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Jose T. Thaiparambil, Emory University
Carrie M. Eggers, Emory University
Adam I Marcus, Emory University

Journal Title: Molecular and Cellular Biology
Volume: Volume 32, Number 16
Publisher: American Society for Microbiology | 2012-08, Pages 3203-3217
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/MCB.00418-12
Permanent URL: http://pid.emory.edu/ark:/25593/f2236

Final published version: http://mcb.asm.org/content/32/16/3203

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Accessed May 28, 2019 7:41 AM EDT
AMPK Regulates Mitotic Spindle Orientation through Phosphorylation of Myosin Regulatory Light Chain

Jose T. Thaiparambil, Carrie M. Eggers, and Adam I. Marcus

Department of Hematology and Medical Oncology, Winship Cancer Institute of Emory University, Atlanta, Georgia, USA

The proper orientation of the mitotic spindle is essential for mitosis; however, how these events unfold at the molecular level is not well understood. AMP-activated protein kinase (AMPK) regulates energy homeostasis in eukaryotes, and AMPK-null Drosophila mutants have spindle defects. We show that threonine172 phosphorylated AMPK localizes to the mitotic spindle poles and increases when cells enter mitosis. AMPK depletion causes a mitotic delay with misoriented spindles relative to the normal division plane and a reduced number and length of astral microtubules. AMPK-depleted cells contain mitotic actin bundles, which prevent astral microtubule-actin cortex attachments. Since myosin regulatory light chain (MRLC) is an AMPK downstream target and mediates actin function, we investigated whether AMPK signals through MRLC to control spindle orientation. Mitotic levels of serine19 phosphorylated MRLC (pMRLCser19) and spindle pole-associated pMRLCser19 are abolished when AMPK function is compromised, indicating that AMPK is essential for pMRLCser19 spindle pole activity. Phosphorylation of AMPK and MRLC in the mitotic spindle is dependent upon calcium/calmodulin-dependent protein kinase kinase (CamKK) activity in LKB1-deficient cells, suggesting that CamKK regulates this pathway when LKB1 function is compromised. Together, these data indicate that AMPK mediates spindle pole-associated pMRLCser19 to control spindle orientation via regulation of actin cortex-astral microtubule attachments.

Precontrol of the cell division plane is achieved through the proper assembly, positioning, and orientation of the microtubule-based spindle. In nonpolarized adherent cells, the spindle orients parallel to the substratum (reviewed in reference 14) and positions itself centrally to ensure an accurate distribution of genetic information and an equal composition of daughter cells (22, 25, 44). When spindles are misoriented, daughter cell placement in tissue is abnormal, potentially leading to tissue disorganization and cancer metastasis (24). Though some of the major components of the spindle (e.g., microtubules and motor proteins) have been intensely studied in spindle orientation, the molecular signaling pathways regulating these events have not been well established.

Astral microtubules emanating from the spindle poles attach to the actin cortex and are essential for proper spindle orientation (6, 8); however, recently it has appeared that the establishment and maintenance of spindle orientation and positioning are more complex than previously believed and involve multiple pathways. The PtdIns-(3,4,5)P3 direct dynein/dynactin forces to orient the spindle parallel to the substratum, a process overseen by the small Rho GTPase cdc42 (35). Transmembrane integrins are essential for spindle orientation by maintaining substrate adhesion contacts during mitosis (36, 37). Actin itself also serves multiple functions that go beyond its role in the cortex, whereby F-actin forms dynamic cables encaging the spindle to function in spindle anchoring and length (48). Furthermore, actin-binding proteins orient and assemble the microtubule spindle. For instance, myosin 10, which localizes to mitotic spindle poles, is required for proper spindle anchoring and length (48), and moesin is required for spindle symmetry and positioning (15). Thus, spindle orientation and positioning are overseen by a complex interplay of signaling proteins, microtubules and associated proteins, and actin and associated proteins.

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase that consists of a catalytic α subunit and regulatory β and γ subunits (33, 47). AMPK regulates energy homeostasis in all eukaryotic organisms and is active when ADP levels are high and ATP levels are low (9). AMPK activity is regulated by phosphorylation at AMPKthr172 (pAMPKthr172) in the α subunit by either the LKB1 kinase or calcium/calmodulin kinase kinase (CamKK) (12, 30). Studies using AMPK-null Drosophila mutants suggest that AMPK functions in mitosis, since mutants have spindle defects that can be partly rescued by a phosphomimetic myosin regulatory light chain (MRLC) (16). Furthermore, pAMPKthr172 localizes to the spindle poles of mammalian cells and human tissues (39, 42). Together, these observations suggest a role for AMPK in mitosis; however, a mitotic function for AMPK has not been defined.

To address this, we have now demonstrated that AMPK depletion results in a mitotic delay, misoriented spindles, and actin bundles surrounding the spindle. These misoriented spindles are likely due to improper astral microtubule-cell cortex interactions caused by actin bundles surrounding the spindle. We propose that actin bundling and consequently misoriented spindles are caused by the inactivity of the previously identified AMPK downstream target, myosin regulatory light chain. MRLC regulates actin turnover, and we show that pMRLCser19 localizes to the mitotic spindle poles and, importantly, that its phosphorylation is hampered when AMPK function is compromised. Furthermore, when MRLC itself is depleted, aberrant, misoriented spindles and actin...
bundling were also observed. Phosphorylation of both AMPK and MRLC in the mitotic spindle is dependent upon calcium/calmodulin-dependent protein kinase kinase activity when LKB1 function is compromised, suggesting that CamKK regulates this pathway in LKB1-deficient cells. However, when LKB1 is present at normal levels, CamKK pharmacological inhibition does not result in spindle misorientation, suggesting that LKB1 and CamKK may have redundant roles in regulating AMPK during mitosis. We propose that AMPK regulates pMRLC at the spindle poles to control spindle orientation via regulation of actin function.

MATERIALS AND METHODS

Tissue culture. H1299 and HeLa cells (purchased from ATCC) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. PTK cells were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% FBS. All cell lines were maintained at 37°C in 5% CO2. H1299 pLKO.1 and H1299 LKB1 short hairpin RNA (shRNA) cells were generated and cultured as previously described (7).

Immunofluorescence and antibodies. Immunofluorescence was performed as previously described (28, 49). Briefly, cells were allowed to adhere overnight on no. 1.5 coverslips placed in tissue culture plates. Cells were fixed in PHEMO buffer, consisting of 3.7% formaldehyde, 0.05% glutaraldehyde, 60 mM PIPES [piperazine-N,N’-bis(2-ethanesulfonic acid)], 25 mM HEPES, 10 mM EGTA, and 4 mM MgSO4, for 10 min. Cells were then washed in phosphate-buffered saline (PBS) and blocked with 10% bovine serum albumin for 15 min. After being washed with PBS, the coverslips were incubated with the appropriate primary antibody overnight at 4°C. Cells were washed again with PBS, and the appropriate Alexa Fluor-conjugated secondary antibody was added at 1:500 for 1 h at room temperature. Antibody incubations were then sequentially repeated for each additional primary-secondary-antibody pair.

Primary antibodies were used as follows: rabbit anti-phospho-AMPKThr172α and total AMPKα, used at 1:500 and 1:1,000, respectively (Cell Signaling Technology); a rabbit anti-phospho-MRLCSer19 and total MRLC, used at 1:250 and 1:1,000, respectively (Cell Signaling Technology). Actin staining was performed with Alexa Fluor 555- or Alexa Fluor 647-conjugated phalloidin (Invitrogen). A mouse anti-CamKK was used at 1:1,000 for Western blotting (BD Biosciences). Secondary antibodies were Alexa Fluor 488, 555, or 647 (Invitrogen) and were used at a dilution of 1:500 and incubated for 1 h at room temperature. Nuclear staining was performed by incubating cells with 0.4 μmol/liter 4’,6-diamidino-2-phenylindole (DAPI) prior to mounting slides with Prolong Gold (Life Technologies).

Cell synchronization. To synchronize HeLa cells in mitosis, a double thymidine block was used. Cells were incubated in thymidine (2.5 mM) for 16 h at 37°C and released from thymidine for 9 h. This was repeated, and cells were harvested at various time points during release.

siRNA treatments. HeLa cells were transfected with 100 nM both AMPK α1 and α2 small interfering RNA (siRNA) or myosin regulatory light chain siRNA (Ambion) using Lipofectamine 2000 (Invitrogen). After 6 h of siRNA incubation, cells were washed with fresh medium and incubated for 48 h. For AMPK siRNA treatment in synchronized cells, cells were incubated in 2.5 mM thymidine overnight (16 h) and then allowed to recover in normal medium for 7 h. AMPK siRNA was then added and incubated for 4 h, followed by overnight incubation in normal medium and a second thymidine block. Cells were then harvested at different time points during the 8-h recovery.

Confocal imaging. Fixed-cell confocal images were acquired using a Zeiss LSM 510 META mounted on a Zeiss Axioplan 200m upright microscope. Images were acquired either using a Zeiss 63 X or 100 X Plan-Apo objective (numerical aperture [N.A.], 1.4) with a zoom of 2. Confocal z stacks were acquired with sections ranging from 0.5 μm to 1.1 μm. In cases where image analysis was performed, image acquisition settings were kept constant. For live-cell confocal imaging, a Perkin Elmer Ultraview ERS spinning disc system was used, which was enclosed at 37°C with 5% CO2. This system is mounted on a Zeiss Axiovert inverted microscope.
with an ASI motorized and programmable stage. Images were acquired using a Zeiss 20× Plan-Apo objective (N.A., 0.75) or Zeiss 40× Plan-Apo objective (N.A., 1.3) using Volocity software. Bright-field imaging of HeLa cells was performed for assessing mitotic duration. For confocal fluorescence imaging, HeLa cells were transfected using Lipofectamine with green fluorescent protein (GFP)-utrophin (a gift provided by Bill Bement, University of Wisconsin) and mCherry tubulin. Cells were imaged at least 24 h posttransfection. In most cases, images were obtained every 5 min across multiple z sections. Basic image processing (e.g., contrast expansion) was performed using Adobe Photoshop CS3.

Spindle orientation, centroid measurements, and image analysis. Spindle angle measurements were adapted from the method given in reference 36, i.e., by measuring the three-dimensional (3-D) distance (across the x, y, and z planes) between the two spindle poles and the 2-D distance (across the x and y planes) of the spindle (see Fig. 3C). The spindle angle was then calculated using the \( \cos^{-1} \) (arccosine). Image analysis was done using Zeiss Axiovision software from confocal z stacks taken on the Zeiss LSM510 META, using the 3-D length tool. Spindle pole centroid measurements were performed using Metamorph software. Images from confocal z stacks of tubulin were first thresholded and then binarized. The binarized image was then outlined by the software, and the centroid was calculated. A line was then drawn between the two centroids of the spindle poles (see Fig. 3B). To measure pMRLCser19 and pAMPKthr172 spindle pole intensity, tubulin staining was first used to demarcate the spindle pole region and a region of interest was drawn around the spindle pole. Images were then equally thresholded, and the mean intensity of the

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**FIG 2** AMPK depletion leads to mitotic delay and arrest. (A) Western blot showing siRNA depletion of AMPK α1 and/or α2 isoforms. GAPDH is shown as a loading control. (B) Immunofluorescence showing successful depletion of pAMPKthr172 at the spindle poles after AMPK α1 and α2 siRNA. Maximum-intensity projections are shown. Scale bar = 5 μm. (C) Representative live-cell bright-field imaging of mitosis in control siRNA and AMPK siRNA. Time is given in minutes postrounding. (D) Bar graph showing the percentages of mitotic cells that show normal mitosis, mitotic delay (>60 min and then cell division), mitotic arrest, or aberrant mitotic exit (i.e., without cytokinesis). (E) Bar graph showing the mean time spent in mitosis for control siRNA- and AMPK siRNA-treated cells (**, P < 0.01; error bars show standard deviations; n = 50 cells for each group).
pMRLC<sub>ser19</sub><sup>19</sup> signal was measured at each spindle pole separately in a single cell. Line profiles for spatial and intensity measurements of actin bundling (see Fig. 5) were performed using Zeiss Axiovision software.

**Pharmacological treatments.** For AMPK inhibition, cells were treated with Compound C at 10 μM (EMD Chemicals) in live cells transfected with GFP-tubulin. Compound C was administered 15 min prior to imaging and was present throughout the duration of the experiment. For jasplakinolide treatment, cells were treated at 500 nM for 1 h and cells were fixed. The number of rotated spindles in jasplakinolide treatment was determined by assessing the relative locations of the spindle poles.
along the z axis. Cells were treated with the CamKK inhibitor STO-609 (Santa Cruz Biotech) for 6 h at 10 µg/ml.

RESULTS

pAMPK\textsuperscript{th\textsubscript{172}} is localized to the spindle poles, and levels increase during mitosis. Immunofluorescence of pAMPK\textsuperscript{th\textsubscript{172}} in three cell lines showed mitotic spindle pole localization (Fig. 1A); specifically, the pAMPK\textsuperscript{th\textsubscript{172}} signal was observed at the spindle poles from prophase to metaphase and became less intense in anaphase (Fig. 1B). Spindle pole-associated pAMPK\textsuperscript{th\textsubscript{172}} was not observed during telophase and cytokinesis, but a weak signal was observed at the cleavage furrow (Fig. 1B). To determine if pAMPK\textsuperscript{th\textsubscript{172}} levels increased during mitosis, HeLa cells were synchronized to enter mitosis and pAMPK\textsuperscript{th\textsubscript{172}} levels were assessed at multiple time points. Basal levels of pAMPK\textsuperscript{th\textsubscript{172}} are relatively low when cells are not in mitosis but rapidly increase when cells prepare for and enter mitosis (as assessed by cyclin B1 levels) and then slowly taper off at the completion of mitosis (Fig. 1C). Total AMPK remained unchanged throughout the time course. These results are consistent with a report showing similar pAMPK\textsuperscript{th\textsubscript{172}} localization at the spindle poles (39, 42) and suggest a role for AMPK in mitosis.

AMPK depletion induces a mitotic delay and arrest. To determine the function of AMPK in mitosis, AMPK siRNA depletion was performed using two siRNA molecules that were separately targeted to the AMPK\textsubscript{1} or AMPK\textsubscript{2} isoform (Fig. 2A). AMPK depletion was confirmed in two cell lines (H1299 and HeLa) using the siRNAs alone or in combination. We continued with the AMPK\textsubscript{1} and AMPK\textsubscript{2} siRNA combination to prevent any potential functional compensation between AMPK isoforms. In parallel, immunofluorescence staining of pAMPK\textsuperscript{th\textsubscript{172}} also showed a reduction of pAMPK\textsuperscript{th\textsubscript{172}} at the spindle poles in mitotic cells, further confirming successful AMPK depletion (Fig. 2B).

To determine the functional impact of AMPK depletion on mitosis, live-cell imaging was performed in control siRNA and AMPK-depleted cells (Fig. 2C). In control HeLa cells, most cells underwent cytokinesis within 30 min of mitotic rounding; however, in AMPK-depleted cells a significant fraction of cells had a mitotic delay lasting about 80 min, while other AMPK-depleted cells never divided and underwent mitotic arrest (Fig. 2C). A bar graph summarizing these results shows that only 40% of cells undergo normal mitosis in AMPK-depleted cells, compared to the control cells, where over 90% of cells have a normal mitosis (Fig. 2D). Approximately 37% of AMPK-depleted cells had a mitotic delay and 12% had a mitotic arrest (Fig. 2D). Furthermore, the average duration of mitosis (defined in this experiment as mitotic rounding to cytokinesis) is 48 min in AMPK siRNA cells but only 25 min in control cells (Fig. 2E). Based upon these data, AMPK knockdown induces mitotic delay in most cells and leads to a significant increase in mitotic duration.

AMPK depletion or inhibition causes misoriented spindles. Since pAMPK\textsuperscript{th\textsubscript{172}} is localized to the spindle poles and AMPK depletion induced a mitotic delay, mitotic spindle function and morphology in AMPK-depleted cells were investigated. Immunofluorescence analysis using confocal planar z sections of tubulin in control cells showed a bipolar spindle that was oriented parallel to the substratum, with spindle poles in similar z planes (Fig. 3A). In contrast, AMPK-depleted mitotic cells had bipolar spindles that appeared to be misoriented relative to the substratum, whereby spindle poles were in drastically different confocal z planes (Fig. 3A). Furthermore, in AMPK-depleted cells, spindle microtubules ap-
AMPK depletion induces actin bundling around the spindle. (A) Two examples of phalloidin (actin) staining in control and AMPK-depleted HeLa cells, where AMPK-depleted cells show actin bundling. (B) Two examples of phalloidin staining in control and AMPK-depleted mitotic H1299 cells, where AMPK-depleted cells show actin bundling. (C) Bar graphs showing the actin phenotype of AMPK-depleted cells. Spindle-associated actin was considered one group with three different categories: filamentous actin, bundled actin, or no actin. Filamentous actin was defined as actin in thin filaments around the spindle, and bundled actin was defined as thick bundles of actin. Values are means from three independent experiments (*, P < 0.05). (D) Line profile through a representative mitotic HeLa cell stained with phalloidin. The gray value intensity of phalloidin is on the y axis and pixel distance on the x axis. The top graph shows a line profile from...
peared poorly organized (Fig. 3A) and spindles were significantly shorter in pole-to-pole length but not width (data not shown).

To further investigate this phenotype, spindle pole orientation along the x-z axes was investigated (Fig. 3B). These data showed that the spindle poles in control cells were in similar z planes but in AMPK-depleted cells spindle poles were offset (3 examples are given for each [Fig. 3B]). To quantitate these differences, the angle of spindle rotation was determined in AMPK knockdown versus control cells (Fig. 3C) by adapting a previously established method (36), whereby perfectly aligned spindle poles would have 0° of rotation and a spindle oriented perpendicular to the substratum would have 90° of rotation. These results show that in control cells the mean angle of spindle pole orientation was 15.4°, compared to 33.5° in AMPK-depleted cells (Fig. 3D) (P < 0.001) confirming that AMPK-depleted cells have misoriented spindles relative to the substratum.

To further test this finding, live-cell mitotic imaging was performed using GFP-tubulin in control and AMPK-depleted cells. Live-cell confocal z sections show that the two spindle poles in control cells are found in similar z sections whereas in AMPK-depleted cells the spindle poles are found in drastically different z planes, indicating spindle misorientation (Fig. 3E). Control cells were then treated with compound C, a pharmacological AMPK inhibitor (50), to determine if AMPK inhibition with a small molecule also induces spindle orientation. Similarly, compound C treatment (10 μM) caused misoriented spindles, with spindle poles located in drastically different optical z sections (Fig. 3E). Therefore, based upon these results, AMPK depletion or pharmacological inhibition induced misoriented spindles relative to the substratum, indicating that AMPK is required for proper spindle orientation.

**Reduced number and length of astral microtubules in AMPK-depleted cells.** Astral microtubules are required for proper spindle orientation; therefore, the length and number of astral microtubules in control and AMPK-depleted cells were assessed. A control cell on average displayed 14.6 distinct astral microtubules at each pole, with a mean length of 4.14 μm (Fig. 4A to C). In contrast, AMPK-depleted cells had on average only 4.5 astral microtubules per spindle pole (P < 0.01), and the astral microtubules were significantly shorter at 3.1 μm (P < 0.005) (Fig. 4A to C). Representative maximum-projection images with contrast expansion show reduced astral microtubules in AMPK-depleted cells compared to control cells (two examples are given [Fig. 4A]). This result indicates that both the length and the number of astral filaments in AMPK-depleted cells are significantly less than in control cells.

**AMPK loss causes increased actin bundling during mitosis and is associated with aberrant astral microtubule-actin cortex interactions.** We showed that AMPK loss leads to a mitotic delay, misoriented spindles, and aberrant astral microtubules. Since actin is important for spindle orientation through astral microtubule-actin cortex attachments, actin morphology in control and AMPK-depleted cells was examined. In control cells, actin is localized to the cell cortex in 99% of all cells (HeLa and H1299) and surrounds the spindle as thin filaments in 74.6% of HeLa and 78.3% of H1299 cells (examples are given in Fig. 5A and B; quantitation is shown in Fig. 5C). Similarly, all AMPK-depleted cells have actin at the cell cortex; however, in contrast, 50.7% of AMPK-depleted HeLa cells and 40.3% of AMPK-depleted H1299 cells have prominent actin bundles surrounding the spindle, which in many cases appeared as a bundled actin ring (Fig. 5A and B; quantitation is shown in Fig. 5C). A representative line profile shows that in control and AMPK-depleted cells there are two peaks at the cellular edges corresponding to the normal actin cortex (Fig. 5D, arrowheads); however, AMPK-depleted cells have an additional two peaks corresponding to the actin-bundled ring, located adjacent to the cortex (Fig. 5D, arrows).

To further assess this phenotype, live-cell imaging was performed by cotransfecting cells with a GFP-utrophin for actin imaging (5) and mCherry-tubulin for microtubule imaging. Control cells primarily showed actin at the cortex actin transiently surrounding a normally oriented spindle (Fig. 5E; see Movie S1 in the supplemental material). Cells divided ~30 min after cell rounding, similar to nontransfected cells (Fig. 2B). In AMPK-depleted cells, actin bundles were observed surrounding a misoriented spindle, and the spindle changed positions frequently throughout mitosis (Fig. 5E; see Movie S2 in the supplemental material). In general, actin bundles appeared within 15 min post-cell rounding, persisted throughout mitosis (Fig. 5E), and were accompanied by a mitotic delay or arrest as shown in Fig. 2. Taken together, these results show that when AMPK is depleted, actin bundles are observed between the cell cortex and the spindle.

To determine if these actin bundles impact the microtubule spindle, we focused on the interactions between astral microtubules and the actin cortex. Confocal images of control siRNA-treated cells showed that in all control cells imaged, most astral microtubules directly contact the actin cortex (Fig. 5F); however, in AMPK siRNA-treated cells, astral microtubules-actin cortex interactions were seemingly impeded by actin bundles in 40% (4 of the 10 cells) of cells imaged for these interactions (Fig. 5F). It appeared that bundles physically prevented astral microtubules from contacting the actin cortex and astral microtubules were either deflected in a different direction or ended at the bundle. Thus, this result suggests that the actin bundling itself may cause the reduced number and length of astral microtubules in AMPK-depleted cells.

**Actin bundling induced by jasplakinolide leads to spindle misorientation.** Since we observed both actin bundling and spindle misorientation in AMPK-depleted cells, we hypothesized that the misoriented spindles were a consequence of actin bundling. To test this, we used jasplakinolide, an actin-stabilizing agent (10), to cause actin bundling and determine if this could in turn induce spindle misorientation. Control cells showed a mean spindle angle of 18.3°, whereas jasplakinolide-treated cells had a mean spindle angle of 27.9° (Fig. 6A and B) (P < 0.0001). Actin bundling was observed around the spindle in 57% of jasplakinolide-
FIG 6 Jasplakinolide induces spindle misorientation and aberrant actin cortex-astral microtubule interactions. (A) Immunofluorescence images of actin and tubulin in control and jasplakinolide (100 nM)-treated cells. Two representative examples are shown in jasplakinolide-treated cells. Green arrows indicate astral microtubule-actin interactions, and green arrowheads indicate astral microtubules impeded by actin bundles. Magnified regions are shown on the right. SP, spindle pole; AC, actin cortex; AB, actin bundle. Scale bar = 10 μm. (B) Scatter plot of control and jasplakinolide-treated (500 nM for 2 h) spindles, showing randomly selected representative spindle angles from the two groups done in triplicate (***, \( P < 0.001 \)). (C) Bar graph showing the actin phenotypes associated with jasplakinolide treatment. Spindle-associated actin was divided into three categories: filamentous actin, bundled actin, and no actin (*, \( P < 0.05 \); error bars show standard deviations). (D) Live-cell imaging of GFP-utr (actin) and mCherry-tubulin in HeLa cells treated with the AMPK inhibitor compound C (10 μM). (E) Bar graph quantifying the effect of compound C (10 μM) on spindle actin bundling. Percentages were obtained from three independent experiments.
treated cells compared to 16% of control cells (Fig. 6A and C). The relationship between actin bundles and astral microtubules was then investigated, and in 5 of 10 jasplakinolide-treated cells imaged, actin bundles seemingly impeded astral microtubules from making normal contact with the actin cortex (Fig. 6A; similar to AMPK depletion in Fig. 5). Overall, these results indicate that pharmacologically induced actin bundling caused spindle misorientation, potentially by preventing astral microtubules from contacting the actin cortex.

Lastly, we examined whether pharmacological inhibition of AMPK with compound C (10 μM) resulted in a bundled actin surrounding a misoriented spindle. Results show that after treatment with compound C, 78% of cells (Fig. 6D and E) imaged by live-cell confocal imaging had actin bundling. A representative example shows persistent actin bundles over time, surrounding a misoriented spindle that is not corrected (Fig. 6D).

AMPK depletion abolishes pMRLCser19 at the spindle poles and reduces mitotic pMRLCser19 levels. Next, we wanted to define the molecular underpinnings of AMPK function in spindle orientation. AMPK regulates myosin regulatory light chain at ser19 (13, 16), which then stimulates the Mg ATPase activity of myosin II leading to actin-based regulation of mitosis, cell migration, and cell polarity (17, 26, 29, 32, 43). Therefore, we hypothesized that AMPK activates MRLC at ser19, to control actin function and consequently spindle orientation during mitosis. To test this, pMRLCser19 localization was first assessed in mitotic cells. pMRLCser19 localized to the actin cortex and also to the spindle poles as previously described (Fig. 7A) (18), similar to pAMPKth172 localization. MRLC depletion using siRNA (Fig. 7B and C) led to rotated spindles with disorganized mitotic microtubules within the spindle (Fig. 7D and E), similar to what occurred with AMPK depletion (Fig. 3). Specifically, control cells had a mean angle of spindle pole orientation of 19.0° and MRLC siRNA cells 30.4° (Fig. 7E). Two examples are shown, and in both cases spindle misorientation is apparent along the z axis, where spindle poles are localized in different confocal z planes (Fig. 7D). Actin bundling surrounding the spindle was observed in 26% of cells, which is less than the 50.7% of AMPK-depleted cells having actin bundling but greater than the 11.3% previously observed in control cells. To determine if AMPK regulates pMRLCser19 during mitosis at the spindle poles, AMPK was depleted with siRNA and MRLCser19 phosphorylation was assessed at the spindle poles. Spindle pole-associated pMRLCser19 was significantly decreased in AMPK siRNA-treated cells compared to control cells (Fig. 8A; a single z section is shown). In many cases, the pMRLCser19 signal was completely abolished at the spindle poles (Fig. 8B). Quantitation of pMRLCser19 intensity shows a nearly 50% decrease in pMRLCser19 spindle pole intensity in all mitotic cells assessed (Fig. 8C). To further test this, pMRLCser19 levels were assessed by Western blotting in control and AMPK siRNA-treated cells synchronized to enter mitosis at 8 h post-thymidine release. Again, in AMPK-depleted cells mitotic pMRLCser19 levels (8 h) were lower than in control cells (Fig. 8D), indicating that loss of AMPK reduced mitotic pMRLCser19 levels. These results show that AMPK regulates pMRLCser19 at the spindle poles and MRLC controls mitotic spindle orientation.

CamKK regulates pAMPKth172 and pMRLCser19 at the spindle poles in LKB1-deficient cells. LKB1 (11, 30, 47) and CamKK (46) phosphorylate AMPK at th172. Since experiments here were done with HeLa cells, and HeLa cells have an LKB1 homozygous deletion (19), we concluded that LKB1 is not essential for pAMPKth172 localization and phosphorylation during mitosis. Therefore, we wanted to determine if CamKK is required for pAMPKth172 at the spindle poles. To do this, we used the specific CamKK inhibitor, STO-609 (1, 13, 34). STO-609 treatment induced severe spindle orientation defects as well as mitotic actin bundling (Fig. 9A), similar to AMPK depletion. Specifically, control (dimethyl sulfoxide [DMSO]-treated) cells have on average a spindle angle of 16°, whereas STO-609-treated cells have a spindle angle of 35.9° (Fig. 9B). Furthermore, the pAMPKth172 spindle pole signal is significantly decreased and multiple pAMPKth172 puncta appear around the spindle pole in maximum-projection images (Fig. 9C and D). Next, pMRLCser19 was examined by performing immunofluorescence assays. In this case, spindle pole-associated pMRLCser19 was reduced in nearly all cells imaged (Fig. 9E). Control cells had a mean fluorescence intensity of 79.2, whereas STO-609-treated cells had a mean fluorescence intensity of 32.9 (Fig. 9F) (P < 0.001). Lastly, we tested whether endogenous CamKK levels increased during mitosis by synchronizing HeLa cells. These data show that when cells begin to enter mitosis CamKK levels increase but not, however, to the extent of pAMPKth172 levels (Fig. 9G). CamKK does not appear to have spindle pole localization but is rather diffuse throughout the mitotic cell (data not shown). Taken together, these data show that pharmacological treatment with the CamKK inhibitor causes spindle misorientation, actin bundling, and reduced pAMPKth172 and pMRLCser19 at the spindle poles, suggesting that CamKK regulates AMPK-MRLC at the spindle poles.

Lastly, we wanted to determine the relationship between the CamKK-AMPK pathway to a potential LKB1-AMPK pathway in mitosis. Therefore, we utilized our parental H1299 pLKO.1 control cells (LKB1 wild type) and their stable H1299 LKB1 shRNA counterpart cells (H1299 shLKB1) (Fig. 10A) (7). Interestingly, stable H1299 shLKB1 cells also had significant spindle orientation defects compared to control cells (Fig. 10B and C), suggesting that LKB1 is also important for spindle orientation. CamKK inhibition with STO-609 in LKB1 shRNA cells caused spindle misorientation as expected (Fig. 10C), which is similar to what occurs in HeLa cells, in which LKB1 is homozygously deleted (Fig. 9); however, when LKB1 was present at normal levels in the parental H1299 pLKO.1 cells, STO-609 had no significant effect on spindle orientation (Fig. 10C). This is consistent with the observation that STO-609 had no effect on spindle orientation in normal PTK1 cells, where wild-type LKB1 is present (Fig. 10D). These data suggest that both LKB1 and CamKK may play redundant roles in regulating AMPK in spindle orientation.

DISCUSSION

Proper spindle orientation is required for development, cell fate, and tissue organization by ensuring an accurate distribution of genetic material and a normal division plane. Based upon our data showing that AMPK depletion or pharmacological inhibition leads to a mitotic delay (Fig. 2) and spindle misorientation (Fig. 3), we conclude that AMPK is essential for normal spindle orientation and when it is defective, mitosis does not proceed efficiently. We propose that spindle misorientation in AMPK-depleted cells is
a direct consequence of a lack of astral microtubules, since astral microtubules are required for proper spindle positioning and orientation by attaching to the actin cortex and stabilizing the spindle (21, 23). In AMPK-depleted cells, the astral microtubules are fewer and shorter and appear to be unable to attach to the normal actin cortex due to actin bundles surrounding the spindle (Fig. 4 and 5). It is therefore interesting to speculate that actin bundling physically impedes astral microtubule elongation and attachment to the cell cortex and consequently astral microtubules are reduced in number and spindles become misoriented. Along those lines, we also observed similar inhibition of astral microtubule-actin cortex attachments and spindle misorientation with jasplakinolide (Fig. 6), indicating that actin bundling itself can induce these phenotypes.

Defects in spindle orientation, astral microtubules, and actin bundling in AMPK-depleted cells are likely due to MRLC inactivation. MRLC is an indirect target of AMPK kinase activity (3, 4), and now we provide data indicating that (i) both pMRLCser19 and pAMPKthr172 are localized to the mitotic spindle poles, (ii) AMPK depletion inhibits spindle pole-associated pMRLCser19 levels and reduces overall mitotic levels of pMRLCser19, and (iii) MRLC depletion itself leads to spindle misorientation and increased actin bundling. Therefore, we conclude that AMPK regulates MRLC activity in the mitotic spindle. Since MRLC controls actin turnover through myosin II in interphase, the cleavage furrow, and neuronal polarity (17, 26, 29, 32, 43, 45), we propose that the lack of pMRLCser19 activity in AMPK-depleted cells leads to reduced actin turnover and, in turn, the observed actin bundling (Fig. 11).

Consequently, the actin bundling prevents normal astral microtubule-actin cortex interactions, causing aberrant spindle orientation and mitotic delay (Fig. 11). Supporting this overall model is recent evidence that AMPK/AMPK2 phosphorylates a network of mitotic substrates, two of them being the MRLC regulators PPP1R12C phosphatase and PAK2 kinase (3). The authors propose that these two AMPK substrates counterbalance MRLC phosphorylation during mitosis, supporting our data showing that AMPK regulates spindle orientation via MRLC activity.

It is possible that the actin bundles in AMPK-depleted cells originate from a revolving actin cluster observed alongside mitotic spindles (20). Though the precise function of this actin cluster has not been established, it is highly dynamic and lends itself to several possible roles in mitosis. One potential role is in the regulation of microtubule dynamics. Actin bundles have been shown to interact with microtubules through the actin microtubule linker (AML) protein, which is essential for proper spindle formation (46). The inhibition of AML by actin bundling could disrupt microtubule assembly and stability, contributing to the defects observed in AMPK-depleted cells. Another role for actin bundles could be in the regulation of cell shape and movement. Actin bundling has been implicated in the formation of the cleavage furrow (47) and the orientation of the mitotic spindle (48). The observed actin bundling and spindle misorientation in AMPK-depleted cells suggest that these bundles may play a role in these processes.

### Figure 7

**MRLC accumulates in mitosis on the spindle poles, and MRLC depletion results in misoriented spindles.** (A) Immunofluorescence of pMRLCser19 in a mitotic cell shows spindle pole and actin cortex localization. Tubulin and actin staining images are also shown along with a merged image. Scale bar = 10 μm. (B) Western blot of pMRLCser19 showing successful depletion with MRLC siRNA. GAPDH shown as a loading control. (C) Representative image showing successful pMRLCser19 depletion at the spindle poles and actin cortex. (D) Confocal z sections of tubulin and actin in control siRNA- and MRLC siRNA-treated cells. Scale bar = 10 μm. Numbers in the top right indicate the z distance from the top. Arrows indicate spindle poles. Two examples of MRLC siRNA-treated cells are shown. (E) Scatter plot showing significantly different spindle angles in control siRNA and MRLC siRNA cells (***, P < 0.001).
additional weight to the observations that microtubules and actin directly interact in the spindle both in vitro and in vivo to control spindle orientation (2, 31, 48). Thus, perhaps loss of MRLC function due to AMPK inactivity reduces MRLC activity in regulating spindle-associated actin, leading to a bundled actin ring.

AMPK is a canonical energy sensor protein, which is activated when ATP levels are low and AMP levels are high (9). Both LKB1 (11, 30, 47) and CamKK (46) activate AMPK by phosphorylation at AMPKθ172. We propose that the AMPK-MRLC pathway is regulated by CamKK in LKB-deficient cells (e.g., HeLa cells in which LKB1 is homozygously deleted [19]), since inhibition of CamKK caused spindle misorientation, reduced pAMPKθ172 at the spindle poles, and reduced pMRLCser19 at the spindle poles (Fig. 9). Furthermore, pAMPKθ172 can also target to the spindle poles in HeLa cells (Fig. 1), showing that LKB1 is not necessary for pAMPKθ172 localization. However, this scenario may be different when LKB1 is present, since CamKK inhibition by STO-609 had no effect on spindle orientation in H1299 pLKO.1 cells (LKB1 wild type) but did induce misorientation in the H1299 LKB1 shRNA clones (Fig. 10). These data support the possibility that LKB1 and CamKK may play redundant roles in regulating spindle orientation or these pathways are separately activated under different conditions. It is suggested that CamKK-induced AMPK activation is independent of changes in adenine nucleotide levels (27); therefore, perhaps CamKK regulation of AMPK in mitosis may not be related to energy sensing as originally proposed (41, 42). Interestingly, LKB1 shRNA cells also show spindle orientation defects relative to their control counterparts, suggesting that LKB1 loss may affect spindle orientation independent of CamKK status. Another possibility is that pAMPKθ172 is also regulated by polo-like kinase, since the PLK1 inhibitor suppresses pAMPKθ172 at the spindle poles (40). Newer technologies like the development of an AMPK sensor (38) should
shed light on these questions and provide additional approaches to studying the activation dynamics and regulation of the AMPK network in mitosis.

In conclusion, we propose that AMPK regulates pMRLC\textsuperscript{ser19} activity at the spindle poles to control spindle orientation via actin cortex-astral microtubule interactions (Fig. 11). It remains to be determined how MRLC regulates spindle-associated actin, but presumably this is through myosin II, which is the major target of inhibiting CamKK in cells without compromised LKB1 does not lead to spindle misorientation. (A) Western blot showing stable LKB1 depletion in H1299 LKB1 shRNA cells (shLKB1) compared to the parental H1299 pLKO.1 control cells. (B) Confocal z sections of the spindle in H1299 pLKO.1 and H1299 shLKB1 cells. Numbers in the upper left of images show the z distances relative to the first section. Arrows show spindle poles. Scale bar = 5 μm. (C) Scatter plot of spindle angle in lung cancer H1299 pLKO.1 cells and H1299 shLKB1 cells treated with STO-609 (*, P < 0.05; **, P < 0.01). (D) Scatter plot of spindle angle in normal PTK1 cells treated with STO-609. No significant difference was observed.
FIG 11 Proposed model depicting the AMPK-pMRLC\textsuperscript{ser19} pathway to maintain proper mitotic spindle orientation. (Left) pAMPK\textsuperscript{thr172} is localized to the mitotic spindle poles and induces phosphorylation of pMRLC\textsuperscript{ser19} at the spindles poles. This presumably results in activation of myosin II (not shown) leading to normal astral microtubule-actin cortex interactions and consequently proper spindle orientation and cytokinesis. (Right) When AMPK function is compromised, pMRLC\textsuperscript{ser19} phosphorylation is reduced at the spindle poles. Consequently, spindle-associated actin bundling ensues, and this hampers normal astral microtubule-actin cortex interactions. As a result of aberrant astral microtubule-actin cortex attachments, spindles are misoriented and a mitotic delay occurs.
active MRLC. Future studies will likely go on to address how pAMPK\textsuperscript{Thr172} itself is regulated at the spindle poles by its upstream kinases during mitosis and whether AMPK serves as a mitotic energy sensor to control spindle orientation.

ACKNOWLEDGMENTS

This work was supported in part by an American Cancer Society Research Scholar grant (RSG-08-035-01-CSM) awarded to A.I.M., grant 1R01CA142858 awarded to A.I.M., a Lung Cancer PO1 grant (5PO1 CA116676), and the Georgia Cancer Coalition.

Images were acquired at the Winship Cell Imaging and Microscopy Shared Resource. We thank Josh Francis for his assistance with the lasjplakinolide experiment and Jennifer Fairman for the artwork in Fig. 11.

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