Oncogenes induce a vimentin filament collapse mediated by HDAC6 that is linked to cell stiffness

Li-Sophie Z. Rathje[a,1], Niklas Nordgren[b,1,2], Torbjörn Pettersson[b,1], Daniel Rönnlund[c,1], Jerker Widengren[c], Pontus Aspenström[a], and Annica K. B. Gad[a,3]

[a]Department of Microbiology, Tumor, and Cell Biology, Karolinska Institutet, SE-171 77 Stockholm, Sweden; [b]School of Chemical Science and Engineering, KTH Royal Institute of Technology, SE-100 44 Stockholm, Sweden; and [c]Department of Experimental Biomolecular Physics/Applied Physics, School of Engineering Sciences, KTH Royal Institute of Technology, AlbaNova University Center, SE-106 91 Stockholm, Sweden

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Oncogenes deregulate fundamental cellular functions, which can lead to development of tumors, tumor-cell invasion, and metastasis. As the mechanical properties of cells govern cell motility, we hypothesized that oncogenes promote cell invasion by inducing cytoskeletal changes that increase cellular stiffness. We show that the oncogenes simian virus 40 large T antigen, c-Myc, and cyclin E induce spatial reorganization of the vimentin intermediate filament network in cells. At the cellular level, this reorganization manifests as increased width of vimentin fibers and the collapse of the vimentin network. At nanoscale resolution, the organization of vimentin fibers in these oncogene-expressing cells was more entangled, with increased width of the fibers compared with control cells. Expression of these oncogenes also resulted in up-regulation of the tubulin deacetylase histone deacetylase 6 (HDAC6) and altered spatial distribution of acetylated microtubules. This oncogene expression also induced increases in cellular stiffness and promoted the invasive capacity of the cells. Importantly, HDAC6 was required and sufficient for the structural collapse of the vimentin filament network, and was required for increased cellular stiffness of the oncogene-expressing cells. Taken together, these data are consistent with the possibility that oncogenes can induce cellular stiffness via an HDAC6-induced reorganization of the vimentin intermediate filament network.

colloidal probe force-mode atomic force microscopy | STED microscopy | cell mechanics | cytoskeleton | cell invasion

Oncogenes induce major changes in cell behavior that can result in the development of tumors and tumor-cell metastases. Although expression of oncogenes promotes the ability of cells to invade the surrounding environment and to form metastases (1–3), the mechanisms behind this remain to be further elucidated. Many tumors show increased tissue stiffness, which can at least in part be explained by an increase in the stiffness of the extracellular microenvironment in tumors (4). Nonetheless, this increase in stiffness might also be due to an increase in the intrinsic stiffness of the cytoplasm of transformed cells. As the mechanical properties of cells govern their motile behavior, we hypothesized that oncogenes promote cell invasion via the control of cellular stiffness.

The cell cytoskeleton is composed of actin filaments, microtubules, and intermediate filaments (IFs), and it is believed to govern the mechanical properties of cells. In particular, the actin filaments have been shown to control cell mechanics (5, 6). In addition, there are a number of observations that indicate that vimentin IFs also contribute to the mechanical properties of cells. Vimentin IFs are a major cytoskeletal component in motile mesenchymal cells and metastatic tumors of epithelial origin. Epithelial-to-mesenchymal transition (EMT) is a diagnostic marker of migration and invasion of cancer cells, and it is characterized by expression of vimentin. Vimentin IFs functionally control the cell shape changes that occur during EMT and are strongly associated with cell invasion and poor tumor prognosis (7–10). This suggests that vimentin IFs have roles in the mechanical and motile properties of cells. In vitro, the mechanical properties of IFs are clearly distinct from the actin microfilament and microtubule systems (11). Under slight deformation, IFs provide compliance to cells. However, under great stress and deformation, IFs provide cells with mechanical strength and stiffness (12, 13). Vimentin fills the entire cytoplasm, and fibroblasts from mice that lack functional vimentin are significantly more pliant than normal fibroblasts (14, 15). Taken together, these observations suggest that the vimentin IF network can contribute to the mechanical strength and stiffness of cells.

In addition to the mechanical properties of individual filaments, the mechanical properties of the IF network depend equally on the geometry and bonds that link individual filaments together in a network (13). Hence, not only the presence, but also the organization of the vimentin IF network will have major effects on the mechanical properties of cells. Therefore, we set out to determine whether, and if so how, oncogenes can change the spatial organization of the vimentin IF network, cellular stiffness, and cell invasion.

Simian virus 40 large T antigen (SV40T), c-Myc, and cyclin E oncogenes are all intimately linked to development and progression of tumors. The SV40T antigen induces cell transformation and promotes tumor formation in various cell systems and animal models through its ability to initiate DNA replication (16). Many cancers show defective c-Myc activity, and aberrant expression of c-Myc induces major changes in gene expression, which result in enhanced cell proliferation (17). Overexpression of cyclin E, which results in accelerated G1 progression and chromosomal instability, is seen in many different types of tumors (18).

Significance

Oncogenes deregulate fundamental cellular functions, which can lead to the development of tumors and metastases. We show that oncogenes change the spatial organization of the vimentin fibers of the intracellular cytoskeleton, induce cell stiffness, and promote the invasive capacity of cells. We further show that this vimentin reorganization and increased cell stiffness requires histone deacetylase 6 (HDAC6). Taken together, these data support the concept that oncogenes can induce cellular stiffness via HDAC6-dependent reorganization of the vimentin filament network. These findings—that key molecules in oncogenic cell transformation, such as oncogenes and HDAC6, can modulate cell stiffness—highlight the importance of the need for further investigation of the mechanical properties of cells to better understand the mechanisms behind tumor and metastasis formation.

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[1]L.-S.Z.R., N.N., T.P., and D.R. contributed equally to the work.

[2]Present address: SP Chemistry, Materials, and Surfaces, SP Technical Research Institute of Sweden, SE-114 86 Stockholm, Sweden.

[3]To whom correspondence should be addressed. E-mail: Annica.Gad@ki.se.

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Many studies have shown that increased activity of histone deacetylase 6 (HDAC6) is intimately linked to tumor formation and the invasive capacity of tumor cells. HDAC6 is up-regulated in many tumor types, and is required for optimal tumor growth; it is also used as a marker for patient prognosis (19). In addition, the Ras oncogene requires a functional HDAC6 to transform cells, as defined through anchorage-independent cell proliferation (20). Also, whereas increased expression of HDAC6 increases the invasive phenotype of cells (21), inhibition of HDAC6 inhibits cell migration (22, 23). Together, this suggests that HDAC6 is required for the cellular changes that lead to cell transformation and tumor-cell invasion. Consequently, HDAC6 is a key target for the development of anticancer drugs (24).

In the present study we analyzed how oncogenes change the organization of the vimentin IF network and how this is linked to changes in cellular stiffness and invasion. Our data indicate that oncogenes cause an HDAC6-mediated reorganization of the vimentin IF network in cells, which is accompanied by increased cellular stiffness and cell invasion.

Results

SV40T, c-Myc, and Cyclin E Oncogenes Promote the Collapse of the Vimentin IF Network. The cytoskeleton is the main determinant of the mechanical properties of cells. Therefore, to determine if oncogenes can control cellular stiffness, we compared the spatial organization of actin filaments, microtubules, and vimentin IFs in cells overexpressing SV40T, c-Myc, and cyclin E with their isogenically matched control cells. We used three specific cell lines without and with expression of these oncogenes, as further detailed in the *SI Materials and Methods*. Increased expression of SV40T, c-Myc, and cyclin E were confirmed in these cell systems (Fig. S1 and ref. 25).

Upon oncogene expression, we observed subtle changes in the spatial organization of the actin filaments and microtubules, and a more pronounced reorganization of the vimentin IF network. Expression of the SV40T, c-Myc, and cyclin E oncogenes resulted in the bundling of thin peripheral vimentin IFs into thicker assemblies, which often showed increased localization toward the perinuclear area of cells (Fig. 1 *A* and *B*). There was a similar clustering of vimentin IFs upon cyclin E expression in the alternative cell system of human dermal fibroblasts (Fig. S2*A*).

To further investigate how oncogenes alter the vimentin IF network, we analyzed actin and vimentin organization at the periphery of the vimentin IF network using a stimulated emission depletion (STED) microscope, which provides eightfold greater resolution than a standard confocal microscope. This ultra-high-resolution analysis showed increased disorganization and entanglement of the vimentin IF network in oncogene-expressing cells (Fig. 1*C*). To quantify this reorganization of vimentin IFs, we analyzed the fiber widths in these images using computational

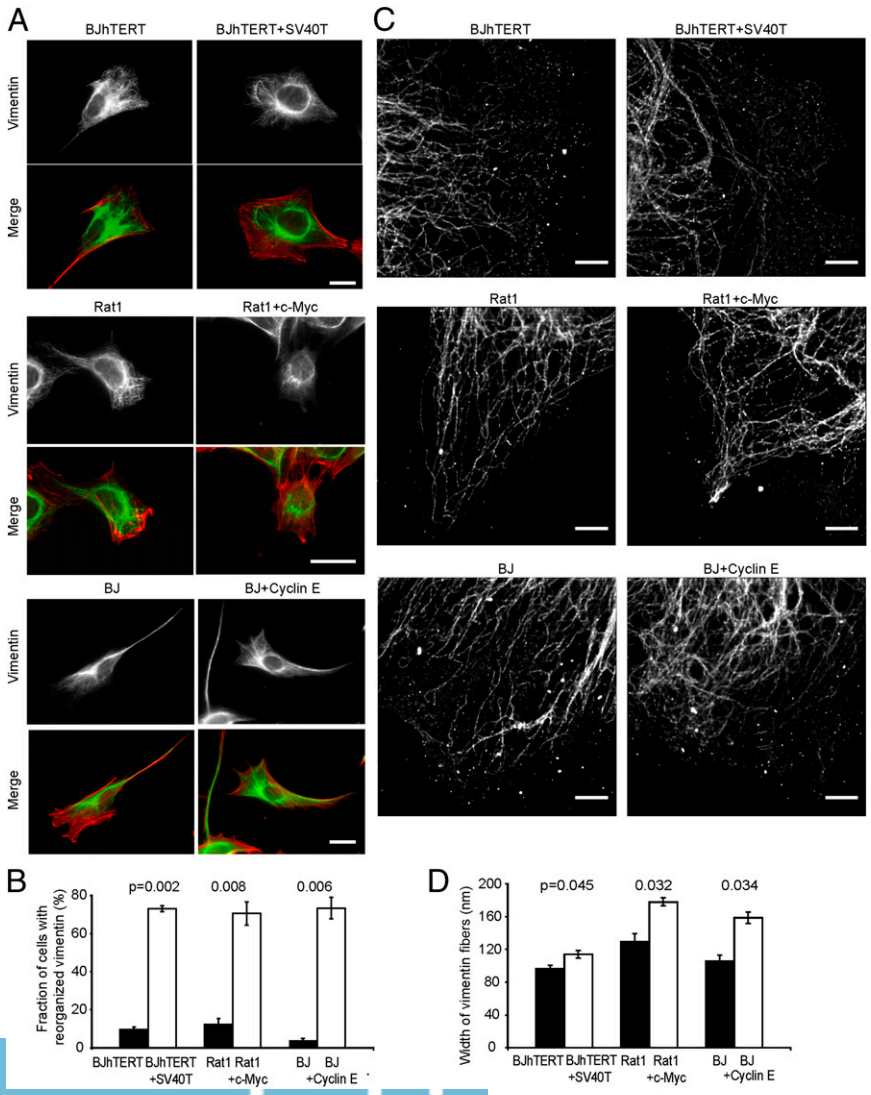

Fig. 1. The SV40T, c-Myc, and cyclin E oncogenes promote the structural collapse of the vimentin IF network. (*A*) Representative normal cells without or with expression of SV40T, c-Myc, and cyclin E (as indicated) were analyzed for spatial organization of vimentin IFs and actin filaments. Black and white images show vimentin and merged images show vimentin (green) and F-actin (red). (*B*) Quantification of spatial organization of vimentin in cells as in *A*. (*C*) Representative cells showing organization of vimentin IFs under ultra-high-resolution STED microscopy. (*D*) Quantification of the width of vimentin IFs in cells as in *C*. (Scale bars: 20 μm in *A* and 2 μm in *C*.) At least three independent experiments were performed under each condition with at least 100 cells (*B*) or five 15 × 15 μm images of different cells (*D*) quantified per experiment. Error bars represent SEM from at least three independent experiments. *P* values indicate significance of differences of treatments versus relevant control.

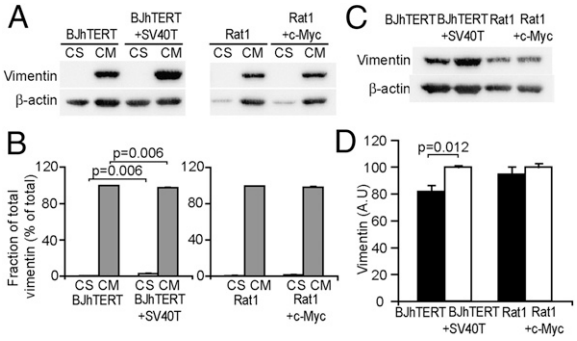

Fig. 2. SV40T- and c-Myc–induced collapse of the vimentin IF network is not linked to major changes in vimentin solubility. Normal cells without or with expression of SV40T and c-Myc were analyzed for total levels and distribution of vimentin and actin in the soluble CS and filamentous CM fractions. (*A*) Representative Western blotting of cells without or with SV40T and c-Myc expression, analyzed for vimentin and actin in the CS and CM fractions as quantified in *B*. (*C*) Total protein levels, as quantified in *D*. Error bars represent SD from at least three independent experiments. *P* values indicate significance of differences of treatments versus relevant control.

image analysis, as described earlier (26). This showed a significant increase in vimentin IF width in these oncogene-expressing cells (Fig. 1*D*). Although our observations often showed increased vimentin IF localization to the perinuclear area of cells (Fig. 1*A*), no clear retraction from the cell border was observed in the high-resolution images (Fig. S2*B*). We conclude that when observed at the nanoscale level, the reorganization of the vimentin network seen at the whole-cell level represented the entanglement of the vimentin fibers.

PDGF and active Rac1 have previously been shown to induce similar, although more pronounced, collapse of vimentin IFs to the perinuclear area, which was biochemically detectable as reduced vimentin solubility (27). The soluble vimentin fractions can be analyzed using a gentle extraction technique and in our cell systems less than 5% of the total vimentin is soluble at physiological salt concentrations. Upon expression of SV40T, the soluble fraction of vimentin was slightly increased (0.4 ± 0.2% to 2.8 ± 0.6%) (Fig. 2 *A* and *B*). There was also an increase in soluble actin and tubulin upon expression of SV40T (Fig. S3 *A* and *B*). In addition, overexpression of SV40T increased total levels of tubulin and vimentin in the cells, but not of actin (Fig. 2 *C* and *D* and Fig. S3 *C* and *D*). These changes were not observed upon c-Myc expression. As c-Myc expression induced vimentin reorganization (Fig. 1), these data indicate that the oncogene-induced reorganization of vimentin IFs does not require any major changes in vimentin solubility and ability to polymerize.

Oncogene-Induced Collapse of the Vimentin IF Network Is Linked to Microtubule Acetylation. The organization of the IFs depends on the integrity of the microtubule network (28–30), and microtubule acetylation has been linked to altered microtubule stability and function (31–33). This led us to hypothesize that the collapse of the vimentin IF network in these oncogene-expressing cells is due to altered acetylation of the microtubules at the periphery of the cells. To test this hypothesis, we analyzed whether SV40T, c-Myc, and cyclin E expression can alter the localization of acetylated tubulin in cells. Upon oncogene expression, we observed

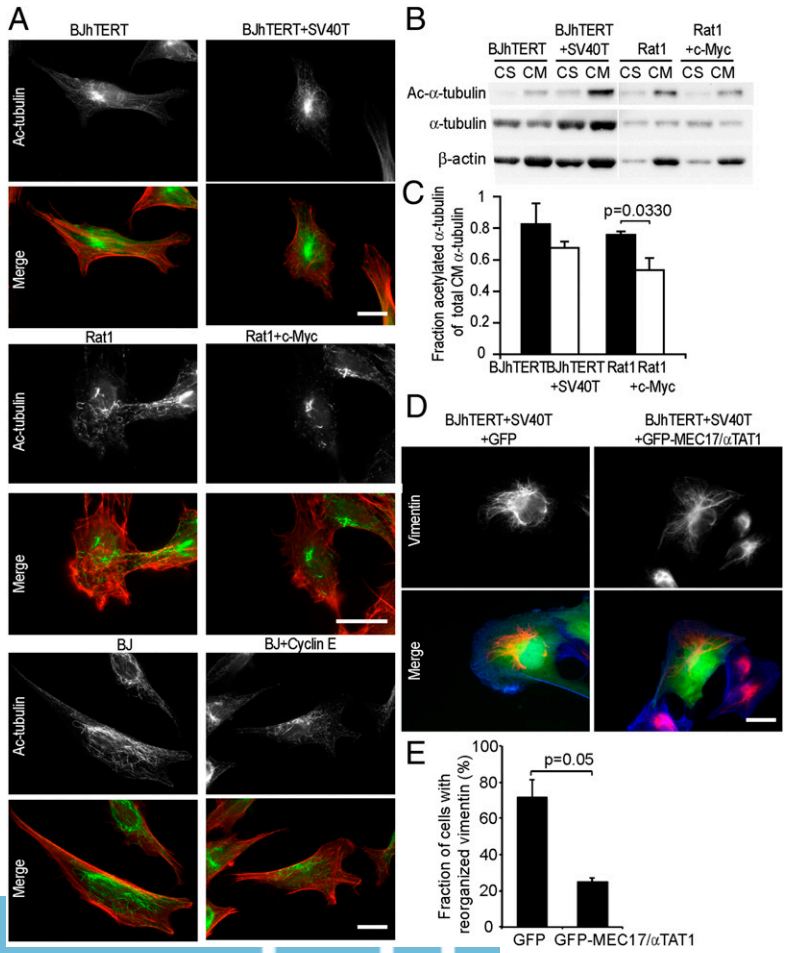

Fig. 3. Microtubule acetylation in the control of vimentin IF organization. (*A*) Representative normal cells without or with expression of SV40T, c-Myc, and cyclin E (as indicated) were analyzed for spatial organization of acetylated microtubules. Black and white images show acetylated tubulin and merged images show acetylated tubulin (green) and F-actin (red). (*B*) Representative Western blotting of cells without or with SV40T and c-Myc expression analyzed for fraction of acetylated tubulin in the CS and CM fractions. (*C*) Quantification of fraction of acetylated tubulin normalized to tubulin in the CM in cells as in *B*. (*D*) The vimentin organization in BJhTERT+SV40T cells expressing GFP–MEC17/α-TAT1 or GFP control. Black and white images show vimentin and merged images show GFP fluorescence (green), vimentin (red), and F-actin (blue). (*E*) Quantification of the vimentin IF collapse in cells as in *C*. At least three independent experiments were carried out for each experimental condition. (Scale bars: 20 μm.) Error bars represent SEM from at least three independent experiments. *P* values indicate significance of differences of treatments versus relevant control.

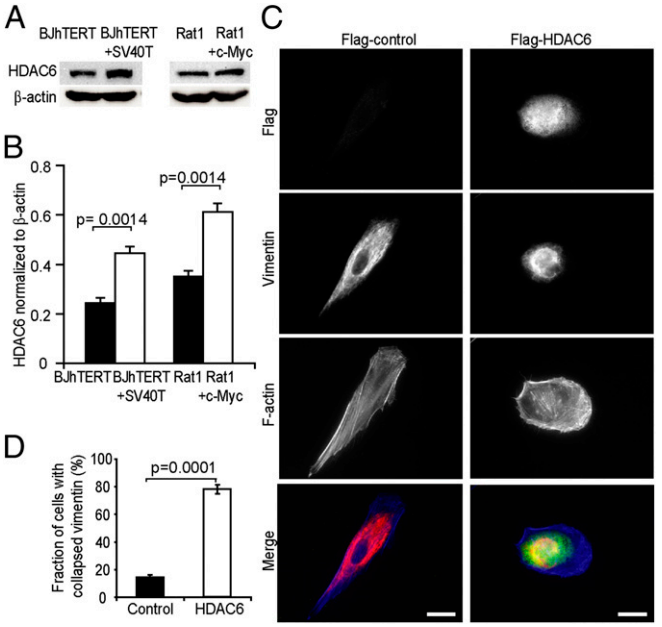

Fig. 4. HDAC6 activity is sufficient for vimentin IF collapse. (*A*) Representative Western blotting for total HDAC6 and actin levels in cells without or with expression of SV40T and c-Myc. (*B*) Quantification of HDAC6 levels in cells as in *A*, normalized to the actin levels. (*C*) Representative images showing the organization of vimentin IFs and F-actin in BJhTERT cells expressing an empty Flag–vector (Flag–control) and Flag–HDAC6. (*D*) Quantification of the collapse of the vimentin IF network in cells as in *C*. Data are representative of at least three independent experiments. (Scale bars: 20 μm.) Error bars represent SEM from at least three independent experiments. *P* values indicate significance of differences of treatments versus relevant control.

a reorganization of the acetylated microtubules toward the perinuclear area of the cell (Fig. 3*A*). We also analyzed the acetylation of microtubules in the nonsoluble/nonextractable cytomatrix (CM) fraction of the cells. Here there was a reduction in the fraction of acetylated tubulin upon expression of c-Myc (Fig. 3 *B* and *C*). To further clarify the link between tubulin acetylation and vimentin IF collapse, we expressed a nonacetylatable variant of tubulin (K40R) in normal cells. Compared with wild-type tubulin, this mutant tubulin induced a reorganization of the vimentin network toward the perinuclear area (Fig. S4). In addition, we observed that expression of the tubulin acetylase MEC17/α-TAT1 inhibited the oncogene-induced vimentin reorganization (Fig. 3 *D* and *E*). Taken together, this indicates that activation of oncogenes can alter the acetylation of microtubules within cells and thereby regulate the organization of the vimentin network.

HDAC6 Is Required and Sufficient for Oncogene-Induced Collapse of the Vimentin IF Network. HDAC6 activity increases the deacetylated form of microtubules (34, 35). We therefore hypothesized that the collapse of the vimentin IF network caused by these oncogenes is due to increased HDAC6 activity. To test this, we first analyzed the HDAC6 protein levels in this oncogene-expressing cell system using Western blotting. The data showed increased levels of HDAC6 in SV40T- and c-Myc–expressing cells, compared with control cells (Fig. 4 *A* and *B*). To determine whether HDAC6 can induce the collapse of the vimentin IF network, we overexpressed Flag–HDAC6 in normal cells and analyzed the effects on vimentin IF organization. Overexpression of HDAC6 in normal cells resulted in the collapse of the vimentin IF network in a similar way to that seen for the oncogene-expressing cells (Fig. 4 *C* and *D*). Transfection of GFP-tagged HDAC6 showed similar results (Fig. S5).

To determine whether HDAC6 is required for this vimentin reorganization, we used the HDAC6-specific inhibitor tubacin to see if it blocked SV40T-induced collapse of the vimentin IF network. This tubacin treatment resulted in significant suppression of SV40T-induced vimentin reorganization (Fig. 5 *A* and *B* and Fig. S6*A*). Also, tubacin treatment suppressed the relocalization of acetylated tubulin toward the center of SV40T-expressing cells and induced a marked increase in acetylated microtubules at the cell periphery (Fig. S6*B*). To further determine whether HDAC6 is required for this oncogene-induced collapse of the vimentin IF network, we investigated vimentin IFs in SV40T-expressing cells transfected with an HDAC6-targeting siRNA. Importantly, this knockdown of HDAC6 resulted in similar recovery of the peripheral vimentin IF network as that seen in tubacin-treated cells (Fig. 5 *C*–*E* and Fig. S6*C*). The transient transfection of a plasmid expressing an HDAC6-targeting shRNA showed similar effects (Fig. S7). Vimentin has been shown to be acetylated (36). To determine whether HDAC6-dependent vimentin reorganization is due to deacetylation of vimentin by HDAC6, we analyzed the levels of acetylated vimentin in cells without and with expression of SV40T or c-Myc. Upon expression of these oncogenes, there was a slight increase, as opposed to a decrease, in the acetylation of vimentin (Fig. S8). This indicates that this vimentin reorganization is not due to deacetylation of vimentin by HDAC6. To determine the role of actomyosin contractility in oncogene-induced vimentin reorganization, we analyzed the vimentin IFs in cells treated with or without the specific myosin inhibitor blebbistatin. The vimentin organization in blebbistatin-treated cells was very similar to control but appeared to be somewhat more disorganized and localized toward the nucleus (Fig. S9*A*), Thus, we conclude that this vimentin reorganization does not require actomyosin-based contractility.

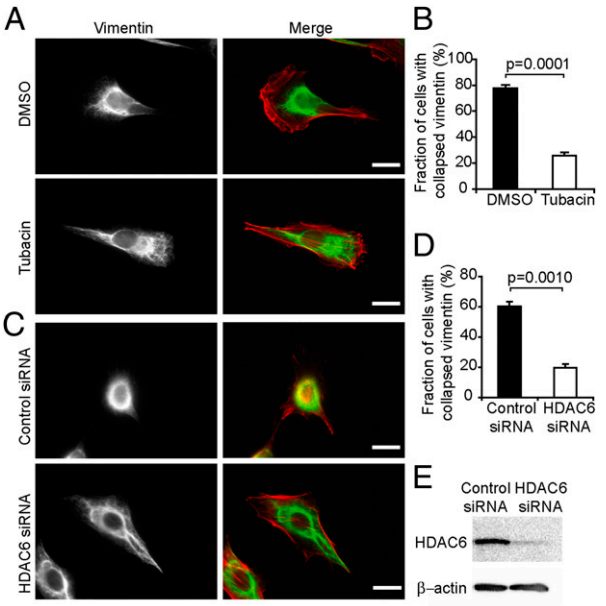

Fig. 5. HDAC6 activity is required for oncogene-induced collapse of the vimentin IF network. (*A*) Representative BJhTERT+SV40T cells treated with DMSO and tubacin analyzed for organization of vimentin IFs. Black and white images show vimentin and merged images show vimentin (green) and F-actin (red). (*B*) Quantification of the collapse of the vimentin IF network in cells as in *A*. (*C*) Representative BJhTERT+SV40T cells treated as controls or with an HDAC6-targeting siRNA analyzed for organization of vimentin IFs. (*D*) Quantification of width of the collapse of the vimentin IF network in cells as in *C*. (*E*) Representative Western blotting of HDAC6 levels before and after treatment with the HDAC6-targeting siRNA. At least three independent experiments were carried out for each experimental condition. (Scale bars: 20 μm.) Error bars represent SEM from at least three independent experiments. *P* values indicate significance of differences of treatments versus relevant control.

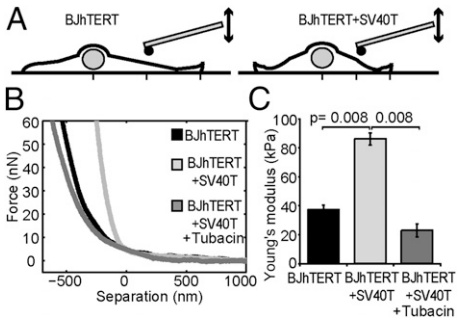

Fig. 6. SV40T induces increased cell stiffness via HDAC6. (*A*) Illustration of the analysis of cell stiffness using colloidal probe force-mode AFM at the location indicated. (*B*) Representative force curves of force upon approach between the sphere and BJhTERT cells (black), BJhTERT+SV40T cells (light gray), and BJhTERT+SV40T cells treated with tubacin (dark gray). (*C*) Quantification of resistance to mechanical deformation (stiffness) of cells as in *B* at an applied load of 30 nN. Ten cells were analyzed for each condition. Error bars represent the SEM. *P* values indicate significance of differences of treatments versus relevant control.

Taken together, these data suggest that this oncogene-induced collapse of the vimentin IF network depends on HDAC6 and on microtubule deacetylation.

Oncogenes Induce Increased Cellular Stiffness via HDAC6. To clarify whether the oncogene-induced, HDAC6-dependent collapse of the vimentin IF network is linked to changes in the stiffness of these cells, we used colloidal probe force-mode atomic force microscopy (AFM). Using this technique, we analyzed the stiffness of normal cells without and with expression of SV40T, at a position between the cell nucleus and the most peripheral border of the cell, *ca*. 5 μm closer to the edge of the cell than to the nucleus (Fig. 6*A*). The SV40T-expressing cells were significantly stiffer than normal cells (Fig. 6 *B* and *C*), which was linked to an increased ability to invade (Fig. 7). Importantly, specific inhibition of HDAC6 by tubacin reversed this SV40T-induced stiffness to the control situation (Fig. 6 *B* and *C*). To determine the role of actomyosin contractility in our system, we analyzed the cell stiffness in cells treated without or with blebbistatin. The blebbistatin-treated cells showed increased stiffness compared with control (Fig. S9*B*). Together, these observations suggest that SV40T increases cell stiffness via mechanisms that require the activity of HDAC6 but not actomyosin-dependent contractile forces.

Discussion

Our data show that expression of oncogenes can lead to reorganization of the vimentin IF network with dissordered arrangement, increased entanglement, and increased width of vimentin fibers. This IF reorganization indicates increased cross-linking of vimentin IFs to each other in oncogene-expressing cells. Given that the cross-linking of individual IFs by glutaraldehyde results in a small increase in mechanical stiffness in vitro (37), it is likely that increased interactions between vimentin IFs can result in increased cytoplasmic stiffness of cells. In agreement with this, a mutant variant of desmin that causes more bundled and less regular IFs and integrates into the endogenous vimentin IF network of cells (38), increases the peripheral stiffness of cells (39). Interestingly, this assembly of vimentin and increased cell stiffness are similar to the vimentin reorganization and local increase in cell stiffness that we observe upon oncogene expression. Our observation that SV40T increases cell stiffness suggests that oncogenically transformed cells will be stiffer than normal cells. This is in agreement with reports that have shown that when directly comparing stiffness of adherent cancer and normal cells, cell stiffness is significantly increased in the cancer cells (40). However, there have also been contradicting reports of decreased cell stiffness and increased deformability of cancer cells (41–44). Nevertheless, as these latter studies analyzed cells in suspension or nonspread cells, this discrepancy in cell stiffness changes might be explained by differences in the spreading of the analyzed cells. Biomechanical analyses of the periphery of well-adhered and spread cells will most likely detect the mechanical properties of the dense and widespread filamentous cytoskeletal networks that fill the cytoplasm. In contrast, analysis of cells in suspension or nonspread cells would be expected to mainly detect stiffness of the cortical cytoskeletal network and the cell nucleus. In agreement with this, previous reports have indicated that cell spreading is a main determinant of the cell stiffness that is detected (45).

The observed increase in cell stiffness upon expression of the SV40T oncogene was linked to increased invasive capacity in our experimental system. Thus, we speculate that increased stiffness of the cell cytoplasm promotes the ability of cells to invade. It would therefore be of interest to analyze the changes in the cellular mechanics and nanoscale organization of the vimentin IF network that accompany EMT.

Our findings are consistent with earlier data that show that vimentin IFs contribute to cellular stiffness (14, 15). It is interesting to note that the increased stiffness that we observed upon oncogene expression did not require actomyosin-based contractility. We hypothesize that oncogene-induced HDAC6 activity reduces the acetylation of the microtubules at the cell periphery, which leads to a reorganization of the vimentin IF network and a consequent increase in stiffness at the cell periphery. In addition, the observation that phosphorylation of vimentin causes vimentin network collapse (46, 47) suggests that also vimentin phosphorylation can alter cell stiffness. The different cytoskeletal systems are closely interconnected and interdependent, and we therefore expect that many components of the cytoskeleton contribute to oncogene-induced stiffness. However, our observation that HDAC6 activity is required for SV40T-induced stiffness indicates that these changes would require a functional HDAC6. As mentioned above, functional HDAC6 is required for oncogenes to transform cells, as measured by the ability of cells to proliferate without any underlying solid, stiff substrate (20). This, together with our observation that HDAC6 is required for oncogenes to increase cell stiffness indicate that the function of HDAC6 is to allow oncogenes to induce a level of intracellular stiffness that promotes cell proliferation, which is independent of extracellular mechanical cues.

Taken together, our data are consistent with the possibility that oncogenes induce an HDAC6-mediated collapse of the vimentin IF network that contribute to cellular stiffness and cell invasion. These findings—that key molecules in oncogenic cell transformation, such as oncogenes and HDAC6, can control cell stiffness—highlight the importance of the need to investigate further the mechanical properties of cells to understand the underlying causes of tumor and metastasis formation.

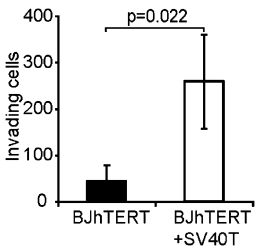

Fig. 7. SV40T promotes cell invasion. Quantification of BJhTERT cells without or with expression of SV40T analyzed for cell invasion. The number of invading cells was normalized to proliferation differences between cell types. The data represent the means of at least three independent experiments. Error bars represent SEM, and *P* values indicate significance of differences of treatments versus relevant control.

Materials and Methods

Cell Culture, Immunfluorescence Staining, and Imaging. Three genetically matched pairs of cell lines were used without and with the expression of SV40T, c-Myc, and cyclin E as further described in *SI Materials and Methods*. These cells were immunostained and analyzed under a Zeiss AxioVert 40 CFL microscope or a custom-built high-resolution STED microscope with a resolution of 40 nm as further detailed in *SI Materials and Methods*.

Preparation of Cytosol Extract and CM and Western Blotting. To separate the soluble cytosolic (CS) fraction of the cells from the nonsoluble filamentous fraction, the cells were extracted using a gentle extraction technique and the two cell fractions were analyzed using Western blotting. This technique is further described in *SI Materials and Methods*.

Colloidal Probe Force-Mode AFM. The stiffness of the single cells was analyzed using colloidal probe AFM. The details of this procedure are provided in *SI Materials and Methods*.

Cell Invasion and Proliferation Assays. In vitro cell invasion assays were performed in duplicate, using BD Biocoat Matrigel Invasion 24-well plate chambers (BD Biosciences) over 22 h. The number of invading cells per 10× objective field of view were counted according to the manufacturer protocol. In parallel, the cells were seeded on tissue-culture plastic at 30 cells per mm^2. Then 22 h later, the cells were trypsinized and counted using a Beckman Coulter cell counter and their proliferation rates were determined. Under these conditions, the proliferation ratio of the BJhTERT:BJhTERT+SV40T cells was 1:1.29 (± 0.24). The differences in invasion between the cell lines were normalized to this difference in proliferation rates.

Statistical Analysis. The statistical analyses were performed using Student's *t* tests with two-tailed heteroscedastic variance.

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