EGF Induced Centrosome Separation Promotes Mitotic Progression and Cell Survival

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Inventory of Supplementary Materials:

Supplementary Figures

Supplementary Movies

Supplementary Experimental Procedures

Supplementary References

Supplementary Figures and Legends:

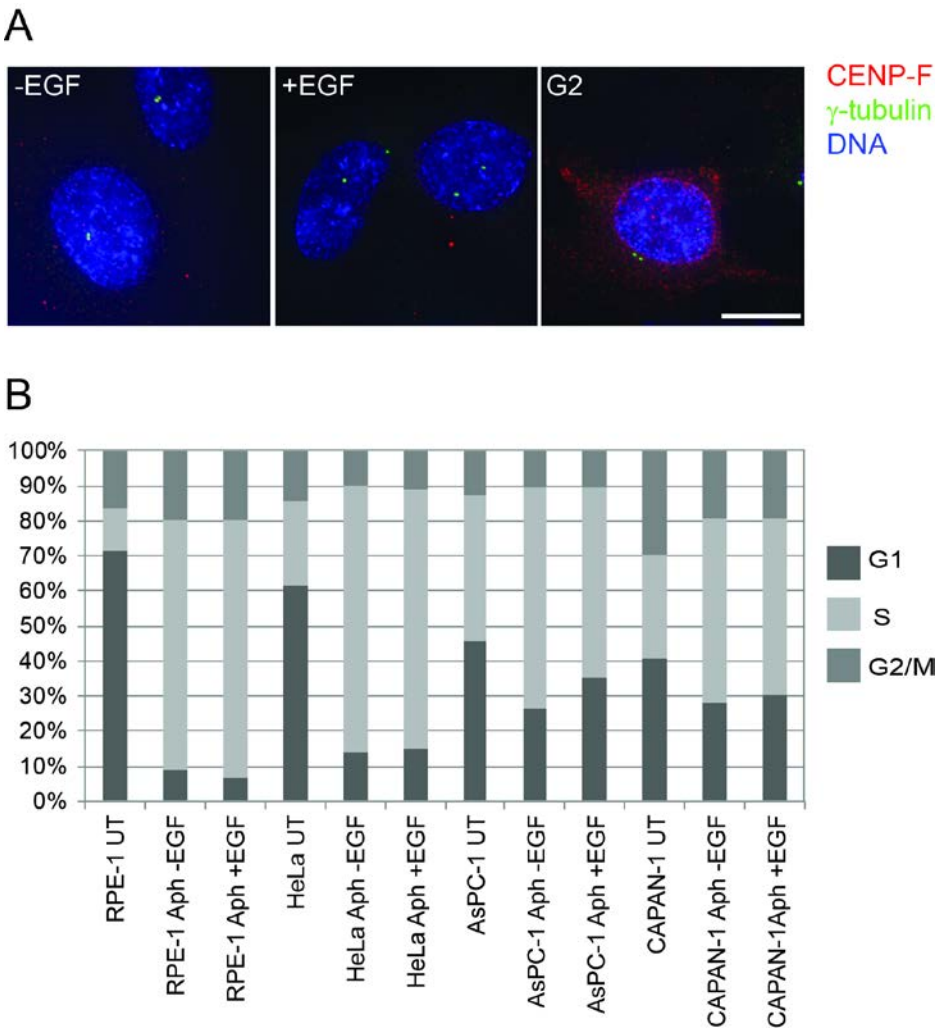


Figure S1. EGF Treated Cells Stay in S Phase in the Presence of Aphidicolin. (Related to Figure 1):

(A) HeLa cells treated as in Figure 1A were fixed and stained with γ-tubulin and CENP-F antibodies. As a control for CENP-F staining (indicated as G2), cells were released from aphidicolin arrest and collected after 4 hours. Scale bar, 10 μm.

(B) RPE-1, HeLa, AsPC-1 and CAPAN-1 cells were either kept untreated or treated with aphidicolin in the presence or absence of EGF (50 ng/ml). Cells were then fixed in ethanol and subjected to FACS analysis by their total DNA content.

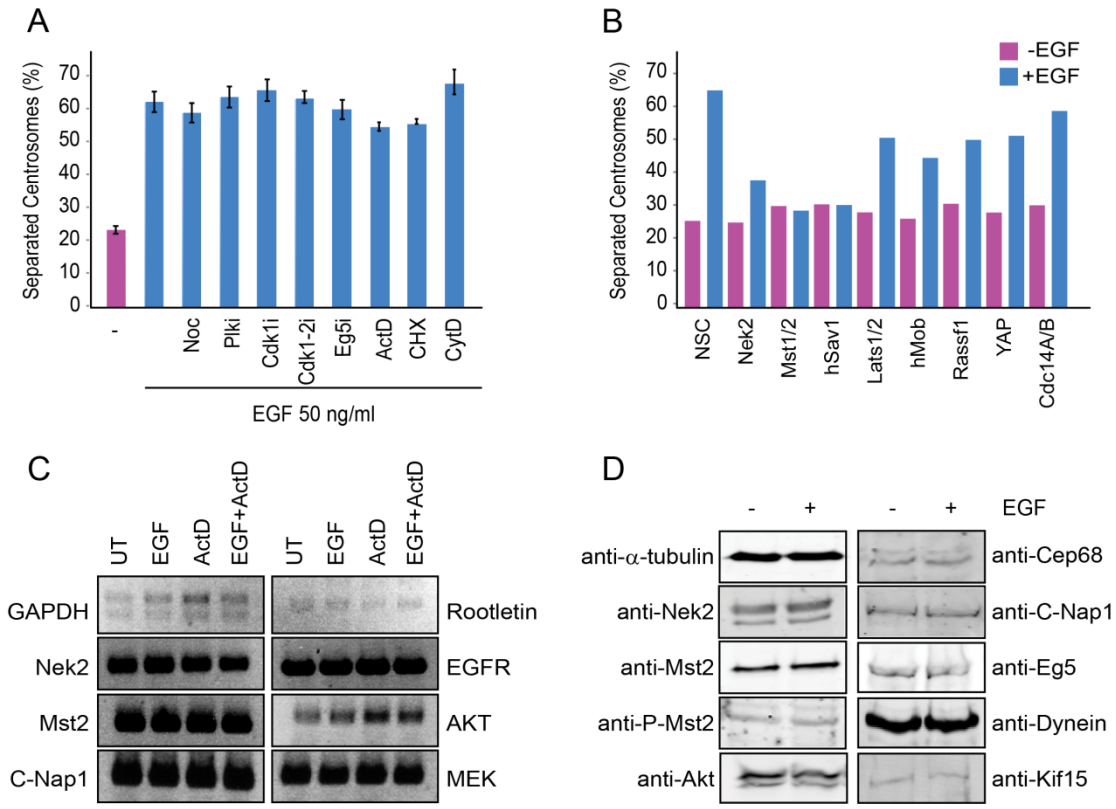


Figure S2. EGF Dependent Centrosome Separation is Independent of Additional Hippo Pathway Components, Major Mitotic Kinases and EGF Addition does not Increase Transcription or Translation of EGFR or Centrosome Separation Pathway Components. (Related to Figure 2):

(A) HeLa cells were arrested in S phase and were incubated with the indicated inhibitors. Cells were then stained with γ-tubulin antibodies and scored for the number of cells with separated centrosomes. Results are from three independent experiments. Data are mean ± SD.

(B) HeLa cells were transfected with indicated siRNA oligos, arrested in S phase and either kept untreated or treated with EGF (50 ng/ml). Cells were then stained with γ-tubulin antibodies and scored for the number of cells with separated centrosomes.

(C) HeLa cells were arrested in S phase, kept untreated or treated with EGF (50 ng/ml) in the absence or presence of ActinomycinD for the inhibition of transcription. Cells were lysed and the level of mRNA was analysed by RT-PCR.

(D) Extracts of HeLa cells that were arrested in S phase. Cells were kept untreated or treated with EGF (50 ng/ml). Immunoblots were analysed with indicated antibodies.

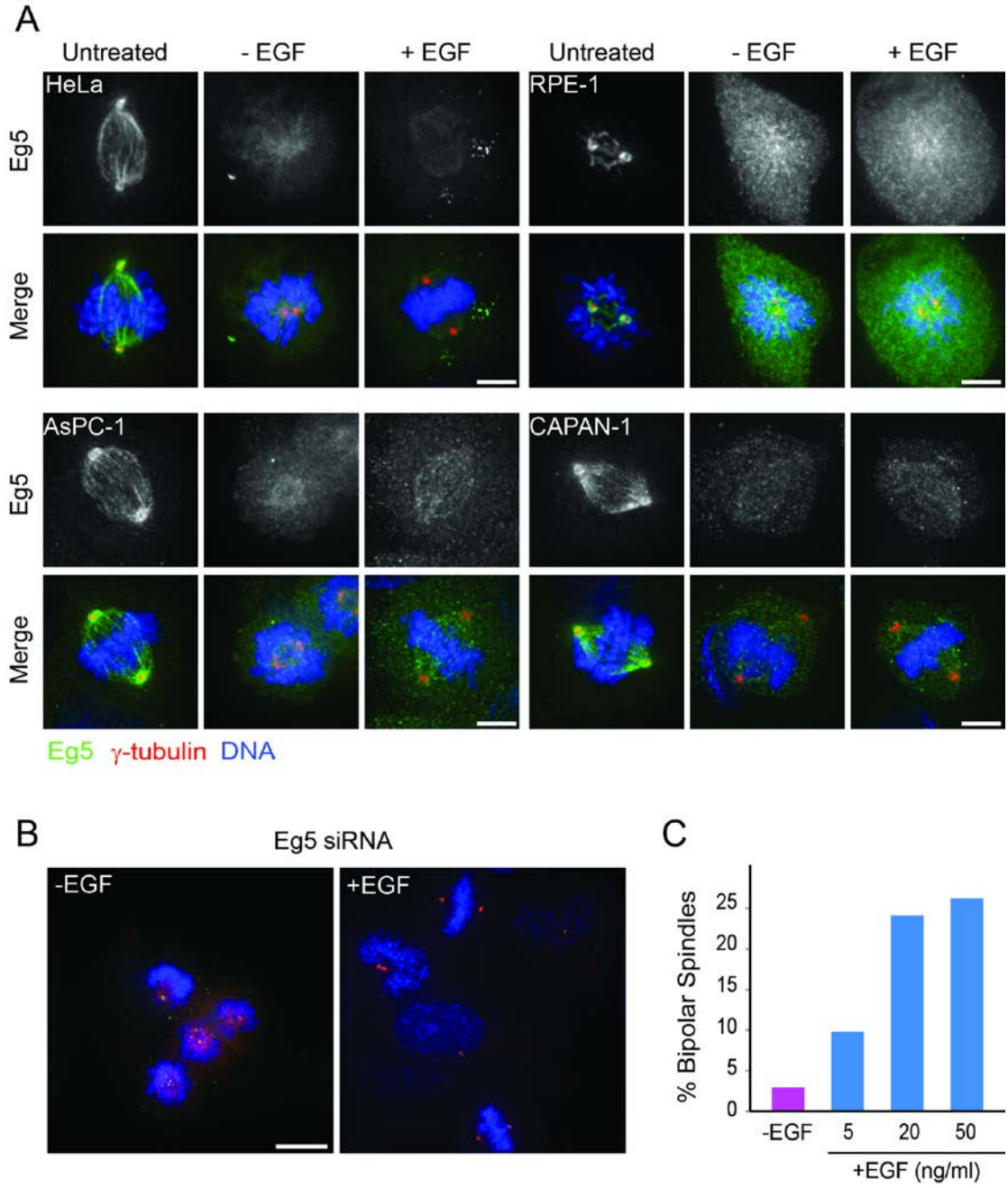


Figure S3. Bipolar Spindle Formation with low or no Eg5 Activity. (Related to Figure 3):

(A) HeLa, RPE-1, CAPAN-1 and AsPC-1 cells were arrested in S phase, released into STLC containing medium with or without EGF. Cells were then fixed and stained with γ-tubulin and Eg5 antibodies. Note that even in cells with bipolar spindles, Eg5

localization is lost from the spindle poles indicating that the spindle bipolarity is established without Eg5 activity. Scale bars, 5 μm.

(B) HeLa cells were transfected with indicated siRNA oligos against Eg5 and kept untreated or treated with EGF (50 ng/ml). The cells were then fixed and stained with centrin and γ-tubulin antibodies. Scale bar, 10 μm.

(C) Cells in Figure S3B were scored for the formation of the bipolar spindles.

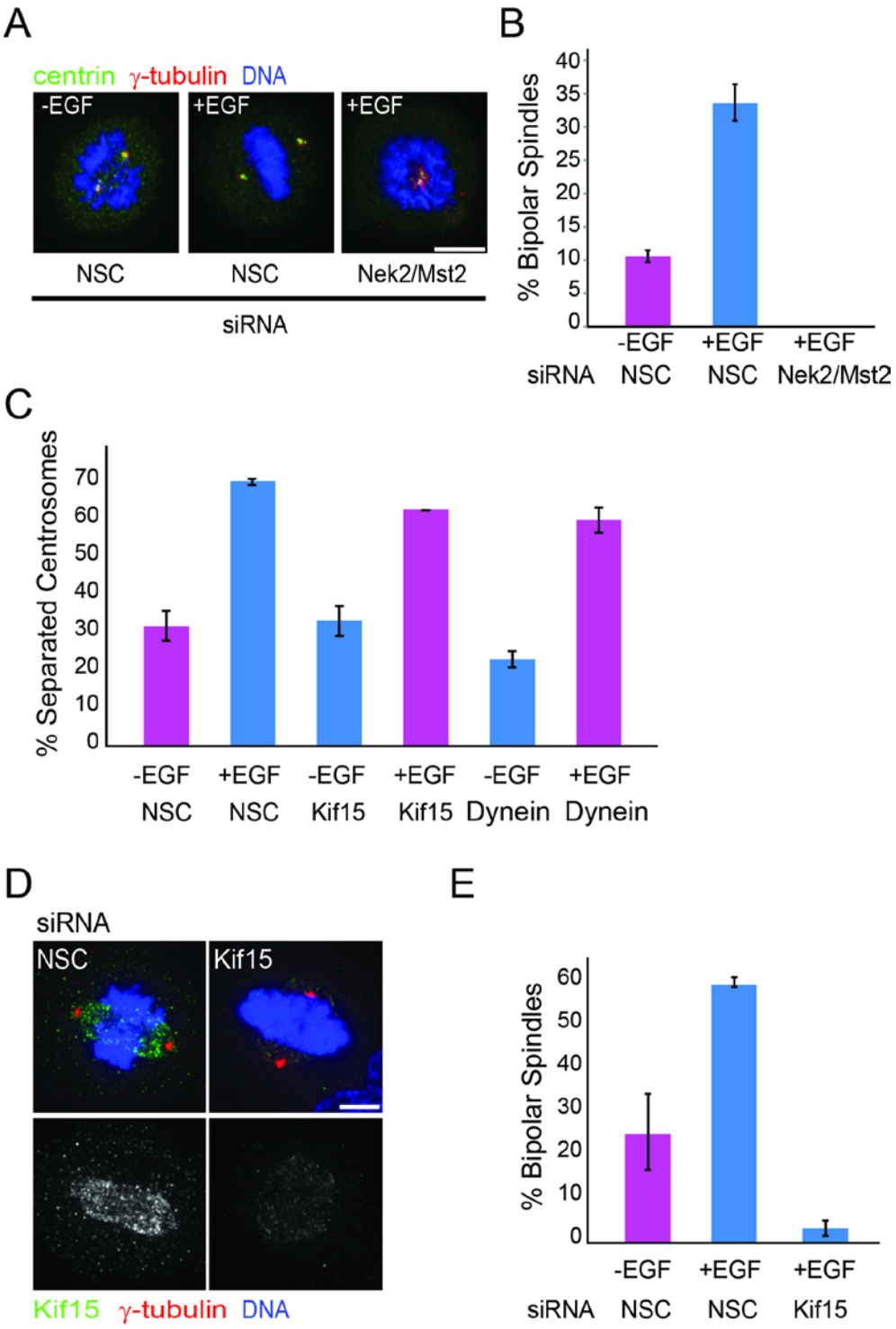


Figure S4. Mitotic Progression in the Absence of Eg5 Activity. (Related to Figure 3):

(A) HeLa cells were transfected with indicated siRNA oligonucleotides, arrested in S phase, released into G2 either with or without EGF addition, and arrested again in

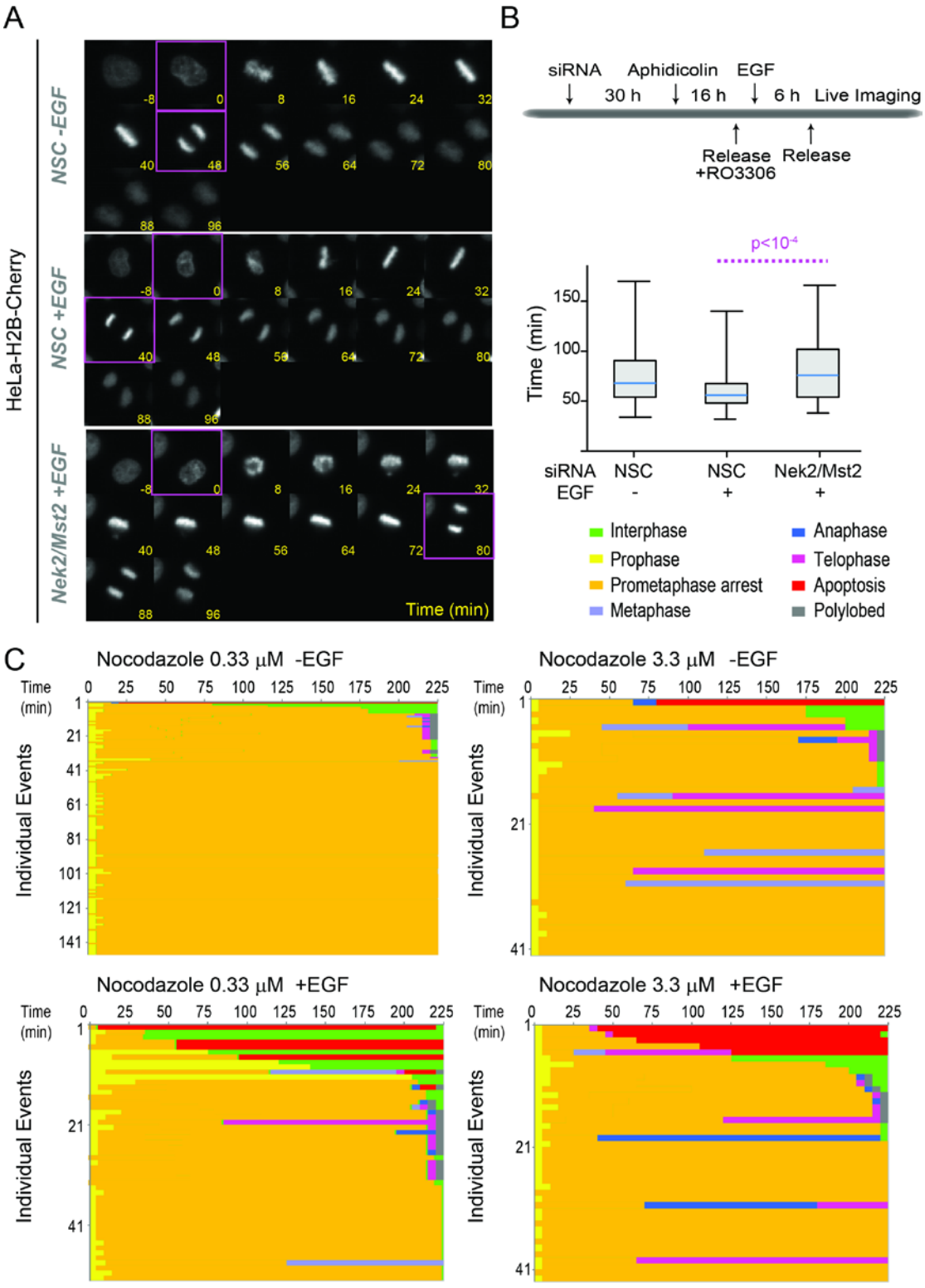
early mitosis by 5 μM STLC for four hours. Cells were then fixed and stained with centrin and γ-tubulin antibodies. Scale bar, 5 μm.

(B) Cells were treated as in Figure S4A. Cells were analysed for their ability to form bipolar spindles. Results are from three independent experiments. n>50 cells counted for each condition. Data are mean ± SD.

(C) HeLa cells that were treated either with non-specific of HKlp2/Kif15 specific siRNa oligos were arrested in S phase by aphidicolin. Cells were then treated with 50 ng/ml (green bars) for four hours in the presence or absence of the dynein inhibitor. The percentage of cells with separated centrosomes was scored. Results are from three independent experiments. n ≥ 150 cells were counted in each case. Data are presented as mean ± SD.

(D) HeLa cells were transfected with the indicated siRNA oligos. Cells were fixed and stained with HKlp2/Kif15 and γ-tubulin antibodies. Scale bar, 5 μm.

(E) HeLa cells were transfected with indicated siRNA oligos, treated with or without EGF (50 ng/ml) and re-arrested in early mitosis with 5 μM STLC for four hours. Cells were then analysed for their ability to form bipolar spindles. Results are from three independent experiments. n>50 cells counted for each condition. Data are mean ± SD.



Supplemental Figure 5. (Related to Figure 4):

Centrosomal Linker is Critical for rapid Mitotic Progression

(A) Still images were taken from movies of HeLa cells stably expressing H2B-Cherry. Cells were transfected with indicated siRNA oligos, arrested in S phase, and

then released into a G2 arrest by Cdk1 inhibition with or without EGF treatment. Upon release from G2 arrest cells were imaged every two minutes. T = 0 was defined as the time point at which NEBD was first observed. The start and the end point of the quantifications are indicated with pink squares.

(B) The average time of cells from chromosome condensation to anaphase was quantified for the cells in Figure S5A. [p (NSC^{-EGF}/NSC^{EGF}) $<10^{-4}$; p (NSC^{EGF}/Nek2-$Mst2^{EGF}$$<10^{-4}$)]. Box-and-whiskers plots: boxes show the upper and lower quartiles (25-75%) with a line at the median, whiskers extend to the minimum and the maximum values.

(C) Spindle Assembly Checkpoint is Active in EGF Treated Cells. HeLa cells stably expressing H2B-mCherry cells were arrested in S phase and released in the absence or presence of EGF. Cells were then treated with different concentrations of Nocodazole and imaged for 15 hours. Mitotic events (n = 141 for 0.33 µM nocodazole –EGF; n = 41-50 for the other conditions) were automatically classified and plotted by CellCognition. Each bar represents one single cell. Most cells remained arrested in prometaphase during the course of the experiment. Note that the differences in mitotic events between untreated and EGF treated cells are not statistically significant.

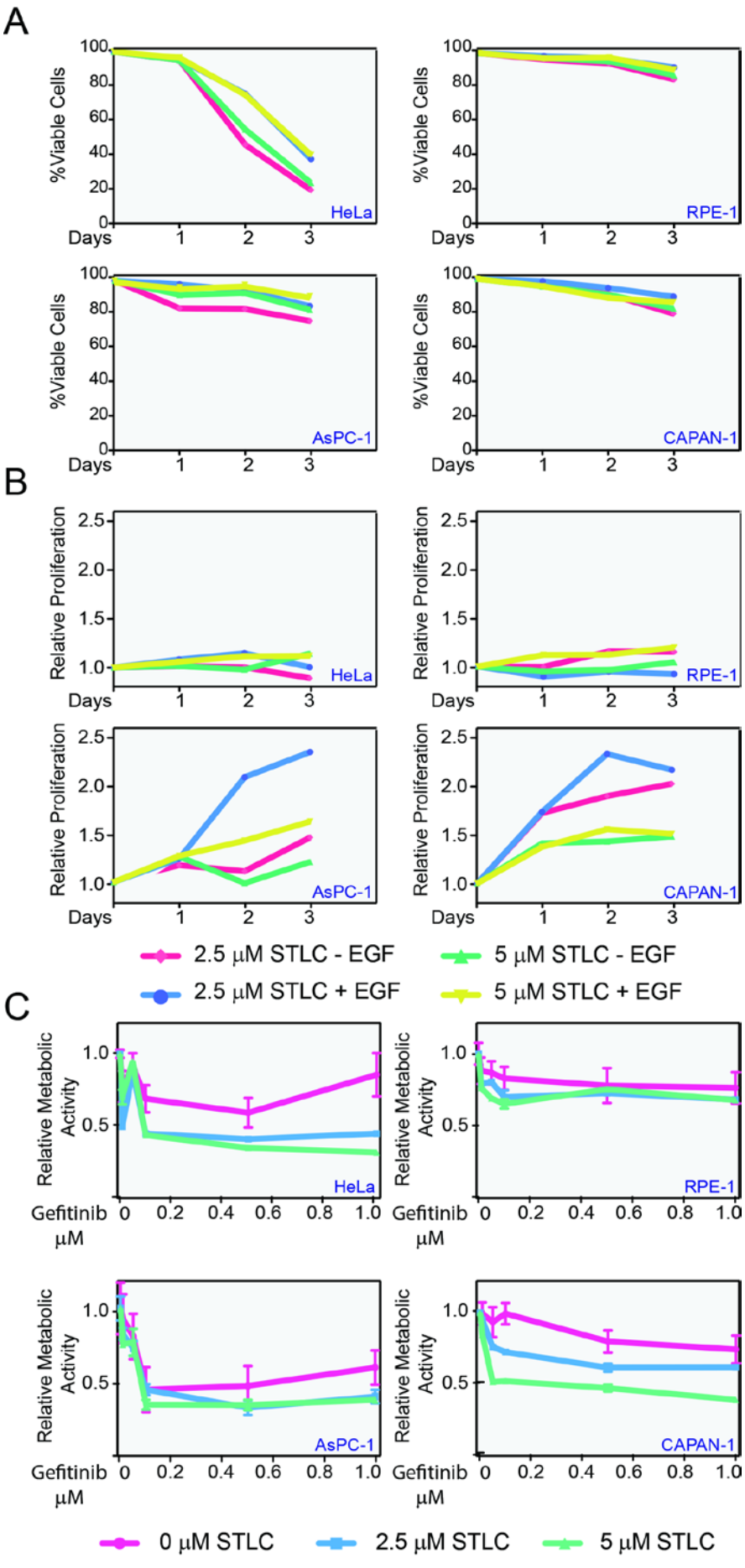

A
%Viable Cells
Days
HeLa
RPE-1
AsPC-1
CAPAN-1
B
Relative Proliferation
2.5 μM STLC - EGF
5 μM STLC - EGF
2.5 μM STLC + EGF
5 μM STLC + EGF
C
Relative Metabolic Activity
Gefitinib μM
0 μM STLC
2.5 μM STLC
5 μM STLC

Figure S6. Cell Line Response to Eg5 and EGFR Inhibition. (Related to Figure 7):

(A, B) RPE-1, HeLa, AsPC-1 and CAPAN-1 cells were treated with or without 50 ng/ml EGF and incubated in the presence of STLC (2.5 or 5 μM) for three days. The percentage of viable cells (A) and relative proliferation of the cells (B) were analysed by the Guava ViaCount. This assay uses a mixture of two DNA binding dyes: a membrane-permeable dye that stains all nucleated cells and a membrane-impermeable dye that only stains damaged cells thus giving a measure of the number of dying cells within a culture.

(C) RPE-1, HeLa, AsPC-1 and CAPAN-1 cells were treated with different combinations of EGFR kinase inhibitor Gefitinib (0, 0.01, 0.05, 0.1, 0.5 and 1 μM) and Eg5 inhibitor STLC (0, 2.5 or 5 μM) for three days. The metabolic activity of the cells were analysed by the MTT assay and is presented relative to the activity of untreated cells.

Supplementary Movie Legends

Movie S1. HeLa cells stably expressing H2B-Cherry were first arrested in S phase, released into G2 without EGF addition, and then imaged every 6 min in the presence of 5 μM STLC. (Related to Figure 3)

Movie S2. HeLa cells stably expressing H2B-Cherry were first arrested in S phase, released into G2 with EGF addition (50 ng/ml), and then imaged every 6 min in the presence of 5 μM STLC. (Related to Figure 3)

Movie S3. HeLa cells stably expressing eGFP-LaminA and H2B-Cherry were arrested in S phase, then released without EGF treatment. 5 hours after release cells were imaged every three minutes. (Related to Figure 4)

Movie S4. HeLa cells stably expressing eGFP-LaminA and H2B-Cherry were arrested in S phase, then released with EGF treatment (50 ng/ml). 5 hours after release cells were imaged every three minutes. (Related to Figure 4)

Movie S5. HeLa cells stably expressing H2B-Cherry were transfected with control siRNA oligos, arrested in S phase, then released into a G2 arrest by Cdk1 inhibition without EGF treatment. Upon release from G2 arrest cells were imaged every two minutes. (Related to Figure S5)

Movie S6. HeLa cells stably expressing H2B-Cherry were transfected with control siRNA oligos, arrested in S phase, then released into a G2 arrest by Cdk1 inhibition with EGF addition (50 ng/ml). Upon release from G2 arrest cells were imaged every two minutes. (Related to Figure S5)

Movie S7. HeLa cells stably expressing H2B-Cherry were transfected with Nek2/Mst2 siRNA oligos, arrested in S phase, then released into a G2 arrest by Cdk1

inhibition with EGF addition (50 ng/ml). Upon release from G2 arrest cells were imaged every two minutes. (Related to Figure S5)

Supplementary Experimental Procedures

Cell Lines and Treatments

For transient transfection of siRNAs, Lipofectamine 2000 or the Neon Transfection System was used as recommended by the manufacturer (Invitrogen). For RNA interference experiments cells were transfected with 50-100 µM siRNA duplexes.

Name	*Sequence*	*Over*	*Company*
NSC-47%GC	AGGUAGUGUAAUCGCCUUG	tt	MWG
Eg5	CCAUCAACACUGGUAAGAA	tt	Ambion
Sav1-48	AAAUUCGGAUGACUCAACUCGUUCC		Invitrogen
YAP1	GACAUCUUCUGGUCAGAGA	tt	MWG
Mst1/STK4-si1	ACAGCUUCUUGCUAAUACA	tt	MWG
Mst2/STK3-si2	ACCUCCUUAUGCUGAUAUA	tt	MWG
Lats1-si2	UAGCAUGGAUUUCAGUAAU	tt	MWG
Lats1-si1	CUAACAACAGAAGUAUAGA	tt	MWG
Lats2-si1	GGUUCUCUAUAGGAACUAC	tt	MWG
Lats2-si2	GGUUCUCUAUAGGAACUAC	tt	MWG
Mob1A	GCACCAAAGTATATTGATT	tt	MWG
Mob1B	GCAGATGGTACTAATATTA	tt	MWG
Nek2-siGendub8	AAACAUCGUUCGUUACUAU	tt	MWG/
Nek2-siGendub6	GAAAGGCAATACTTAGATG	tt	MWG/
C-Nap1-si	OnTarget Smart Pool		Dharmacon

siRNA duplexes used in this study

Cells were arrested in S phase with 1.6 µg/ml aphidicolin (Sigma). To arrest cells in G2, cells were first incubated with aphidicolin for 16 hours, released into fresh medium containing the cyclin dependent kinase (Cdk) inhibitor RO3306 (Calbiochem) at 5 µM. For inhibition of Eg5 motor activity, STLC (Sigma) was used at concentrations of 2.5 or 5 µM. Mitotic kinases were inhibited with 100 nM BI2536

(Plk1), 5 µM RO3306 (Cdk1) and 5 µM Roscovitine (Cdk1/Cdk2).

For the induction of centrosome separation, EGF (Life Technologies) was used at concentrations ranging from 1 ng/ml to 100 ng/ml. To inhibit kinases of the EGF signalling pathway following inhibitors were used: Gefitinib to inhibit EGFR (kind gift of O. Sahin) at a concentration of 10 µM, U0126 to inhibit MEK1/2 kinases (kind gift of O. Sahin) at 10 µM, SB203580 (Tocris) to inhibit p38 MAPK, InSolution™ Akt Inhibitor IV (Calbiochem) and LY294002 (Cell Signalling Technologies) to inhibit PI3K at 10 µM, PTP45 to inhibit PTEN phosphatase and Raf Inhibitor IV, V and VI to inhibit Raf kinases (Calbiochem). ActinomycinD (Sigma) was used at 2.5 µg/ml to inhibit transcription, Cycloheximide (Sigma) was used at 100 µM to inhibit translation, MTs were depolymerized with 1 µg/ml nocodazole (Sigma) and CytochalasinD (Sigma) at 5 µg/ml to interfere with the actin cytoskeleton.

Antibodies

Following antibodies were used: anti-centrin (Mardin et al., 2010), anti-Mst2 (this study), anti-P-Mst1/2-T183/T180 (Cell Signalling), anti-Nek2 (this study), anti-C-Nap1 (this study), anti-rootletin (this study), anti-γ-tubulin (Sigma-Aldrich and Abcam), anti-alpha-tubulin (Sigma-Aldrich), anti-CENP-F (BD Transduction), anti-CENP-A (MBL), anti-Eg5 (kind gift from T. Mayer), anti-dynein (Milipore), anti-HKlp2/Kif15 (kind gifts of I. Vernos and R. Medema), anti-Akt anti-P-Akt(S473), anti-P-ERK (p44/p42), anti-P-MEK1/2, anti-EGFR and anti-GAPDH (Cell Signalling Technology). Secondary antibodies were anti-rabbit or anti-goat IgG coupled to Alexa Fluor 680 (Invitrogen), anti-mouse IgGs coupled to IRDye 800 (Rockland). Secondary antibodies for indirect immunofluorescence were donkey anti-rabbit IgGs coupled to Alexa Fluor 488, Alexa Fluor 594 or Alexa Fluor 647, donkey anti-mouse

IgGs coupled to Alexa Fluor 555 or Alexa Fluor 488 or Alexa Fluor 647and donkey anti-goat IgG coupled to Alexa Fluor 555 (Invitrogen).

Flow Cytometry

Cell cycle profiles were determined by measuring cellular DNA content via flow cytometry. Briefly, cells were washed twice with ice-cold PBS and resuspended in 1.5 ml of 70% ice cold-ethanol. Fixed cells were collected by centrifugation, washed twice with PBS, treated with 0.1 mg/ml of RNaseA for 30 min, 37°C and stained with 0.01 mg/ml propidium iodide solution. Profiles were determined with a FACS Canto instrument (BD Biosciences) and analyzed using FACS Diva Software (BD Biosciences).

Immunofluorescence Microscopy

For indirect immunofluorescence, cells were fixed with ice-cold methanol for 5 min or 3% PFA for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min, blocked with 10% FCS for 30 min and stained with antibodies in 3% BSA-PBS. DNA was stained with 0.2 g/ml Hoechst 33342 (Calbiochem).

Sample Preparation for Live Cell Imaging

Cells were seeded into chambered coverglasses (LabTEK: Thermo Fisher Scientific) whose lids were sealed with baysilone paste (Neolab). At least 30 min before imaging, culture medium was exchanged by prewarmed imaging medium (CO_2 independent medium without phenol red with 20% FCS, 2 mM glutamine, 100 mg/ml penicillin and streptomycin). Live cell microscopy was performed in 37˚C microscope incubators (EMBL GP106) on the microscopes indicated in the different assays.

FISH Experiments

Cells were grown on coverslips and fixed in ice-cold methanol. Fluorescence in situ hybridization was carried out following standard procedures. Briefly, the coverslips were washed in SSC 2X, incubated with pepsin, and fixed in 1% formaldehyde, followed by dehydration in ethanol series. Nuclei were denatured for 5 minutes at 75°C and hybridization was performed at 40°C overnight. Subsequently, cells were washed at 66°C in 2X SSC buffer (0.30 M sodium citrate and 0.030 M NaCl) for 10 minutes and twice in 0.4X SSC for 7 minutes. Cells were mounted in Vectashield with DAPI (H 1200; Vector Laboratories, Burlingame, CA, USA). Probe labelling for chromosomes 1, 3, 8 and 17 centromeric sequences was carried out by nick translation with DY-495-aadUTP or DY-547-aadUTP (Dyomics, Jena, Germany). Capturing of fluorescence images and counting of signals was performed on an Axiovert 200 M epifluorescence microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with a Plan-Apochromat 63X/1.4 objective.

RT-PCR and Immunoblotting

For RT-PCR analyses, cells were seeded in 6-well plates and after indicated treatments, they were collected and the total mRNA was isolated by column based RNA purification kit (Qiagen). Both cDNA synthesis and PCR were combined with one-step PCR kit as described by the manufacturer (Invitrogen). Gene-specific primers that were used for the amplification of particular transcripts are summarized below

Name	Sequence
Mst2-RT-fw	AGTACTGTGGCGCTGGCTCTGTCT
Mst2-RT-rev	CTGGCTTTCTGAATGTTGGTGGTG
Nek2-RT-fw	TGGGGAAAGTAAAGAGAACATCAT
Nek2-RT-rev	TTAAAAGCCCAACCAAGAAAGTAT
hSav1-RT-fw	CAGGGGAGGCATGCTTCAGGTATT
hSav1-RT-rev	ACAGGGGCTCGTGCGTAAAC
Mst2-RT-fw	AGTACTGTGGCGCTGGCTCTGTCT
Mst2-RT-rev	CTGGCTTTCTGAATGTTGGTGGTG
Nek2-RT-fw	TGGGGAAAGTAAAGAGAACATCAT
Nek2-RT-rev	TTAAAAGCCCAACCAAGAAAGTAT
hSav1-RT-fw	CAGGGGAGGCATGCTTCAGGTATT
hSav1-RT-rev	ACAGGGGCTCGTGCGTAAAC
C-Nap-RT-fw	GAGGCCCAGCAGAGACAAGCAACC
C-Nap-RT-rev	CACAACCTCCCGCCATAGACTGA
root-RT-fw	TGGCGCTGCAGGAGGAGAGTGT
root-RT-rev	CTTAGCCAGGGCCCCATTCAGC
EGFR-RT-1	TCCTGGGCAAAGAAGAAAC
EGFR-RT-2	TTGGGGTGATGGCTAAAGGAGATT
EGFR-RT-3	CCCCCAGCGCTACCTTGTCATTCA
EGFR-RT-4	AGTGCTGTGGGGGTCCTGGTAGTG
AKT-RT-fw	GGGGGATGGGCCAGGGTTTA
AKT-RT-rev	TTGAAGAATTTGGAGGGAAGGTT
MEK-RT-fw	GCCTTAACCAGCCCAGCACACC
MEK-RT-rev	ATCCCGAAAATACAGGCAGACAGC

RT-PCR primers used in this study

For immunoblotting, cells were seeded in 6-well plates and after extraction with complete Tris lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) the amounts of protein in the extract were determined by Bradford assay and equal amount of extracts were loaded on pre-cast SDS gradient gels (Bio-Rad). After immunoblotting, signals were measured using fluorescently labeled secondary antibodies on a LiCOR Odyssey scanner (LI-COR Biosciences), which is able to linearly measure signals over a 10000 range.

Supplementary References:

Mardin, B.R., Lange, C., Baxter, J.E., Hardy, T., Scholz, S.R., Fry, A.M., and Schiebel, E. (2010). Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. Nat Cell Biol *12*, 1166-1176.