RHAMM promotes interphase microtubule instability and mitotic spindle integrity through MEK1/ERK1, 2 activity.

C. Tolg1, S.J. Hamilton1, L. Morningstar1 J. Zhang1, S. Zhang1, K. Esguerra2, P.G. Telmer1, LG. Luyt2, R. Harrison3, JB. McCarthy4, E. Turley2*

1 Depts. Oncology/Biochemistry and London Regional Cancer Program, University of Western Ontario and London Health Sciences Center; 2 Depts Oncology and Chemistry and London Regional Cancer Program, University of Western Ontario and London Health Sciences Centre 3 Department of Biological Sciences, University of Toronto at Scarborough, Toronto ON CA; 4 Dept. Lab. Medicine and Pathology, Tumor Biology and Research Program, Masonic Comprehensive Cancer Center/University of Minnesota.

Corresponding author: E. Turley, PH: 519-685-8600 ext-53677 FX: 519-685-8616 EMAIL: eva.turley@lhsc.on.ca

Running Title: RHAMM affects interphase/mitotic spindle microtubule stability

An oncogenic form of RHAMM (Receptor for Hyaluronan Mediated Motility, mouse, aa163-794 termed RHAMMΔ163) is a cell surface hyaluronan receptor and mitotic spindle protein that is highly expressed in aggressive human cancers. Its regulation of mitotic spindle integrity is thought to contribute to tumor progression but the molecular mechanisms underlying this function have not previously been defined. Here, we report that intracellular RHAMMΔ163 modifies the stability of interphase and mitotic spindle microtubules through extracellular regulated kinase 1, 2 (ERK1, 2) activity. RHAMM−/− mouse embryonic fibroblasts (RHAMM−/− MEF) exhibit strongly acetylated interphase microtubules, multi-pole mitotic spindles, aberrant chromosome segregation and inappropriate cytokinesis during mitosis. These defects are rescued by either expression of RHAMM or mutant active mitogen-activated protein kinase kinase 1 (MEK1). Mutational analyses show that RHAMMΔ163 binds to α-, β-tubulin protein via a carboxyl terminal leucine zipper but in vitro analyses indicate this interaction does not directly contribute to tubulin polymerization/stability. Co-immunoprecipitation and pull-down assays reveal complexes of RHAMMΔ163, ERK1, 2/MEK1, and α - and β-tubulin, and demonstrate direct binding of RHAMMΔ163 to ERK1 via a D-site motif. In vitro kinase analyses, expression of mutant RHAMMΔ163 defective in ERK1 binding in MEF, and blocking MEK1 activity collectively confirm that the effect of RHAMMΔ163 on interphase and mitotic spindle microtubules is mediated by ERK1, 2 activity. Our results suggest a model wherein intracellular RHAMMΔ163 functions as an adaptor protein to control microtubule polymerization during interphase and mitosis as a result of localizing ERK1, 2/MEK1 complexes to their tubulin-associated substrates.

INTRODUCTION

The interphase microtubule network is composed of α- and β-tubulin heterodimers that form tubules, which are highly dynamic structures participating in cell morphology/polarity, signaling, migration, proliferation and protein trafficking (1-3). The effects of microtubules depend on their dynamic nature comprised of polymerization and depolymerization cycles. These are primarily controlled by post-translational modification of microtubule stabilizing (MAPs) and destabilizing proteins (e.g. Stathmin) (4-6). The microtubules of mitotic spindles, which also contain heterodimers of α - and β-tubulin, are particularly dynamic. This property is essential for appropriate chromosome segregation and, consequently, genomic stability (7-10). The microtubule functions that depend upon dynamic cycles of polymerization/depolymerization are increasingly targeted as a means for controlling cancer growth and spread, and the progression of
other diseases in which microtubules play a role. For example taxanes, which promote microtubule stability, are widely used as adjuvant treatment for cancer (11-13). However, an understanding of the molecular mechanisms controlling microtubule turnover in cancer cells is still incomplete.

RHAMM (RHAMM/HMMR is the human gene designation while Rhamm is the mouse gene designation. RHAMM is used here to describe the protein product of both species.) is an oncogenic protein that has been implicated in the progression of many human cancers including breast, AML, multiple myeloma, gastric and prostate cancers (14-16). Most studies to date suggest that RHAMM overexpression promotes tumor progression. Thus, high RHAMM expression in breast cancer is predictive of poor clinical outcome (17,18) and polymorphisms in this gene have been linked to breast cancer susceptibility in some human populations (18,19). SERA analyses have identified RHAMM as a tumor marker for AML and clinical trials are ongoing to assess the use of RHAMM peptide vaccines for control of AML and multiple myeloma (20).

RHAMM expression in adult mammals is largely restricted to sites of tissue injury and to pathological processes involved in chronic inflammation and neoplasia (15,21). RHAMM is distributed within several intracellular compartments and it is also localized to the surface of certain normal and transformed cells (21,22). Intracellular RHAMM proteins are found in the cell nucleus (15) on interphase microtubules (23), mitotic spindles, centrosomes (24) and within mitochondria (25). RHAMM is one of a number of proteins, which can be exported to the cell surface by unconventional mechanisms (15,26). This structurally diverse group of proteins is characterized by the lack of an identifiable signal peptide for export through the golgi/ER.

Extracellular RHAMM promotes cell motility and invasion through sustained stimulation of MEK1/ERK1, 2 kinases (gene designation for MEK1 is MAP2K1, for ERK1 is MAPK3, and for ERK2 is MAPK1. MEK1, ERK1 and ERK2 are used here for protein products of these genes) activity resulting from its association with integral receptors such as CD44 and PDGFR, and with hyaluronan (15,22,27). The functions of intracellular RHAMM are less well understood but its association with mitotic spindles requires a leucine zipper located in the carboxyl terminus that facilitates RHAMM/tubulin interactions. These interactions are required for Ran-driven, acentrosomal, mitotic spindle pole formation in Xenopus egg extracts (15,28,29). Forced high expression of RHAMM results in multi-pole spindles and this effect has been linked to genomic instability in multiple myeloma (30). RHAMM’s pole stimulating function is restricted in human cell lines by Breast cancer gene 1 (BRCA1)/BRCA1 association ring domain 1 (BARD1) complexes, which safeguard the formation of focused bipolar mitotic spindles (28). Recently, intracellular RHAMM has also been implicated in abscission during cytokinesis as a result of its association with supervillin, a member of the gelsolin family of proteins (31). The molecular mechanisms underlying these two RHAMM functions in the cell cycle has not, to our knowledge previously been reported.

Full length RHAMM (RHAMMFL) is expressed in cultured cells and contains two microtubule-binding regions (23,29). Injured, subconfluent or neoplastic cultured cells express additional RHAMM protein isoforms resulting from alternative splicing and/or post-translational processing (32, 33). Most of these are N-terminal truncations of the full-length protein and therefore contain only the carboxyl terminal tubulin binding sequence (27). At least one of the truncated forms (e.g. RHAMMΔ163) is transforming when overexpressed in 10T1/2 MEF cell lines (32). Therefore understanding the mechanisms by which RHAMM affects microtubule structures via its carboxyl terminal sequence may help to clarify its role(s) in neoplastic diseases.

We have shown that intracellular RHAMM proteins, in particular RHAMMΔ163, complex with MEK1 and ERK1, 2 (33). We were prompted to investigate whether or not intracellular RHAMM protein controls
interphase and mitotic microtubule stability/integrity through MEK1/ERK1,2 since we reported that ERK1,2 activity is necessary for dynamic instability of interphase microtubules in RAS-transformed fibroblasts (34), others have shown that microtubule stability results in multi-pole spindles (35) and BRCA1/ERK1,2 form complexes during mitosis (36,37). Here, our results suggest that MEK1/ERK1,2 complexes mediate the effects of intracellular RHAMMΔ163 on interphase and mitotic spindle structure and that RHAMM performs scaffolding functions to control both activity and targeting of MEK1/ERK1,2 complexes to tubulin.

EXPERIMENTAL PROCEDURES

Reagents
α- and β-tubulin heterodimers and MAP-enriched tubulin were purchased from Cytoskeleton Inc (Denver, CO). Mouse anti-tubulin monoclonal antibodies, and anti-phospho-ERK1,2 antibodies were purchased from Sigma (St. Louis, MO) and Santa Cruz (Santa Cruz, CA). An anti-pan ERK1,2 polyclonal antibody was purchased from Transduction labs (Mississauga, ON). Alexa dye secondary antibodies were obtained from Invitrogen (Burlington, ON). Nocodazole and the MEK1 inhibitor (PD98059) were purchased from Sigma. The mutant active MEK1 expression vector was the kind gift of Natalie Ahn (U. Colorado, Boulder, CO). Polyclonal anti-RHAMM antibodies were prepared against the peptide sequence (mouse RHAMM)727KLKDENSQKLSEVS740 by ProSci (Poway CA). Additional peptides,715HQNLKQKIKHVVKLKDENSQKSVKLRSQ745, as well as 728KLKDENSQKSVKL742, which contains the leucine zipper required for an association of RHAMM with the mitotic spindle (28), and control peptides,194KLQATKDLTESKGLVQLEGKL217 and control peptide 2: 218VSIEKEKIDEK228 were used in MAP kinase binding assays, and were synthesized at the London Regional Cancer Program Proteomics Center. Biotinylated hyaluronan was purchased from Hyalose (Oklahoma City, OK).

Cells and cell lines
10T1/2 and C3 10T1/2 fibroblasts were purchased from ATCC (Manassas, VA). 10T1/2 fibroblasts were transfected with RHAMM cDNA constructs shown in Fig. 1A as previously described (32). Constructs contained either an HA or Myc epitope tag (32,33). RHAMM +/+ , RHAMMΔ163-rescued, RHAMMΔ163-rescued and mutant active MEK1-rescued MEF cell lines were prepared as previously described (33). Primary mouse embryonic fibroblasts with genotypes RHAMM +/+ and RHAMM +/+ (wildtype) were obtained from RHAMM +/- heterozygote matings and were thus litter-matched. MEF were isolated from ED14 mouse embryos as described (34).

Immunofluorescence
Cells were plated overnight on sterile glass coverslips at 50% sub-confluence in DMEM supplemented with 10% FBS. Cells were fixed with buffered 3% paraformaldehyde then permeabilized with 0.1% Triton X-100 in PBS. Non-specific binding sites were blocked with 3% BSA in PBS for 1 hr at 20°C. Anti-tubulin, anti-acetylated tubulin and anti-phospho-ERK1,2 antibodies were diluted 1:100, Anti-RHAMM antibodies were diluted 1:1000. Fixed cultures were incubated with primary antibodies for 2 hrs at 20°C. Cultures were washed in 3% BSA in PBS then incubated with Alexa dye labeled secondary antibodies, which were diluted 1:150. Cultures were washed again in 3% BSA/PBS then mounted in a Vectashield mountant containing DAPI (1.5 µg/ml).

Western and far western blots
Cell lysates were prepared using RIPA buffer as described previously (33). Equal amounts of cell lysate or GST-RHAMM recombinant protein were resolved by electrophoresis on a 10% SDS-PAGE gel. Separated proteins were transferred to nitrocellulose membranes in a buffer containing 25 mM Tris-HCL (pH 8.3), 192 mM glycine and 20% methanol using electrophoretic transfer cells (Bio-Rad, Mississauga, ON) at 100V for 1.5 hrs at 4°C. Membranes were incubated in TBST + 5% defatted milk to block non-specific protein binding sites then the membranes were incubated with primary antibodies at dilutions
recommended by the manufacturer for 2 hrs at 4°C. Polyclonal anti-RHAMM antibodies were used at a dilution of 1:15,000. Membranes were washed then immunodetection was performed using secondary antibodies provided in an ECL kit (Invitrogen).

For far western analyses, recombinant GST-RHAMM was separated on SDS-PAGE and transferred to nitrocellulose membranes as above. Microtubule protein (Cytoskeleton Inc, 99% α- and β-tubulin heterodimers) was incubated with the nitrocellulose membrane at 0.2 µg/ml as described for primary antibodies, washed and then incubated with an anti-α-tubulin mouse monoclonal antibody (1:100 dilution). Bound tubulin antibodies were detected using goat anti-mouse-HRP secondary antibody. Bound antibody was detected with reagents in an ECL kit as above.

Pull-down assays
Recombinant mouse GST-RHAMM proteins and GST protein by itself were produced in bacteria as described (22) then subsequently purified using glutathione Sepharose 4B beads (GE healthcare). ERK1, ERK2 and MEK1 recombinant proteins (human) were purchased from Enzo LifeSciences Inc (Plymouth Meeting, PA). Based on a Coomassie blue stained SDS-PAGE gel, 100 µl GST-RHAMM706-767 beads and 50 µl GST beads were used for this assay. The beads were washed with 1xPBS before blocking with 100 mM lactose overnight at 4°C. Purified bovine α- and β-heterodimeric tubulin (Cytoskeleton Inc) was diluted in tubulin buffer to a concentration of 50 µg/ml and 500 µl was mixed with lactose-blocked GST-RHAMM706-767 or GST beads alone at 4°C for 3 hours. The beads were then washed with 1 ml wash buffer (50 mM Tris, pH 8.0 with 0.1% Triton X-100) 5 times at 4°C. 100 µl of 2X SDS loading buffer was added to beads, which were boiled at 95°C for 10 min, then 20 µl of the supernatant were loaded on a 10% SDS-PAGE gel. Separated proteins were transferred to a nitrocellulose membrane and incubated with anti-tubulin antibodies as described above for western blots. To confirm binding of α- and β-tubulin to RHAMM in cells, cell lysates were prepared from primary RHAMM−/− and wildtype MEF as described above and then pull-down assays were performed using recombinant RHAMM706-767 linked to Sepharose beads. Beads were washed and associated proteins were separated on a gradient SDS-PAGE (5-12%), lightly stained with silver and clearly separated bands in the range of 45-65 kDa were cut out, protein eluted and identified with MALDI-TOF analysis (Emili and Greenblatt Proteomics Research Center at the University of Toronto, Toronto, CA).

To assess direct binding of RHAMM to ERK1, ERK2, and MEK1, purified GST-RHAMM recombinant protein (mouse, aa164-794) was immobilized on glutathione-Sepharose as a GST fusion protein or covalently linked to Sulfolink gel as per manufacturer’s instructions (Pierce Biotechnology, Rockford IL). Recombinant MAP kinases were incubated with GST-RHAMM beads in binding buffer (25 mM HEPES, pH 7.2; 50 mM NaCl; 10 mM MgCl2) for 1 hr at 4°C on a nutator. Beads were sedimented by centrifugation, washed 10X with 1 ml cold binding buffer/wash then boiled in SDS-PAGE loading buffer before electrophoresis on 10% SDS-PAGE and transferred to a nitrocellulose membrane for western blot analysis. For competition analyses, 1 µg MAP kinase recombinant protein was incubated with 10 µg peptide or soluble recombinant wildtype or mutant RHAMM protein at 4°C on a nutator for 1 hr then GST-RHAMM-beads were added and the mixture was incubated an additional 1 hr. GST-RHAMM-beads were captured by centrifugation and analyzed as above for bound MAP kinases.

Microtubule pelleting assays
Pelleting assays were performed using GST-RHAMMΔ373 and GST-RHAMMΔ163 recombinant proteins or GST alone used as a control. These proteins were incubated with taxol-stabilized porcine microtubules, prepared according to manufacturers instructions (MAP-spin-down assay kit, Cytoskeleton Inc). 1-2 µg/ml recombinant RHAMM proteins were dissolved in microtubule cushion buffer (PEM) supplemented with 2 mM DTT and 20 µM taxol. The solution was pre-cleared by centrifugation at 80,000 g for 45 min at 4°C. The supernatant was decanted and incubated with either 50 µM of
taxol-polymerized microtubules or buffer alone. Samples were layered over 350 µl of 10% glycerol in PEM and microtubules were sedimented from soluble protein by centrifugation at 80,000 g for 40 min at RT. Equal amounts of the supernatant and pellet were separated on 10% SDS-PAGE gels and tubulin was detected by western blot analysis as described above.

**Immunoprecipitation assays**
Immunoprecipitations were performed using 500 µg protein from cell lysates pre-cleared with 10 µl of protein A/G Sepharose beads (GIBCO BRL, Gaithersburg, MD). The pre-cleared lysate was incubated with primary antibodies for 12 hrs at 4˚C on a nutator at concentrations recommended by the manufacturer. Anti-RHAMM polyclonal antibodies were used at 5 µg/400 µg cell lysate. The protein/antibody complexes were captured with protein A/G Sepharose beads, which were pelleted and washed. Bound protein was released by boiling in 25 µl Laemmli buffer. Released proteins were separated on a 10% SDS-PAGE as described above and western blots conducted as described above.

**Analysis of acetylated tubulin levels in soluble and insoluble fractions**
1x10^5 cells were plated on fibronectin-coated (10 µg/ml) cell culture dishes in DMEM/10% FCS containing antibiotics/antimycotics. 24 hrs later, cells were washed with PBS. The soluble fraction was isolated by treating cells with 300 µl microtubule stabilizing buffer (0.1 M Pipes pH 6.9, 1 mM EGTA, 2.5 mM GTP, 4% PEG 6000, 0.2% TritonX-100) plus proteinase and phosphatase inhibitor on ice. Following removal of the soluble fraction, the insoluble fraction was isolated by treating cells with 300 µl RIPA lysis buffer (50 mM TrisHCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 % NP-40, 0.1 % SDS) on ice and scraping the plate with a cell scraper. Soluble and insoluble fractions were stored at 4˚C (short term) or −80˚C (long term) prior to quantifying the protein concentration using an Advanced Protein Assay (Cytoskeleton). Equal amounts of protein were loaded on an SDS-PAGE gel and separated by electrophoresis as described above. Western analyses were performed using 1:1000 anti acetylated and 1:1000 total (α and β) tubulin antibodies (Sigma).

**Resistance of interphase microtubules to nocodazole**
Cells were plated at 50% confluence on fibronectin-coated coverslips as above. 24 hrs later, cells were treated with culture medium containing different concentrations of either nocodazole or DMSO for 30 min. After treatment, cells were washed with PBS and incubated with microtubule stabilizing buffer for 10 min on ice. Cell monolayers were washed again with PBS then fixed in 4% paraformaldehyde in PBS for 10 min on ice. After fixation, cells were washed for 5 min with PBS then blocked with 3% BSA/PBS for 1 hr at room temperature. Cells were incubated overnight at 4˚C with either 1:200 diluted (1% BSA/PBS) anti-α-tubulin antibody or isotype matched non-immune IgG (Cytoskeleton Inc). Monolayers were washed 3 times at 5 min intervals with PBS followed by 30 min incubation with 1:200 diluted (1% BSA/PBS) secondary anti mouse or anti rabbit secondary antibody (labeled with Alexa Fluor 647). Monolayers were washed 3 times at 5 min intervals with PBS to remove unbound secondary antibody then incubated with a 1:20,000 dilution (PBS) of DAPI for 10 min to detect nuclei. Monolayers were washed 3 times in PBS at 5 min intervals then mounted in Dako fluorescent mounting medium and examined with an Olympus confocal microscope.

**Nocodazole-induced cell cycle block**
Cells were plated at 50% confluence on sterile coverslips in DMEM, 10% FCS plus antibiotic/anti-mycotic and incubated at 37˚C, 5% CO2, humidified atmosphere and allowed to adhere for 5-6 hrs then incubated for 24 hrs in 60 ng/ml of nocodazole. Cells were released from the nocodazole block by washing monolayers 3 times with DMEM+10% FCS. Monolayers were fixed in 3% paraformaldehyde at 2, 4 and 6 hours following the removal of nocodazole and stained for α-tubulin using anti-tubulin antibodies as described above. Cells were photographed with an Olympus confocal microscope.
Quantitative PCR (QPCR)
RNA was isolated from 50% confluent cells using TRIZOL (Invitrogen, Burlington, ON) following the manufacturers instructions. 1 µg of RNA was reverse-transcribed using SuperscriptII (Invitrogen, Burlington, ON) following the manufacturers instructions. Oligo dT (Invitrogen, Burlington, ON) was used as primer for cDNA synthesis. CYBR Green PCR master mix was purchased from SA Biosciences (Frederick, MD). PCR amplification was performed on a Stratagene MX 3000 instrument. Primer sequence: RHAMM left primer: GGA AGCAGCTGGAAGAGAAA; RHAMM right primer: CTGTTCCTCGGCTTCAAGG β-actin left primer: CTCTTTGATGTCACG CACGATTTC; β-actin right primer: GTGGGC CGCTCTAGGCACCAA Cycle conditions: 10 min 94˚C, 30 sec 94˚C, 45 sec 55˚C, 45 sec 77˚C, 10 min 72˚C. Relative expression levels were calculated as ΔCt. Amplification of β-actin was used for standardization.

RESULTS
RHAMMΔ163 decorates both interphase and mitotic spindle microtubules.
RHAMMFL was previously shown to decorate both interphase and mitotic spindles of epithelial and immune cells (23,30). We wanted to confirm the co-localization of RHAMM proteins with microtubule structures in 10T1/2 fibroblasts, which express both endogenous RHAMMFL and truncated RHAMM forms, including small amounts of RHAMMΔ163 (Fig. 1A, B). In mouse, these RHAMM proteins are 95 and 70 kDa respectively. As previously noted for the other cell types (23, 29), these endogenous RHAMM proteins decorated the poles of mitotic spindles in 10T 1/2 fibroblasts as well as the length of interphase microtubules (Fig. 2A). Primary wildtype MEF expressed lower levels of RHAMM protein than 10T1/2 fibroblasts, and expression was most easily detected in these primary cells by mRNA analysis (Fig. 1B, graph). Analysis showed that the predominant RHAMM protein was FL. Therefore, immortalized RHAMM Δ/Δ MEF lines (22) were transfected with either RHAMMFL or RHAMMΔ163 in order to compare the tubulin binding properties of both RHAMM protein forms. Expression of these two RHAMM forms in transfected cells was confirmed with QPCR and immunohistochemistry (Fig. 1B graph, C). When photographed at the same laser intensity, primary RHAMMΔ/Δ MEF as well as immortalized RHAMMΔ/Δ MEF lines exhibited large bundles of brightly staining interphase microtubules in contrast to the interphase microtubules of either immortalized RHAMM- rescued lines or primary wildtype MEF, which stained less brightly (Fig. 2B). Primary and immortalized RHAMMΔ/Δ MEF exhibited a high frequency of multi-pole mitotic spindles (Fig. 2B and SUPPL. Fig. 1A) and chromosome misalignment on these aberrant spindles. Expression of RHAMMFL rescued both interphase microtubule abnormalities and mitotic spindle/chromosome segregation defects (Fig. 2B). These results suggested that, in addition to decorating microtubule structures, RHAMM plays an essential and non-redundant role in microtubule integrity.

RHAMM associates with both interphase and mitotic spindle microtubules
Previous studies identified microtubule-binding sequences in both exon 4 and 16 of RHAMMFL (23,29) (Fig. 1A). In these studies, deletion of aa1-103 resulted in loss of RHAMM on interphase microtubules and its accumulation in the nucleus (23). An additional microtubule-binding site was identified in the carboxyl terminus of RHAMM that co-immunoprecipitated with γ-tubulin (29), and mediated an association with centrosomes and mitotic spindles (24,29). This second binding site contained a leucine zipper (mouse, 728LKDENSQLKSEVSKL 742), which was required for decoration of RHAMM on mitotic spindles (28,29). These collective results were interpreted as evidence that the two tubulin-binding sequences of RHAMMFL performed separate functions: the N-terminal sequence was proposed to regulate interphase microtubules while the carboxyl terminal sequence was proposed to regulate mitotic spindle/centrosomes integrity.

In order to confirm this separation of tubulin binding sites, we first prepared Myc-tagged N-
terminal truncations of RHAMM FL that lacked the tubulin-binding site in exon 4 (e.g. RHAMMΔ173, Fig. 3A and RHAMMΔ163, data not shown). These constructs were expressed in 10T1/2 fibroblasts and their ability to decorate interphase and mitotic spindle microtubules was assessed with immunofluorescence assays (Fig. 3A, interphase microtubules shown). Results unexpectedly showed that the carboxyl terminal tubulin-binding region alone was sufficient to locate RHAMM to both interphase and mitotic spindle microtubules. The Myc tag did not modify the association of RHAMM with microtubules since untagged truncated RHAMM cDNA’s expressed in RHAMM−/− MEF also decorated interphase microtubules (data not shown). These results predicted that the leucine zipper bound to both interphase and spindle microtubules, raising the possibility that short RHAMM forms such as RHAMMΔ163 similarly affect interphase and mitotic spindle microtubules by a common mechanism.

RHAMMΔ163 promotes microtubule instability
Since loss of RHAMM expression resulted in the appearance of brightly staining, large microtubule networks (e.g. Fig. 2B), we assessed the possibility that RHAMM expression affects microtubule stability. Stability was compared in primary RHAMM−/− and wildtype MEF by quantifying resistance of interphase microtubules to disruption by nocodazole and by measuring α-tubulin acetylation levels, the latter used as a marker for stable microtubules. Interphase microtubules of primary RHAMM−/− MEF were more resistant to disruption by nocodazole than wildtype MEF (Fig. 3B and SUPPL. Fig. 2). Alpha-tubulin was also significantly more acetylated in primary and immortalized RHAMM−/− MEF compared to immortalized RHAMM-rescued MEF (Fig. 4A) and primary wildtype MEF (Fig. 4B). The expression of RHAMM containing either one (RHAMMΔ163) or two (RHAMMFL) microtubule binding sites rescued this RHAMM−/− microtubule phenotype equally well (Fig.4A, B), predicting that the carboxyl-terminal mitotic spindle/tubulin binding site was able to mediate interphase microtubule interactions.

Spindle microtubules resemble their interphase counterparts in that they are dynamically regulated, at least in part, by similar mechanisms. To further characterize the mitotic functions of RHAMMΔ163 and to assess if RHAMM expression affected mitotic spindle stability, we quantified mitotic processes that reflect the dynamic nature of mitotic microtubules such as spindle integrity (10,35,36), chromosome segregation (37) and abscission during cytokinesis (38) in immortalized RHAMM−/−, RHAMMΔ163 and RHAMMFL rescued MEF (Fig. 5, 6 and SUPPL. Fig. 3). The occurrence of bi-pole vs. multi-pole spindles and abnormal chromosome alignment on the mitotic spindle were used as indicators of aberrant spindle formation (35). Abnormal cytokinesis was detected by time-lapse analysis of mitotic cells (31) and by the presence of multinucleated cells.

Immortalized RHAMM−/− MEF exhibited a high percentage (almost 40% of mitotic cells) of multi-pole spindles and this defect was most strongly reduced by the expression of RHAMMFL (Fig. 5) and, interestingly, to a lesser extent by expression of RHAMMΔ163 (data not shown). Chromosome segregation was aberrant on multi-pole spindles and this defect was also rescued by expression of RHAMMFL (e.g. Fig. 5). A similar finding was observed when primary RHAMM−/− MEF were compared to primary wildtype MEF (SUPPL. Fig. 1B). Large, multinucleated cells were common in RHAMM−/− MEF populations and their presence was reduced by either RHAMMFL or RHAMMΔ163 expression (Fig. 6 and SUPPL. Fig. 3B). Time-lapse analysis of RHAMM−/− MEF revealed a high percentage of cells with aberrant abscission during cytokinesis (SUPPL. Fig. 3). Abscission defects ranged from failure of the cleavage furrow to form in mitotic cells, resulting in giant multinuclear cells, to formation of multiple cleavage furrows, resulting in many small daughter cells, most of which lacked nuclei. These defects were equally rescued by the expression of RHAMMFL or RHAMMΔ163. Collectively, data suggested that RHAMM was required for regulating stability of interphase and mitotic microtubules and that this function resided in the carboxyl-terminal

728LKDENSQLKSEVSKL 742 microtubule-binding site.
RHAMM directly binds to α- and β-tubulin
To begin to identify the mechanisms by which the carboxyl terminus of RHAMM affected interphase and mitotic spindles, we identified binding partners for LKDENSQLKSEVSKL. We first determined that recombinant RHAMM fragments containing this sequence (SUPPL. Fig. 4A) bound equally well to both soluble tubulin and pelleted, taxol stabilized microtubules (SUPPL. Fig. 4B). We subsequently used soluble tubulin extracts for our assays. Alpha- and β-tubulin are common to both mitotic spindle and interphase microtubules while γ-tubulin is uniquely present in centrosomes and mitotic spindles (5,6). We therefore first performed pull-down assays using recombinant carboxyl terminal RHAMM fragments (SUPPL. Fig. 4A) and 10T1/2 fibroblast lysates. MALDI TOF analysis was performed on isolated proteins that were separated on SDS-PAGE. Analysis showed that RHAMM∆163 bound to α- and β-tubulin (data not shown). γ-tubulin was not detected in these assays although this may have been due to limiting amounts in 10T1/2 cell lysates relative to the other tubulin isoforms. To assess if interactions were direct or indirect, pull-down assays were performed using purified α-, β-tubulin heterodimers and a recombinant RHAMM706-767 fragment linked to Sepharose beads (Fig. 7A). Bound proteins were separated on SDS-PAGE and identified using anti-α or β-tubulin antibodies with western blots. Sepharose-GST served as a negative control. RHAMM706-767 bound to tubulin heterodimers (Fig. 7A) while GST alone did not. The ability of truncated RHAMM forms that are represented in cells (e.g. RHAMM∆163 and RHAMM∆373) to bind directly to these tubulin isoforms was confirmed with a far western assay using soluble α-, β-tubulin heterodimers as probes (SUPPL. Fig. 4C). The laddering of recombinant RHAMM proteins in the assay shown in SUPPL. Fig. 4C was due to protease activity. The interaction between recombinant RHAMM706-767 and tubulin heterodimers was strongly reduced by the presence of a synthetic peptide (LKDENQLKSEVSKL) mimicking the RHAMM carboxyl terminal leucine zipper (Fig. 7B). Collectively, these results indicated that the interaction between the carboxyl-terminal binding site in RHAMM and tubulin was direct and mediated by the highly conserved mitotic spindle binding LKDENSQLKSEVSKL sequence (28,29).

MEK1/ERK1, 2 mediate the effects of RHAMM on interphase and mitotic microtubules
We next investigated how RHAMM∆163 affected microtubule stability. Previous studies had suggested RHAMMFL directly modified tubulin stability by promoting polymerization, similar to many other microtubule-associated proteins (MAPs) (23). To assess this possibility, the ability of GST-RHAMM to modify formation of taxol stabilized tubulin polymers was assessed in vitro (SUPPL. Fig. 5). Recombinant RHAMM∆163 and RHAMM∆373 fragments did not significantly increase or decrease the amount of pelleted microtubules. These results suggested that RHAMM fragments such as RHAMM∆163 indirectly affected microtubule stability.

We previously showed that a H-RAS/ERK1, 2 pathway promoted dynamic turnover of interphase microtubules, using α -tubulin acetylation as a marker for microtubule stability (33,34). ERK1, 2 were originally isolated from microtubules and these kinases decorate both interphase (34,39) and mitotic spindles (28,29,40). Since we reported that intracellular RHAMM∆163 complexed with MEK1/ERK1, 2 kinases and was required for activation of these MAP kinases through H-RAS (32,33), we assessed the role of ERK1, 2 activity in RHAMM-mediated effects on microtubule stability.

The effect of a MEK1 inhibitor, PD98059 on acetylation of α -tubulin in RHAMM∆163-transfected and H-RAS-transformed 10T1/2 cells, both of which expressed high levels of RHAMM∆163, was assessed. PD098059 significantly increased acetylated tubulin levels in these cell lines although this inhibitor had little effect on parental 10T1/2 cells, which expressed low levels of RHAMM∆163 (SUPPL. Fig. 6, Fig. 1B). Conversely, expression of mutant active MEK1 in immortalized RHAMM−/− MEF reduced acetylated tubulin to levels similar
to RHAMM\textsuperscript{Δ163} transfected MEF, thus phenocopying the effects of RHAMM\textsuperscript{Δ163}-rescue on interphase microtubules (Fig. 4).

Although a direct role for ERK1, 2 in somatic cell centrosome-driven mitosis is still controversial (40-42), the above results and evidence that ERK1, 2 phosphorylate protein substrates during G2/M (43), prompted us to examine the role of these kinases in RHAMM-mediated events of mitosis. Mitotic spindle integrity, chromosome segregation and cytokinesis fidelity were compared in immortalized RHAMM\textsuperscript{-/-} MEF following stable expression of either RHAMM\textsuperscript{FL} or mutant active MEK1. Mutant MEK1 increased ERK1, 2 activity as expected [(22), data not shown], reduced the frequency of cells with multi-pole mitotic spindles and restored bi-pole spindles similar to that observed with RHAMM-rescue (Fig. 5). Activated MEK1 also restored the fidelity of cytokinesis in immortalized RHAMM\textsuperscript{-/-} MEF to the same degree as RHAMM-rescue, as detected by time-lapse analyses and quantification of multinucleated cells (Fig. 6, SUPPL. Fig. 3). However, in spite of promoting normal mitotic spindle morphology, activated MEK1 did not restore normal chromosome alignment and segregation on the mitotic plate to the extent of RHAMM rescue (e.g. Fig. 5).

**RHAMM binds directly to ERK1 and mutation of its ERK docking sequence phenocopies RHAMM loss**

Our previous work showed that cell surface RHAMM regulated ERK1, 2 activation through an association with the integral hyaluronan receptor, CD44 (22,27). To exclude a possible involvement of cell surface RHAMM-activated ERK1, 2 in controlling microtubule dynamics, we added recombinant RHAMM\textsuperscript{Δ163}-beads to RHAMM\textsuperscript{-/-} MEF and quantified their effect on mitosis using time-lapse analysis (22). Extracellular RHAMM\textsuperscript{Δ163} did not rescue the mitotic defects of immortalized RHAMM\textsuperscript{-/-} MEF (data not shown) indicating that cell surface activation of ERK1, 2 through RHAMM was not sufficient for driving alterations in microtubule dynamics. These and data described above raised the possibility that intracellular RHAMM proteins, in particular RHAMM\textsuperscript{Δ163} scaffolded MEK1/ERK1, 2 to tubulin.

Protein kinase anchoring proteins generally bind directly to their target kinase (44). We therefore determined if GST-RHAMM bound directly or indirectly to MEK1, ERK1 and ERK2 recombinant proteins using pull-down assays. Surprisingly, only ERK1 bound directly to recombinant RHAMM\textsuperscript{Δ163} (Fig. 8B) suggesting that previously noted interactions of RHAMM with MEK1 and ERK2 were indirect (e.g. Fig. 9C). Binding to ERK1 was specific in that soluble GST-RHAMM competed with ERK1/RHAMM-bead interactions (Fig. 8B). Examination of RHAMM\textsuperscript{Δ163} sequence revealed a highly conserved MAP kinase “D” docking-site (Fig. 8A). These sites are composed of positively charged and hydrophobic clusters of amino acids separated by 2-6 amino acids and are common to many of ERK1, 2 scaffolds and substrates (45). To determine if the sequence K\textsuperscript{721}-L\textsuperscript{728} acted as a docking site for ERK1, we used two experimental approaches. In the first, both K\textsuperscript{721} and K\textsuperscript{727} were mutated to E\textsuperscript{721} and E\textsuperscript{728} and recombinant mutant GST-RHAMM was assayed for binding to ERK1 in pull-down assays: binding was reduced by approximately 50% (Fig. 8C). In the second approach, a synthetic peptide containing the putative D-site sequence (H\textsuperscript{715}-Q\textsuperscript{745}, Fig. 8A) was used to compete for RHAMM\textsuperscript{Δ163}/ERK1 interactions (Fig. 8B). This peptide reduced binding of ERK1 to RHAMM\textsuperscript{Δ163} by approximately 90% (Fig. 8C). Unrelated synthetic RHAMM peptides (Fig. 8A) had no effect on binding (Fig. 8C) and served as controls.

Since intracellular hyaluronan-RHAMM interactions have been suggested to play a role in mitosis (46) and to also activate and target ERK1, 2 (22), the above mutant RHAMM\textsuperscript{Δ163} protein was assessed for its hyaluronan binding ability. Mutant RHAMM\textsuperscript{Δ163} retained an ability to bind to biotinylated hyaluronan consistent with evidence that V\textsuperscript{747}-K\textsuperscript{750} are essential for this interaction (47,48), and peptide H\textsuperscript{715}-Q\textsuperscript{745} did not block binding of biotinylated hyaluronan to recombinant RHAMM\textsuperscript{Δ163} (data not shown). These results allowed us to clearly interpret a role of direct RHAMM\textsuperscript{Δ163}/ERK1 interactions in
microtubule dynamics in cells. We therefore next assessed if the D-site also mediated binding of ERK1 to RHAMM in cells. The association of ERK1 and MEK1 with RHAMMΔ163 and the ERK1 docking mutant RHAMMΔ163 (Fig. 8A) was compared by immunoprecipitation assays following transient expression of these RHAMM constructs in 10T1/2 cells (Fig. 1B) (33). ERK1/MEK1 co-associated with RHAMMΔ163 but not with the mutant RHAMMΔ163 (Fig. 9A) confirming that the D site was necessary for ERK1/MEK1/RHAMM interactions in cells.

We then assessed if RHAMMΔ163 anchored ERK1, 2 to microtubules, providing these MAP kinases with access to their microtubule MAPs, which then directly modified microtubule dynamics. To begin to assess this possibility, mutant RHAMMΔ163 was expressed in H-RAS-transformed 10T1/2 cells, which exhibited high levels of microtubule associated, active ERK1, 2 (34). We expected that mutant RHAMMΔ163 would behave as a dominant negative suppressor of endogenous RHAMM proteins since RHAMM proteins dimerize and trimerize (data not shown) and since we successfully blocked the hyaluronan binding function of cell surface RHAMM with this approach (32,33). Alpha- and β–tubulin heterodimers were immunoprecipitated and associated active ERK1, 2 (p-ERK1, 2) were detected with western blots. Expression of mutant RHAMMΔ163 in H-RAS transformed 10T1/2 fibroblasts resulted in loss of detectable phospho-ERK1, 2 from tubulin (Fig. 9B). Total cellular levels of p-ERK1, 2 were also reduced in mutant RHAMMΔ163-transfected cells and therefore values were normalized by calculating the percent of tubulin associated p-ERK1, 2 to total cellular p-ERK in both cell lines (Fig. 9B, graph). Expression of mutant RHAMMΔ163 thus reduced the percentage of tubulin-associated p-ERK1, 2 by approximately 2.5 fold. We next assessed if mutant RHAMMΔ163 also affected acetylated tubulin levels. While acetylated tubulin levels were low in H-RAS or RHAMMΔ163 10T1/2 fibroblasts, the expression of mutant RHAMMΔ163 in H-RAS 10T1/2 fibroblasts strongly increased levels of α-tubulin acetylation (Fig. 10 A, B). These results were consistent with a model in which intracellular RHAMMΔ163 functioned as an adaptor protein that bound directly to ERK1 and to tubulin but indirectly to MEK1/ERK2, thus targeting this activated kinase complex to microtubules, which phosphorylated MAPs to modify microtubule stability (Fig. 9C).

**DISCUSSION**

Our data suggest that RHAMM proteins control the structure of interphase and mitotic spindle microtubules and that these effects are driven by MEK1/ERK1, 2 kinase activity. Previous reports had established an involvement of ERK1, 2 in interphase microtubule dynamics resulting from their ability to phosphorylate both microtubule stabilizing and destabilizing proteins such as MAP’s and stathmin (49,50). Our results additionally and unexpectedly reveal a role for MEK1 in restricting multi-pole mitotic spindles and promoting normal cytokinesis during mitosis. Evidence presented in this report further suggests that RHAMM/MEK1/ERK1, 2 complexes affect microtubule function by promoting dynamic instability. We therefore propose that RHAMM targets and anchors MEK1/ERK1, 2 to tubulin, where these MAP kinases phosphorylate the tubulin-associated proteins that regulate microtubule dynamics (5). Since the dynamic nature of microtubules has been linked to functions associated with cancer progression including cell cycle progression and motility/invasion, our results raise the possibility that microtubules are an important oncogenic target of transforming RHAMM protein forms such as RHAMMΔ163.

The ERK1, 2 MAP kinases decorate both interphase microtubules and poles of mitotic spindles (50-53), and modify interphase microtubule stability (34). Although ERK1, 2 kinase activity is clearly required for progression through G1/S, the direct vs. indirect role of these kinases in somatic cell mitosis (G2/M) is still controversial (40,41,51). On the one hand, proteomic analyses have identified G2/M targets for ERK1, 2 kinases (43) and blocking MEK1 with kinase inhibitors can result in aberrant mitotic spindles and a G2/M block (50,51,53). On the other hand, acute blocking of MEK1 activity during mitosis to prevent the direct
substrate effects of this kinase pathway resulted in very minor consequences to mitosis and in particular did not influence mitotic spindle integrity of treated cells (41). The authors of this last study concluded that the MEK1/ERK1, 2 kinase pathway controls expression of genes necessary for progression through G2/M [e.g. cdc25C (42), cyclinB1/cdc-2 (54)] but does not play a major role in normal G2/M by directly phosphorylating substrates. While RHAMM may affect events in mitosis by MEK1/ERK1, 2-regulated gene expression it also appears to have direct effects on mitotic spindles since its addition to Xenopus egg extracts controls mitotic spindle pole formation and number (28). Further studies will be required to dissect the roles of direct vs. indirect effects of RHAMM/MEK1/ERK1, 2 complexes in mitotic spindle integrity of somatic cells.

Mitotic spindle formation is driven by multiple, cooperative, microtubule nucleation and capture sites. Centrosomes play a dominant role in microtubule capture in somatic cells but are absent from germ cells and plant cells. Cells lacking centrosomes form mitotic spindles in a chromatin dependent manner, a process that requires formation of Ran-GTP gradients (36,55,56). However, Ran-GTP gradients are also thought to provide kinetic stimulus but not the driving force for mitotic spindle formation in somatic cells that contain centrosomes (55). Intracellular RHAMM proteins have, to date, been most strongly implicated in Ran-dependent spindle assembly (28). Intriguingly, Ran, like RHAMM, is over-expressed in human cancers in vivo and a number of human cancer cell lines exhibit dependence on Ran-GTP for successful mitosis. Silencing Ran expression in tumor cells results in aberrant mitotic spindle formation and apoptosis while mitosis and survival of normal cell lines are largely unaffected (57,58). Therefore Ran-directed mitosis may predominate in diseased and/or stressed tissues, and RHAMM may also participate in spindle formation under these conditions. This possibility is consistent with evidence that RHAMM expression is primarily limited to tissue injury and neoplasia, that it associates with TPX2, a spindle pole protein required for Ran-driven mitosis (24) and that Ran-directed mitosis requires several ERK1, 2 substrates including survivin (57) and Ran-binding protein (59-61).

The physiological and pathological processes that require RHAMM for cell division in vivo are understudied. RHAMM−/− mice are fertile and adults do not have obvious defects that can be associated with aberrant cell proliferation during embryogenesis or adult homeostasis. Loss of RHAMM reduces desmoid tumor initiation and invasion in a mouse model of tumor susceptibility but the consequences of RHAMM loss on tumor cell division was evident only when cell-cell contact was limited in culture (62). Furthermore, although cell division was not the major focus of the study, differences in mesenchymal cell proliferation during excisional skin wound repair of RHAMM−/− vs. wildtype siblings were not observed (22). Thus, a major challenge for future studies will be to define the conditions under which RHAMM plays a role in mitosis in vivo.

In the present study, RHAMM-loss resulted in a high percentage of multi-pole spindles in mitotic cells. These results are consistent with a previous study showing that microinjection of function-blocking RHAMM antibodies also promoted multi-pole spindles (24). However, an in vitro study utilizing Xenopus egg or HeLa cell extracts showed that excess carboxyl terminal RHAMM protein fragments promoted multiple spindle poles while anti-RHAMM antibodies focused spindle poles in Ran-driven spindle formation. These effects depended upon the presence of BRCA1/BARD1 complexes, which were proposed to block the pole-stimulating function of RHAMM protein (28). This apparent discrepancy with our present results and those of Maxwell et al (24) predicts that the mitotic functions of RHAMM are complex and may depend upon cell background, RHAMM protein levels and possibly RHAMM isoform expression. For example, our results showing that RHAMM controls several apparently mechanistically distinct processes during mitosis is consistent with functional complexity. Thus, RHAMM loss affects not only spindle integrity but also chromosome segregation and cytokinesis: while the effects of RHAMM on spindle integrity and cytokinesis are mediated by
MEK1, chromosome segregation appears to be mediated through other mechanisms.

In conclusion, we show that RHAMM associates with both interphase and mitotic spindle microtubules by directly binding to α- and β-tubulin through a highly conserved leucine zipper in its carboxyl-terminus. This interaction promotes dynamic instability of interphase microtubules and is associated with mitotic spindle defects that can also arise from altered microtubule stability. These RHAMM-mediated effects require MEK1/ERK1, 2 activity. Since RHAMM binds directly to both α/β-tubulin and ERK1, and complexes with MEK1/ERK2, we propose that intracellular carboxyl terminal fragments of RHAMM perform scaffolding terminal functions linking active MEK1/ERK1, 2 to their microtubule substrates.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Cancer Research Society (Montreal, Canada) to ET and by the Canadian Breast Cancer Society (partial salary to ET). The study was also partially supported by NIH Grant NCI 5R0119092 to JBM and ET. KVNE is supported by a Translational Breast Cancer Studentship from the London Regional Cancer Program.

REFERENCES

1. van der Vaart, B., Akhmanova, A., and Straube, A. (2009) Biochem Soc Trans 37, 1007-1013
2. Alberti, C. (2009) Eur Rev Med Pharmacol Sci 13, 13-21
3. Arce, C. A., Casale, C. H., and Barra, H. S. (2008) FEBS J 275, 4664-4674
4. Holmfeldt, P., Sellin, M. E., and Gullberg, M. (2009) Cell Mol Life Sci 66, 3263-3276
5. Hammond, J. W., Huang, C. F., Kacch, S., Jacobson, C., Banker, G., and Verhey, K. J. (2009) Mol Biol Cell
6. Verhey, K. J., and Gaertig, J. (2007) Cell Cycle 6, 2152-2160
7. Kotwaliwale, C., and Biggins, S. (2006) Cell 127, 1105-1108
8. Crasta, K., Lim, H. H., Zhang, T., Nirantar, S., and Surana, U. (2008) Cell Cycle 7, 2960-2966
9. Tillement, V., Remy, M. H., Raynaud-Messina, B., Mazzolini, L., Haren, L., and Merdes, A. (2009) Mol Biol Cell
10. Mapiato, H., DeLuca, J., Salmon, E. D., and Earnshaw, W. C. (2004) J Cell Sci 117, 5461-5477
11. Bedard, P. L., Di Leo, A., and Piccart-Gebhart, M. J. (2010) Nat Rev Clin Oncol 7, 22-36
12. Perez, E. A. (2009) Mol Cancer Ther 8, 2086-2095
13. Schwartz, J. (2009) Am J Health Syst Pharm 66, S3-8
14. Greiner, J., Bullinger, L., Guinn, B. A., Dohner, H., and Schmitt, M. (2008) Clin Cancer Res 14, 7161-7166
15. Maxwell, C. A., McCarthy, J., and Turley, E. (2008) J Cell Sci 121, 925-932
16. Giannopoulos, K., and Schmitt, M. (2006) Leuk Lymphoma 47, 2028-2036
17. Wang, C., Thor, A. D., Moore, D. H., 2nd, Zhao, Y., Kerschmann, R., Stern, R., Watson, P. H., and Turley, E. A. (1998) Clin Cancer Res 4, 567-576
18. Pujana, M. A., Han, J. D., Starita, L. M., Stevens, K. N., Tewari, M., Ahn, J. S., Rennert, G., Moreno, V., Kirchhoff, T., Gold, B., Assmann, V., Elshamy, W. M., Rual, J. F., Levine, D., Rozeck, L. S., Gelman, R. S., Gunsalus, K. C., Greenberg, R. A., Sobhian, B., Bertin, N., Venkatesan, K., Ayivi-Guedehoussou, N., Sole, X., Hernandez, P., Lazaro, C., Nathanson, K. L., Weber, B. L., Cusick, M. E., Hill, D. E., Ofit, K., Livingston, D. M., Gruber, S. B., Parvin, J. D., and Vidal, M. (2007) Nat Genet 39, 1338-1349
19. Kalmyrzaev, B., Pharoah, P. D., Easton, D. F., Ponder, B. A., and Dunning, A. M. (2008) *Cancer Epidemiol Biomarkers Prev* **17**, 3618-3620
20. Schmitt, M., Schmitt, A., Rojewski, M. T., Chen, J., Giannopoulos, K., Fei, F., Yu, Y., Gotz, M., Heyduk, M., Ritter, G., Speiser, D. E., Gnjatic, S., Guillaume, P., Ringhofer, M., Schlenk, R. F., Liebsch, P., Bunjes, D., Shiku, H., Dohner, H., and Greiner, J. (2008) *Blood* **111**, 1357-1365
21. Slevin, M., Krupinski, J., Gaffney, J., Matou, S., West, D., Delisser, H., Savani, R. C., and Kumar, S. (2007) *Matrix Biol* **26**, 58-68
22. Tolg, C., Hamilton, S. R., Nakrieko, K. A., Kooshesh, F., Walton, P., McCarthy, J. B., Bissell, M. J., and Turley, E. A. (2006) *J Cell Biol* **175**, 1017-1028
23. Assmann, V., Jenkinson, D., Marshall, J. F., and Hart, I. R. (1999) *J Cell Sci* **112 ( Pt 22)**, 3943-3954
24. Maxwell, C. A., Keats, J. J., Crainie, M., Sun, X., Yen, T., Shibuya, E., Hendzel, M., Chan, G., and Pilarski, L. M. (2003) *Mol Biol Cell* **14**, 2262-2276
25. Lynn, B. D., Li, X., Cattini, P. A., Turley, E. A., and Nagy, J. I. (2001) *J Comp Neurol* **439**, 315-330
26. Radisky, D. C., Stallings-Mann, M., Hirai, Y., and Bissell, M. J. (2009) *Nat Rev Mol Cell Biol* **10**, 228-234
27. Hamilton, S. R., Fard, S. F., Paiwand, F. F., Tolg, C., Veiseh, M., Wang, C., McCarthy, J. B., Bissell, M. J., Koropatnick, J., and Turley, E. A. (2007) *J Biol Chem* **282**, 16667-16680
28. Joukov, V., Groen, A. C., Prokhorova, T., Gerson, R., White, E., Rodriguez, A., Walter, J. C., and Livingston, D. M. (2006) *Cell* **127**, 539-552
29. Groen, A. C., Cameron, L. A., Coughlin, M., Miyamoto, D. T., Mitchison, T. J., and Ohi, R. (2004) *Curr Biol* **14**, 1801-1811
30. Maxwell, C. A., Keats, J. J., Belch, A. R., Pilarski, L. M., and Reiman, T. (2005) *Cancer Res* **65**, 850-860
31. Fang, Z., Takizawa, N., Wilson, K. A., Smith, T. C., Delprato, A., Davidson, M. W., Lambright, D. G., and Luna, E. J. (2010) *Traffic*
32. Hall, C. L., Yang, B., Yang, X., Zhang, S., Turley, M., Samuel, S., Lange, L. A., Wang, C., Cupen, G. D., Savani, R. C., Greenberg, A. H., and Turley, E. A. (1995) *Cell* **82**, 19-26
33. Zhang, S., Chang, M. C., Zylka, D., Turley, S., Harrison, R., and Turley, E. A. (1998) *J Biol Chem* **273**, 11342-11348
34. Harrison, R. E., and Turley, E. A. (2001) *Neoplasia* **3**, 385-394
35. Mohan, R., and Panda, D. (2008) *Cancer Res* **68**, 6181-6189
36. Karsenti, E., and Vernos, I. (2001) *Science* **294**, 543-547
37. Waleczak, C. E., Cai, S., and Khodjakov, A. (2010) *Nat Rev Mol Cell Biol* **11**, 91-102
38. von Dassow, G. (2009) *Trends Cell Biol* **19**, 165-173
39. Zhou, F. Q., and Snider, W. D. (2006) *Philos Trans R Soc Lond B Biol Sci* **361**, 1575-1592
40. Matkovic, K., Lukinovic-Skudar, V., Banfic, H., and Visnjic, D. (2009) *Int J Hematol* **89**, 159-166
41. Shinohara, M., Mikhailov, A. V., Aguirre-Ghiso, J. A., and Rieder, C. L. (2006) *Mol Biol Cell* **17**, 5227-5240
42. Wang, R., He, G., Nelmann-Gonzalez, M., Ashorn, C. L., Gallick, G. E., Stukenberg, P. T., Kirschner, M. W., and Kuang, J. (2007) *Cell* **128**, 1119-1132
43. Roberts, E. C., Hammond, K., Traish, A. M., Resing, K. A., and Ahn, N. G. (2006) *Proteomics* **6**, 4541-4553
44. Ramos, J. W. (2008) *Int J Biochem Cell Biol* **40**, 2707-2719
45. Bardwell, L. (2006) *Biochem Soc Trans* **34**, 837-841
46. Evanko, S. P., Parks, W. T., and Wight, T. N. (2004) *J Histochem Cytochem* **52**, 1525-1535
47. Ziebell, M. R., and Prestwich, G. D. (2004) *J Comput Aided Mol Des* **18**, 597-614
48. Yang, B., Yang, B. L., Savani, R. C., and Turley, E. A. (1994) *EMBO J* **13**, 286-296
49. Richter-Landsberg, C. (2008) *J Mol Neurosci* **35**, 55-63
FIGURE LEGENDS

Fig. 1. RHAMM is expressed by primary and immortalized MEF
A. Diagram of RHAMM transcripts expressed by 10T1/2 cells. RHAMM^FL contains two microtubule-binding sequences; one located in exon 4 and the other in exon 16 (arrows). The latter contains a leucine zipper responsible for the association of RHAMM with mitotic spindles (29). An N-terminal truncation of RHAMM (RHAMM^∆163) contains only the mitotic spindle binding sequence, yet retains the ability to bind to interphase microtubules. B. Left panel: RHAMM protein expression of 10T1/2 cells and 10T1/2 cells transfected with RHAMM^∆163. An anti-RHAMM polyclonal antibody recognizing sequences in exon 8 was used to detect RHAMM by western analysis. Arrows indicate RHAMM^FL and RHAMM^∆163. Right panel: RHAMM mRNA expression of wildtype, RHAMM^∆163-transfected RHAMM^-/-, RHAMM^FL-transfected RHAMM^-/- and RHAMM^-/- MEF. Quantitative real-time PCR using primers common to both RHAMM^∆163 and RHAMM^FL was employed to quantify mRNA expression. RHAMM^-/- MEF do not express RHAMM mRNA. Transfection of RHAMM^-/- cells with RHAMM^∆163 or RHAMM^FL restores RHAMM mRNA expression. C. RHAMM protein expression of RHAMM^-/-, RHAMM^FL and RHAMM^∆163 MEF was detected by immunofluorescence using RHAMM exon 8 specific polyclonal antibodies. As expected, RHAMM^-/- MEF do not express RHAMM protein whereas RHAMM^∆163 and RHAMM^FL rescued RHAMM^-/- MEF do. Images were taken with a Zeiss confocal microscope at 40X magnification. Laser settings were kept constant.

Fig. 2. RHAMM decorates microtubules and its expression affects microtubule structures.
A. RHAMM decorates interphase microtubules and mitotic spindles. Immunofluorescence staining with anti-RHAMM exon 8 specific polyclonal antibodies was used to analyze RHAMM expression and distribution in 10T1/2 cells. Tubulin was detected by staining with an anti α-tubulin antibody. RHAMM co-distributes with tubulin in interphase microtubules of 10T1/2 fibroblasts. RHAMM also co-localizes with tubulin in mitotic spindle microtubules (lower three panels) of 10T1/2 cells and is particularly concentrated at the spindle apex. Images were taken on a Zeiss confocal microscope. B. Immunofluorescence staining of RHAMM^-/-, RHAMM^FL-rescued and wildtype MEF shows that microtubules stain more brightly in RHAMM^-/- MEF than in either RHAMM-rescued or wildtype MEF (images were taken at the same laser setting). Small insert in image of RHAMM-rescued MEF shows tubulin staining taken at higher laser setting. Nuclei of RHAMM^-/- MEF are often larger and cells more highly spread than RHAMM^-/- MEF. RHAMM loss also results in aberrant mitotic spindle
formation and defective chromosome alignment/segregation compared to RHAMM-rescued or wildtype MEFs. Arrows indicate poles of mitotic spindles. Images were taken with an Olympus Confocal microscope at 40X magnification.

**Fig. 3.** Myc-RHAMM<sup>373</sup> binds to interphase microtubules and loss of RHAMM expression increases interphase microtubule resistance to nocodazole.

A. 10T1/2 cells were transfected with a RHAMM expression construct lacking the first N-terminal 373 amino acids (RHAMM<sup>373</sup>). To distinguish between expression of the transfected construct from endogenous RHAMM, RHAMM<sup>373</sup> included an N-terminal Myc epitope tag. RHAMM<sup>373</sup> transfected 10T1/2 cells were stained with Myc tag (green) and anti α-tubulin (red) antibodies. RHAMM<sup>373</sup> and α-tubulin co-localize on interphase microtubules (yellow). Images were taken with a Zeiss confocal at 40X magnification. B. Nocodazole-resistance of interphase microtubules is increased in RHAMM<sup>−/−</sup> MEF. Primary Wildtype and RHAMM<sup>−/−</sup> MEF were exposed to 3ng/ml nocodazole or buffer containing DMSO alone. Soluble and insoluble protein fractions were isolated and separated on a 10% SDS PAGE gel. The amount of α-tubulin in both fractions was quantified by western analysis using an α-tubulin antibody. Values represent the mean and S.E.M. of n=6 samples Statistical significance was assessed by a Student’s “t” test and significant results (p<0.01) are marked by asterisks.

**Fig. 4.** Stability of interphase microtubules is modified by RHAMM expression and by activated MEK1.

A. Acetylated and total tubulin levels in 50 % confluent RHAMM<sup>−/−</sup>, RHAMM<sup>Δ163</sup>-rescued and RHAMM<sup>MEK1</sup>-rescued MEF were detected by western analysis using anti α-acetylated tubulin and α-tubulin antibodies. Graph shows the ratio of acetylated tubulin to total tubulin. B. Comparison of microtubule stability between primary RHAMM<sup>−/−</sup> and wildtype MEF. 50 % confluent wildtype and RHAMM<sup>−/−</sup> MEF were serum-starved overnight in defined medium. 30 min after stimulation with 10% FBS, total protein was isolated and levels of acetylated and total α-tubulin levels were determined by western analysis. Values in both A. and B. are the mean and S.E.M. of n=3 replicates. Statistical significance was assessed by a Student’s “t” test and significant results (p<0.01) are marked by asterisks.

**Fig. 5.** RHAMM loss results in aberrant mitotic spindles.

Genetic loss of RHAMM results in a high percentage of multi-pole mitotic spindles and aberrant chromosome alignment. Arrows denote spindle poles and arrowheads denote chromosomes. Cells were treated with nocodazole overnight to increase the number of cells undergoing mitosis. 2 hrs after nocodazole removal, cells were fixed and stained with α-tubulin antibody and DAPI. Expression of RHAMM<sup>FL</sup> or mutant active MEK1 significantly reduces the number of multi-pole spindles. RHAMM<sup>FL</sup> restores chromosome alignment on mitotic spindles to a greater degree than mutant active MEK1. Images were taken with an Olympus confocal microscope at 40x magnification and 1.6 x Zoom. Graph depicts percentage of cells with two, three or more than three spindle poles. Values in the graph are the Mean and S.E.M. of n=150 cells. Statistical significance was assessed by a Student’s “t” test. Significant results (p<0.01) are marked by asterisks.

**Fig. 6.** RHAMM loss results in increased multinucleated cells

RHAMM<sup>−/−</sup> MEF exhibit a higher percentage of multinucleated cells than RHAMM<sup>Δ163</sup>, RHAMM<sup>FL</sup> or activated MEK1-rescued MEF. Expression of either RHAMM<sup>FL</sup> or RHAMM<sup>Δ163</sup> rescued to a similar extent as mutant active MEK1. Cells were cultured overnight, 50 % confluent cultures were fixed and nuclei were stained with DAPI. Cells with single and multiple nuclei were counted per microscopic field. Graph depicts percentage of cells with multiple nuclei. Values are the mean and S.E.M. of n=160 cells pooled from 2 separate experiments. Statistical significance was assessed by a Student’s “t” test and significant results (p<0.01) are marked by asterisks.
Fig. 7. RHAMM binds directly to heterodimeric α-, β-tubulin.
A. Pull-down assays were performed using Sepharose-GST-RHAMM Δ163 and purified α-, β-tubulin heterodimers. Tubulin that bound to RHAMM was identified with western blots using an anti-pan tubulin antibody. B. Binding of GST-RHAMM Δ163 to tubulin heterodimers is blocked by a synthetic peptide mimicking the leucine zipper (mouse, L<sup>728</sup>-L<sup>742</sup>), which is required for an association of RHAMM with the mitotic spindle. Pull-down assays using Sepharose-GST-RHAMM Δ163 and tubulin heterodimers were performed in the presence of varying amounts of synthetic peptide. Values in the graph are the Mean and S.E.M. n=3 separate experiments. Asterisks denote statistical significance (Student’s “t” test, p<0.01).

Fig. 8. RHAMM binds directly to recombinant ERK1.
A. Diagram of mouse RHAMM carboxyl terminal sequence containing the leucine zipper (bold letters) necessary for RHAMM Δ163/tubulin interactions and an upstream, highly conserved sequence resembling a D-site for docking ERK (dotted red line). Residues that were mutated in mutant RHAMM Δ163 are indicated in red and the sequences of the peptide used for competing ERK1/RHAMM interactions and control peptides are indicated. B. Western blots of pull-down assays using Sepharose-GST-RHAMM Δ163 beads and recombinant ERK1 demonstrate direct binding of recombinant RHAMM Δ163 to recombinant ERK1 protein. Increasing concentrations of excess, soluble recombinant RHAMM Δ163 was included in pull-down assays to block RHAMM Δ163/ERK1 binding by competition and to demonstrate specificity of binding. Similar pull-down assays were used to assess interactions of RHAMM Δ163 with MEK1 and ERK2 but binding was not detected. C. The binding region required for RHAMM Δ163/ERK1 interactions is identified by pull-down assays using mutant recombinant RHAMM Δ163 (721K/E, 727K/E) and competition with a peptide containing the wildtype sequence. Mutation of two lysine residues reduces binding by approximately 60% while a peptide representing the entire putative docking region reduces binding by approximately 90%.

Fig. 9. Mutation of RHAMM Δ163 D site reduces RHAMM/ERK1 interactions and association of p-ERK1, 2 with tubulin.
A. Native and mutant (loss of ERK docking) RHAMM Δ163 were expressed in 10T1/2 fibroblasts and the association of RHAMM with ERK1 and MEK1 were assessed by immunoprecipitation using anti RHAMM antibodies. While native RHAMM Δ163 immunoprecipitates with ERK1, ERK2 (data not shown) and MEK1, mutant RHAMM Δ163 does not. Immunoprecipitations using anti ERK1 and non-immune IgG were used as positive and negative controls, respectively. B. H-RAS transformed cells express high levels of endogenous RHAMM Δ163 and display abundant tubulin-associated p-ERK1, 2, as detected by immunoprecipitation assays using anti α-tubulin antibodies. Expression of mutant RHAMM Δ163, which acts as a dominant negative suppressor of endogenous RHAMM Δ163 function, ablates the association of p-ERK1, 2 with tubulin. Densitometry values represent the Mean and S.E.M. of n=4 samples. Asterisks denote statistical significance (Student’s “t” test, p<0.01). C. Diagram of proposed interactions amongst RHAMM, MEK1, ERK1, 2 and tubulin. RHAMM is predicted to scaffold MEK1/ERK1, 2 to tubulin in mitotic spindle and interphase microtubules. RHAMM binds directly to ERK1 through its D site and to tubulin through its carboxyl terminal leucine zipper but indirectly complexes with MEK1 and ERK2 via as yet unidentified proteins or as a result of a direct association of ERK1 with both MEK1 and ERK2.

Fig. 10. Interactions of RHAMM Δ163 with ERK1 are required for microtubule instability in H-RAS transformed cells.
A. Immunofluorescence using acetylated tubulin specific antibodies in parental 10T1/2 cells and in 10T1/2 cells transfected with either RHAMM Δ163 or H-RAS shows that expression of these two proteins reduces microtubule stability. Expression of mutant RHAMM Δ163 in H-RAS transformed cells blocks the effect of RAS on microtubules. Images were taken with a Zeiss confocal microscope at 40X magnification. B. Acetylated α-tubulin is quantified by densitometry analysis of western blots. As predicted from immunofluorescence images, expression of either H-RAS or oncogenic RHAMM Δ163...
significantly reduces acetylated $\alpha$-tubulin levels relative to parental 10T1/2 cells, while conversely, expression of mutant RHAMM$^{\Delta163}$ restores acetylated $\alpha$-tubulin to parental 10T1/2 levels. Values represent the Mean and S.E.M. of n=3 assays. Statistical significance was assessed using a Student’s “t” test and statistically significant results (p<0.01) are marked by an asterisk.
Figure 1.

A.

Tubulin binding sequences

ATG

1  2  3  4  5  6  7  8  9  10  11  12  13  14  15  16  17  18

aa1  RHAMM\textsuperscript{FL}  aa\textsuperscript{794}

aa\textsuperscript{163}  RHAMM\textsuperscript{\textDelta 163}  aa\textsuperscript{794}

B.

10T1/2-RHAMM\textsuperscript{\textDelta 163}  10T1/2 (parental)

RHAMM\textsuperscript{FL}  $\approx$ 95 kDa

RHAMM\textsuperscript{\textDelta 163}  $\approx$ 70 kDa

C.

RHAMM\textsuperscript{\textDelta 163}-rescued  RHAMM\textsuperscript{FL}-rescued

Scale bar: 30 μm
Figure 3.

A. Myc epitope tag

Leucine zipper (LKDENSEMKLSEVK)^742

\[ \text{aa}^{373} \text{RHAMM}^{1373} \text{aa}^{794} \]

\[ \text{\alpha-tubulin} \quad \text{myc-RHAMM}^{1373} \quad \text{merge} \]

30 \mu m

B. Graph showing insoluble/total tubulin levels.

- Wildtype
- RHAMM^+^
Figure 5.
Figure 6.
Figure 7.

A. 

IB: tubulin antibody

B. 

IB: tubulin antibody

| Concentration of peptide (LKDENSQKSEVSKL) | % binding |
|------------------------------------------|----------|
| buffer                                   | 100%     |
| 10 µg                                    | 80%      |
| 100 µg                                   | 40%      |

* Indicates significant difference.
Figure 8.

A. 

PUTATIVE DOMAIN

715HQNLKQKIQHVVLKDENSLLKSEVSLRSQ745

715HQNLKQEIKHVVELKDLNSLKLSEVSKLRSQ745

715HQNLKQKIQHVVLKDENSLLKSEVSKLRSQ745

195KLQATQKDLTESKGIQVEQKL217

218VSIIEKEKIDEK228

72kDa RHMM4163

aa163

aa794

MUTANT RHMM4163

BLOCKING PEPTIDE

CONTROL PEPTIDE 1

CONTROL PEPTIDE 2

B. 

RHMM4163-bead pulldowns

+ soluble RHMM4163

ERK1 alone

control beads + ERK1

ERK1

ERK1 + 0.5 mg

ERK1 + 2.5 mg

ERK1 + 10 mg

IB: ERK1

C. 

IB: ERK1

IB: ERK2

IB: MEK1
Figure 9.

A. **IP: α-RHAMM**

![Western Blot Images](image)

- IP: α-ERK1
- IP: IgG
- Empty Vector
- RHAMMΔ163
- mut RHAMMΔ163

**IB: ERK1, ERK2**

- O.D. (arbitrary units)

B. **IP: tubulin**

![Western Blot Images](image)

- IP: tubulin
- IB: ERK1,2
- H-RAS
- H-RAS/mut RHAMMΔ163

**cell lysate**

- % tubulin-associated p-ERK (arbitrary units)

C. **Mitotic spindles** **Interphase microtubules**

![Diagram](image)
RHAMM promotes interphase microtubule instability and mitotic spindle integrity through MEK1/ERK1, 2 activity
Cornelia Tolg, Sara J. Hamilton, Lyndsey Morningsar, Jing Zhang, Kenneth V. Esguerra, Patrick G. Telmer, Len G. Luyt, Rene Harrison, James B. McCarthy and Eva A. Turley

J. Biol. Chem. published online June 17, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.121491

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/06/16/M110.121491.DC1