coTitle: Kinesin Molecular Motor Eg5 Functions During the Elongation Phase of Polypeptide Synthesis

Kristen M. Bartoli, Jelena Jakovljevic, John L. Woolford Jr, and William S. Saunders

1Department of Biological Sciences, University of Pittsburgh, Pittsburgh PA 15260
2Biochemistry and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh PA 15260
3Department of Biological Sciences, Carnegie Mellon University, Pittsburgh PA 15213

Corresponding author: William S. Saunders, 274 Crawford Hall, 4249 5th Ave., Pittsburgh, PA 15260 (412)624-4320, FAX (412)624-4759; wsaund@pitt.edu
Abstract: The kinesin-related molecular motor Eg5 plays a well-defined role in cell division, promoting spindle assembly, but here we show that during interphase Eg5 primarily associated with ribosomes and both Eg5 and the microtubule cytoskeleton were required for optimal nascent polypeptide synthesis. When Eg5 was inhibited, ribosomes no longer bound to microtubules in vitro, ribosome elongation rates slowed and polysomes increased in intact cells, indicating an inhibition of the elongation phase of polypeptide synthesis. Together these results demonstrate for the first time that the molecular motor Eg5 is a motile link between the ribosome and the cytoskeleton enhancing the efficiency of translation elongation.

Introduction: Protein translation can be reproduced in cell free extracts, but it is likely that additional regulatory and structural mechanisms influence polypeptide synthesis in vivo. One mechanism that may serve to enhance translation in intact cells is the association of the translational machinery with the linear cytoskeleton filaments of the cytoplasm. These structural elements may support directionality, cellular localization or efficiency of translation compared to cell-free systems. An association of various translational components with the cytoskeleton has been observed in vivo; these components include mRNA, aminoacyl-tRNA synthetases, initiation and elongation factors. Ribosomes have been previously shown to functionally associate with both actin and microtubules in many different eukaryotic cell types (reviewed in Kim and Columbe, 2010). In sea urchin embryos, an association of ribosomes with microtubules occurs via a short stalk (Suprenant et
al., 1989) reminiscent of cargo association to microtubules by molecular motors. Consistent with this interpretation, ribosomes migrate along microtubules in hemipteran oocytes (Macgregor and Stebbings, 1970), the kinesin motor KIF4 (kinesin 4) is required for anterograde transport of ribosomes in rat neurons (Bisbal et al., 2009), and KIF3A is found in periaxoplasmic ribosomal plaques (Sotelo-Silveira et al., 2004). In addition, both kinesin and dynein molecular motors are shown to function in mRNA transport and localization (Jansen, 1999; Suprenant, 1993) and play roles in the formation of mRNA-silenced stress granules and P-bodies (Loschi et al., 2009). However, roles for molecular motors directly in protein synthesis has not yet been demonstrated.

The Eg5 motor plays well-described roles in spindle assembly and bipolarity (Kapitein et al., 2005; Ohsugi et al., 2008). Here we show that during interphase, Eg5 associated with ribosomes and was required for efficient elongation of protein synthesis. In the absence of Eg5 activity, or when the Eg5 ATPase was inhibited, polypeptide synthesis decreased ~ 40%. Eg5 was shown to associate with ribosomes through sucrose gradient sedimentation and co-immunoprecipitation and was required for the association of ribosomes with microtubules. Inhibition of the Eg5 ATPase caused a classical elongation delay in translation with an accumulation of polysomes and a reduction in 80S ribosomes. These results show that Eg5 has additional roles in the cell during interphase to promote translation elongation during polypeptide synthesis.
Results:

_Eg5 associates with ribosomes and is essential for normal levels of polypeptide synthesis._

A role for the Eg5 motor in protein synthesis was assayed by examining $^{35}$S Met/Cys incorporation into nascent polypeptides. Protein synthesis was significantly reduced by ~40% when RPE1 cells were given a 4 hr treatment of monastrol, a specific inhibitor of Eg5s ATPase activity, or after a 24 hr siRNA knockdown (Fig. 1A, B). Cycloheximide was used as a positive control for translational inhibition. When monastrol was washed away and replaced with fresh medium, translation returned to normal levels, showing the inhibition of translation was reversible and not due to cell death (Fig. 1A). The reduction in translation from Eg5 inhibition was observed in multiple cell lines showing the inclusiveness of this phenotype (Fig. 1A Supplemental).

To confirm the specificity of the Eg5 phenotype, we repeated the $^{35}$S Met/Cys incorporation assay with two additional small molecule inhibitors of Eg5, S-Trityl-L-cysteine and dimethylenastron and also observed a similar decrease in protein synthesis (Fig. 1B Supplemental). Similarly, the rate of polypeptide synthesis, which is the rate of $^{35}$S Met/Cys incorporation over time, decreased when Eg5 was knocked-down by siRNA or after treatment with monastrol (data not shown). Microtubule depolymerization with nocodazole or Colcemid also produced a reduction of protein synthesis, consistent with a requirement for microtubule-based motors in translation (Fig. 1C).

Protein synthesis occurs in both cytosolic and endoplasmic reticulum (ER)-associated compartments. RPE1 cells labeled for $^{35}$S Met/Cys incorporation were fractionated into
cytosolic or ER fractions and nascent protein synthesis compared with and without Eg5 inhibition. Monastrol treatment reduced protein synthesis in both cellular compartments (Fig. 2A Supplemental). To further confirm the specificity of the Eg5 inhibition we repeated the assay with two siRNA oligonucleotides to Eg5 from nonoverlapping regions of the gene and also observed decreased protein synthesis in both cytosolic and ER fractions (Fig. 2B,C Supplemental). In summary, five different specific agents of Eg5 inhibition caused a reduction in protein synthesis in the cell. However, this was not a general phenotype of interference with mitotic motors. For example, knockdown of the chromokinesin Kid reproducibly resulted in an increase in protein translation (Fig. 2D Supplemental).

*Decrease in translation after Eg5 inhibition is not due to mitotic arrest or cell death*

Translation is known to be diminished when cells enter mitosis (Sivan et al., 2007), and prolonged inhibition of Eg5 can cause mitotic arrest (Mayer et al., 1999). So we took care to be certain that the translational phenotype after loss of Eg5 we are describing was not due to mitotic arrest. In all experiments with Eg5 inhibitors, we used only short 1-4 hr exposures that did not increase the mitotic index of the cells (Fig. 3A Supplemental, times used marked with asterisk). Mitotic cells remained less than 5% of the population, and remained the same in both control and the inhibited population and therefore cannot explain the observed phenotypes. Similar results were observed whether the mitotic index was measured by DAPI staining (Fig. 3A, B Supplemental) or by phosphorylated histone-H3 immunofluorescence (data not shown). Similarly, the two Eg5 siRNAs used produced little or no increase in mitotic cells at the 24 hr time point chosen for the translational
analysis (Fig. 3B Supplemental). For all other siRNA experiments, except Fig. 2B Supplementary, siRNA #2 was used, which gave no change in the mitotic index at 24 hrs. Therefore, the phenotypes described in this paper after loss of Eg5 are not a result of mitotic arrest.

We also tested whether the loss of translation was due to cell death. When cells were treated with monastrol there was no caspase 3-associated apoptotic cell death observed (Fig. 4A Supplemental). Additionally, neither of the siRNA knockdown oligos caused a detectable decrease in metabolic activity of the cells (Fig. 4B Supplemental). Monastrol treatment caused a partial inhibition of metabolic activity, but it was reversible, indicating the inhibition was not due to cell death (Fig. 4C Supplemental). These results confirm that a reduction of protein translation was observed before any indication of cell death could be documented.

*Eg5 is a component of ribosomes*

To determine if Eg5 could associate with ribosomes, cellular lysates were fractionated on 10 to 45% sucrose gradients. The eukaryotic 40S and 60S ribosomal subunits assemble on mRNA to form the 80S ribosome. The association of multiple 80S ribosomes on a single mRNA constitutes a translating polysome complex. Sucrose gradient fractionation of RPE1 lysates revealed that Eg5 could be found in each of the 40S, 60S, 80S and polysome fractions (Fig. 2A).

If a 10-25% sucrose gradient was used, to provide further separation of the 80S ribosomes from the 40S and 60S subunits without resolving polysomes, under these
conditions we could see co-sedimentation on the gradient of Eg5 and the 40S, 60S and 80S ribosomes (Fig. 2B). This is in contrast to other microtubule motors, such as cytoplasmic dynein, which did not co-localize with ribosomes in our experiments and indicates that Eg5 co-sediments with ribosomes (Fig. 2B). Note that virtually no Eg5 was found at the top of the gradient, free of ribosomes and subunits, suggesting that much of the cytoplasmic pool of the Eg5 motor may be associated with ribosomes.

To confirm an association of Eg5 with ribosomes, we immunoprecipitated Eg5 and tested for the presence of rpL10A, a marker for the 60S subunit (Fig. 2C). rpL10A is present in the pellet at low levels without any antibodies added, but substantially more rpL10A was pelleted when Eg5 was immunoprecipitated. rpS5, a marker for the 40S subunit, was present in the pellet only when Eg5 was immunoprecipitated (Fig. 2C). rpS5 was not present in the pellet when an unrelated protein, MYPT1, was immunoprecipitated. These results further demonstrate that Eg5 associates with ribosomes.

*Ribosomes associate with microtubules through Eg5*

Next we tested if Eg5 promoted association of the ribosome with microtubules. Purified cytosolic 40S, 60S subunits, or 80S ribosomes were mixed with *in vitro* assembled microtubules and centrifuged. Both the 40S subunits and 80S ribosomes partially associated with the microtubule pellet, while the 60S subunits did not under these conditions (Fig. 2D). These observations confirm that ribosomes can
associate with microtubules in mammalian cells, as demonstrated previously in other cell types (Hamill et al., 1994; Han et al., 2006; Suprenant et al., 1989). However, when Eg5 was knocked-down, ribosomal association with the microtubule pellet was lost (Fig. 2D). If microtubules were omitted from the binding reaction, neither the 40S subunit or 80S ribosome were found in the pellet, demonstrating the specificity of the binding reaction (Fig. 2E). These results indicated that Eg5 was required to maintain ribosome association with the insoluble microtubule cytoskeleton.

_Eg5 is essential at the elongation phase of translation_

To examine how Eg5 activity was important for protein synthesis, polysome profiling analysis was completed. Distinctive changes in polysome profiles can be observed to differentiate between initiation and elongation defects. Defects in translation initiation reveal a reduction in polysomes with a corresponding increase in 80S ribosomes, while defects during translation elongation lead to an increase in the polysome fraction, while 80S monosomes are reduced, because slower moving ribosomes accumulate on the mRNA (Shin et al., 2009).

Monastrol treatment of RPE1 cells produced a clear elongation phenotype in the polysome profile, with both an increase in polysomes and a decrease in 80S ribosomes (Fig. 3A). In each of these profiles, we measured the area under the curves to calculate a polysome/80S monosome (P/M) ratio to avoid variance between data sets due to sample size. The P/M ratio increased when Eg5 is inhibited by monastrol, consistent with an elongation defect (Fig. 3A). Other Eg5 inhibitors, such as S-Trityl-L-cysteine, (or
dimethylenastron, data not shown) confirmed this data by producing a similar phenotype of enhanced polysome levels and P/M ratios (Fig. 3C).

Cycloheximide (CHX) is often used while isolating ribosomes to preserve the polysomes from ribosome “run-off” during purification (). In the absence of CHX, in control cells ribosomes continue to elongate, run-off the mRNA, and accumulate as free 80S ribosomes, (Fig. 3B, left most panel). However, in the presence of an elongation defect and in the absence of CHX, ribosomes are slower to run-off and polysomes persist. 4 hrs after monastrol treatment in the absence of CHX, polysomes and the P/M ratio increased (Fig. 3B) further supporting an elongation defect when Eg5 function is compromised (Fig. 3B).

These results after Eg5 inhibition are in contrast to translation initiation inhibitors, as treatment with arsenite resulted in decreased P/M ratios and increased 80S ribosomes at the expense of polysomes (Fig. 3A, B, right most panels). The absence of this profile change when Eg5 is inhibited suggests that Eg5 may not participate in initiation. These phenotypic changes after monastrol treatment are also distinct from that observed when cells become senescent and nondividing, as observed when RPE1 are arrested by serum starvation (Fig. 5 Supplemental).

To further confirm Eg5 function during translation elongation, the ribosome half-transit time was measured, an indication of the time it takes for one ribosome to traverse an average-sized mRNA (Fan and Penman, 1970). If Eg5 was required for optimal elongation, we should see an increase in the ribosome half-transit time
because ribosomes are slower to move along the mRNA. After a 4 hr monastrol inhibition of Eg5, the ribosome half-transit time in RPE1 cells increased on average ~ 2.8 fold (Fig. 4), showing that ribosome movement during elongation slowed when Eg5 was inhibited demonstrating Eg5’s requirement for translation elongation.

We can independently test this conclusion by evaluating whether Eg5 is preferentially required for 5’cap-dependent initiation. U2OS cells, chosen for their high transfection efficiency, were transiently transfected with a bicistronic protein expression plasmid allowing 5’cap-dependent and independent translation initiation to be monitored simultaneously (Nie and Htun, 2006). Translation of the YFP marker is controlled via the traditional 5’cap-dependent pathway, while translation of CFP is constitutive via an internal ribosome entry site (IRES). If Eg5 functions in cap-dependent initiation, then a preferential decrease in YFP expression would be expected. However, if Eg5 functions during elongation, a decrease in both markers would be anticipated. After Eg5 knockdown, expression of both markers was significantly reduced by roughly equal amounts, 52% (CFP) and 55% (YFP), suggesting that Eg5 functioned independent of the initiation step of translation (Fig. 5A), consistent with a role primarily in elongation.

**Discussion:** This manuscript makes two related conclusions: the Eg5 molecular motor functions in protein translation and protein translation requires molecular motors during elongation. We conclude that Eg5 function is important during the elongation phase of polypeptide synthesis because we see 1) decreased protein synthesis, 2) reduced translation from both cap-dependent and cap-independent transcripts, 3) delayed ribosomal half-transit times, and 4) enhanced polysomes and
P/M ratios when Eg5 is inhibited, all consistent with a defect in elongation and mostly inconsistent with deficiencies in other stages of translation such as initiation and termination.

A ~40% reduction in cellular polypeptide synthesis was observed with five specific inhibitors of Eg5 ruling out primarily off-target affects. Additionally, translation inhibited phenotypes were seen in as little as one hour after inhibition of Eg5, with fewer than 5% of the cells in mitosis, and no increase in the mitotic frequency over controls, showing that the phenotypes were not a result of mitotic arrest. Eg5 expression is diminished after mitosis, but Eg5 remains present throughout the cell cycle () consistent with a role in interphase, as indicated by our observations.

Our discovery of a role for Eg5 in protein synthesis has important implications for phase I and phase II clinical trials targeting Eg5 for cancer treatment (Bergnes et al., 2005; Burris et al.; Duhl and Renhowe, 2005; Huszar et al., 2009; Lad et al., 2008; Nakai et al., 2009; Sakowicz et al., 2004; Sarli and Giannis, 2008; Zhang and Xu, 2008). Critical to the rationale of choosing mitotic motors as targets is the belief that they function only in mitosis and their inhibition should only minimally interfere with the processes in interphase cells (Bergnes et al., 2005; Burris et al.; Duhl and Renhowe, 2005; Huszar et al., 2009; Lad et al., 2008; Sarli and Giannis, 2008) as cancer cells typically have a higher mitotic index than noncancer cells. The results reported here indicate an important role for Eg5 during interphase that needs to be considered in the design and application of anti-Eg5 therapeutics.
Eg5 is required to link ribosomes to microtubules in in vitro binding assays, and the ATPase activity of Eg5 is essential, and we propose that Eg5 serves as a motile link between the ribosome and cellular microtubules to enhance the efficiency of translation (Fig. 5B). Eg5 could serve as a traditional elongation factor to promote synthesis of the polypeptide chain, but there is no evidence to support that conclusion and our preferred model is that Eg5 interacts with ribosomes as a macromolecular cargo to maintain association of ribosomes with the cytoskeleton. While the structural aspects of Eg5 association with ribosomes and the molecular details of Eg5's role in translation remain to be determined, these results for the first time define a direct role for molecular motors in ribosomal movement and Eg5 in polypeptide synthesis.

**Methods:**

Cell lines:

RPE1 (human retinal pigmented epithelial cells stably transfected with human telomerase reverse transcriptase (hTERT)) cells were cultured in DMEM/F-12, (#D6421). UPCI:SCC103 (a gracious gift from Susan M. Gollin (University of Pittsburgh were cultured in MEM (M4655) with 2mM L-Glutamine, and 1% MEM non-essential amino acids (Invitrogen). HFF-hTERT and NIH-3T3 were maintained in DMEM with 2mM L-Glutamine. U2OS cells were maintained in McCoy's media (#16600082, Invitrogen).

Antibodies:
The following antibodies were used in this study: Eg5 – rabbit anti-Eg5 (AKIN03), Actin – rabbit anti-Actin (AAN01) (Cytoskeleton); Eg5 – mouse anti-Eg5 (#627802, Biolegend); RPS5 – mouse anti-RPS5 (#AB58345), RPL10A mouse anti-RPL10A (#Ab55544), GAPDH – rabbit anti-GAPDH (14˚C10) #2118 (Cell Signaling); Calnexin (Stressgen); Caspase-3 – rabbit anti-caspase 3 (8G10) #9665 (Cell Signaling); FLAG – mouse anti-FLAG #4049 (Sigma); HA – mouse anti-HA #1583816 (Roche).

Culture of cell lines:
All cultures were grown at 37˚C with 5% CO₂. All culture media and supplements were purchased from Sigma, most cell lines were obtained from the American Type Culture Collection (ATCC), and were supplemented with 10% FBS (#511150, Atlanta Biologicals) unless otherwise stated.

Small molecule treatments:
The following times and concentrations were used: 4 hr monastrol-130 M (Tocris Bioscience; batch #3), 2 hr nocodazole-12 M, 2 hr Colcemid-0.002mg/mL, 30 min of low levels of puromycin treatment (100 g/mL), CHX (0.1mg/mL), 1 hr 0.05 mM arsenite, 1 hr 3 M dimethylenastron (Alexis biochemicals), 4 hr 1.5 M S-Trityl-L-cysteine (Alexis biochemicals). All reagents were purchased from Sigma, unless otherwise noted and dissolved in DMSO, except for puromycin and arsenite, which is pre-dissolved. For washout experiments, cells were treated for 4 hrs with monastrol, followed by 3 washes in FBS, and a 4 hr recovery.

Serum starvation:
RPE1 cells were serum starved for 32 hrs in DMEM media, without FBS. Fresh DMEM was added every 6 hrs, prior to cell lysis and polysome profiling.
Transfections:

siRNA:

Cells were reverse-transfected for 24 hrs with 1.5 g/60mm tissue culture plate of siRNA using HiPerfect transfection reagent following the manufactures’ protocol. Fluorescently labeled scrambled siRNA was used (cat# 1022563) as the control. All reagents were purchased from Qiagen unless specified. Eg5 siRNA #1 (SI02653770) and Eg5 siRNA #2 (s7904) (Ambion), and Kid siRNA (Hs_Kif22_6).

Plasmids:

Bicistronic plasmid transfection (Addgene plasmid 18673) into U2OS cells was completed using FuGene6 (#1814443, Roche) transfection reagent for 24 hrs following the manufactures’ protocol, after a 12 hr knockdown of Eg5. Eg5 was knocked-down for a total of 36 hrs. Antibodies to HA or FLAG were used for immunoblot analysis, and quantitation of immunoblots were completed using Photoshop.

35S Met/Cys incorporation assays:

Cells were grown in 60 mm plates and media was changed to DMEM without methionine and cysteine (#D0244, Sigma) plus 5% dialyzed FBS (#F0392, Sigma) and 2 mM L-glutamine for 30 min at 37°C, with the addition of 100 Ci/mL of 35S Met/Cys (#NEG072007MC, Perkin Elmer). To stop reactions, 0.1 mg/mL of CHX was added, cells were trypsinized, and washed prior to cell lysis in RIPA buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1% NP40). Protein samples were equalized prior to splitting them: one half were subjected to scintillation counting, where duplicate samples of each lysate was placed on GF/C filters (#28497-743, VWR), washed with
10% TCA, once with 100% ethanol, and dried before analysis. The second half was subjected to a Lowry assay and/or separated on SDS-PAGE, for confirmation of equal loading or of knockdown of the specified protein.

Fractionation of cell lysates:
For fractionation of cell lysates into cytosolic and membrane fractions, a digitonin fractionation protocol was used. Briefly, cells were trypsinized, washed, and the cell membrane was broke open by pipetting 25x’s with a cut-pipette tip in digitonin buffer solution (10 mM Pipes [pH 6.8], 300 mM Sucrose, 3 mM MgCl₂, 5 mM EDTA, 0.01% digitonin, 1 mM PMSF). Lysates were incubated for 8 min on ice, centrifuged at 3,000 x g for 4 min, and the cytosolic fraction was removed. Pellet was washed once in PBS, centrifuged and resuspended in RIPA buffer to retain the membrane fraction.

Polysome profiling:

10-45% Sucrose gradients:
20-30 million RPE1 cells were incubated with 0.1 mg/mL of cycloheximide (CHX) for 10 min, prior to lysis. (Samples which were treated with CHX are labeled +CHX, whereas samples which were not treated with CHX are labeled -CHX.) Cells were lysed (20 mM Tris-HCl (pH 7.2), 130 mM KCl, 30 mM MgCl₂, 2.5 mM DTT, 0.2% NP-40, 0.5% sodium deoxycholate, 10 mg/mL of cycloheximide, 0.2 mg/mL of heparin, 1 mM PMSF), and DNA pellet was omitted by centrifugation. The lysates were placed on a 10-45% (wt/wt) sucrose gradient (10 mM Tris-HCl (pH 7.2), 60 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.1 mg/mL of heparin) and samples were centrifuged at 27,000 g for 2.5 hrs at 4°C using a Beckman L7 Ultracentrifuge (Model L7-65) in a
Sorval AH629 rotor, fractionated by upward displacement through an ISCO UA-5 with constant UV monitoring at an absorbance of 260 nm,

For immunoblot analysis of 10-45% sucrose gradient, fractions representing each of the ribosomal subunits and/or ribosomes were pooled together. For extraction of proteins, a final concentration of 20 mM Tris pH 7.5 was added, followed by the addition of 15 L of Stratagene resin (Stratagene). Samples were then rotated at room temperature for 30 min, prior to centrifugation; pelleted beads were resuspended in 2X SDS loading dye and samples were boiled for 10 min to elute proteins.

10-25% sucrose gradients:

Sucrose gradient fractionation was completed similar to above with the following exceptions. Lysates were layed on 10-25% sucrose gradients and centrifuged for 4 hr prior to upward displacement and constant UV monitoring.

For immunoblot:

1 mL fractions were collected and were precipitated using 10% final concentration of TCA for immunoblot analysis.

Polysome/monosomes ratio calculations:

For calculation of polysome/monosomes ratios, each polysome profile graph was photocopied and enlarged at 151%. Next, the area under each ribosomal peak (40S, 60S, 80S, and polysomes) was estimated by weighing paper cutouts of the profiles. Each peak was cutout (in triplicate) and weighed (in triplicate) on an analytical balance (Ohaus Adventurer SL AS64). Averages of the area under each ribosomal peak were calculated and the average weight of the polysomes was
divided by the average weight of monosomes (80S ribosomes) per profile in order to calculate the P/M ratio.

Immunoprecipitation:
Immunoprecipitation was completed following the manufacturers protocol with these exceptions: 10 million RPE1 cells were lysed (50 mM Hepes [pH 7.5], 150 mM NaCl, 0.1% Triton X-100, 1mM EDTA, 2.5 mM Glycerol, 1 mM NaF, 0.1 mM Na3VO4, 10 mM Beta-glycerophosphate, 1 mM PMSF), and the DNA pellet was removed by centrifugation. 10% of the supernatant was saved for WCL analysis, while the other 90% was used for the IP. Each tube received either 2 g of rabbit anti-Eg5 antibody, MYPT1 or 4 L of 30% glycerol, (Eg5 antibody was reconstituted in 30% glycerol) for the immunoprecipitation. Samples were loaded on a 12% SDS-PAGE. Each sample represented 50% of the total except for the WCL which represented 10% of the initial sample. MYPT1 was used as a negative control.

Ribosome ½ transit time assay:
30 million cells were trypsinized, washed, and resuspended in BME (#B1522, Sigma) plus 10% dialyzed FBS and 2 mM L-glutamine for 20 min prior to the addition of 10 Ci/mL of 35S Met/Cys. At each time point, 500 L of cells were removed, placed in an ice cold tube, with 500 g/mL of CHX, and incubated on ice. Cells were centrifuged, washed with ice cold PBS containing CHX, recentrifuged, lysed (0.02 M Tris [pH 7.2], 0.130 M KCl, 0.03 M MgCl2, 1% NP40, 0.05% Sodium deoxycholate, 0.2 mg/mL Heparin, 0.25 mg/mL CHX, 1 mM DTT, 1 mM PMSF, RNAsin Inhibitor (Promega)) and the DNA was omitted by centrifugation prior to splitting the lysates: 500 L of the lysate was saved (PMS fraction) containing total
proteins, while another 500 L was placed on a stepwise 20% sucrose buffer and 60% sucrose cushion. Samples were centrifuged in a S100-AT5 ultra-centrifuge rotor at 55,000 g for 27 min, after which 500 L of the sample was removed (PRS) containing completed proteins released from the ribosomes. The PMS and PRS fractions were then TCA-precipitated on GF/C filters and subjected to scintillation counting. Half-transit times were calculated by comparing the incorporation of radioactivity into total proteins and completed proteins.

In vitro microtubule binding assays:

Purified tubulin (isolated from bovine brains) was thawed on ice with the addition of an equal volume of 1X PM (10 mM PIPES (pH 7.0), 5 mM Mg Acetate, and 1 mM EGTA (pH 7.0)) buffer and 2 mM GTP, followed by a 30 min centrifugation at 10,000 g (4°C). Supernatant was removed, grow buffer (1X PM buffer, 0.1 mM Taxol (Sigma), 5 mM GTP) was added to it in a 4:1 ratio (tubulin:grow buffer), followed by a 15 min incubation (34°C) with rotation. Fractions for each ribosomal subunit from the polysome profiling were pooled together, inverted and split: half received the binding reaction (5X PM buffer, 100 mM NaCl, 0.04 mM Taxol, 1 mM GTP) plus 12% tubulin and was incubated with rotation for 45 min (34°C), while the other half received the binding reaction without tubulin, Taxol, or GTP and remained at 4°C. After incubation, cells were centrifuged at 13,000 g for 30 min at 34°C or 4°C. The supernatant was removed (containing non-microtubule bound proteins), pellets were washed in PBS, re-centrifuged, and the supernatant discarded. The pellet contained microtubules and microtubule-bound proteins; 20% of the supernatant and 50% of the pellet were loaded on 12% SDS-PAGE gels.
Mitotic Index analyses:
Cells were fixed in 0.02% Triton-X 100 in 4% paraformaldehyde, for 15 min prior to the addition of DAPI. A minimum of 300 cells were counted per trial and the experiment was completed in triplicate. All cells were analyzed on an Olympus BX60 epifluorescence microscope with 100x oil immersion objectives, unless specified. Hamamatsu Argus-20 CCD camera was used to capture images.

Apoptosis assay:
Caspase-3 antibody was used to determine apoptosis after a 4-16 hr monastrol treatment; 16 hrs of 1 M staurosporine was used as a positive control to demonstrate caspase-3 cleavage.

Metabolic activity assay:
MTS cell proliferation assays were performed following the manufactures’ protocol (Promega).

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Author’s contributions: K.B. performed all of the experiments shown. J.J. provided essential technical assistance in the execution of the sucrose gradients that were done in the laboratory of J.W. Both J.W. and J.J. also provided intellectual support interpreting various data. The remaining experiments were performed in the laboratory of W. S., who also provided scientific guidance in the development of the
project. The paper was written primarily by K. B. and W. S., with extensive editing and revision by J.J. and J.W.

Figure Legends:

Fig. 1. Loss of Eg5 or disruption of microtubules caused a defect in protein synthesis. 

\(^{35}\text{S Met/Cys incorporation assays were performed in RPE1 cells as indicated (A) 4 hrs after 0.13 mM monastrol or DMSO (solvent control) treatment or a 4 hr monastrol or DMSO treatment followed by a 4 hr release into the same medium without monastrol, (B) 24 hrs before or after Eg5 knockdown, or (C) a 2 hr treatment with 0.012 mM nocodazole or 0.002 mg/mL Colcemid (microtubule disruptors) or the DMSO solvent control. CHX (0.1 mg/ml) was used as a positive control in A. Results are shown as means ± s.d. and are representative of at least three independent experiments, }P\text{ values represent Students’ t-test (null hypothesis).}

Fig.2. Eg5 associates with ribosomes and links ribosomes to microtubules. A Polysome profiling of mature ribosomes from RPE1 cells. Whole cell lysates were layered on a 10-45% continuous sucrose gradient, centrifuged for 2.5 hrs at 27,000 x g, and fractionated with constant monitoring at an absorbance of 260 nm. The positions of the 40S, 60S subunits and the 80S ribosomes and polysomes are indicated. Fractions representing each of the subunits and/or ribosomes or polysomes were pooled together and proteins were concentrated using Strataclean resin, as described in materials and methods. Purified proteins were subjected to
SDS-PAGE. Immunoblots are representative of at least six independent experiments.

B Sucrose gradient fractionation of whole cell lysates on 10-25% sucrose gradients to allow further separation of the 40S, 60S subunits and 80S ribosomes without resolving the polysome fractions. Sucrose gradients were centrifuged for 4 hrs at 27,000 xg and fractionated as mentioned above. Portions of the 40S, 60S and 80S ribosome is indicated. Fractions were TCA-precipitated prior to SDS-PAGE. Immunoblots are representative of at least three independent experiments. C IP of Eg5 in RPE1 cells was conducted and immunoprecipitates were probed for the presence the 60S ribosomal subunit, as represented by rpL10A (top panel), or for the presence of the 40S ribosomal subunit, as represented by rpS5 (bottom left panel). IP of MYPT1 (bottom, right panel), an unrelated ribosomal protein, was used as a negative control; IP of Eg5, in the same panel, was completed at the same time and was used as a positive control. For each sample, 10% of the WCL and 50% of IP were loaded on the gel. Results shown are representative of at least 3 independent experiments. D Microtubule binding assays shows Eg5 required for ribosomal association with microtubule pellet. Sucrose gradient purified 40S, 60S or 80S ribosomal fractions were added to preformed taxol-stabilized microtubules for 45 min, followed by a 30 min centrifugation at 13,000 x g. Immunoblots in the presence of Eg5 are representative of at least five independent experiments and in the absence of Eg5, are representative of at least three independent transfection and/or binding assay experiments. P=microtubule pellet, S=supernatant. Assays completed in the presence (left panel) or absence (right panel) (E) of polymerized microtubules.
Fig. 3. Loss of Eg5 leads to a decrease in the 80S ribosome and increase in polysomes indicative of a delay in elongation. (A) Polysome profiling before or after a 4 hr monastrol treatment (n=6), after, or in the presence of 0.5 mM arsenite (n=2). CHX was added 10 min prior to cell lysis. Positions of the 40S subunit, 60S subunit, 80S ribosomes, and polysomes are indicated. (B) Same experiment as (A) except done in the absence of cycloheximide. These experiments were performed 4-6 times with similar results. (C) Same experiment but with S-Trityl-L-cysteine added for 2 hrs (n=4). Polysome profiling was completed as described in Fig. 2A. P/M=polysome/monosomes ratios are were calculated as described in Methods.

Fig. 4. Inhibition of Eg5 slows the rate of ribosome movement. Ribosome half-transit assays after Eg5 inhibition, as described further in Materials and Methods. Briefly, incorporation of \(^{35}\)S Met/Cys into total proteins (post mitochondrial supernatants; black diamonds) and completed proteins (post ribosomal supernatants; white diamonds) are graphed. The half-transit time was determined by linear regression analysis from the displacement in time between the two lines. Graphs shown (left) is representative of four independent experiments and bar graph (right) represents the average half-transit time before and after Eg5 inhibition.
Fig. 5. Eg5 functions during elongation. (A) U2OS cells were transfected with siRNA to Eg5 for a total of 36 hours and a bicistronic expression plasmid for a total of 24 hours. Quantification of immunoblots from five independent experiments with antibodies to HA or FLAG epitopes on the CFP (cap-independent translation) or YFP (cap-dependent translation) marker proteins are shown. Results are represented as means ± s.d., $P$ values represent Students’ t-test (null hypothesis). (B) Eg5 is proposed to support translational elongation in the cell by linking ribosomes to microtubules.

Suppl. Fig. 1. Inhibition of Eg5 causes decreased protein synthesis in various cell lines and also occurs with the use of different Eg5 inhibitors. $^{35}$S Met/Cys incorporation assays were completed after (A) a 4 hr monastrol treatment in four different cells lines, (B) after a 4 hr treatment with 0.00015 mM S-Trityl-L-cysteine (left panel), or after a 1 hr treatment with 0.003 mM dimethylenastrone (right panel). Results are shown as means ± s.d. and represent at least three representative experiments. $P$ values represent Students’ t-test (null hypothesis).

Suppl. Fig. 2. Inhibition of Eg5 causes decreased translation in proteins translation in both cytosolic and membrane compartments, while knock-down of kinesin motor Kid causes an increase in protein synthesis.

A-C $^{35}$S Met/Cys incorporation assays were completed and cells were lysed into membrane and cytosolic fractions, (A) after a 4 hr monastrol, or (B,C) 24 hrs after
one of two different siRNAs targeted to different regions of the Eg5 gene were used.

D Kinesin motor Kid was knocked-down by siRNA for 24 hrs prior to $^{35}$S Met/Cys incorporation assays. Results are shown as means ± s.d. and represent at least three representative experiments. P values represent Students’ t-test (null hypothesis).

Suppl. Fig. 3. Decrease in protein synthesis after loss of Eg5 is not due to mitotic arrest. A Examination of mitotic indices after monastrol treatment (left panel), S-Trityl-L-cysteine (middle panel) or dimethylenastron (right panel). Asterisks represent the timepoint at which the $^{35}$S Met/Cys incorporation assays were completed. B Examination of mitotic indices after a 24 hr after Eg5 knockdown (by siRNA #1 or #2) in comparison to a 4 hrs after monastrol treatment. In all experiments, a minimum of 300 cells were counted per experiment and at least three independent experiments were completed. Results are shown as means ± s.d. and represent at least three representative experiments.

Suppl. Fig. 4. Decrease in protein synthesis after loss of Eg5 is not due to cell death or decrease metabolic activity. A Apoptosis in RPE1 cells after a 0.13 mM monastrol treatment at various time points was investigated. Staurosporine was used as a positive control for caspase-3 cleavage. B Metabolic activity after a 24 hr knockdown of Eg5 by two different siRNAs. C Metabolic activity assay after a 4 hr monastrol treatment, or after a 4 hr monastrol treatment followed by a 4 hr washout. Results are shown as means ± s.d. and represent at least three representative experiments.
Suppl. Fig. 5. Decrease in 80S ribosome and increase of polysomes after Eg5 inhibition is not due to cellular stress. Polysome profiling of cells serum starved for 32 hr resulted in decreased 80S ribosomes as well as decreased polysomes and is not consistent with that seen after Eg5 inhibition.