAC6 activity. Whether HDAC6

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interacts with TOG domains or another domain in CLASPs has not yet been investigated. It will be thus, interesting to do a detailed analysis on +TIP binding profiles to deacetylated or hyperacetylated MTs with or without the presence of HDAC6 inhibitors to elucidate a mechanism for altered p150glued and CLASP2 binding to hyperacetylated MT plus ends. Thus, HDAC6 activity or the physical presence of HDAC6 on MTs may recruit specific subsets of MT +TIPs to the MT plus ends by direct interaction.

In general, though, our results with localization of MT +TIPs make it hard to pinpoint a common mechanism of altered localizations of these proteins at MT +TIPs in cells with loss of HDAC6 activity. Surprisingly, p150Glued, another CAP-Gly +TIP, is localized normally in TTL-null neurons (Erck et al., 2005). This is in sharp contrast to the finding that three CAP-Gly +TIPs (CLIP170, CLIP-115, and p150Glued) are mislocalized in TTL-null fibroblasts (Peris et al., 2006) Thus, there are likely different molecular mechanisms for CAP-Gly +TIP-mediated regulation of MT dynamics in neurons and non-neuronal cells. Moreover, while CLASP2 is involved in fibroblast migration (Akhmanova et al., 2001), EB1 has recently been reported to be involved in HDAC6-mediated directional migration of endothelial cells (Li et al., 2011a). These results may also indicate cell-specific differences between fibroblast and endothelial cells in migration.

Hyper-acetylation of virtually all cellular MTs could also delocalize signaling pathways involved in MT-capture pathways. This hypothesis is supported by the finding that HDAC6-inhibited fibroblasts did generate stabilized MT arrays, even though they failed to reorient these or other organelles towards the leading edge. Stabilized MT arrays can be generated by one of two pathways, either by the +TIPs EB1 and APC (Eng et al., 2006; Wen et al., 2004) or via the cortical docking of CLASPs to their cortical receptors (Lansbergen et al., 2006). Although,

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CLASP2 MT stabilization to the leading edge is defective in HDAC6 KO MEFs, EB1

localization was not altered suggesting that the EB1 MT stabilization is active and functional in

these cells. Indeed, Importantly, EB1 does not redistribute to the leading edge upon the onset of

directional migration as reported for CLASPs which are known to be selectively localized to the

leading edge (Lansbergen et al., 2006). Thus it is possible that these proteins are differentially

regulated by upstream localization factors and that the localization of CLASPs but not the EB1

family is specifically required to be at the leading edge. Of course, we did not investigate the

localization of APC, an EB1 partner and MT stabilizer in the same pathway, in our HDAC6 KO

MEFs. Thus, it is possible that while binding of EB1 to MTs is not regulated by signaling

networks in cellular polarization, APC's binding to MTs is. This is a possibility that bears further

investigation.

While our data point to possible mechanisms by which MT acetylation may play a role in

cellular polarization of migrating cells, we cannot determine whether our results are attributable

to the loss of HDAC6 activity on MT hyperacetylation as we were unable to definitively

conclude whether the acetylation status of α-tubulin directly regulates intracellular transport in

cell motility as both a WT and a non-acetylatable tubulin mutant equivalently rescued

bidirectional vesicular transport. Expression of the non-acetylatable tubulin mutant has however,

previously been shown to rescue focal adhesion turnover in HDAC6 KO MEFs (Tran and

Bulinski, unpublished data). Although our result clearly demonstrates that a MT-based

mechanism is responsible for inhibited cellular polarization and bidirectional intracellular

trafficking in HDAC6 null fibroblasts, one explanation for this discrepancy can be that

expression of tubulin in HDAC6 KO MEFs results in an increase in unmodified tubulin residues

along polymerized MTs, or a general increase in MT polymerization, which would both also

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rescue MT-based motor transport. Therefore, determining whether modulation of the acetylation state of tubulin is specifically required for intracellular transport in motile cells is the focus of current efforts. Analysis of the acetylation-mimetic mutant, with lysine mutated to glutamine, which should not rescue intracellular transport unless expression results in increased unmodified tubulin residues along MTs or MT polymerization, will be included in this analysis.

Alternatively, it is possible that another HDAC6 substrate or interacting partner modulates the effects on MT-based transport and polarization in migrating cells. HDAC6 has several other identified substrates (see Table 1, Chapter 1). From the known substrates only α-tubulin, Hsp90, and cortactin are cell motility modifiers. Though we were able to rule out Hsp90 and cortactin, the two major HDAC6 substrates known to be involved in actin-based cell motility, we cannot discount the possibility of other HDAC6 substrates contributing to the motility defect we observe here. However, none of these other substrates are known to be direct cell motility modifiers and whether altering the acetylation state of these substrates contributes to altering directional migration remains to be determined. It is also likely that HDAC6 has substrates involved in the regulation of cell motility that have not yet been identified. For example, presence of HDAC6 in protein complexes containing the formin, mDia (Destaing et al., 2005), GSK3β (Chen et al., 2010), PP1 (Brush et al., 2004), or SIRT2 (North et al., 2003), suggests that HDAC6 may deacetylate these or other substituents of these protein complexes, many of which are known to interact with and impact the function of MTs. The identification of such a factor in migrating cells that coordinates the HDAC6 and MT networks will be the subject of future studies.

We find that treating WT MEFs with TSA recapitulated the behavior of HDAC6 KO MEFs in rate of directional migration, extent of cellular polarization, and MT hyperacetylation

suggesting that it is the partial or complete loss of activity of HDAC6, specifically, and not decreased level of HDAC6 or activity of other HDACs, that inhibits MT-dependent intracellular polarization and directional migration. However, there is also a possibility that, in addition to its deacetylase activity which we have shown is required for MT-dependent polarization in motile cells, HDAC6 plays a deacetylase independent or scaffolding role in the directional migration of fibroblasts as well as that reported for lymphoctyes (Cabrero et al., 2006). This is supported by the fact that in fibroblasts, the deletion of the BUZ domain caused a similar inhibition in directional migration as the catalytically inactive HDAC6 mutant (Gao et al., 2007). Moreover, the BUZ domain is implicated in linking ubiquitinated cargo to the MT motor dynein in MT-motor dependent transport (Kawaguchi et al., 2003). Thus, it is likely that this domain has an important contribution in the transport of membrane vesicles and other organelles important in membrane turnover.

Furthermore, as HDAC6 interacts with many proteins (see Chapter 1, Table 2) that impact the MT cytoskeleton, including the dynein motor (Kawaguchi et al., 2003); GSK3β (Chen et al., 2010), GRK2 (Lafarga et al., 2012), mDia ((Destaing et al., 2005), as well as many MT MAPs, including the neuronal tau (Ding et al., 2008), p150glued (Hubbert et al., 2002; Kawaguchi et al., 2003), Arp1 and EB1 (Zilberman et al., 2009), as well as β-tubulin (Zhang et al., 2003b), the scenario that HDAC6 additionally plays a deacetylase-independent role in fibroblast motility is likely and remains to be investigated. The direct interaction of HDAC6 with the MT motor dynein and MT +TIPs EB1 and p150glued (Kawaguchi et al., 2003; Zilberman et al., 2009) could thus explain our results with altered intracellular transport and binding of +TIPs. Furthermore, tubacin has been shown to disrupt the binding between dynein and MTs (Hideshima et al., 2005), thus the loss of HDAC6 or its activity could either directly decrease

dynein loading onto MTs, or indirectly by inhibiting p150glued plus-end binding, causing the

decreased dynein-based motility. It is possible that HDAC6 also directly interacts with and

regulates kinesins, as has been shown for HDAC1 (Kim et al., 2010). Future studies will thus

focus on determining whether our results are due to MT hyperacetylation caused by an inhibition

or loss of HDAC6 activity or by a structural role of HDAC6 and will involve the use of catalytic

inactive mutants and the BUZ domain mutant of HDAC6. We will also investigate whether +TIP

and MT motor binding is affected by loss of a structural role of HDAC6 or its catalytic activity.

mDia is a likely HDAC6-interacting candidate as recent studies have implicated mDia1

in MTOC reorientation in fibroblasts and lymphocytes (Gomez et al., 2007; Yamana et al.,

2006). Furthermore, mDia1 is also shown to be involved in regulating Golgi structure formation,

by influencing all three downstream processes, actin polymerization, myosin contractility and

MT-based intracellular transport (Zilberman et al., 2011). GSK3β is another likely candidate to

mediate HDAC6-dependent effects as it interacts with HDAC6 and also inhibits its deacetylase

activity (Chen et al., 2010). Inhibition of GSK3β or its inhibition of HDAC6 activity was

reported to enhance kinesin-1 affinity for mitochondria and increase kinesin-1-mediated

anterograde mitochondrial transport in neurons, a result consistent with previous studies that

showed increased affinity of the MT motor kinesin1 with Ac MTs and cargoes leading to

enhanced polarized transport along the modified MTs (Dompierre et al., 2007; Reed et al., 2006).

It will be interesting to see whether the polarization and intracellular transport defect in HDAC6

KO MEFs can be rescued by the introduction of mDia or GSK3β. Interestingly, GSK3β, which

interacts with HDAC6 (Chen et al., 2010) is involved in the regulation of both MT stabilization

pathways involving MT +TIPs that polarize stabilized, modified MTs towards the leading edge

in migrating cells. GSK3β is negatively regulated by mDia1 in the MT stabilization pathway

involving EB1 and APC which bind to and stabilize MT plus ends (Eng et al., 2006; Wen et al., 2004). The inhibition of GSK3β, which inhibits HDAC6 (Chen et al., 2010) is also involved in the regulation of CLASP2 (Akhmanova et al., 2001; Kumar et al., 2009; Lansbergen et al., 2006; Watanabe et al., 2009; Wittmann and Waterman-Storer, 2005). The phosphorylation of CLASP2 by active GSK3β disrupts the localization of CLASP2 and EB1 from MT plus ends (Watanabe et al., 2009). However, we observed that loss of HDAC6 only affected CLASP2 localization, not EB1. Thus GSK3β and HDAC6 may function to coordinate these separate MT stabilization pathways. Furthermore, GSK3β may coordinate the actin and the MT networks required for efficient cell migration via regulating the interaction between CLASP2 and IQGAP1 which can bind both actin and MTs.

Though we could not investigate all the substrates and HDAC6 interacting partners, we have ruled out most substrates involved in cell motility and investigated the obvious interacting proteins that regulate MT-dependent effects, namely MT motors and +TIPs. It is also puzzling that HDAC6 null mice are viable and do not exhibit any major defects (Zhang et al., 2008). However these results reflect experiments performed in homeostatic conditions. We predict that further studies with specific cancer and disease models will reveal a more severe defect upon the loss of HDAC6 in developmental, wound-healing and aberrant invasive migration in vivo. While there are many open questions meriting further investigation, our novel data help significantly advance the understanding of how HDAC6 activity modulates cell motility via MT cytoskeleton dependent processes thereby filling a big gap extant in this field of study.

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Appendix A

Related Publication Tran et al., 2007

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 α-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 α-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 α-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 α-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
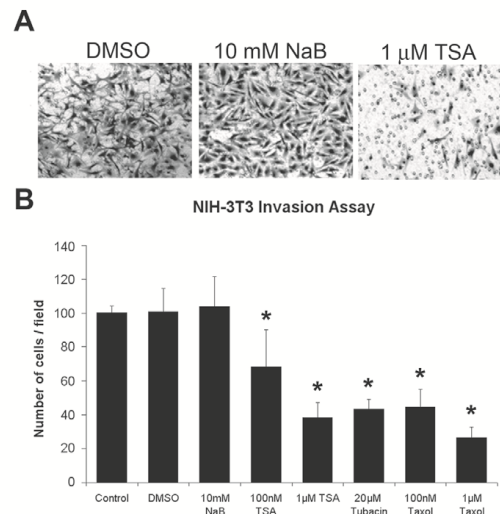
Fig. 1. Inhibition of tubulin deacetylation decreases cell motility in Transwell chemotactic invasion assays of NIH-3T3 cells. (A) Micrographs of typical fields. (B) Quantification of invasion migration. Treatments were as indicated. Standard deviation is shown. Asterisks mark conditions statistically 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; Verschueren 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 μM; e.g. for 0.1 μM TSA, 1 μM TSA and 20 μM tubacin, P values relative to 0.1 μM taxol were $P=0.09$, $P=0.3$ and $P=0.3$, respectively). However, treatment with a high concentration of taxol (1 μM) decreased invasion motility to a significantly greater degree than either 1 μM TSA or 0.1 μM taxol ($P=0.01$ or $P=0.004$, respectively). These data

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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
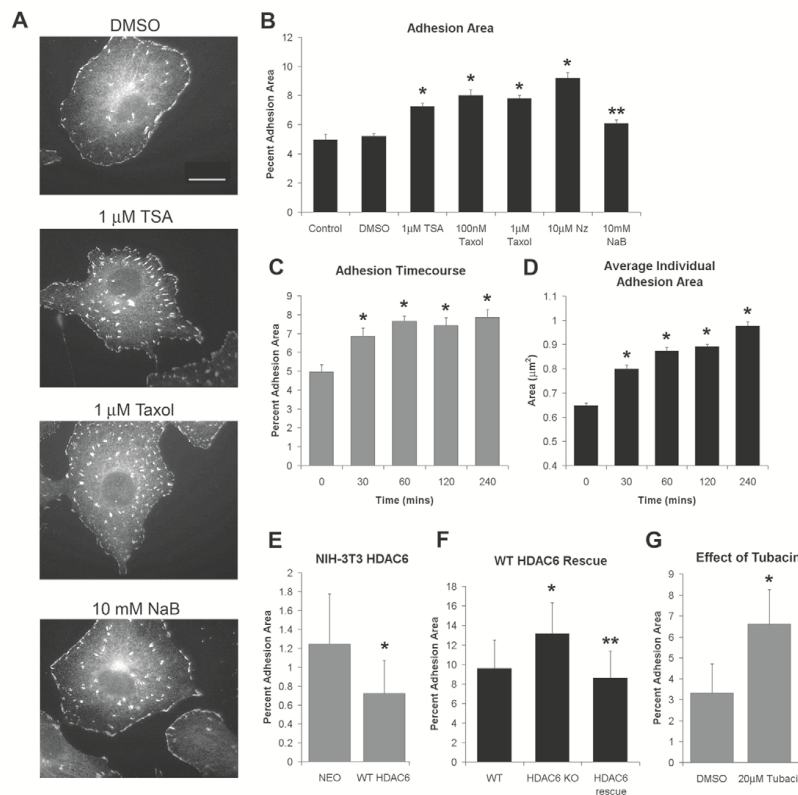
Fig. 2. Altered level or activity of HDAC6 alters focal adhesion area. (A) Paxillin immunostaining of typical TC-7 cells, following 2-hour treatments as indicated. Bar, 20 μm. (B) Quantification of adhesion area in TC-7 cells (shown in A) treated with TSA, taxol and Nz (nocodazole), and NaB; see Materials 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
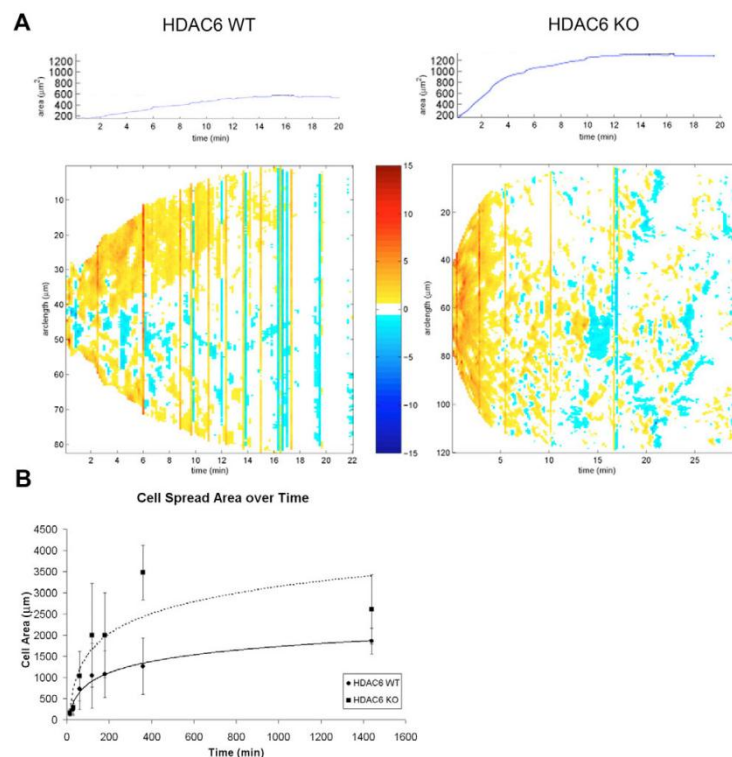
Fig. 3. Cell spreading is altered by HDAC6 level. (A) Early events in spreading of HDAC6 WT and HDAC6 KO MEFs, measured using computer-assisted TIRF microscopy to track the boundaries of the cell during spreading on fibronectin-coated coverslips. Top two panels show quantification of spread cell areas 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) ÷ (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 μM TSA were roughly comparable to cells treated with 0.1 μM 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

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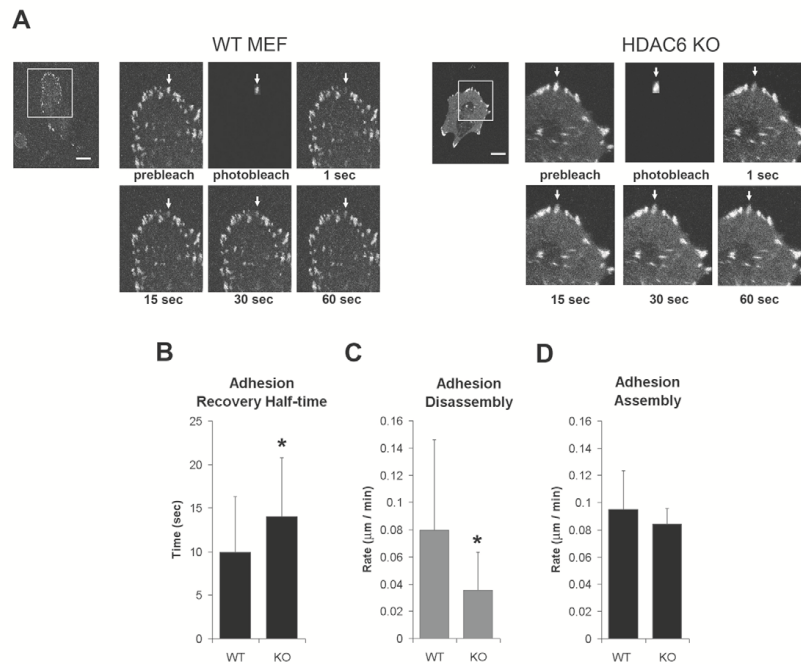
Fig. 4. Focal adhesion turnover and disassembly is affected by MT acetylation. (A) Focal adhesion turnover was calculated from FRAP images of WT and HDAC6 KO MEFs expressing paxillin-GFP. Bars, 20 µm. (B) Average half-time ($t_{1/2}$) of FRAP of adhesions in HDAC6 WT and HDAC6 KO MEFs expressing GFP-paxillin; $n>8$ 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 µM) 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,
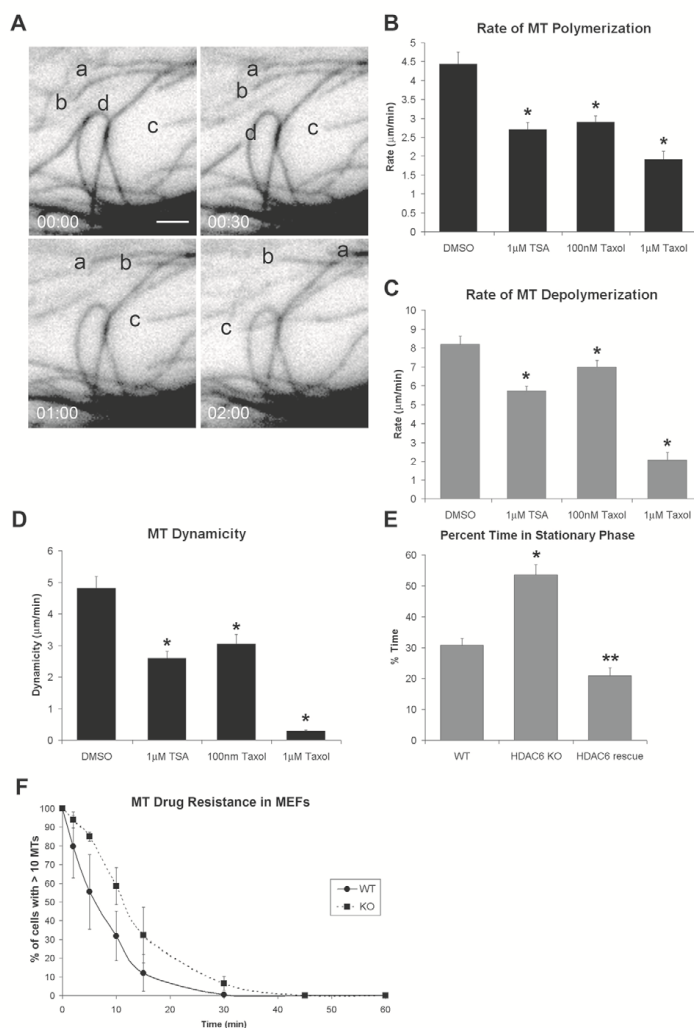
Fig. 5. MT acetylation dampens MT dynamics. (A) Typical micrographs used to measure MT dynamics in 3x GFP-EMTB-TC-7 cells by MT end-tracking; an edge of a cell is shown with elapsed time in minutes:seconds, and four sample MT ends are shown (a-d). Bar, 10 μm. (B-C) Rates of MT (B) polymerization, (C) 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 μM) 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 α-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 α-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 cofilamentous 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 of activity 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 μM 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 α-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 β-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_2 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, 2.5×10^5 cells were applied to each 12-well Transwell membrane insert or, in some experiments, 0.65×10^5 cells per 24-well Transwell insert (both having 8-μm 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 μM 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× 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)] ÷ (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 μM 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 μM ATP in 0.1 M PIPES pH 6.9, 1 mM EGTA, and 1 mM MgSO$_4$, 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 μM 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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Appendix B

Related Publication Chang et al., 2002

Alteration of the C-terminal Amino Acid of Tubulin Specifically Inhibits Myogenic Differentiation*

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Detyrosination is an evolutionarily conserved post-translational modification of microtubule polymers that is known to be enhanced during early morphological differentiation of cultured myogenic cells (Gundersen, G. G., Khawaja, S., and Bulinski, J. C. (1989) *J. Cell Biol.* 109, 2275–2288). We proposed that altering the C terminus of α-tubulin by detyrosination plays a role in morphological differentiation. To test our hypothesis, we treated L6 myoblasts with 3-nitrotyrosine (Eiserich, J. P., Estevez, A. G., Bamberg, T. V., Ye, Y. Z., Chumley, P. H., Beckman, J. S., and Freeman, B. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 6365–6375), a nontoxic inhibitor that resulted in high level inhibition of microtubule detyrosination and low level incorporation of nitrotyrosine into microtubules. Even though microtubule stabilization or modification by acetylation still occurred normally, morphological differentiation was blocked; myoblasts neither elongated significantly nor fused. Nitrotyrosine treatment prevented synthesis or activation of markers of myogenic differentiation, including muscle-specific myosin, α-actin, integrin α_7, and myogenin. Consistent with this, myoblast integrin β_{1A} remained highly expressed. In contrast, the increase in β-catenin level characteristic of early myogenesis was unaffected by treatment. These results show that the identity of the C-terminal residue of α-tubulin modulates microtubule activity, possibly because binding to or signaling from modified microtubules is required for the myogenic program.

Microtubules (MTs)[1] participate in cell division, motility, transport, and morphogenesis. The ability of MTs to quickly polymerize and depolymerize, a process known as dynamic instability, places regulation of MT dynamics at the center of active research (1). Tubulin undergoes a host of post-translational modifications, including detyrosination, acetylation,

generation of Δ2-tubulin, phosphorylation, polyglutamylation, and polyglycylation (2). Of these, detyrosination has been most extensively studied, yet its functions remain to be determined.

Detyrosination is a unique modification involving cleavage of the C-terminal tyrosine residue of α-tubulin within MTs by a tubulin-specific carboxypeptidase (TCP), leaving Glu tubulin, named for its newly exposed C-terminal residue. Detyrosination is reversible: tubulin tyrosine ligase (TTL) adds a tyrosine residue to the C terminus of protomeric Glu-tubulin, re-forming tyrosinated (Tyr) tubulin (2, 3). MTs enriched in Glu tubulin (called Glu MTs) have been shown to be enhanced in cellular longevity or stability (4–6). However, detyrosination is known to be insufficient to stabilize MTs (7, 8), rendering detyrosination an effect, not a cause, of MT stability.

Glu MTs are found in a distinct subset of MTs in undifferentiated cultured cells (9). Usually the Glu MT subset largely overlaps with the subset enriched in post-translationally acetylated subunits (10, 11), suggesting that post-translationally modified subunits within a stable MT may function to establish functionally distinct MT populations. For example, modified MTs may function in cellular morphogenesis or cell polarization (3). Antibody microinjections suggest that Glu tubulin subunits anchor vimentin filaments to MTs in migrating 3T3 fibroblasts (12) and may be involved in directed organelle transport (13, 14).

The hypothesis that stable Glu MTs are involved in cellular morphogenesis is suggested by their increased abundance during differentiative events (3, 15). However, to date no study has demonstrated that Glu MTs are necessary for differentiation. During myogenesis, cells undergo striking morphological changes; they elongate, align with neighbors, and finally fuse into multinucleated myotubes. In L6 cells, Glu MTs accumulate upon induction of myogenesis, coincident with formation of stable MTs and preceding myoblast fusion, accumulation of post-translationally acetylated MTs, and appearance of muscle-specific myosin (16). A test of the functional consequences of inhibiting MT detyrosination during myogenesis has not been straightforward, largely because no direct inhibitors of TCP are available.

Eiserich *et al.* (17) showed that a modified amino acid, 3-nitrotyrosine (NO2Tyr), which is generated by reaction of tyrosine with nitric oxide-derived species, including nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$) (18–20), may act as an indirect inhibitor of TCP. That is, NO2Tyr can be taken up by cells, where the tyrosinating enzyme, tubulin tyrosine ligase (TTL), can incorporate it into the C terminus of Glu tubulin to yield nitrotyrosinated tubulin (21). Eiserich *et al.* (17) found that, when NO2Tyr was incorporated, Glu MTs were decreased,

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[1] The abbreviations used are: MT, microtubule; TCP, tubulin-specific carboxypeptidase; TTL, tubulin tyrosine ligase; Tyr, tyrosinated; DMEM, Dulbecco's modified Eagle's medium; NO2Tyr, 3-nitrotyrosine; MOPS, 4-morpholinepropanesulfonic acid.

and *in vitro* studies with carboxypeptidase A suggested that the nitrotyrosinated MTs might work by blocking the generation of Glu MTs by TCP. In this study, we show that NO_2Tyr-MTs inhibit TCP; consequently, NO_2Tyr can be used as an indirect inhibitor of MT detyrosination during differentiation of L6 myoblasts. Perturbation of Glu MT formation prevents myogenic morphogenesis, and accumulation and activation of muscle-specific factors, revealing a link between MT modification and progression of the myogenic program.

EXPERIMENTAL PROCEDURES

Materials—Unless noted, tissue culture supplies were from Invitrogen, and sera were from HyClone, Inc. Other chemicals and antibodies were from Sigma Chemical Co., immunochemicals were from Organon Teknika, and 4-methyl-2-nitrophenol was from Aldrich Chemical Co.

Cell Culture and Myogenic Differentiation—Rat L6 myoblasts (ATCC), maintained in DMEM with 10% fetal bovine serum, were induced to differentiate by shifting to DMEM with 2% horse serum (heat-inactivated, 56 °C, 30 min), and the extent of differentiation was scored (16). To test media of various pH levels, proliferating cells were incubated in DMEM with serum, lacking sodium bicarbonate, containing 30 mM HEPES buffer at each pH. Note that the pH of each medium refers to the pH of the HEPES buffer added, and may not reflect the pH maintained in the incubator. The pH of unbuffered control media was ~7.3.

The pH shift protocol (see Fig. 1*B*) entailed adding pH 7.0 medium, at 37 °C (*i.e.* maximum Glu tubulin content, see Fig.1*A*) to sub-confluent plates of L6 cells for 10 h to increase Glu MT level. Plates were rinsed once in cold pH 7.8 medium and placed at 4 °C for 1 h to depolymerize Glu MTs. Next, cells were incubated at 37 °C for 12 h at pH 7.8 (*i.e.* minimum Glu tubulin content) allowing TTL to re-tyrosinate Glu tubulin protomers, thus reducing Glu tubulin level significantly. Finally, differentiation medium was added. 400 μM NO_2Tyr was present in half the plates (+NO_2Tyr plates) throughout the experiment, and both control plates and those with added NO_2Tyr underwent the pH shift protocol. To prevent overgrowth of cultures during the time course, differentiation-defective cells were killed at 2 days with 18 μM β-D-arabinofuranoside, which has been shown not to interfere with myogenesis (22).

Antibodies—Antibodies were used at dilutions shown parenthetically: β-catenin, C2206 (1:6000); α-sarcomeric actin, 5C5 (1:1500); vimentin, V9 (1:400); desmin, D8281 (1:1000); and acetylated tubulin, 6–11B-1 (1:1000). MF20 hybridoma against muscle-specific myosin heavy chain was from the Developmental Studies Hybridoma Bank; undiluted culture supernatant was used. Anti-NO_2Tyr (1:800) was from Upstate Biotechnology, and anti-myogenin, M-225 (1:100) was from Santa Cruz Biotechnology. Anti-β-tubulin, 3F3 (1:2500), was generously provided by Dr. J. Lessard (University of Cincinnati) and anti-integrins β_{1A} (1:1000) and α_{7A} (1:500) were obtained courtesy of Dr. G. Tarone (University of Torino). Anti-Glu tubulin, SG (1:6000) was previously described (9).

Immunoblotting, Immunofluorescence, and Northern Blots—Preparation of cell lysates, SDS-PAGE, Western blotting, and immunostaining were described in Chapin and Bulinski (23). For enhanced chemiluminescence (ECL), blots were blocked with 1% bovine serum albumin; bound antibodies were detected with SuperSignal chemiluminescent substrate (Pierce). Northern blots were performed exactly as described in Gruber *et al.* (24), using actin and glyceraldehyde-3-phosphate dehydrogenase probes from Ambion (the latter for normalization of RNA loads).

Immunofluorescence was performed as described in Gundersen *et al.* (16). Antibodies were diluted as follows: anti-β-tubulin, 3F3, 1:100; anti-Glu, SG, 1:400; and secondary antibodies, 1:100. Images, captured with an Orca-cooled charge-coupled device camera (Hamamatsu Photonics) equipped with a Uniblitz Shutter (Vincent Associates), and attached to a Nikon Optiphot microscope, were processed with Meta-Morph software (Universal Imaging).

In Vitro Assay of Tubulin Carboxypeptidase Activity—Preparation of brain TCP, [^{14}C]tyrosine-labeled MT substrate, and the TCP assay, were described previously (25, 26). Briefly, [^{14}C]tyrosine-labeled tubulin dimers were self-assembled in MHM (25 mM MOPS, 25 mM HEPES, pH 7.5, 5 mM $MgCl_2$) for 20 min, stabilized by Taxol (20 μM, 5 min), and centrifuged, all at 37 °C (436,000 $\times$ *g*, 4 min, Optima TL centrifuge; Beckman Instruments) to yield a MT substrate pellet. For the first incubation step, *i.e.* binding of TCP to MT substrate, the pellet from the previous step was incubated in MHM (10 min, 37 °C) with 5 volumes of

A) NO_2 Tyr does not inhibit binding of TCP to MT substrate[a]	
NO_2 Tyr concentration	TCP activity
mM	*pmol / min / mg*
0	10.2 ± 0.3
10	10.5 ± 0.4

B) NO_2 Tyr does not inhibit activity of TCP bound to MT substrate[b]	
NO_2 Tyr concentration	TCP activity
mM	*pmol / min / mg*
0	12.0 ± 0.8
1	11.4 ± 0.3
10	9.1 ± 1.1[c]

C) Nitrotyrosination of MT substrate inhibits TCP[d]		
Substrate	TCP activity	*N*
	pmol / min / mg	
Untreated MT substrate	8.3 ± 1.7	2
Solvent-treated MT substrate	7.7 ± 1.9	3
TMN-treated MT substrate[e]	0.20 ± 0.15[c]	3

[a] NO_2 Tyr was added to TCP-substrate mixture during the first incubation (the binding step; see "Experimental Procedures"). $N = 3$ for all incubations.
[b] NO_2 Tyr was added to TCP-MT substrate mixture during the second incubation step, measuring TCP activity (see "Experimental Procedures"). $N = 3$ for all incubations.
[c] Significant inhibition, using unpaired *t* test ($p < 0.05$).
[d] See "Experimental Procedures" for details of the nitrotyrosinated MT substrate.
[e] MT substrate was incubated in 4% ethanol for 1 h at 37 °C.

crude brain TCP and 20 μM Taxol (26), and the mixture was centrifuged as before, except at 25 °C. For the second incubation step, measuring TCP activity, the pellet from the previous step was resuspended in MHM (30 min, 37 °C), proteins were precipitated with 10% trichloroacetic acid (w/v) at 4 °C, centrifuged (245,000 $\times$ *g*, Optima TL), and radioactivity of trichloroacetic acid-soluble (*i.e.* containing [^{14}C]tyrosine cleaved by TCP), and insoluble fractions were counted.

Nitrotyrosinated MT substrate was prepared by resuspending [^{14}C]tyrosine MT substrate (above) in 100 mM Tris, pH 8.2 (alkaline pH to allow tyrosine nitration but no cysteine oxidation), and incubating with 4 mM tetranitromethane, 4% ethanol (1 h, 37 °C). [^{14}C]Nitrotyrosinated MTs (NO_2Tyr content, 2 mol/mol tubulin dimer, quantified from $A_{430 nm}$), were centrifuged (436,000 $\times$ *g*, 4 min), washed with Tris buffer, centrifuged as before, and used in the TCP assay above. Release of [^{14}C]NO_2Tyr into the supernatant during the second incubation was measured.

RESULTS

Nitrotyrosine (NO_2Tyr) Inhibits TCP Activity in Vitro—To ascertain the mechanism by which NO_2Tyr inhibits generation of Glu MTs, we assayed its effects on TCP activity *in vitro*. MT substrate containing [^{14}C]tyrosine-labeled α-tubulin was first incubated with TCP preparations to allow binding of enzyme to MT substrate. TCP·MT substrate complex was then incubated, and TCP activity was measured as [^{14}C]tyrosine released from the C terminus of the substrate's α-tubulin (Refs. 24 and 26, see "Experimental Procedures"). We first tested whether free NO_2Tyr affected binding of TCP to MT substrate. Table I shows that adding NO_2Tyr to the initial incubation of MT substrate with TCP did not affect TCP activity measured, suggesting that free NO_2Tyr does not inhibit the binding of TCP to the MT substrate.

Next, we added NO_2Tyr during the second incubation, which quantified activity of TCP to generate Glu tubulin. We detected no difference without and with 1 mM NO_2Tyr, indicating that, at a concentration of 1 mM (*i.e.* 2.5 times that used *in vivo*), free NO_2Tyr did not interfere with activity of TCP once it was bound to MT substrate. However, in samples containing 10 mM NO_2Tyr, 25 times the concentration used *in vivo*, we did measure a small but significant ($<25\%$, $p < 0.05$) decrease in TCP activity (Table I). Because TCP activity was inhibited only at

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such high NO$_2$Tyr concentrations, and TCP-MT substrate binding was not inhibited, it is unlikely that free NO$_2$Tyr interferes significantly with detyrosination *in vitro* or *in vivo*.

Next, we tested whether nitrotyrosinated MT (NO$_2$Tyr-MT) substrate (*i.e.* MTs with incorporated NO$_2$Tyr) could inhibit detyrosination by TCP. NO$_2$Tyr-MT substrate, prepared by reacting ^{14}C-labeled MT substrate with tetranitromethane (see "Experimental Procedures"), inhibited TCP activity effectively (Table I), and this inhibition was due to nitrotyrosination, rather than denaturation, of MT substrate, because activity was unaffected by solvent alone (Table I). The facts that 1) TCP activity bound to and sedimented with NO$_2$Tyr-MT substrate (data not shown) and 2) NO$_2$Tyr-MT-bound TCP cleaved neither ^{14}C-labeled-NO$_2$Tyr nor ^{14}C-labeled Tyr suggested that TCP was effectively sequestered on NO$_2$Tyr-MTs in the enzyme-substrate mixtures. One would expect similar inhibition *in vivo*; that is, TCP would bind NO$_2$Tyr-MTs tightly and would neither cleave NO$_2$Tyr from the MTs nor readily dissociate to cleave other Tyr-MTs in the cell. In addition, these results predict that the species inhibiting TCP *in vivo* would be NO$_2$Tyr-MTs rather than free NO$_2$Tyr.

NO$_2$Tyr Specifically Inhibits MT Detyrosination in Vivo—A suitable *in vivo* detyrosination inhibitor must satisfy three criteria: First, the inhibitor must be nontoxic to L6 cells during the experimental time course. Second, incorporation must be efficient enough either to block α-tubulin detyrosination completely or to prevent increased detyrosination during myogenesis. Third, the compound has to be incorporated only post-translationally and only into α-tubulin. Although all three criteria were met for use of NO$_2$Tyr in undifferentiated cells (17), satisfying these criteria in differentiating cells demanded further efforts.

For the first criterion, we examined possible toxicity by incubating either proliferating or differentiated L6 cells with NO$_2$Tyr in culture medium for 10 days. No detectable effects on mitosis or other deleterious effects such as cell death were observed (data not shown), and proliferation and/or differentiation rates were nearly normal ($\geq$90% of controls; data not shown).

The second criterion, achieving efficient incorporation of the inhibitor, posed a significant challenge. Merely incubating L6 cells for 7 days in culture medium with 400 μM or even 800 μM NO$_2$Tyr did not result in diminution of Glu tubulin level, or incorporation of NO$_2$Tyr, as detected with Glu or NO$_2$Tyr antibodies, respectively (data not shown). The inefficiency of NO$_2$Tyr to perturb detyrosination was not surprising, because proliferating mammalian cells possess highly dynamic MTs (10), Glu MTs compose only a small subset (<5%), and Glu tubulin resides exclusively in polymer (5, 23), where it is unavailable to TTL. Thus, in proliferating cells <5% of the tubulin is available for detyrosination and subsequent re-tyrosination by TTL. To prevent the ~20-fold increase in Glu MTs during myogenesis, we had to improve NO$_2$Tyr incorporation into MTs and, thus, inhibition of TCP before inducing differentiation.

One strategy for effecting increased NO$_2$Tyr incorporation would be to perturb *in vivo* MT dynamics in NO$_2$Tyr-containing culture medium, allowing TTL to incorporate NO$_2$Tyr, in lieu of tyrosine, into Glu protomers (21). For example, transiently stabilizing MTs would generate Glu MTs (5); destabilizing these Glu MTs in the presence of NO$_2$Tyr would release Glu tubulin protomers that could be nitrotyrosinated by TTL.

We initially attempted to develop a MT stabilization-destabilization protocol using Taxol to stabilize MTs (5); however, Taxol treatments proved irreversible. Instead, we explored using pH to influence MT stability, based on reports that pH
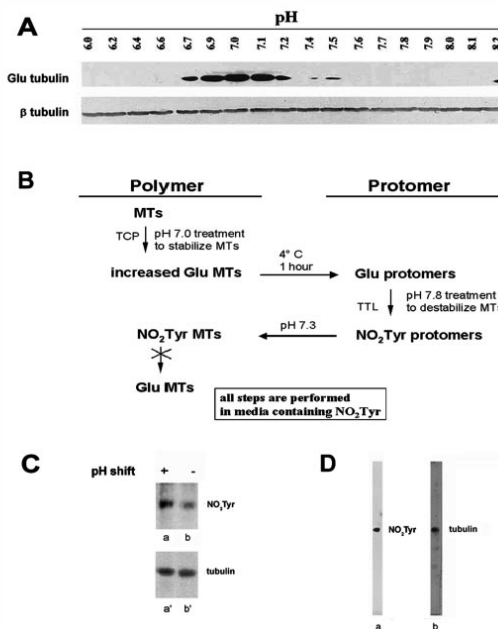
FIG. 1. **Enhanced incorporation of NO$_2$Tyr into cellular MTs.** *A*, Glu tubulin level of L6 myoblasts can be manipulated by altering extracellular pH. Proliferating L6 myoblasts, grown to half confluency in bicarbonate-buffered medium at pH 7.3, were incubated (10–12 h) in bicarbonate-free media containing 30 mM HEPES at each pH level. Cells reached steady-state Glu tubulin levels after $\leq$10 h of incubation at each pH (not shown). Cell extracts (30 μg) were blotted, and detyrosinated α (Glu tubulin) and β tubulin were immunolabeled. Note that, while Glu tubulin content varied, total tubulin (β-tubulin) was constant at all pH levels. *B*, pH shift protocol promotes efficient incorporation of NO$_2$Tyr into L6 myoblasts. To maximize NO$_2$Tyr incorporation into MTs, L6 cells were subjected to a pH shift protocol, as depicted (see "Experimental Procedures" for details). The pH shift scheme was carried out with or without NO$_2$Tyr; only the +NO$_2$Tyr version is shown. First, the level of cellular Glu MTs was raised by exposure to medium buffered at pH 7.0, an MT-stabilizing pH that increases Glu MTs (*A*), then cells were chilled to depolymerize the resulting Glu MTs. This releases Glu tubulin protomer, which is a substrate for TTL. Next, cells were re-warmed and incubated in medium buffered at pH 7.8, an MT-destabilizing pH (Fig. 1*A*) at which Glu tubulin content reaches its nadir. Subsequently, cells were re-equilibrated in unbuffered medium and used immediately in differentiation experiments. *C*, NO$_2$Tyr is incorporated into tubulin efficiently, following the pH shift protocol. L6 cell extract (60 μg) was immunoblotted with anti-NO$_2$Tyr antibody (α and *b*), and anti-β-tubulin antibody (α' and *b'*). *Lanes α* and *b* are from cells that were (+) and were not (−) subjected to the pH shift protocol, which increases NO$_2$Tyr incorporation (compare α and *b*) but does not alter tubulin content (compare α' and *b'*). *D*, NO$_2$Tyr is incorporated specifically into tubulin. Differentiated L6 cell extract (60 μg) was blotted and immunolabeled with α, anti-NO$_2$Tyr antibody, and overexposed via ECL. *b* shows anti-tubulin immunolabeling of the same lane. Note in *lane α* that no nontubulin bands incorporated detectable NO$_2$Tyr. From densitometric scanning we conclude that >99% of NO$_2$Tyr was incorporated into tubulin, because even a species that incorporated 1% as much NO$_2$Tyr would have been detected.

affects MT polymerization *in vivo* (27). As shown in Fig. 1*A*, lower extracellular pH enhanced detyrosination, reaching a maximum Glu tubulin level at pH 7.0. Glu tubulin level was lower at media pH levels of 7.2–7.5 and was not detected at media pH 7.6–8.0 (Fig. 1*A*). Overexposure of blots revealed a nadir in Glu tubulin level at pH 7.8 (data not shown). Staining of blots with anti-acetylated tubulin, a standard indicator

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of stable MTs (10), suggested that changes in Glu tubulin level were correlated with changes in MT stability (data not shown).

Fig. 1*B* shows the pH shift protocol devised from these data, which satisfied the second criterion for use of NO_2Tyr, that is, maximizing its incorporation into α-tubulin. Briefly, we first exposed L6 cell cultures to pH 7.0 medium for 10–12 h to stabilize MTs and increase Glu MT level. We then cold-treated the cells and incubated them in pH 7.8 medium for 12 h to destabilize MTs. Finally, we applied differentiation medium to start myogenesis. With or without added NO_2Tyr, all cells underwent the pH shift protocol, which markedly improved NO_2Tyr incorporation (Fig. 1*C*, *lane a*), compared with simply incubating cells in NO_2Tyr (Fig. 1*C*, *lane b*). In fact, densitometry of anti-NO_2Tyr-labeled blots revealed >8-fold more NO_2Tyr incorporation in *lane a* than in *lane b*. Moreover, other means of reversibly stabilizing MTs, *e.g.* transient sodium azide treatment, also increased NO_2Tyr incorporation into tubulin, and myogenesis was altered identically (data not shown).

The third criterion, that the inhibitor must be incorporated strictly post-translationally, and only into α-tubulin, was met in nonmuscle cells (17). Likewise, in L6 cells we could not detect translational incorporation of NO_2Tyr. However, concerned that the slightly slower cell growth rate (<10%) we observed in NO_2Tyr-treated *versus* control cells could result from translational incorporation of NO_2Tyr, albeit at a level not detectable even on the most sensitive Western blots (*e.g.* Fig. 1*D*), we treated cultures with an identical concentration (400 μM) of 2-nitro-*p*-cresyl (4-methyl-2-nitrophenol). This deaminated/decarboxylated version of NO_2Tyr can neither be used in protein synthesis nor serve as a TTL substrate, and it was similarly nontoxic; we observed no mitotic defects or apoptosis. 2-Nitro-*p*-cresyl treatment yielded the same paltry inhibition of cell growth rate (<10%) as other nitrated phenol compounds that are incapable of translational incorporation (28). Thus, NO_2Tyr appears to be incorporated only post-translationally.

Exposing L6 cells to transient pH shifts in the continuous presence of NO_2Tyr yielded incorporation only into α-tubulin; even the most sensitive Western blots revealed no other NO_2Tyr-labeled species (Fig. 1*D*). We also noted that the severity of NO_2Tyr effects on cell differentiation (see below) was strictly dependent upon the efficiency of NO_2Tyr incorporation into tubulin. For example, proliferating or differentiated myoblasts or mature myotubes were all unaffected by a 10-day incubation with NO_2Tyr if they were not first subjected to the pH shift protocol, and little NO_2Tyr incorporation could be detected in cells that were NO_2Tyr-treated without pH shift (Fig. 1*C*, *lane b*). The pH shift procedure changes MT dynamics (Fig. 1*B*), heightening the incorporation of NO_2Tyr by TTL into protomeric Glu tubulin; however, it is unlikely that changing the dynamics of MTs would change incorporation of NO_2Tyr into nontubulin proteins. Taken together, these data strongly suggest that in our experiments NO_2Tyr was added only post-translationally and only to α-tubulin.

NO_2Tyr Inhibits Detyrosination, but Not MT Stabilization, during Myogenesis—To inhibit Glu MT formation during myogenesis, we first subjected L6 myoblasts to the pH shift protocol and then placed them in differentiation medium and monitored their myogenesis for a 10-day period. Fig. 2 documents the depletion of myogenesis-induced detyrosination. Note that a high level of Glu tubulin persisted throughout the pre-treatments with cold and pH 7.8, such that the Glu tubulin level was still elevated and NO_2Tyr incorporation was undetectable at the time differentiation was induced (Fig. 2, *lanes marked 0*). However, by the 2-day time point, NO_2Tyr incorporation could
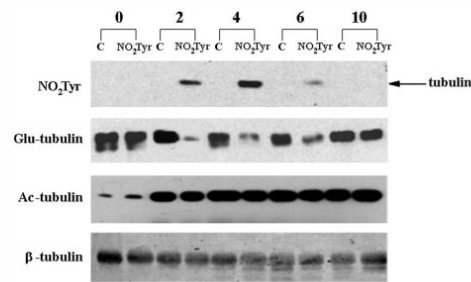

Fig. 2. **NO_2Tyr incorporation reduces detyrosination but not acetylation.** Extracts of pH-shifted L6 cells (see "Experimental Procedures") without (*control lanes, C*) or with NO_2Tyr (*NO_2Tyr lanes*) and placed in differentiation medium for 0–10 days were blotted to reveal NO_2Tyr, Glu-tubulin, acetylated tubulin (*Ac-tubulin*), and total tubulin (*β-tubulin*). A hapten antibody against NO_2Tyr was used to visualize all proteins with covalently incorporated NO_2Tyr. As shown in Fig. 1*C* for a single time point, no proteins other than α-tubulin were labeled with anti-NO_2Tyr. The quantities of total protein loaded to detect each antigen were: NO_2Tyr, 60 μg; Glu-, Ac-, and β-tubulin, 30 μg.

easily be detected and Glu tubulin level was greatly diminished (Fig. 2, *lanes marked 2*). Fig. 2 shows that NO_2Tyr dramatically reduced Glu tubulin level (*NO_2Tyr lanes*). In contrast, Glu tubulin was abundant in control cells (Fig. 2, *C lanes*) or cells exposed to NO_2Tyr without prior pH shift (not shown). With or without NO_2Tyr, cells commencing differentiation (Fig. 2, *0 lanes*) often contained a high Glu tubulin level, albeit markedly reduced in NO_2Tyr-treated cells.

Although NO_2Tyr treatment markedly decreased Glu tubulin level for the first week of differentiation, the level of Glu tubulin increased during the time course, such that its level was similar in cells with or without NO_2Tyr at the 10-day time point (Fig. 2, *10 lanes*, compare *C* and *NO_2Tyr*). Eventual failure of the inhibitor could arise in one or both of two ways: First, even optimal incorporation efficiency would not result in nitrotyrosination of all tubulin molecules. Remaining Tyr tubulin, polymerized into Tyr MTs, could be detyrosinated during the differentiation time course. Second, Tyr tubulin synthesized and polymerized into Tyr MTs during the 10 days could also be detyrosinated.

Detyrosination is a post-polymerization modification (5); Glu MTs that appear during myogenesis in L6 cells represent a stable MT population, compared with their dynamic Tyr MT counterparts (16). We used antibodies specific for another modification enriched in stable MTs, acetylated α-tubulin (29), a standard indicator of stable MTs (10), to ask whether NO_2Tyr treatment not only decreased Glu content but also stability of MTs during differentiation. Fig. 2 shows that acetylation increased equivalently during differentiation, regardless of NO_2Tyr treatment, suggesting that MTs were stabilized normally in NO_2Tyr-treated cells, despite the reduction in Glu tubulin level.

NO_2Tyr Inhibits Glu MT Accumulation and Myogenic Morphogenesis—L6 cells treated with NO_2Tyr for up to 10 days did not differentiate, unlike untreated cells (Fig. 3*A*) or cells treated with NO_2Tyr without prior pH shift (not pictured). The low magnification micrographs in Fig. 3*A* show that, initially, with or without NO_2Tyr treatment, L6 myoblasts were largely unpolarized, and many mitotic cells were seen (Fig. 3*A*, *panels a* and *b*). At 2 days, untreated L6 cells had elongated and begun to align with one another (Fig. 3*A*, *panel c*, *arrows*), although little fusion had yet occurred. In contrast, few NO_2Tyr-treated cells had become polarized, even fewer appeared to be aligned with one another (Fig. 3*A*, *panel d*, *arrow*), and none had fused
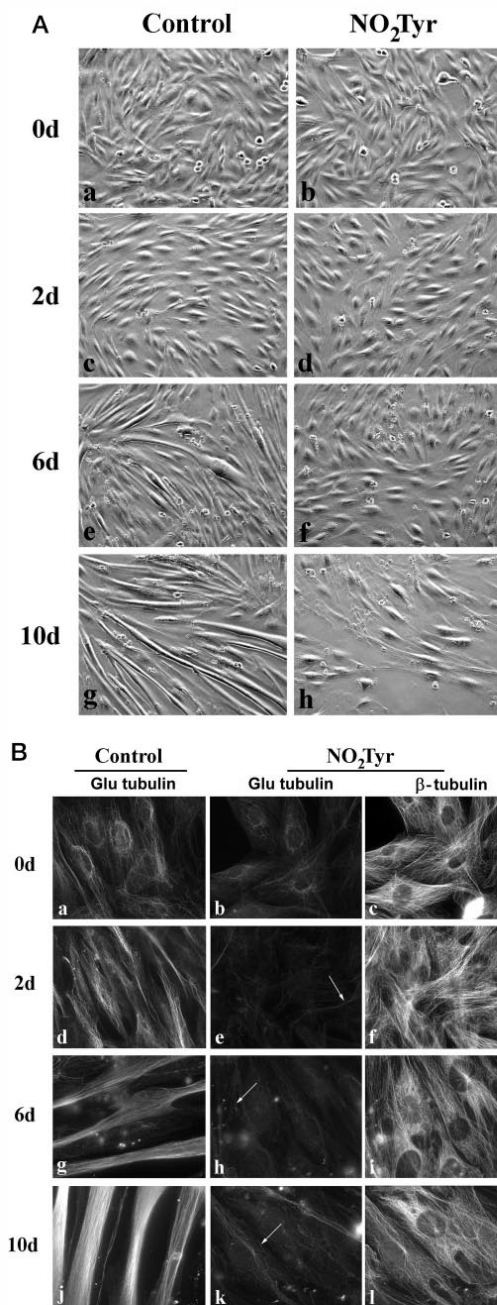
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A

Control   NO$_2$Tyr

0d   a   b

2d   c   d

6d   e   f

10d   g   h

B

Control   NO$_2$Tyr

Glu tubulin   Glu tubulin   β-tubulin

0d   a   b   c

2d   d   e   f

6d   g   h   i

10d   j   k   l

TABLE II
NO$_2$Tyr inhibits myogenesis of L6 cells

Time of differentiation	% nuclei in myotubes	
	Control[a]	NO$_2$Tyr
days	*%*	
0[a]	6.9	2.5
2	6.6	1.8
4	22	0.70
6	28	1.6
10	60	5.2

[a] Cells grown in proliferation medium for 2 days, without pH shift.

(Fig. 3A, *panel d*). After 6 days and 10 days of differentiation, untreated L6 cells had formed long, compact myotubes that were often branched tubes. Many contained as many as 100 nuclei. In contrast, NO$_2$Tyr-treated cells were well-spread and unpolarized; they had not changed in morphology significantly during the time course (Fig. 3A, compare *panels e* and *g* to *f* and *h*). We quantified the extent of myogenic differentiation by scoring the percentage of fused cells (Table II). In control cultures at 10 days, a majority of cells (60%) had fused with their neighbors, whereas in NO$_2$Tyr-treated cultures fusion was insignificant at any time point (~5%, not statistically different from undifferentiated control cells).

We used immunofluorescence to monitor Glu MT distribution during myogenesis. Before differentiation, both NO$_2$Tyr-treated and untreated cells contained a significant number of Glu MTs (Fig. 3B, *panels a–c*), but these were fewer in number and dimmer in NO$_2$Tyr-treated than in control cells (Fig. 3B, *panels a* and *b*). Glu MTs in undifferentiated cells were frequently curly, and they clustered nearby or circumscribed the nucleus. By 2 days of myogenesis, Glu MTs in untreated cells had begun to form small, straight bundles aligned parallel to the long axis of the cell (Fig. 3B, *panel d*). At 6 days and 10 days (Fig. 3B, *panels g* and *j*), larger bundles of Glu MTs coursed along the length of highly developed myotubes. In contrast, at 2 days, NO$_2$Tyr-treated cells contained almost no Glu MTs, and the total MT array was neither aligned nor polarized (Fig. 3B, *panels e* and *f*, respectively). By 6 and 10 days, NO$_2$Tyr-treated cells showed only a few MTs dimly labeled with Glu antibody (Fig. 3B, *panels h* and *k*, *arrows*). NO$_2$Tyr treatment did not change the total cellular array of MTs in proliferating cells (not shown) or in differentiating cells (Fig. 3B, compare *panels i* and *l* to *c*). Thus, the paucity of Glu MTs did not stem from a decrease in the total MT array, of which they represent a subset.

Lack of Glu MTs Blocks Expression of Muscle-specific Structural and Regulatory Proteins—As major components of assembling myofibrils, muscle-specific myosin and sarcomeric α-actin are abundantly expressed at late stages of muscle differentiation, concomitant with myoblast fusion (30). Because NO$_2$Tyr-treated L6 cells showed morphological deficits early in myogen-

FIG. 3. *A*, NO$_2$Tyr treatment blocks early morphogenetic events of myogenesis. L6 cells were pH shifted without (*a*, *c*, *e*, and *g*) or with NO$_2$Tyr (*b*, *d*, *f*, and *h*), allowed to differentiate for 0–10 days, and photographed under phase illumination. Note the paucity of cells elongated and aligned parallel to one another in NO$_2$Tyr-treated, compared with control cells at 2 days (*arrows*, *d* and *c*). Note that 6 days and 10 days after induction of differentiation, myoblasts had fused to form

highly developed, multinucleated myotubes in control samples (*e* and *g*), whereas in NO$_2$Tyr-treated cells morphological differentiation was perturbed; that is, no fusion was observed. Note the flat, highly spread single cells (*f* and *h*), in contrast to the narrow myotubes (*e* and *g*). *Bar* in *h*, 100 μm for all panels. *B*, NO$_2$Tyr treatment blocks elaboration of Glu MTs during myogenesis. L6 cells were pH shifted without (control: *a*, *d*, *g*, and *j*) or with NO$_2$Tyr (*b*, *c*, *e*, *f*, *h*, *i*, *k*, and *l*), differentiated for 0–10 days, and immunostained to visualize Glu-enriched MTs (Glu tubulin: *a*, *b*, *d*, *e*, *g*, *h*, *j*, and *k*) or total tubulin (β-tubulin: *c*, *f*, *i*, and *l*). At 0 days, Glu MTs were abundant in control (*a*) but less so in NO$_2$Tyr-treated (*b*) cells. Although Glu MTs increased in abundance and staining intensity from 2–10 days in controls (*d*, *g*, and *j*), little staining was seen in NO$_2$Tyr-treated cells (*e*, *h*, and *k*). In contrast, the total MT array was unaffected by NO$_2$Tyr treatment (β-tub: *c*, *f*, *i*, and *l*). Note that staining intensity with anti-Glu tubulin antibody here correlates with Western blots (Fig. 2). *Arrows* indicate a few Glu MTs visible in NO$_2$Tyr-treated cells at 2, 6, and 10 days (*e*, *h*, and *k*). *Bar* in *l*, 20 μm.
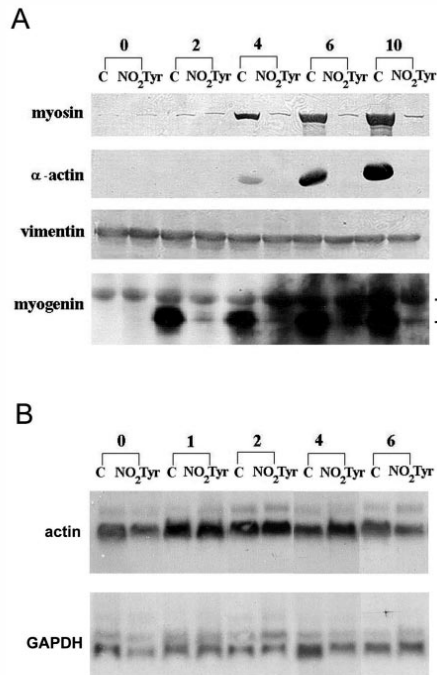
FIG. 4. *A*, NO₂Tyr blocks accumulation of muscle-specific proteins. Extracts of pH-shifted L6 cells without (*control lanes, C*) or with NO₂Tyr, differentiated for 0–10 days, were immunoblotted with antibodies to muscle-specific myosin heavy chain (*myosin*), sarcomeric α-actin, vimentin, and myogenin. 60, 60, 65, and 65 μg of extract protein were used to detect each antigen, respectively. *p* and *h* mark the electrophoretic position of phosphorylated and hypophosphorylated (active) myogenin, respectively. *B*, NO₂Tyr treatment does not affect accumulation of muscle-specific actin transcript. Total RNA (20 μg) from pH-shifted L6 cells without (*control lanes, C*) or with NO₂Tyr, differentiated for 0–10 days, were hybridized with a muscle actin probe (*actin lanes*) and a glyceraldehyde-3-phosphate dehydrogenase probe, as a loading control. Notice that NO₂Tyr treatment does not change the muscle actin transcript level detectably, in contrast to effects of the treatment on actin protein (compare *B* and *A*).

esis, we tested whether these late myogenic markers were properly expressed. As shown in Fig. 4, in control cells, accumulated muscle-specific myosin increased from a low, basal level in undifferentiated cells to a high level by 4 days (*C lanes*). In NO₂Tyr-treated cells, myosin expression remained at basal levels throughout the 10-day time course (Fig. 4, *NO₂Tyr lanes*). Similarly, sarcomeric α-actin was first detected at 4 days of differentiation in control cells (Fig. 4, *C lanes*), whereas it was not detected at any stage in NO₂Tyr-treated cells (Fig. 4, *NO₂Tyr lanes*). From these gene expression results and those of Fig. 3, which showed NO₂Tyr inhibition of early morphological changes, we conclude that NO₂Tyr treatment prevented both early and later events of myogenesis.

Fig. 4*B* shows that actin transcript accumulation, which precedes accumulation of α-actin, was not inhibited by NO₂Tyr treatment, in contrast to the striking inhibition of accumulation of muscle actin protein. NO₂Tyr most likely inhibits actin expression by inhibiting its translation or its accumulation, rather than its transcription. Because NO₂Tyr prevents myofibril assembly, actin turnover could conceivably be affected by its assembly state.

The intermediate filament protein, vimentin, was previously

shown to colocalize with Glu MTs in fibroblasts and to depend upon this MT subset for extension into peripheral areas of the cell (12). To determine whether a decrease in Glu MT level affected the level of vimentin, we probed blots of differentiating L6 cells with anti-vimentin (Fig. 4). The level of accumulated vimentin remained constant during differentiation regardless of treatment, indicating that vimentin level was insensitive to either the increase in Glu MTs during differentiation (in control cells) or the absence of Glu MTs (in NO₂Tyr-treated cells). This result presents the possibility that vimentin filaments may not utilize Glu MTs as interaction partners in muscle cells.

During differentiation of muscle tissue, expression of desmin intermediate filaments is induced. We hypothesized that desmin, which is highly homologous to vimentin, might be affected by NO₂Tyr depletion of Glu MTs. However, L6 cells were reported not to express desmin (31). Consistent with this finding, we were unable to detect either desmin protein or mRNA on Western or Northern blots, with or without NO₂Tyr treatment.

Our findings, that altering a cytoskeletal protein affected expression of muscle-specific cytoskeletal proteins, actin and myosin, prompted us to ask whether expression of a regulatory protein might be similarly affected. If transcription factors were affected by NO₂Tyr, expression of a subset of muscle-specific genes might then be blocked. Myogenin is the most relevant transcription factor in our system. Of the known myogenic basic helix-loop-helix transcription factors, L6 muscle cells express abundant myogenin, a low level of Myf-5, and no detectable MyoD (32, 33). In addition, myogenin synthesis is known to up-regulate its expression level, whereas myogenin phosphorylation negatively regulates its DNA binding activity (34). As shown in Fig. 4, we were able to assay both active and inactive forms, because myogenin separates electrophoretically into two bands: the active, hypophosphorylated form that migrates more rapidly (*labeled h*) than the inactive, phosphorylated form (*labeled p*) (35–37).

In control cultures (*C lanes*), myogenin expression was up-regulated at the onset of myogenesis (Fig. 4, compare *2d* to *0d*). Blots revealed that the active, hypophosphorylated species was more abundant than the phosphorylated species (Fig. 4, compare *h* and *p* bands in *lanes C*), and the intensity of both *h* and *p bands* increased during differentiation (*C lanes*). In contrast, NO₂Tyr treatment lowered the level of the lower molecular weight h band (*i.e.* active myogenin), throughout differentiation, whereas it did not have a consistent effect on the level of inactive phosphorylated myogenin (Fig. 4, *NO₂Tyr lanes, p band*). The finding that NO₂Tyr-treated cells possessed less of the active form of myogenin suggested that Glu MTs are necessary for synthesis or accumulation of a muscle-specific transcription factor.

Expression of Cell Adhesion Molecules Is Perturbed by NO₂Tyr Treatment—Because inhibition of Glu MT formation impacted upon both muscle-specific structural and regulatory proteins, as well as on morphological changes in myoblasts, we asked whether the lack of Glu MTs might also affect cell adhesion molecules involved in myogenic morphogenesis or signaling of myogenic gene expression. Integrins α₇A and β₁ form transmembrane heterodimeric receptors that mediate association of the extracellular matrix protein, laminin, to the intracellular actin cytoskeleton in myotubes (38, 39). α₇A integrin level increases during skeletal muscle differentiation (40, 41), whereas β₁A integrin level decreases as myoblasts switch to expressing integrin β₁D (42).

Accordingly, we examined expression of integrins α₇A and β₁A during differentiation of control and NO₂Tyr-treated L6 cells. Fig. 5 shows that, in control L6 cells, α₇A integrin level

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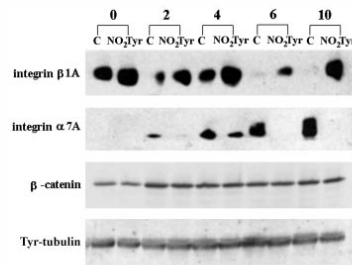
FIG. 5. **Expression of cell surface adhesion molecules in NO₂Tyr-treated cells.** Extracts of pH-shifted L6 cells without (*control lanes*, *C*) or with NO₂Tyr (*NO₂Tyr lanes*) and differentiated for 0–10 days were immunoblotted with antibodies to integrin β_{1A}, integrin α_{7A}, and β-catenin. 65 μg of extract protein was loaded for integrin detection, 60 μg for β-catenin. Values were normalized by labeling each blot with anti-Tyr tubulin antibody, as shown.

increased dramatically as myoblasts underwent differentiation (α_{7A} blot, *C lanes*); conversely, β_{1A} level decreased from high to undetectable during differentiation (Fig. 5, β_{1A} blot, *C lanes*) in agreement with Belkin *et al.* (42). NO₂Tyr-treated cells did not exhibit this integrin switch (Fig. 5, *NO₂Tyr lanes*): α_{7A} integrin was not abundant in NO₂Tyr-treated cells at any time from 0 to 10 days, with only a minor amount detected at 4 days. At the same time, β_{1A} level remained high at all stages examined. Constitutive expression of integrin β_{1A} was previously reported to be sufficient to block the cell cycle withdrawal requisite for myogenic differentiation in primary quail muscle cells (43). In contrast, our NO₂Tyr-treated L6 cells withdrew from the cell cycle (*i.e.* they survived β-D-arabinofuranoside treatment) even though they continued to express abundant integrin β_{1A}. Myogenin up-regulation has been correlated with up-regulation of integrin α_7 (31, 44), and lack of myogenin activity in NO₂Tyr-treated cells (Fig. 4) might contribute to their failure to induce integrin α_7. Thus, proper switching of integrins was perturbed in post-mitotic L6 myoblasts in which Glu MT formation was inhibited.

N-cadherin, a cell-cell adhesion molecule expressed in differentiating skeletal muscle cells, forms cell surface clusters that are thought to promote activation of myogenesis by helping align cells for fusion (45, 46). N-cadherin-dependent adhesion was reported to up-regulate myogenin (47), and exogenously expressed N-cadherin was shown to increase myogenesis and abundance of the N-cadherin binding partner, β-catenin, which functions in intracellular signaling (45). Because inhibition of Glu MT formation by NO₂Tyr prevented myogenin up-regulation, we asked whether the inhibitor altered β-catenin level. However, the β-catenin level increased ∼2-fold during myogenesis, and this increase was not affected by NO₂Tyr treatment (Fig. 5).

DISCUSSION

In this study, we tested the role of post-translational detyrosination during myogenesis. Previous studies had been hampered by the lack of direct inhibitors of TCP, which has not yet been purified or cloned. In other systems, Glu MT function had been inhibited by microinjecting anti-Glu antibodies (12, 14) or Glu tubulin protomers (13), but neither approach was amenable to studies of myogenic cells. A report by Eiserich *et al.* (17), that carboxypeptidase A could not cleave NO₂Tyr from tubulin *in vitro*, suggested that TCP might possess similar substrate specificity. Our demonstration that, indeed, NO₂Tyr-MT substrate inhibits TCP *in vitro* pointed to NO₂Tyr-MTs as an effective inhibitor of Glu MT formation in muscle cells. Successfully obtaining enough NO₂Tyr incorporation into MTs

to inhibit detyrosination effectively was predicated on the use of a pH shift protocol that altered cells' Glu tubulin content reversibly.

The fact that Glu tubulin level was altered in response to extracellular pH suggested that MT stability was also altered. Analysis of acetylated tubulin and examination of MT behavior *in vivo* by time-lapse microscopy corroborated this hypothesis (data not shown). Although it is unclear how extracellular pH affects *in vivo* MT stability, a putative mechanism is pH-induced changes in tubulin polymerization. The effects we measured coincide with evidence from other investigators that the quantity and, thus presumably, the stability of MTs polymerized is exquisitely sensitive to pH *in vitro* (48, 49) and *in vivo* (27). Instead of or in addition to, pH could affect activity of stathmin/Op18, an MT-destabilizer. For example, pH 6.8 enhances complexes of stathmin/Op18 with tubulin protomer, rather than with MT protofilaments, favoring persistence of stable MTs. In contrast, alkaline pH increases interaction of stathmin/Op18 with protofilaments, thus destabilizing MTs (50–52). Future work will elucidate how extracellular pH modifies MT dynamics; at present, we merely exploited this phenomenon as a convenient means of optimizing NO₂Tyr incorporation.

NO₂Tyr specifically inhibits detyrosination, as judged by several criteria: First, the degree of NO₂Tyr incorporation into MTs, rather than the length of treatment or concentration of NO₂Tyr applied, predicted the severity of myogenic inhibition. Furthermore, 2-nitro-*p*-cresyl, a nitrophenol compound similar to NO₂Tyr, neither affected myogenesis nor became incorporated into tubulin, further supporting our conclusion that NO₂Tyr was effective only when incorporated into MTs (see above). Third, NO₂Tyr was detected only in tubulin, suggesting that its incorporation was catalyzed only by the tubulin-specific enzyme, TTL. Fourth, with or without NO₂Tyr, MT acetylation occurred normally. Thus, NO₂Tyr administration inhibits only a single post-translational modification, detyrosination.

Our data corroborated results of Khawaja *et al.* (7) and Webster *et al.* (8), who showed that detyrosination is a result of, rather than a contributor to, MT stabilization. The specificity of NO₂Tyr allowed us to test independently the role of a single post-translational modification. Acetylation and detyrosination occur along distinct but overlapping populations of stable MTs (10, 11, 29), and both are up-regulated during myogenesis (16). Myogenesis was prevented when MT detyrosination was inhibited and acetylation was unaffected; therefore, the two modifications are not functionally redundant. No experiments to date address whether MT acetylation is also required for myogenesis. That different post-translational modifications affect functions or localizations of different MT subsets was suggested by Moreno and Schatten (53), who showed that post-translationally glutamylated MTs (54) and acetylated MTs were found in different compartments of developing spermatids.

Our finding that NO₂Tyr treatment of L6 cells irreversibly inhibited early myogenic events was not surprising, because the early elaboration of Glu MTs had implicated them in events prior to fusion (16). Our data showing early defects in cell elongation suggest that inhibition of Glu MTs inhibits signaling required for myogenesis rather than physically inhibiting fusion. Like myogenesis, early morphogenesis during neurite outgrowth occurs concomitant with MT stabilization and detyrosination (55, 56). Thus, by analogy, Glu MTs may be required for neurite outgrowth, as well.

Our experiments demonstrate that Glu MTs are *necessary* for myogenic differentiation. Other data, though, suggest that they may not be *sufficient*. For example, although the MT-stabilizing drug, Taxol, increases Glu MT level (5), attempts to

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use low doses of Taxol to re-induce myogenesis after NO_2Tyr treatment were unsuccessful (data not shown). Failure to rescue NO_2Tyr-treated cells implies that Glu MTs are not sufficient for myogenic differentiation. Alternatively, the failure to restart some events of myogenesis with other interdependent events already in progress might have precluded differentiation that could have otherwise been induced by Glu MTs.

NO_2Tyr treatment of L6 myoblasts generated pleiotropic effects upon differentiation. For example, NO_2Tyr severely compromised accumulation of active myogenin, which functions in early myogenic transcription; this might lead to defects in protein expression later in the myogenic time course. Although NO_2Tyr treatment had no discernible effect on the early accumulation of muscle actin mRNA transcript, it prevented accumulation of actin protein, along with myosin, another late myogenic marker assembled into sarcomeres. A switch in integrins thought to mediate cell adhesion changes was also prevented, in this case, resulting from either a transcriptional or post-transcriptional block. Taken together, our results suggest that Glu MTs play a critical primary role early in the myogenic pathway and exert secondary effects at later myogenic stages.

Previous studies have focused on signal transduction events leading to the generation of Glu MTs (57, 58). In contrast, our data implicate a signaling pathway that is activated by generation of Glu MTs; failure of this signal perturbs myogenesis. Possible mechanisms by which Glu MTs could signal their presence include changes in motor activity, *i.e.* if Glu MTs change kinesin-based transport functions (59), or changes in binding of signal transduction molecules, *i.e.* if Glu MTs alter signaling to the nucleus. In the latter scenario, if detyrosination were prevented, Glu MT-binding molecules would fail to bind MTs, and changes in their targeting and/or activity would result. Alternatively, without Glu MTs, molecules bound specifically to Tyr MTs might fail to dissociate from MTs. The latter is unlikely, though, because the increase in Glu subunits within the MTs is more dramatic than the decrease in Tyr subunits.

It is a formal possibility that the presence of NO_2Tyr-MTs may exacerbate the effects of inhibiting Glu MTs. Incorporation of a novel residue, NO_2Tyr, may structurally perturb binding of molecules that normally bind either to Glu- or Tyr-MTs but not to NO_2Tyr-MTs. This will be tested in future experiments. Alternatively, NO_2Tyr-tubulin could have downstream effects by blocking further modification, *i.e.* removal of the C-terminal residue of Glu tubulin to form Δ2-tubulin (54). The latter possibility is unlikely, because Δ2-tubulin accumulates mainly in neuronal MTs and has not been detected in muscle (60). In any case, Western blots (Fig. 2), performed with equally reactive antibodies (not shown) document that NO_2Tyr treatment resulted in a Glu-MT *decrease* that was more significant than the NO_2Tyr-MT *increase*. This is probably because, as shown *in vitro* in TCP assays, NO_2Tyr-MTs bind tightly to TCP and prevent its subsequent activity to detyrosinate Tyr subunits. Even if a minuscule amount of NO_2Tyr-MTs is somehow responsible for potently affecting myogenic MTs, our work shows that changing the C-terminal residue from Glu to NO_2Tyr, or to Tyr, abrogates functions required for myogenesis.

Our data suggest the existence of Glu MT-binding molecules; candidates for these include proteins that show muscle-specific expression and/or specific interaction with stable MTs. An interesting candidate is MURF, a muscle-specific RING-finger protein required for myoblast differentiation, which has been shown to interact with stable MTs (61). Involvement of MURF in transcription may provide an important link between MT properties and myogenic gene expression. Other proteins such as CLASPS, APC, and EB1, whose expression is not limited to

muscle, bind to MTs stabilized by plus-end capping (62–64). Plus-end binding species may exert MT-stabilizing effects early in the myogenic pathway, possibly upstream of MT detyrosination. These may be distinct from Glu MT-binding molecules that signal the stabilization of certain MTs. Future studies will be needed to identify mechanisms by which Glu MTs function in the signaling of myogenic events.

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