Extra centrosomes and/or chromosomes prolong mitosis in human cells

Control of mitotic duration

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Using laser microsurgery and cell fusion we have explored how additional centrosomes and/or chromosomes influence the duration of mitosis in human cells. We find that doubling the chromosome number adds ~10 minutes to a 20 minute division while doubling the number of centrosomes adds ~30 minutes more, and extra centrosomes and/or chromosomes prolong mitosis by delaying satisfaction of the spindle assembly checkpoint. Thus mitosis can be prolonged by non genetic means and extra chromosomes and centrosomes likely contribute to the elevated mitotic index seen in many tumors.

The spindle assembly checkpoint (SAC) prolongs mitosis until all kinetochores are stably attached to spindle microtubules (MTs). In organisms with low chromosome numbers, like flies[1] or fission yeast, the SAC is not essential because spindles form rapidly. However, in higher eukaryotes where the attachment of all chromosomes can take hours[2] the SAC is essential.

In animals kinetochore attachment to the spindle is envisioned to occur via a stochastic exploration of space by dynamic MTs nucleated from the centrosomes[3]. This "search-and-capture" mechanism predicts that spindle assembly would be delayed in cells with extra chromosomes and accelerated in cells with extra centrosomes. Surprisingly, the little data existing on this topic is counterintuitive suggesting that extra chromosomes[4,5] and/or centrosomes[6] have little influence on the duration of mitosis.

To systematically explore this issue we followed cultures of diploid human cells at 37^{o}C by time-lapse video LM. These records revealed that the duration of mitosis, defined as the interval between nuclear envelope breakdown (NEB) and anaphase onset, is 19 ± 3 minutes in telomerase-immortalized retinal pigment epithelial (RPE-1) cells (mean ± SD; n = 200; throughout the text n represents the number of cells pooled from ≥3 independent experiments). Next we inhibited cytokinesis in RPE-1 cultures with 0.2 μM cytochalasin D. After 16 hrs these cultures contained binucleated cells that after a thorough washing[7] entered the next mitosis within 24 hours. Since binucleated cells contain twice the normal number of centrosomes (and chromosomes) they formed multipolar spindles during mitosis. Relative to 2N controls in these cultures, which averaged 20 ± 4 (n = 130) minutes in mitosis, binucleated cells averaged 49 ± 17 (n = 90) minutes (Fig. 1a). Thus, doubling the chromosome number, or doubling the centrosome number, or both, prolongs mitosis >2X. Although binucleated RPE-1 cells initially form tetrapolar spindles, these subsequently transform into bipolar or tripolar spindles so that 52% of the cells ultimately divided into two daughters (Fig. 1a, Supplementary Information, Fig. S1 online) while 48% divided into three. This prolongation of mitosis in binucleated RPE-1 cells is due to a delay in satisfying the SAC because binucleated cells entered anaphase 17-22 minutes (Fig. 1b, n = 6) after microinjection with Mad2-ΔC, a dominant-negative form of Mad2 that abrogates the SAC[8].

Since cancer cells often contain extra chromosomes and centrosomes one would expect their mitosis to be prolonged. Indeed, we found that HeLa (modal chromosome number 80-85, ATCC catalog) average 46 ± 19 (n = 200) minutes in mitosis (see also Supplementary Table 1 online). As in RPE-1, this prolongation is due to the SAC because depleting Mad2 with siRNA induces HeLa to exit mitosis ~15 minutes after NEB [9]. We also found that mitosis in binucleated HeLa containing 2X more chromosomes and centrosomes averages 88 ± 35 (n = 104) minutes. However, unlike RPE-1 cells, 97% of binucleated HeLa cells formed stable tri- or tetra-polar spindles that produce three or four daughter cells.

The duration of mitosis in binucleated rat kangaroo (PtK_1) cells is reported to be similar to that of mononucleated cells (57 versus 50 minutes[6]). This contrasts sharply with our finding that mitosis in binucleated RPE-1 cells is prolonged >2X relative to mononucleated controls. The reasons for this discrepancy are unknown. However, since the 2N chromosome number in PtK_1 is 12, binucleated cells enter mitosis with 24 chromosomes which is 4 times fewer than in

binucleated RPE-1 cells (and the earlier data do show that, relative to mononucleated PtK_1 cells, mitosis takes 7 minutes longer in binucleated cells).

Is it the extra centrosomes, extra chromosomes, or both, that prolongs mitosis in binucleated RPE-1cells? To answer this question we inhibited cytokinesis in RPE-1 expressing human centrin-1/GFP to generate binucleated G1 cells containing two labeled centrosomes[10]. We then removed one centriole pair (i.e., a centrosome) by laser microsurgery[11]. To eliminate the possibility that the cytochalasin used to induce bi-nucleation triggered the p38 stress checkpoint [10,7] in our experimental cells, we prophylactically treated the cultures continuously, starting 30 minutes before adding cytochalasin D, with a p38 inhibitor (20-μM SB203580)[12]. With time these binucleated cells entered mitosis in the presence of two centrosomes and formed bipolar spindles (Fig. 1c) that took 33 ± 5 minutes (n = 10) to enter anaphase. Each division produced two daughter G1 cells containing one centrosome and a 4N nucleus that cycled into the next mitosis which lasted 29 ± 5 minutes (n = 6; Fig. 1d). By contrast, surrounding mononucleated 2N cells with 2 centrosomes, and binucleated cells with 4 centrosomes averaged, respectively, 22 ± 3 minutes (n = 100) and 56 ± 20 minutes (n = 80) in mitosis. Thus, doubling the chromosome number increases the duration of mitosis by ~50%.

Mitosis in RPE-1 cells with twice the normal chromosome number requires ~30 min in the presence of 2 centrosomes but ~50 min in the presence of 4 centrosomes. This implies that doubling the centrosome number in diploid cells will prolong mitosis by ~100%. To confirm this we fused diploid G1 RPE-1 cells with G1 cytoplasts to create normal 2N G1 cells with an extra centrosome (Fig. 2). After DNA and centrosome replication these cells entered a mitosis that lasted 53 ± 21 min (n = 11), similar to binucleated cells that enter mitosis with 4 centrosomes (49 ± 17 min). Of the eleven cells followed for this experiment, ten formed bipolar spindles and divided into two progeny after first forming tetrapolar spindles. Clearly, doubling the centrosome number in human cells prolongs mitosis ~3X more than doubling the chromosome number.

Intuitively extra chromosomes are expected to prolong mitosis because each contains sister kinetochores that must become stably attached to the spindle. It is unknown why extra centrosomes delay satisfaction of the SAC. In RPE-1 this delay could result from the progressive reorganization of a tetrapolar spindle into a tri- or bipolar spindle (Fig. 1a, Supplementary Information Fig. S1). However, as in RPE1, mitosis is also prolonged ~2X in HeLa cells containing 4 centrosomes that do not undergo this spindle reorganization. Possibly the presence of multiple MT asters negatively affects the stability of kinetochore attachments on a transient basis.

Our findings partly explain why tumors exhibit an enhanced mitotic index relative to surrounding tissues. Usually this phenomenon is ascribed to an abnormally accelerated cell cycle[13]. However, our data suggest that mitosis is prolonged in cancer cells because many are hyper-diploid (polyploid) and/or contain extra centrosomes[14,15]. Indeed, a strong positive correlation exists between the average duration of mitosis in cancer cell lines and the incidence of multipolar chromosome distribution (Supplementary Information, Table 1). The same is true for transformed lines: on average fully transformed BJ-ELR cells spend ~ 50% longer in mitosis than their parental BJ cells (33 versus 20 min), and BJ-ELR form multipolar spindles 12X more frequently than BJ cells (~70% of BJ-ELR cells are also hyper-diploid). The occurrence of bipolar and multipolar mitotic populations also explains the high cell-to-cell variability in the duration of mitosis in cancer cell lines (Supplementary Online Fig. S2). In contrast, cancer cells having normal chromosome and centrosome complements (HT1080) can divide in less than 20 minutes (Supplementary Information Fig. S2 and Table 1 online). Thus, mitosis can be prolonged by non-genetic means.

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Competing financial interests

The authors declare that they have no competing financial interests.

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Figure Legends

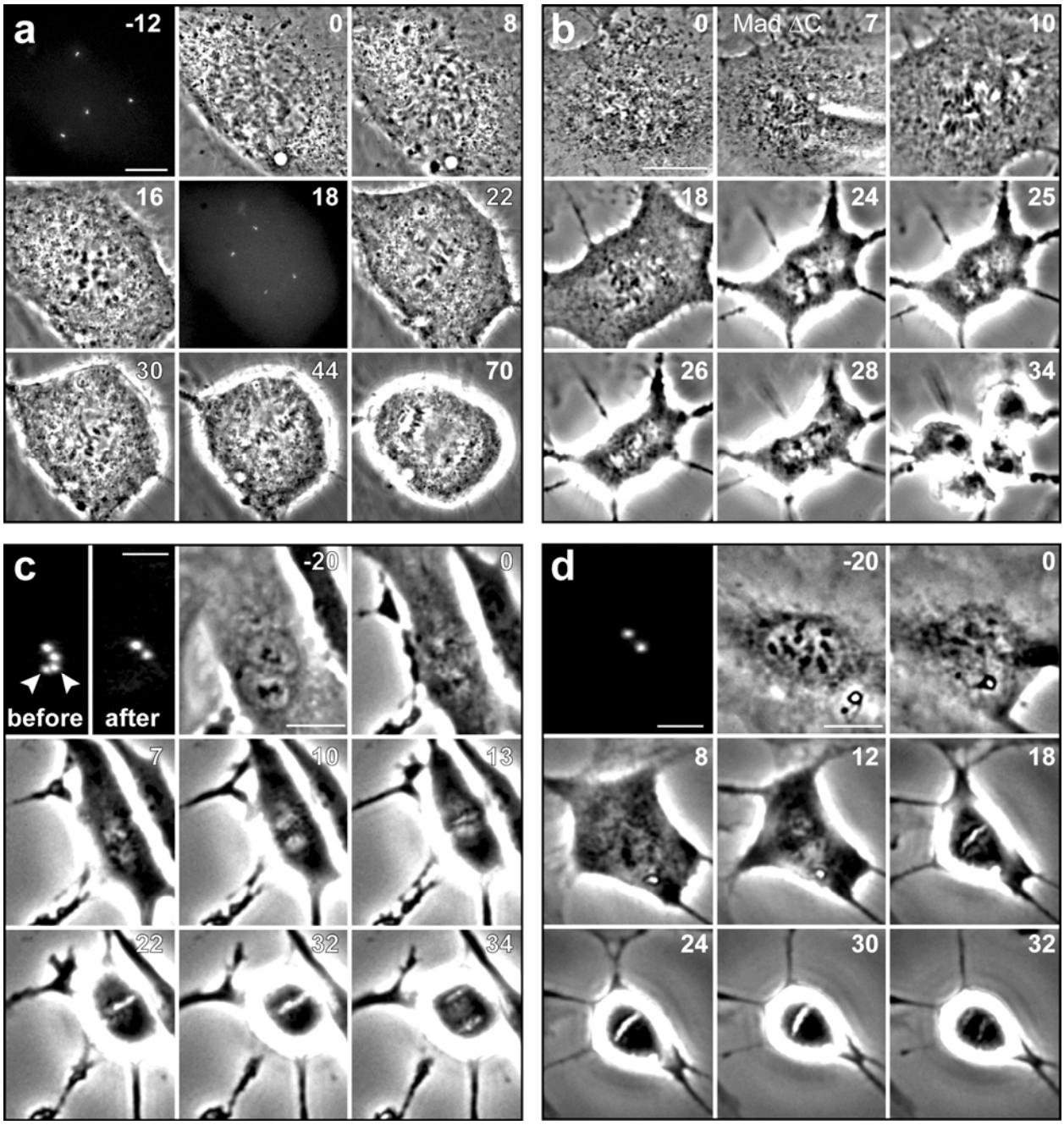

Figure 1. Doubling the chromosome number in human RPE-1 cells prolongs mitosis by delaying satisfaction of the spindle assembly checkpoint. (**a**) The duration of mitosis in binucleated (4N) RPE-1 cells expressing human centrin-1/GFP (a centriolar tag) averaged 49 ± 17 minutes, compared to 20 ± 3 minutes in neighboring mononucleated (2N) controls, and binucleated cells initially formed tetrapolar spindles that reorganize into bipolar or tripolar spindles before anaphase. (**b**) Injecting binucleated cells shortly after nuclear envelope breakdown (7') with purified Mad2-ΔC induces anaphase (25') within 17~22 min. (**c**) Destroying one centrosome in a binucleated G1 cell by laser microsurgery produces cells that form normal

bipolar spindles that average 33 ± 5 minutes in mitosis. (**d**) The 4N mononucleated G1 cells produced from the division in (**c**) contained a single centrosome, and averaged 29±5 min in the next mitosis. The fluorescence images in (c) and (d) were acquired in G1 and scale bars = 2 μm. Scale bars = 10 μm in (a) and 20 μm in the phase images (b, c, d).

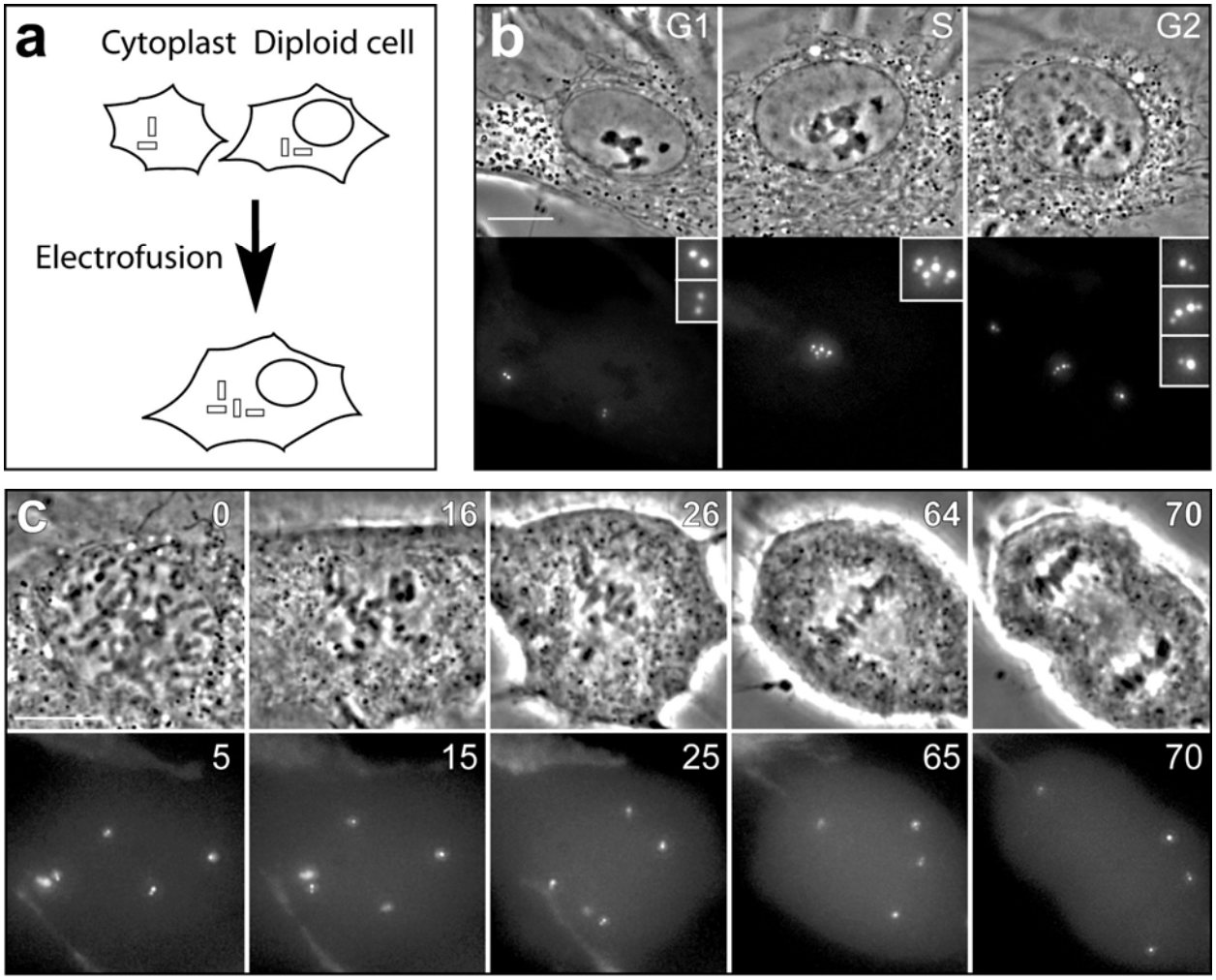

Figure 2. Extra centrosomes prolong mitosis in mononucleated (2N) cells. (**a**) Enucleated G1 RPE-1 cytoplasts containing one centrin-1/GFP labeled centrosome were electrofused with surrounding G1 cells. (**b**) This produced G1 cells containing a 2N nucleus and 2 centrosomes, which subsequently replicated into a 4N nucleus and 4 centrosomes. (**c**) As this cell entered mitosis it formed a tetrapolar spindle (16') that became bipolar (26', 64') so that the chromosomes were segregated into two cells. In this example the duration of mitosis was 64 minutes (the average from 11 cells was 53 ± 21 minutes). Time is in minutes. Scale bars = 10 μm.

Supplemental Online Information

Methods

Cell Culture. Telomerase-immortalized human retinal pigment epithelia (RPE-1), RPE-1 constitutively expressing centrin-1/GFP, HeLa, U2OS, HT1080, CF-PAC, MCF-7 and T-HEP3 cells were cultured in Dulbeco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). BJ and BJ-ELR cells were cultured in a mixture of DMEM and Medium 199 (4:1) with 10% FBS [1]. Cell cultures were maintained in a humidified incubator at 37ºC with a 5% CO_2 atmosphere and seeded onto glass coverslips 24-48 hr prior to experimentation.

Bi-nucleation and Cell Fusion. Binucleated cells were generated by treating coverslip cultures for 16 hrs with 0.2 □M cytochalasin D (Sigma). The drug was then removed by washing with fresh medium six times over a 30 min period. RPE1 centrin-1/GFP cytoplasts were created by treating coverslip cultures with 2 □M cytochalasin D for 15 minutes, after which they were placed vertically into a slot-shaped holder mounted in a centrifuge tube. Approximately 70% of the cells were then enucleated by a 20 min centrifugation at 12,000 rpm. Two to three hrs after enucleation neighboring cells were electrofused as described previously [2].

Light Microscopy/Laser microsurgery/Microinjection: Coverslip cultures were assembled into Rose chambers with phenol-free L-15 medium (GIBCO) supplemented with 10% FBS [3]. Time-lapse phase-contrast images were acquired at 1 min intervals with a 20X (0.50 NA) PlanFluor objective mounted on a Nikon Eclipse TE2000-U or a 10X (0.25 NA) PlanFluor objective mounted on a Nikon DIAPHOT 200 microscope. These microscopes were equipped with an ORCA-ER cooled-CCD camera (Hamamatsu, Japan) or a Micromax camera (Roper Scientific, Trenton, NJ), and the imaging systems were driven by Image-Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD). Cells were maintained at 37ºC throughout the recordings using heated microscope enclosures [3].

For laser microsurgery experiments coverslip cultures of centrin-1/GFP RPE1 cells were treated for 30 min with 20 □m of the p38 inhibitor SB 203580 (Calbiochem), after which they were treated with 20 □m SB 203580 and 0.2 μM cytochalasin D. The cytochalasin D was then removed 16 hrs later by washing 6X in media containing SB203580 after which the coverslips were mounted in Rose chambers in the presence of SB203580. After an overnight incubation at 37°C they were mounted on the stage of our laser microsurgery system and selected centrosomes were ablated as previously described ([4])

For microinjection studies His-tagged Mad2□C (from Dr. E.D. Salmon, Chapel Hill, N.C.) was purified and concentrated to 3 □g/□l. Coverslip cultures of binucleated RPE1 cells were mounted in Rose chambers lacking a top coverslip and selected cells were then injected ~5 minutes after NEB with purified Mad□C containing rhodamine-dextran 3000 (0.25 □g/□l; Molecular Probes, Eugene, OR) using a Narishige IM 300 Microinjector system. Immediately after injection the top coverslip was added to the Rose chamber and the cell of interest filmed by phase-contrast until it exited mitosis.

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Supplemental Figures

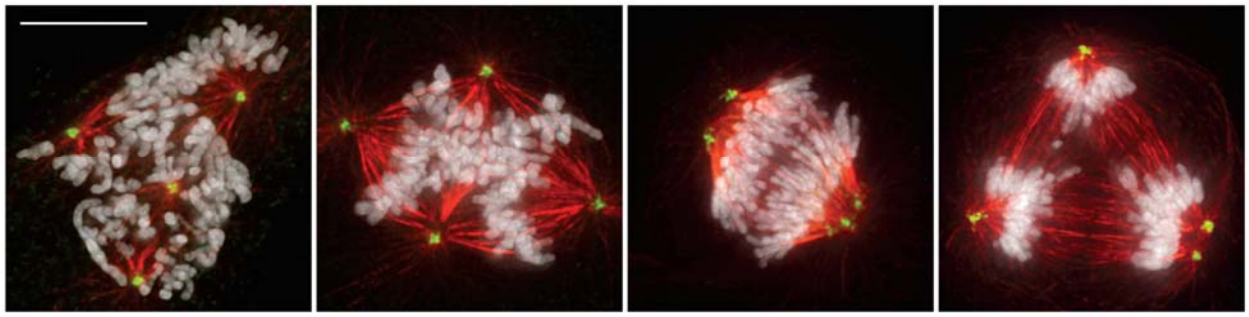

Figure S1. Binucleated RPE1 cells initiate the formation of a tetrapolar spindle that ultimately is converted into a bipolar or tripolar spindle. Live cell studies revealed that during the early stages of spindle assembly in binucleated RPE1 cells all four centrosomes participate in the formation of a tetrapolar spindle. However, these spindles ultimately reorganize into bipolar or tripolar spindles before anaphase. Indirect immunofluorescent studies using anti-α and anti-γ-tubulin antibodies revealed that in fixed RPE-1 cell cultures the percentage of bipolar versus tripolar anaphase cells, in cells that contained 4 centrosomes, was, respectively, 74±4% versus 26±4% (n=150, anaphase cells, 3 experiments). Scale bar = 10 μm.

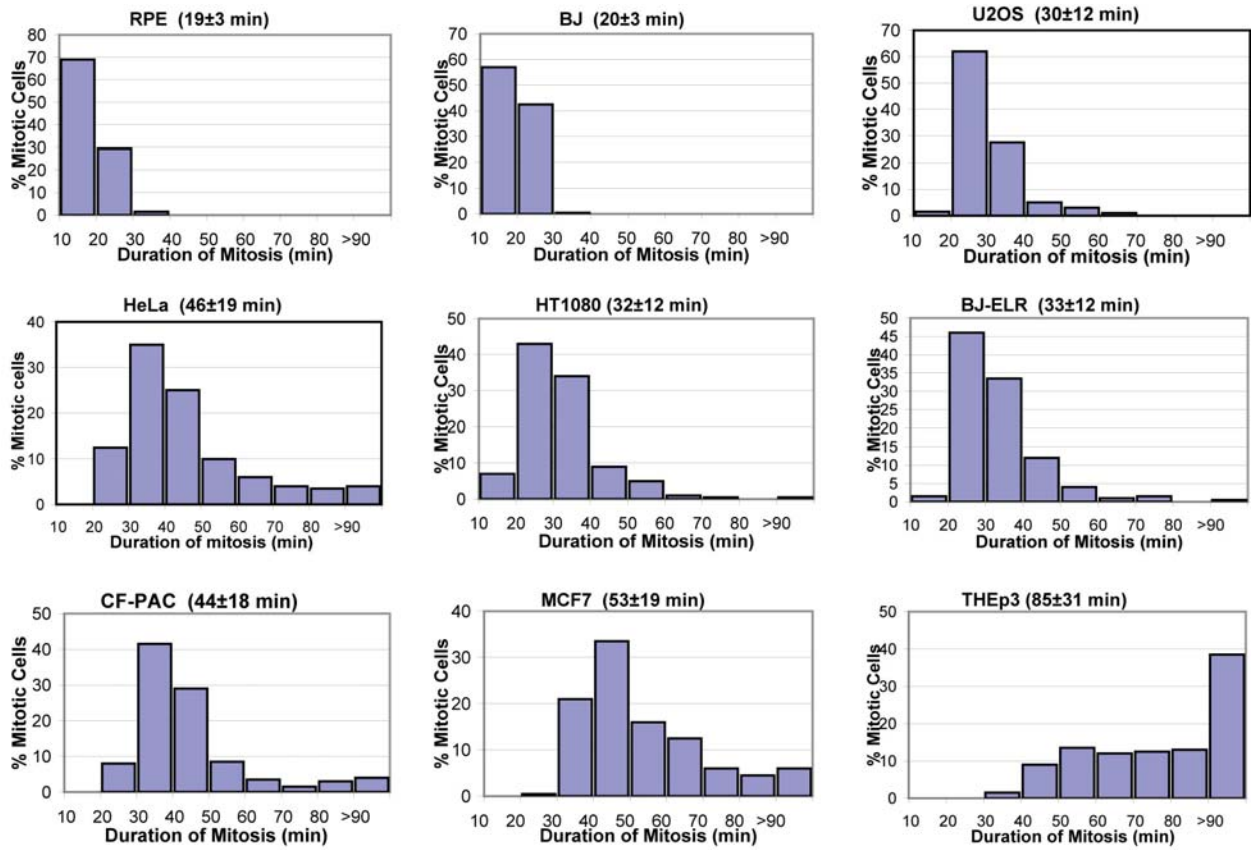

Figure S2. Histograms depicting the distribution of mitotic durations in various normal, transformed and cancer cell lines (see Table S1). For our study we defined the duration of mitosis as that

period between nuclear envelope breakdown and anaphase onset. Here the percentage of cells exhibiting similar durations of mitosis within each data set is plotted against the duration of mitosis. Normal (BJ and RPE1) cells show a tight grouping in their mitotic timing whereas cells from transformed (BJ-ELR) and cancer lines (U2OS, HeLa, HT1080; CF-PAC, MCF7 and THEp3) show significantly broader distributions.

Table 1

Characteristics of Mitosis in some Normal, Transformed and Cancer Cell Lines

Cell Line Designation	***Cell Type***	***2N***	***Duration of M[1,2]***	***Multi-polar Spindles (%)[3]***	***Duration of Multipolar division (n)[3]***
BJ	foreskin fibroblasts	46	20±3	0.8	NA
hTERT RPE-1	Telomerase immortalized retinal pigment epithelia	46	19±3	0.2	NA
HT1080	fibrosarcoma (activated N-ras)	46 (44-48)	32±12	2.2	60±35 (6)
U-2OS	osteosarcoma	>96	30±12	10	51±29 (8)
BJ-ELR	BJ (above) – p53, -pRb + Ras	74-91[4]	33±12	12.2	55±14 (3)
CF-PAC1	Pancreatic ductal carcinoma	73 (65-75)	44±18	12.8	71±25 (4)
HeLa	Adenocarcinoma	~82	46±19	6.5	75±21 (13)
MCF-7	Breast ductal carcinoma	69-85	53±19	16.4	79±23 (11)
T-HEp3	Epidermoid carcinoma	69-90	85±31	20.6	114±32 (5)

[1] Duration in minutes from nuclear envelope breakdown to anaphase onset ± standard deviation; [2] N = 200 mitotic cells collected from ≥3 independent cultures; [3]Cells in the live recordings that exhibited multiple cleavage furrows during anaphase; [4] Li et al., PNAS 97:3236-3241, 2000.