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Active Erk Regulates Microtubule Stability in H-ras–Transformed Cells[1]

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Abstract

Increasing evidence suggests that activated erk regulates cell functions, at least in part, by mechanisms that do not require gene transcription. Here we show that the map kinase, erk, decorates microtubules (MTs) and mitotic spindles in both parental and mutant active ras-transfected 10T1/2 fibroblasts and MCF10A breast epithelial cells. Approximately 20% of total cellular erk decorated MTs in both cell lines. A greater proportion of activated erk was associated with MTs in the presence of mutant active H-ras than in parental cells. Activation of erk by the ras pathway coincided with a decrease in the stability of MT, as detected by a stability marker. The MKK1 inhibitor, PD98059 and transfection of a dominant negative MKK1 blocked ras-induced instability of MTs but did not modify the association of erk with MTs or affect MT stability of the parental cells. These results indicate that the subset of active erk kinase that associates with MTs contributes to their instability in the presence of a mutant active ras. The MT-associated subset of active erk likely contributes to the enhanced invasive and proliferative abilities of cells containing mutant active H-ras. *Neoplasia* (2001) **3,** 385–394.

Keywords: Erk, ras oncogene, microtubule stability, breast epithelia fibroblast.

Introduction

Microtubule (MT) reorganization and functional specialization is required for the cellular remodeling that occurs during proliferation, migration, and invasion of normal and cancer cells [1–6]. Drugs that hyperstabilize MTs, such as paclitaxel, can block the progress of cells through mitosis and have been reported to inhibit the invasion of breast and ovarian cancer cells [7–9]. The initial clinical success of reagents such as paclitaxel has focused attention on the extracellular factors and signaling cascades that regulate the stability and turnover of MTs during the transformation process.

Microtubule-associated proteins (MAPs), such as tau and MAP-1, bind directly to tubulin and are members of a group of proteins that regulate MT stability. They promote both the polymerization of tubulin and the stability of MTs by reducing disassembly rate [10]. The ability of MAPs to bind to tubulin is controlled, in part, by posttranslational modification including serine threonine phosphorylation [11]. Cdc2, MARKs, erk, and GSK-3β [12–14] kinases have been shown to phosphorylate MAPs *in vitro* and to modify MAP-mediated MT polymerization *in vitro* [11–14]. However, the role that kinases play in determining MT dynamics *in vivo* has not been well studied.

Recently, erk, has been shown by several groups to decorate interphase MTs [15–18] and to associate with the kinetochore of the mitotic spindle *in vivo* [19]. Erk also associates with dynamic structures, such as focal adhesions, that are associated with the actin cytoskeleton [20]. Ras, which can activate erk [24,26–28], has previously been reported to promote cytoskeletal remodeling and to contribute to highly dynamic processes such as vesicular transport and cell motility [21,22]. Several studies have shown that erk kinase activity results in disassembly of actin stress fibers [15,23]. However, little is known about the role of erk–MT associations in MT function *in vivo* [19]. Erk is predicted to contribute to the dynamic instability of MT subsets because it phosphorylates both MAPs *in vitro* [24,25] as well as proteins such as stathmin that are involved in MT turnover [26].

Because of the role of MTs in proliferative, motile, and invasive processes [6–9], and recent studies linking erk and its upstream activator kinase MKK1 [27–29] to breast cancer progression, we have assessed the role of the ras–erk cascade in regulating MT stability both in fibroblasts and in breast epithelial cells. A CIRAS-3 fibroblast line was derived from the stable transfection of H-ras into 10T1/2 cells, which show lung colonization that is indicative of highly metastatic cells [30]. The MCF10aNeo breast epithelial cells were transformed with a mutant *H-ras* oncogene, which has served as a model for the study of the early stages of breast cancer [31]. We show that erk kinase is required for dynamic MT turnover in both cell backgrounds in the presence of mutant active ras but not in parental cells.

Abbreviations: MT, microtubule; MAPs, microtubule-associated proteins; erk, extracellular signal regulated kinase

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Materials and Methods

Antibodies and Other Reagents

Monoclonal anti–acetylated α-tubulin (6-11B-1), anti–α-tubulin (B-5-1-2), and anti–active erk antibody (clone MAPK-YT) were purchased from Sigma Chemical (St. Louis, MO). An antipan-erk polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY) and an anti–HA polyclonal antibody (HA.11) was purchased from BaBCO (Richmond, CA). Secondary antibodies, polyclonal FITC/TRITC-conjugated goat anti-mouse or anti-rabbit secondary antibodies, were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and HRP-conjugated donkey anti-mouse and anti-rabbit antibodies were purchased from BioRad (Hercules, CA). DMEM and F12 media were purchased from Gibco-BRL (Burlington, ON, Canada); and fetal bovine and equine serum, from Hyclone (Logan, UT) and Gibco-BRL, respectively. All other reagents, unless otherwise indicated, were purchased from Sigma.

Cell Culture

Human breast cancer cells MCF10aNeo (transfected with neomycin vector) and MCF10a-H-ras (transfected with T24-Ha-ras oncogene), obtained from Dr. C. J. Der (University of North Carolina, Chapel Hill, NC), were grown in supplemented DMEM/F12 (1:1) media with 5% equine serum, as described [31]. The 10T1/2 and CIRAS-3 (10T1/2-H-ras) transfected murine fibroblasts, kindly donated by Dr. S. Egan (The Hospital for Sick Children, Toronto, ON, Canada), were cultured in DMEM containing 10% fetal calf serum. Mitotic cells were enriched by the treatment of sparse cell cultures with 0.03 μg/ml nocodazole (Sigma) for 12 to 16 hours in growth medium. For all other experiments, equivalent cell numbers of each cell type were plated for 8 hours in growth medium to allow attachment to the substratum, then cells were serum-starved for 18 hours in serum-free medium. For immunofluorescence, 1×10^4 cells were plated on glass coverslips in six-well plates. For Western blot analysis, 1×10^5 cells were plated onto 150-mm tissue-culture plates. For analysis of erk activity and MT stability, cells were pretreated with 50 μm of the MKK inhibitor PD 98059 (Calbiochem Biosciences, Mississauga, ON, Canada) for 2 hours, then stimulated with 10% serum in the presence of PD 98059 for 2 hours [32]. Controls received DMSO carrier alone, followed by serum stimulation.

Preparation of MT Fraction

The MT cytoskeleton was enriched, as described [33], for indirect immunofluorescence studies and Western blot analyses. Soluble proteins were extracted by two consecutive 5-minute treatments of cells with 0.2% Triton X-100 in MT-stabilizing buffer [0.1 M PIPES (pH 6.9), 1 mM EGTA, 2.5 mM GTP, 4% PEG 6000] supplemented with protease and phosphatase inhibitors [33]. For immunofluorescent studies, these extracted cells were fixed as described in the next section. For Western blot analysis, the remaining cytoskeletal and nuclear matrix was scraped off the plate into 300 μl/plate TBST [10 mM Tris (pH 7.5), 150 mM NaCl, 0.2% Triton X-100, 10 mM EDTA, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM microcystin LR, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 200 μg/ml PMSF]. The collected fraction was chilled on ice for 1 minute to depolymerize MTs, vortexed, and then centrifuged to separate the nuclear matrix and intermediate filament-containing fraction [33]. The supernatant containing the MT-enriched fraction [15] was then analyzed with Western blots.

Western Blot Analysis

For analysis of total cell lysates, monolayer cultures containing equal cell numbers were washed with phosphate-buffered saline (PBS) and then lysed in a 300 μl/plate RIPA buffer containing protease inhibitors, as described [33]. Laemmli buffer was added to the cell lysates and to the MT-enriched fractions, prepared as described above; these were then boiled and loaded equally by volume and separated by electrophoresis on a 10% SDS-PAGE gel, with prestained molecular weight standards (Sigma). Separated proteins were transferred to nitrocellulose membranes. Blots were blocked in TBST containing 5% defatted milk on a gyrating shaker for 3 hours. Primary and secondary antibodies were diluted to 1 μg/ml in TBST with 1% milk, and were incubated with blots for 3 hours and 1 hour, respectively. Immunodetection of bound antibody was done with secondary antibodies conjugated to HRP (1 μg/ml) and visualized with ECL (Amersham Pharmacia Biotech, Piscataway, NJ), according to the protocol of the supplier.

Transient Transfections

H-ras–transformed cells were transiently transfected with 1 μg of a pMCL-HA–tagged dominant negative (K97M) form of MKK plasmid DNA [34] with the Lipofectin reagent (Life Technologies, Gaithersburg, MD). Cells were washed twice in defined medium after a 4-hour treatment, then allowed to recover for 24 hours in growth medium (10% fetal calf serum) before being processed for immunofluorescence, as described below.

Indirect Immunofluorescence

For immunofluorescent analyses, subconfluent cells were washed with PBS, fixed with buffered 3% paraformaldehyde, and then permeabilized with 0.1% Triton X-100 in PBS (PBST). Fixed cells were incubated for 1 hour with PBS containing 3% bovine serum albumin (BSA) to block nonspecific binding sites, then incubated with primary antibodies diluted to 10 μg/ml in 1% BSA/PBS for 2 hours. Cells were washed with BSA/PBST and incubated with fluorochrome-conjugated secondary antibodies (10 μg/ml) in the same buffer, then washed before coverslips were mounted on glass slides with Vectashield (Vector Laboratories, Burlington, CA) mounting media. Cells were examined with a Zeiss Axiovert 100 M confocal LSM510

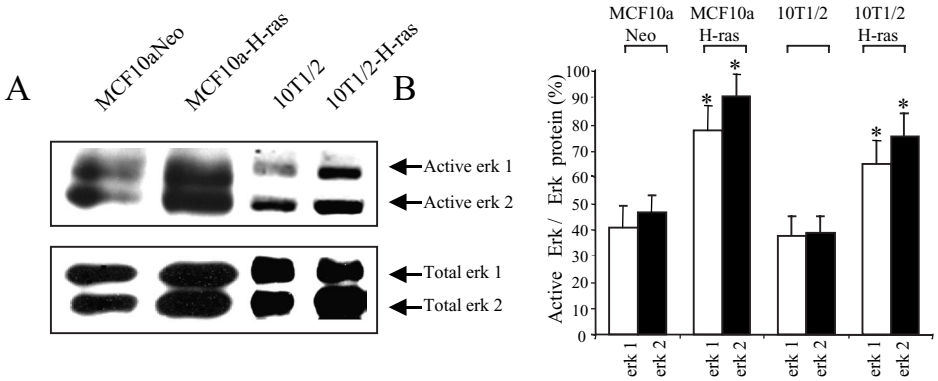

Figure 1. *Stable transfection of fibroblasts and epithelial cells with mutant H-ras results in an increase in the percentage of active erk found in total erk protein levels. (A) Active erk and total cellular erk levels assayed by Western blot analysis of cell lysates from MCF10aNeo, MCF10a-H-ras, parental 10T1/2, and 10T1/2-H-ras cells. Total erk protein levels slightly increased in the presence of a mutant H-ras whereas active erk levels increased by approximately two-fold compared with parental cells after serum stimulation, as detected by densitometry analysis of replicate Western blot analyses in both human breast epithelial cells (asterisks, P<.007 and .002, for active erk 1 and 2, respectively) and murine 10T1/2 fibroblasts (asterisks, P<.01 and .004 for active erk 1 and 2, respectively), relative to vector-control cells (B). Blots shown here are representative of three independent experiments.*

microscope at constant laser settings. Mitotic cells were mounted with Vectashield containing DAPI (1.5 μg/ml) and examined with epifluorescence on a Nikon Eclipse E1000 microscope.

Densitometry Analysis

Quantitative evaluation of optical densities of the reactive protein bands in Western blot analysis was done with NIH Image (Scion Image 1.62a). Protein bands were analyzed from replicate experiments after ECL and exposure of blots at equal time points to autoradiographic film. The calculation for relative amounts of active erk and acetylated α-tubulin in whole cell lysates from equal cell numbers was normalized to total erk and total α-tubulin, respectively. Active erk in MT-enriched fractions was normalized to total erk or to total active erk and to tubulin yield after extraction. Acetylated α-tubulin levels after extraction were normalized to total α-tubulin. Unless otherwise stated, blots were representative of three independent assays. Data from each experiment were pooled and expressed as mean ± SD. Statistical analysis was done with a two-tailed Student's *t* test, where $P<.05$ was considered significant.

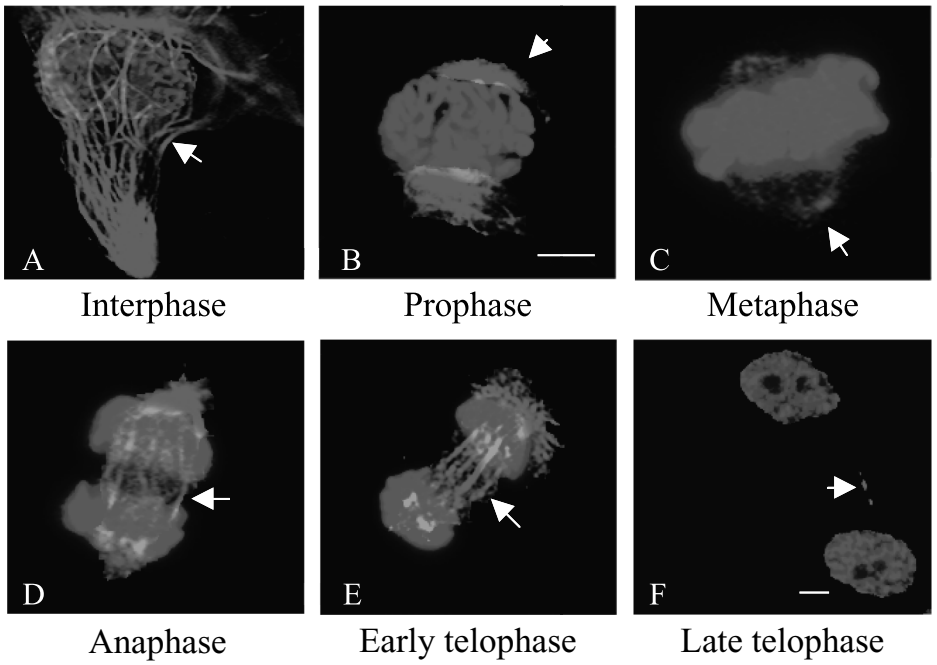

Figure 2. *Anti–acetylated α-tubulin antibody detects stable MT subsets. Immunofluorescence of MCF10aNeo cells with the anti–acetylated α-tubulin antibody and DAPI confirm that the tubulin posttranslational modification detected by this antibody is associated with the presence of stable MT subsets in interphase (A, arrows) and during mitosis (B–F, arrows). Thus, staining is restricted to stable MT structures such as centrosomes (B), spindle poles (C), polar MTs (D and E), and midbodies (F) during cell division (bars, 10 μm).*

Results

Cells transfected with mutant active ras express two-fold more activated erk kinase and MTs are less stable, as detected by a stability marker. For these studies, we used mutant H-ras–transfected and parental human epithelial cells and murine fibroblasts, and compared the levels of erk protein expression and activation (Figure 1). The level of erk protein expression was slightly higher in both H-ras–transfected cells lines than in parental cells (Figure 1*A*) but the proportion of activated erk 1 and 2 kinases, standardized to total cellular erk 1 and 2, was approximately two-fold higher in both the H-ras–transfected breast epithelial and fibroblast cells (Figure 1, *A* and *B*). To begin to assess the role of erk kinase in MT stability of these cell lines, we used a marker for MT stability that detected posttranslational modification of tubulin subunits [14,35,36]. We confirmed that this marker detected the stable interphase MT of confluent 10T1/2 fibroblasts but not the highly dynamic MTs of late telophase (Figure 2) [35]. Prominent acetylated MT arrays were observed in

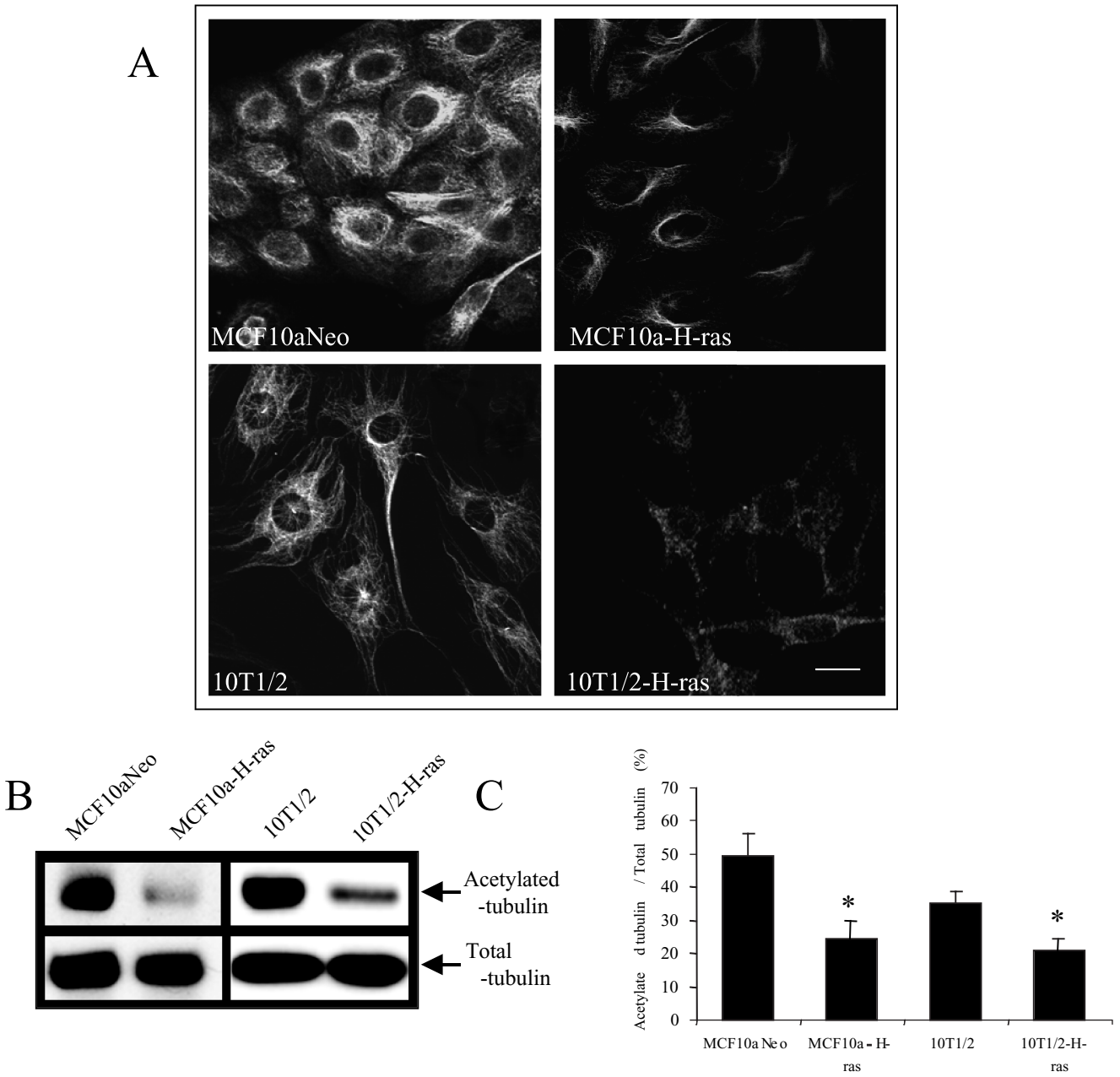

Figure 3. *Stable transfection of fibroblasts and breast epithelial cells with mutant active H-ras results in a significant decrease in acetylated MTs. (A) Indirect immunofluorescence of MCF10a cells stably transfected with vector alone, or H-ras, parental murine 10T1/2 fibroblasts, and 10T1/2 cells transfected with H-ras, by means of an anti–acetylated α-tubulin antibody. Confocal analysis at constant laser settings show fewer acetylated MTs in both H-ras–transfected cell types, compared with parental cells (bar, 30 μm), an observation that was confirmed by Western blot analysis of cell lysates (B). The Western blots shown here are representative of four independent assays. (C) Densitometry analysis of Western blots quantifying the reduced acetylated α-tubulin protein in H-ras–transfected cells (asterisks; P<.012 and .006, respectively). Acetylated α-tubulin levels were normalized to total α-tubulin levels for each cell type.*

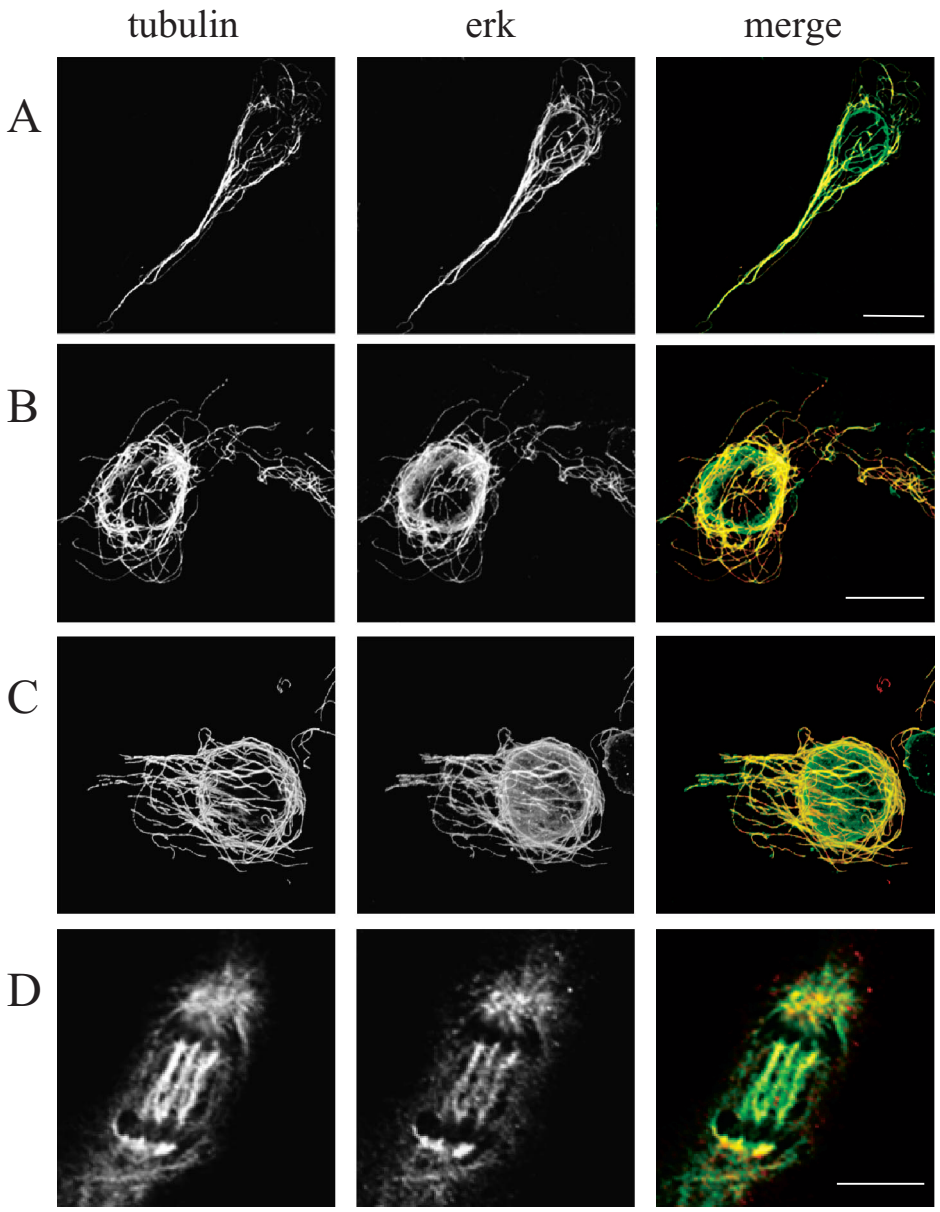

Figure 4. *Erk colocalizes with extracted MTs in both breast epithelial cells and murine fibroblasts. These three-dimensional reconstructions of confocal images show representative attached interphase cells after double immunofluorescence with a pan-α-tubulin monoclonal antibody and a pan-erk polyclonal antibody after the removal of the cytosol and preservation of MTs. (A) MCF10aNeo, (B) MCF10a-H-ras, and (C) 10T1/2-H-ras interphase cells. (D) MCF10a-H-ras mitotic cell. Colocalization of MTs and erk in both epithelial and fibroblast cell types was observed (bars, 25 μm).*

interphase MCF10aNeo and 10T1/2 cells (Figure 3*A*). However, at constant confocal laser settings, a visible decrease in acetylated MTs was seen in H-ras–transfected MCF10a and embryonic 10T1/2-H-ras cells (Figure 3*A*). These results suggest that MTs are less stable in both H-ras–transfected cell types. This observation is consistent with previous observations [27] showing that MTs of H-ras–transfected fibroblasts were short and poorly organized, characteristics typical of MTs with a high turnover. To quantify the difference in acetylation of tubulin among the cell lines, tubulin was separated from other proteins in cell lysates using SDS-PAGE and the degree of acetylation of tubulin subunits was detected in Western blots using the anti–acetylated tubulin antibody. As shown in Figure 3, *B* and *C*, levels of acetylated tubulin, normalized to total α-tubulin, were significantly lower in both the H-ras–transfected MCF10A breast epithelial cells and in the H-ras–transfected 10T1/2 fibroblasts.

Erk Colocalizes with MTs in Human-Breast Epithelial Cells and Murine Fibroblasts

Erk has previously been reported to decorate interphase MTs in nontransformed cells, including macrophages, neurons, and NIH 3T3 fibroblasts [15]. Erk has also been shown to associate with the kineticore of the mitotic spindle of NIH 3T3 fibroblasts [19]. We assessed whether human breast epithelial and murine embryonic cells exhibited a similar codistribution of erk with interphase and mitotic MTs

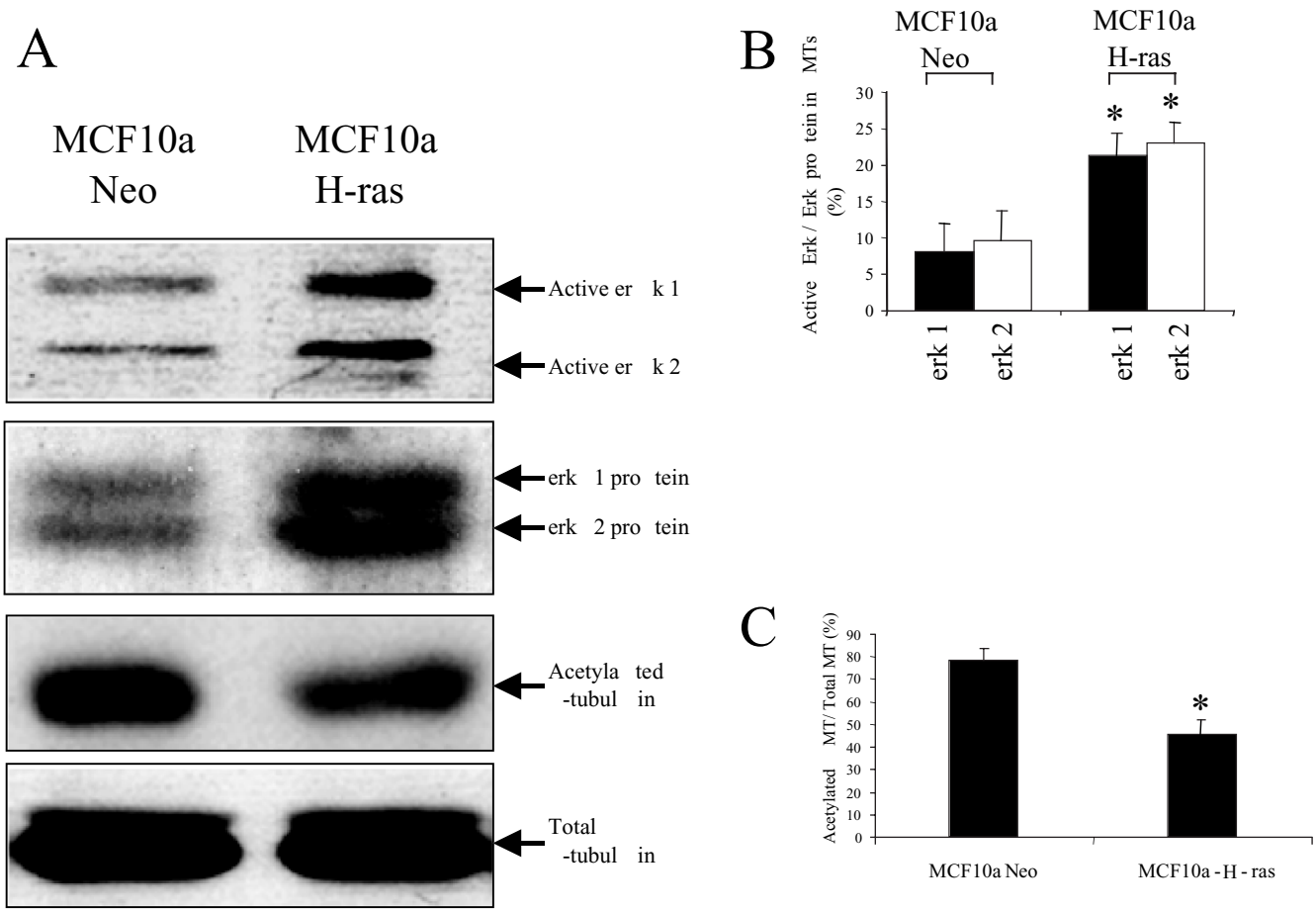

Figure 5. *H-ras–transfected cells exhibit increased levels of MT-associated active erk. (A) Representative Western blot analysis of active erk, total erk, acetylated α-tubulin, and total α-tubulin present in the MT-enriched fraction of MCF 10aNeo and MCF10a-H-ras cells. (B) Densitometry analysis of replicate Western blot experiments typified by (A) reveal that H-ras–transfected MCF10a cells have a significantly higher (approximately three-fold) ratio of active erk to total erk protein, associated with polymerized MTs than MCF10aNeo cells (asterisks, $P < .015$ and .008 for active erk 1 and 2, respectively). These values were normalized to a total α-tubulin yield after extraction. (C) A higher amount of active erk is associated with MTs in ras-transformed cells (25% to 28%) than with vector-control cells (19%) when MT-associated active erk levels are compared with total cellular active erk levels (see), representing about a 35% increase in active erk levels. (D) Densitometry of the acetylated and total α-tubulin protein bands in (A) show that the isolated polymerized MT subsets in H-ras–transfected cells have significantly lower levels of acetylated α-tubulin in proportion to total α-tubulin than parental control cells when soluble tubulin is extracted (asterisks; $P < .03$ and .047 for MCF10a and 10T1/2, respectively). Blots shown here are representative of three independent experiments.*

and also assessed whether mutant active ras modified this association. Cells were extracted in a detergent containing buffer to remove the cytosol and to preserve MTs [33]. The MTs were fixed and examined by double immunofluorescence for erk and α-tubulin distribution. Extensive colocalization of erk with MTs was observed in both H-ras–transfected and parental cell lines in interphase (Figure 4, *A* – *C*) and mitosis (Figure 4*D*). These results confirmed that erk associated with MTs independent of the cell backgrounds and that this association was maintained in the presence of mutant active ras.

H-ras–Transfected Cells Have Higher Levels of MT-Bound Active Erk than Parental Cells

To quantify the amount of active erk associated with MTs, we separated an MT-enriched fraction from the nuclear and intermediate filament fraction, as previously described using parental and H-ras–transfected MCF10A breast epithelial cells [33]. This technique removed most other cytoskeletal components and preserved MTs and bound proteins [33]. A pan-α-tubulin antibody confirmed that MTs were abundant in this fraction (Figure 5*A*). Densitometry analysis of replicate Western blot analyses revealed a slight increase in MT-associated total erk protein in H-ras–transfected MCF10 A over that in parental cells, even though total erk 1 and 2 protein expression of these two cell types is similar (Figures 1*A* and 5*A*). H-ras–transfected MCF10a cells exhibit two- to three-fold higher levels of MT-associated activated erk 1 and 2 kinase than parental cells (Figure 5*A*) when values were normalized to total MT-associated erk, a ratio similar to that observed for total cellular activated erk kinase in these cell lines (Figure 1*A*). The percentage of total erk in the MT-enriched fraction ranged from 15% to 25%, in both the ras-transfected and control cells, comparable to reports for other cell lines [15–17]. The MT fraction of ras-transfected and parental cell lines were also compared for acetylated α-tubulin levels. The amount of acetylated α-tubulin,

normalized to total α-tubulin, was significantly less in the ras-transfected cells than in the empty vector-transfected cells (Figure 5*D*, *asterisks*), confirming results shown in Figure 1 where no attempt was made to isolate MT.

Inhibition of Erk Activation Enhances MT Stability in H-ras–Transfected Cells

To assess whether association of active erk with MTs was directly linked to the significantly decreased acetylated MT

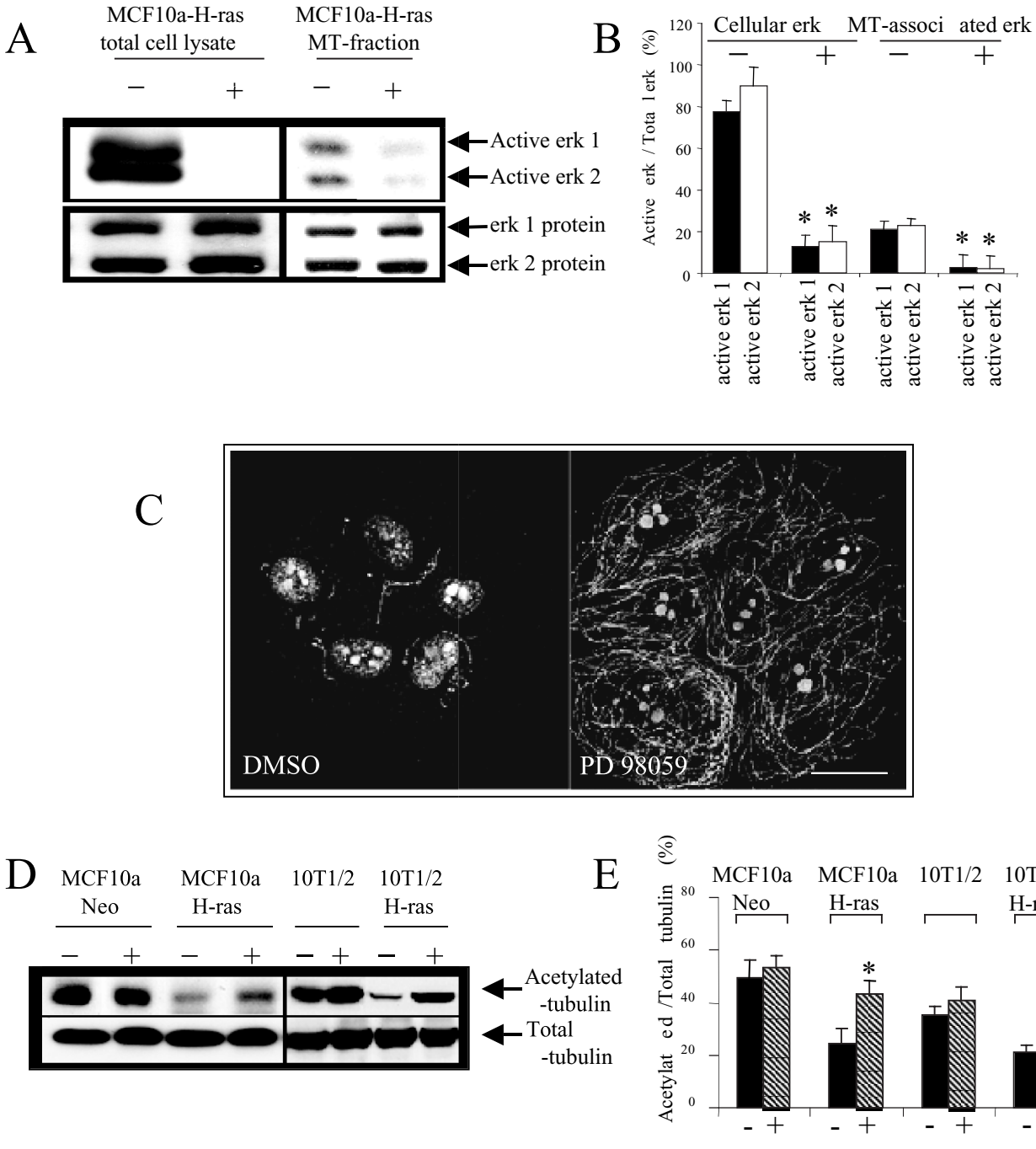

Figure 6. *Addition of PD 98059 increases acetylated α-tubulin levels in ras-transfected human epithelial and murine fibroblast cells. (A) Representative Western blot analysis comparing active erk in cell lysates and MT-enriched lysates in MCF10a-H-ras cells, treated with either DMSO (–) or PD 98059 (+). (B) Densitometry of Western blots of three separate assays confirm a significant decrease in active erk levels in both cell lysate and in MT-enriched fractions after MKK inhibition (asterisks, P<.0007 and .0004 for active erk 1 and active erk 2 in cell lysates, respectively; P<.009 and .004 for active erk 1 and active erk 2 in MT-enriched fractions, respectively). (C) Immunofluorescence of MCF10a-H-ras cells stained with the anti–acetylated α-tubulin antibody after treatment with DMSO alone or 50 μm PD 98059. An increase in acetylated MT levels was observed after inhibition of MKK1 (bar, 30 μm). (D) Levels of acetylated α-tubulin and total α-tubulin in parental control, H-ras–transfected 10T1/2 and MCF10a cell lysates after treatment with PD 98059 or DMSO vehicle alone were assessed with Western blot analysis. (E) Densitometry of three independent Western blot analyses of acetylated α-tubulin levels, expressed as a percentage of total α-tubulin levels, reveal that H-ras–transfected 10T1/2 and MCF10a cells had significantly higher acetylated α-tubulin levels in treated cells than control (asterisks, P<.012 and .006 for MCF10a and 10T1/2 cells, respectively). Neither parental cell line showed a significant difference in acetylated α-tubulin levels for PD 98059- or DMSO-treated cells. (+) denotes addition of 50 μm PD98059, (–) denotes DMSO carrier alone.*

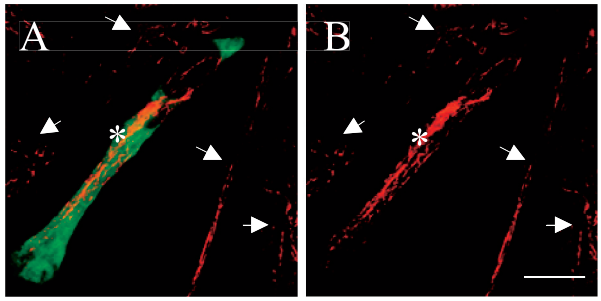

Figure 7. *Transient transfection of a dominant negative HA-tagged MKK1 increases acetylated α-tubulin immunostaining in H-ras–transfected murine 10T1/2 cells. (A and B) Immunofluorescence of 10T1/2-H-ras cells with an anti–HA epitope tag antibody (A, green) and an anti–acetylated α-tubulin antibody (A and B, red), after transient transfection of an HA-tagged dominant negative MKK cDNA. The asterisk indicates a transfected cell, surrounded by untransfected cells (arrowheads). Pronounced acetylated cytoplasmic MT arrays were observed only in transfected cells.*

levels in H-ras–transfected cells, parental and H-ras–transfected 10T1/2 and MCF10a cells were treated with a MKK1 inhibitor, PD 98059, or DMSO carrier vehicle alone. The activity of total cellular and MT-bound erk 1 and 2 levels were significantly and similarly reduced after acute treatment with PD 98059, in the absence of detectable changes in the expression of erk protein and in the absence of a decrease in the association of erk with MTs in both ras-transfected (Figure 6, *A* and *B*) and parental cells (data not shown). However, acetylated α-tubulin levels, detected both by immunofluorescence and Western blot analyses, were significantly increased in treated H-ras–transfected cells (Figure 6, $C - E$) but not in parental cells (Figure 6*E*).

To confirm results with H-ras transfected cells treated with PD 98059, 10T1/2-H-ras cells were transiently transfected with an HA-tagged mutant of MKK 1 that functions as a dominant negative inhibitor [41,42]. An increase in acetylated MTs was detected in transfected cells, marked by reactivity to an anti–HA-tag antibody (Figure 7; *asterisk*). Surrounding nontransfected cells (Figure 7, *arrowheads*) did not exhibit this increase in acetylated α-tubulin staining and served as internal controls.

Discussion

Tumor progression involves enhanced cancer cell invasion and proliferation, events that require MT reorganization, which arises from a regulated but dynamic instability. Many factors regulate MT stability but the most well studied is the role of MAPs that promote MT stability and enhance turnover when they are released from MTs as a result of phosphorylation by an orchestra of kinases [6]. ERK kinases have previously been shown to decorate both interphase and mitotic spindle MTs [15–19] and this kinase family has also been proposed to be one of the many kinase regulators of MT stability, based primarily on their ability to phosphorylate MAPs *in vitro* [14]. To our knowledge, we provide the first *in vivo* evidence that activation of ERK1 and 2 is required for destabilization of MTs in cells containing activated ras signaling pathways. We show that H-ras–mediated activation of erk results in an increased association of activated erk with MTs, noted previously by others [16], and that erk activity mediates a significant amount of MT instability in two cell backgrounds. Inhibition of erk kinase activity in H-ras MCF10a breast cells restored MT stability, as detected by acetylation of α-tubulin, to approximately 30% that of parental breast epithelial cells. In contrast, inhibition of erk kinase in H-ras-10T1/2 fibroblasts restored stability to approximately 80% of parental 10T1/2 cells. In H-ras–transfected breast cells, there must exist multiple additional mechanisms that regulate MT stability whereas the erk pathway is the predominant pathway for controlling MT in H-ras–transfected fibroblasts. Possible alternative pathways could include ras-regulated expression of proteins that modify MT stability such as strathmin [26] and/or other kinases that phosphorylate MAPs and that are activated by ras [7]. Possibly, such additional pathways are restricted in fibroblasts. In this context the kinetics of MT disassembly have been reported to differ between fibroblasts and epithelial cells [37].

Our results suggesting that erk only mediates MT stability in cells expressing an activated ras could mean that other pathways cooperate with erk kinases to effect MT turnover that are not activated in the parental cells. At present, we cannot exclude the possibility that α-tubulin subunits in parental cells are maximally acetylated and any erk-mediated changes in MT stability in parental cells are simply not detected with this marker. Use of other assays of MT stability such as recovery of MT polymers following a nocodazole block could resolve these possibilities [37,38]. If parental and ras-transfected cells do indeed differ in their reliance on erk for MT stability, the subset of erk kinases that associate with MTs in tumor cells that either hyperexpress ras or bear a mutant active ras, or other disease conditions where ras is constitutively activated by inappropriate signaling through cytokines/growth factors, might provide a useful therapeutic target [5,39].

ERK kinases have primarily been studied for their role in regulating activation of AP-1 transcription factors and consequent regulation of genes involved in invasion and motility, for instance specific metalloproteinases. This property is consistent with the localization of activated erk kinase to the cell nucleus in proliferating cells [21]. Several recent reports, however, have noted the presence of activated erk kinases in nonnuclear compartments including focal adhesions [20], cell lamellae [40], and as we and others [15–19] have noted, MTs. These observations and the known ability of erk kinases to phosphorylate proteins associated with the cytoskeleton including myosin light-chain kinase [41] and MAPs raise important questions as to the role of erk kinases in these additional cellular compartments. The ability of erk kinases to phosphorylate MAPs *in vitro* [6] provides support for the existence of an erk-mediated mechanism of MT turnover that could be independent of gene expression. The effect of erk kinases on MT stability could contribute to the documented role of these kinases in regulating cell motility, invasion, and proliferation [21]. MTs serve as tracks for cellular transport and regulate

cell shape, polarity, and cell process formation, all of which are required functions for cell motility [4]. Mutant active H- and K-ras promote MT reorganization within polarized active lamellae and processes [42], whereas N-ras stimulates MT-dependent particle translocation [43]. Decreased MT stability is likely required for the rapid formation of extensions and lamellae during migration and invasion, and for the exocytosis of sequestered molecules important for invasion, such as matrix metalloproteinases (MMPs). Enhanced MT turnover is also required for targeting and promoting focal adhesions involved in cell motility and growth responses [4]. MTs have also recently been reported to regulate the formation of podosomes, which are located under the cell body and which are sites of release for metalloproteinases [44,45]. When podosomes are located at the tips of cell protrusions, they promote cell motility and invasion [44,45]. Enhanced proliferation may be mediated by decreased MT stability, as formation of the mitotic spindle requires an estimated 20- to 100-fold decrease in MT stability of interphase MTs [7].

Hyperexpression of erk and MKK have recently been linked to the progression of human breast carcinoma. In human tumor biopsies, the overexpression of MKK and erk 1 and 2 has been noted in breast cancer tumors [27–29]. As well, human breast-cancer cells expressing H-ras exhibit up to 15-fold enhanced erk activity and their anchorage-independent growth is strongly inhibited by PD 98059 [32]. We confirm an enhanced activation of erk in breast cancer cells and extend this to show an enhanced association of erk with MTs. Previous reports have shown that erk decorates MTs in NIH 3T3 fibroblasts, neurons, macrophages, and mouse oocytes [15–19]. These results are extended here to include human breast epithelial cells and 10T1/2 murine fibroblasts.

Understanding the role of mutant ras in MT stability and identifying accessory upstream or downstream proteins associated with erk activation may ultimately permit the development of combination therapies and/or paclitaxel-like drugs that exhibit enhanced efficacy and reduced toxicity.

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