Supplementary Information for

Shi and King, "Chromosome Nondisjunction Yields Tetraploid Rather than Aneuploid Cells in Human Cell Lines"

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Supplementary Methods

Creation of H2B-GFP expressing cell lines

To obtain H2B-GFP positive HeLa cells, cells were grown on a 6-well plate ($1x10^5$ cells per well) for 24 h and transfected with pBOS-H2BGFP (BD Pharmingen 559241) using FuGENE 6 transfection reagent (Roche Diagnostics Corp. 1814443) followed by selection using blasticidin S (2 µg/ml, Calbiochem 203350) for 3 weeks. To obtain H2B-GFP positive N/TERT-1 cells, a retroviral vector expressing an H2B-GFP fusion protein was constructed by replacing the EGFP fragment in retroviral vector pLEGFP-N1 (BD Clontech 6059-1) with the fragment H2BGFP from pBOS-H2BGFP (BD Pharmingen 559241) cut out with Not I and Sal I. Packaging cells PA317 (ATCC) were transfected with the resulting vector using FuGENE 6 (Roche Diagnostics Corp. 1814443) followed by selection in Geneticin (500 µg/ml, Gibco 11811-031) for 10 days. Nearly confluent cultures of PA317 packaging cells producing the above vectors were incubated for 7 h in serum-free keratinocyte medium (Gibco). The resulting retroviral supernatants were passed through a 0.45-µm filter and stored in 2-ml aliquots at -80 °C. To obtain cell lines that stably express H2B-GFP, N/TERT-1 cells were grown on 6-well plates ($2x10^5$ per well) for 48 h and transduced by refeeding for 10 h with retroviral supernatant plus 2 µg of Polybrene (Sigma) per ml. The transduced cells were subcultured the next day into 75-cm^2 flasks. Drug selection (200 µg/ml G418) was started 2 days after transduction and continued for 2 weeks.

Fluorescence *In Situ* hybridization procedures

Plasmids encoding chromosome-specific centromeric probes were obtained from ATCC (Chromosome 8: pJM128, Cat No. 61398; Chromosome 12: pA12H8, Cat No. 59904). Plasmid DNA was labeled with SpectumRed dUTP (Vysis 30-803400) or SpectrumGreeen dUTP (Vysis 30-803200) using a nick translation system (Invitrogen 18160-010). Probes specific for chromosome 6 and 17 were obtained from Vysis (Cat. Nos. 32-132006 and 32-130017 respectively) and were prelabeled with SpectrumGreen and SpectrumOrange.

Cells were fixed in methanol or methanol:acetic acid (3:1) at -20ºC overnight. Coverslips were allowed to dry, affixed to microscope slides, and incubated sequentially in 2xSSC, 1% paraformaldehyde in PBS and 0.1% Triton-100 in PBS for 10 min each at room temperature. Slides were then incubated in increasing concentrations of ethanol (70%-90%-100%) for 2 min each. Coverslips were allowed to dry completely and then 10 µl of hybridization solution containing hybridization buffer MM2.1 (5.5 mL formamide, 1g dextran sulfate, 0.5 mL 20X SSC, adjusted to 7 ml with H_2O, pH>7) and labeled probes at a concentration of 1 ng/µl per probe were added to each coverglass. Coverslips were sealed with a new coverglass and heated at 78ºC for 8 min on a hot plate, then moved to a humidified chamber and incubated at 37ºC for 2 to 4 days. The top coverglass was removed and slides washed in 50% formamide in 2xSSC at 45ºC for 30 min to remove unbound probes. Slides were then incubated in 0.1% Triton-100 in 2xSSC for 30 min at 45ºC. Nuclei were stained in 100 ng/ml DAPI for 10 min and rinsed once in PBS. Cells were covered with a new coverglass in Vectashield mounting medium (Vector Laboratories H-1000).

FISH slides were examined using a Nikon E800 Eclipse Upright Microscope

fitted with a triple band-pass filter (No. 61000 Chroma Technology, Brattleboro, USA) for simultaneous visualization of blue (DAPI), green (SpectrumGreen), and red (SpectrumRed and SpectrumOrange) fluorescent signals**,** and individual filters for each of the fluorochromes (DAPI: Ex, 330-380; DM 400; Em 420; SpectrumGreen: Ex 455-495; DM 505; Em 515-555; SpectrumOrange: Ex, 510-560; DM 565; Em 590; SpectrumRed: Ex 535-585; DM 595; Em 610-685) to digitize the images. Images were acquired with a Hamamatsu Orca 100 Cooled CCD Camera using MetaMorph software (Universal Imaging Corporation).

Immunocytochemical staining

To distinguish mono-, bi- or multinucleated cells after treatments with nocodazole, VP16, ICRF-193 or siRNA targeting Mad2 or Separase or in concurrent controls, HeLa cells were stained with FITC-Conjugated monoclonal anti-α-tubulin antibody (Sigma F2168).

Calculation of the predicted overall rate of chromosome missegregation

The estimated overall rate of chromosome missegregation in cells with a normal bipolar mitosis was estimated by multiplying the average of the rates obtained in Fig. 1c,d for anaphase/telophase cells by the total number of different chromosomes in the cell (twenty-three). For N/TERT-1 cells, the average rate of missegregation was 0.075% per chromosome, whereas for HeLa cells the average rate was 0.315% per chromosome. Multiplying each of these rates by twenty-three yields overall rates of 1.73% for N/TERT-1 cells and 7.25% for HeLa cells. This calculation assumes that all chromosomes have a similar nondisjunction rate and segregate independently.

Chromosome loss versus nondisjunction

We noted that chromosome loss was very rare compared to nondisjunction. No loss was found in anaphase or telophase N/TERT-1 cells, and only 1 out of 456 binucleated N/TERT-1 cell lost a chromosome 8 (0.22%); no binucleated cells lost chromosome 12. Only 1 out of 2059 anaphase HeLa cell was found to lose one copy of chromosome 12 (0.05%) and no cells lost chromosome 8. Seven and six out of 649 binucleated HeLa cells lost chromosome 8 (1.08%) and 12 (0.92%), respectively.

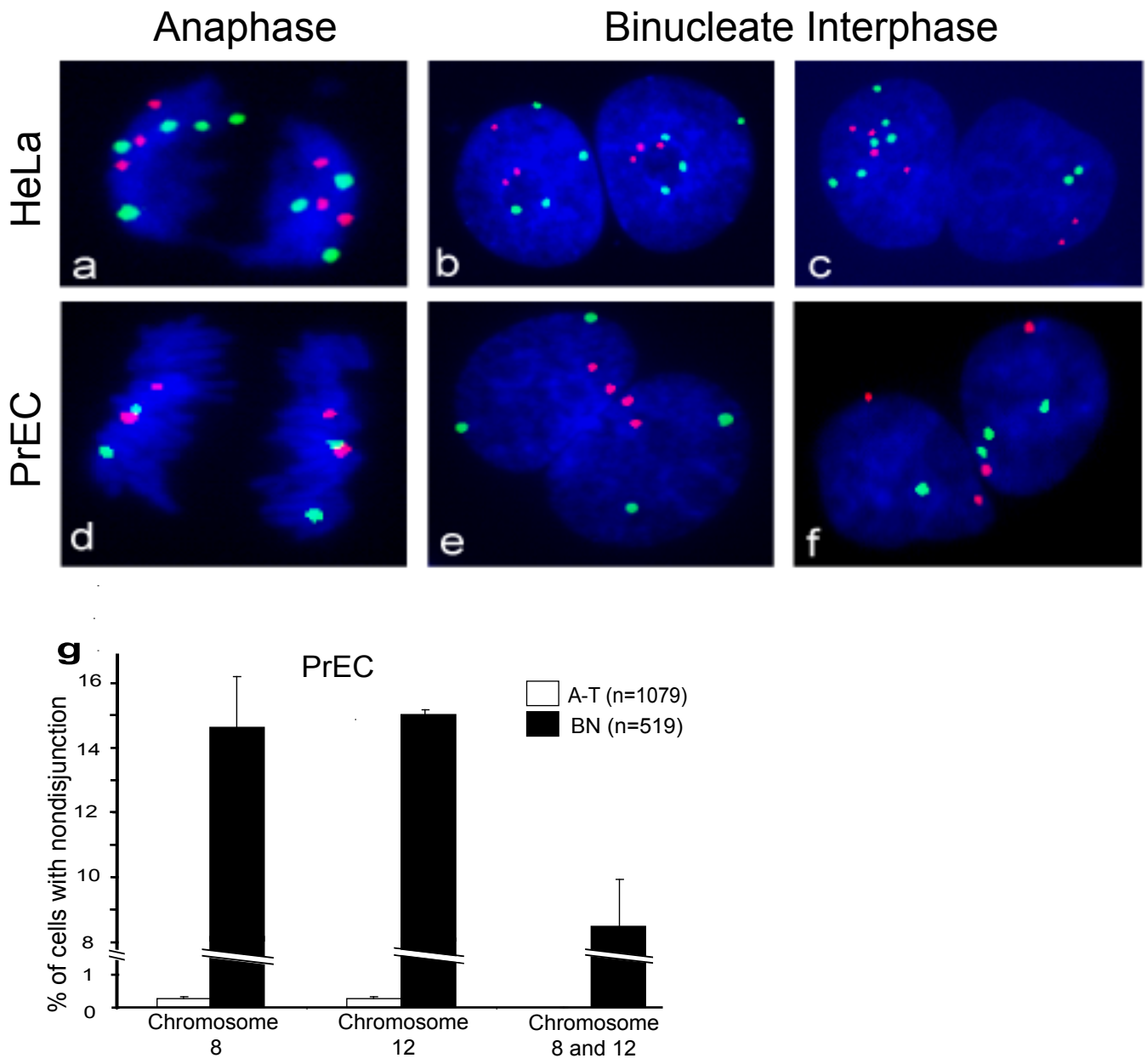
Additional details regarding Figure 2

FISH data was marked as “not determined” in Figure 2 (white bars in the nondisjunction column) if the FISH results were not interpretable (according to the exclusion criteria described in Methods, or if the cell moved out of the field before the end of the movie, or if hybridization was not analyzed). We attempted to interpret hybridization data for all of the binucleated cells analyzed in Figure 2a, but in 9/125 cells signals could not be interpreted or the cell moved out of field. In Figure 2b, we attempted to interpret the FISH results for 240 of the 395 cells followed by time-lapse imaging. Of the 240 cells, FISH results could not be interpreted for 29 cells or the cell moved out of the field. For the remaining 211 cells, the FISH data were interpretable and no nondisjunction events were observed.

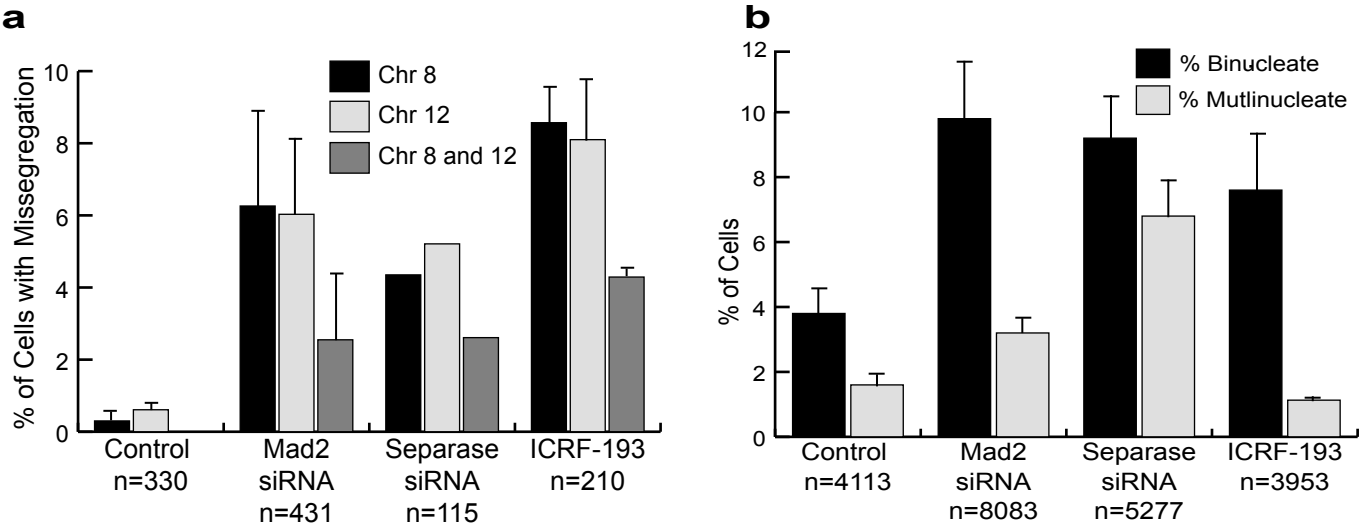
Additional details regarding Figure 3

In Figure 3a and 3b, those cells marked as “persistent link” were cells in which the cytoplasmic bridge appeared intact through a full cell cycle (until the cell entered mitosis) or until it underwent apoptosis. Cells marked “cannot be determined” either moved out of the field or overlapped, making it impossible to ascertain their status.

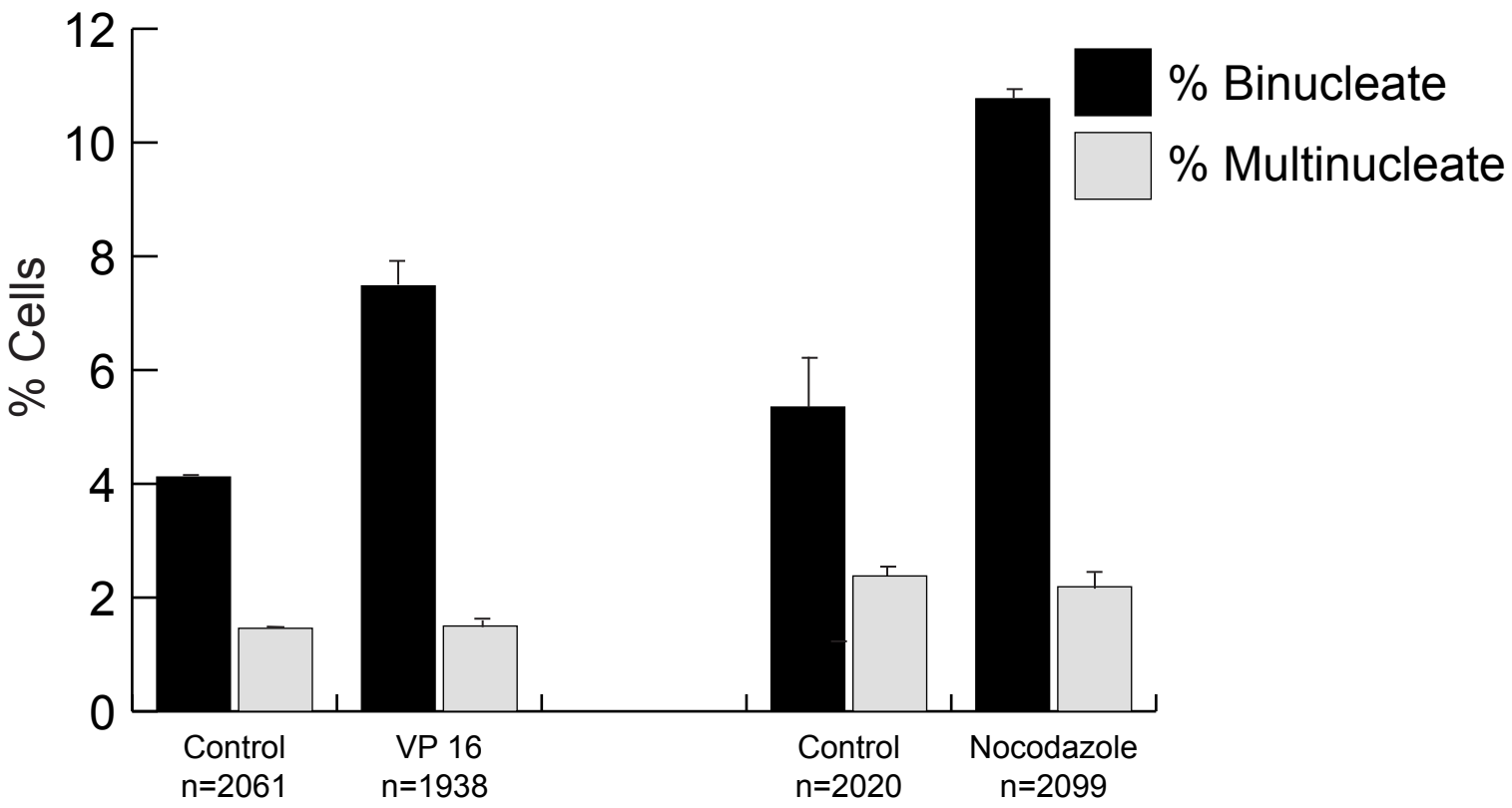
In Figure 3c and 3d, cells were scored as remaining in interphase if they did not divide or undergo apoptosis for a period longer than one cell cycle (22.5 h for N/TERT-1 cells and 26 h for HeLa cells). The cell cycle delay in binucleated N/TERT-1 cells that did not enter mitosis was substantial, as cells were followed for an average of 40.1 hours without evidence of division. A total of 57 N/TERT-1 cells were assigned as “remaining in interphase.” Of these cells, 16 (28%) were followed for 25-30 hours, 10 were followed for 30-45 hours (17%), and 31 (55%) were followed for >45 hours. In binucleated N/TERT-1 cells, the average time from cytokinesis initiation to furrow regression was 144+/-24 minutes, whereas the time from cytokinesis initiation to abscission was 46+/-38 minutes in cells that completed cytokinesis. Thus the delay in cell division appears to be much longer than the additional amount of time required to undergo furrow regression compared to abscission. These data suggest a prolonged cell cycle delay or cell cycle arrest in this population of cells.



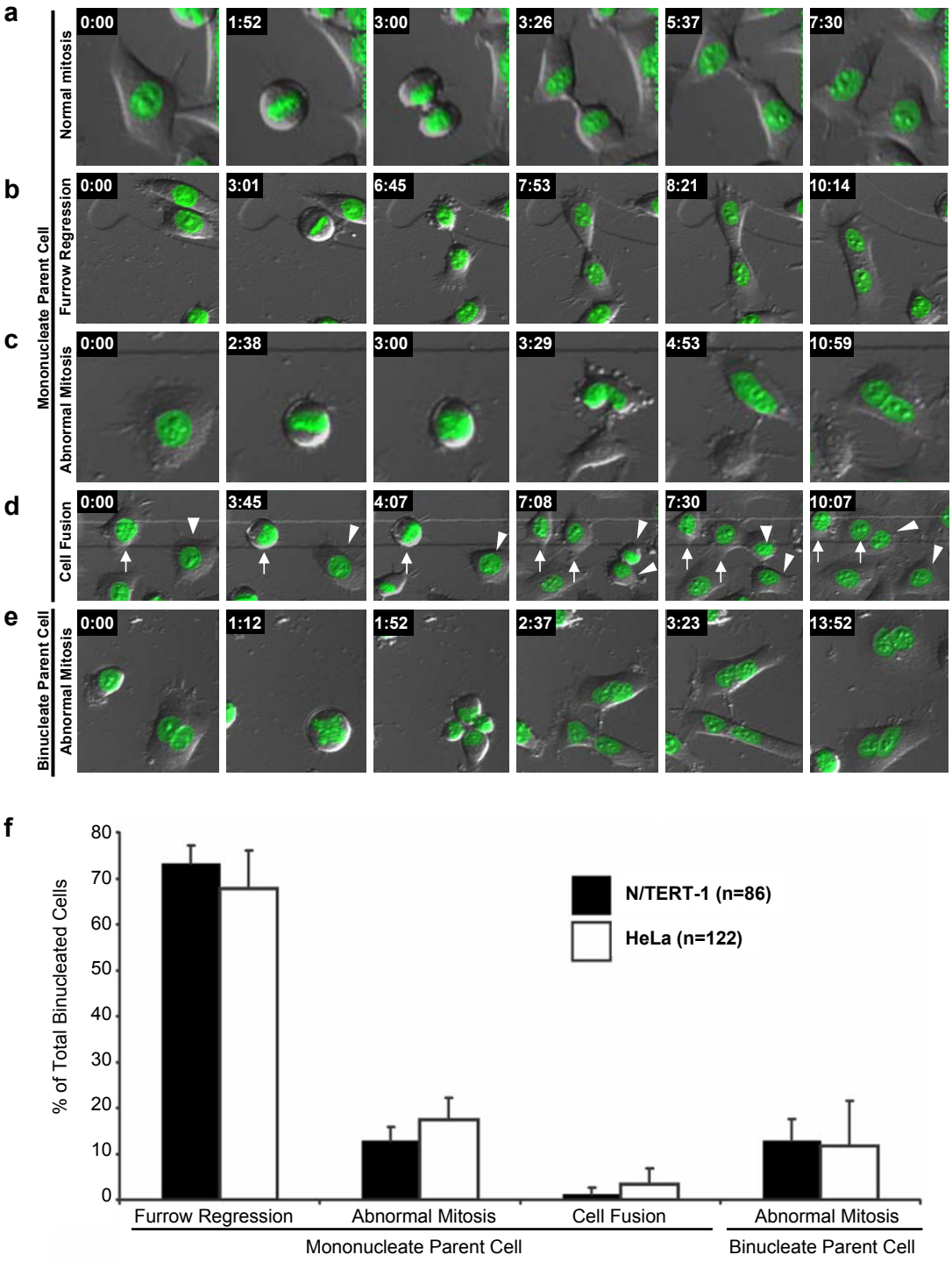
Supplementary Figure 1. Analysis of chromosome segregation by fluorescence *in situ* hybridization in HeLa and PrEC cells. Examples of mitotic (**a,d**) and binucleated interphase (**b,c,e,f**) HeLa and PrEC cells analyzed by fluorescence *in situ* hybridization (FISH) using chromosome 8-(red) and 12-(green) specific centromeric probes. Note that HeLa cells are aneuploid, with a modal number of 3 copies of chromosome 8 and four copies of chromosome 12 (see Supplementary Figure 5 for more details). **b,d,e,** Examples of normal segregation. **a**, Chromosome 12 nondisjunction in an anaphase cell. **c**, Nondisjunction of both chromosomes 8 and 12 in a binucleated cell. **f**, Nondisjunction of chromosome 12 in a binucleated cell. **j**, Quantitation of chromosome nondisjunction in binucleated (BN, solid bar) and anaphase/telophase PrEC cells with normal bipolar spindles (A-T, clear bar). Results are the average of analysis of 519 binucleated and 1079 ana-telophase PrEC cells from 5 experiments. Error bars are standard deviatiation.



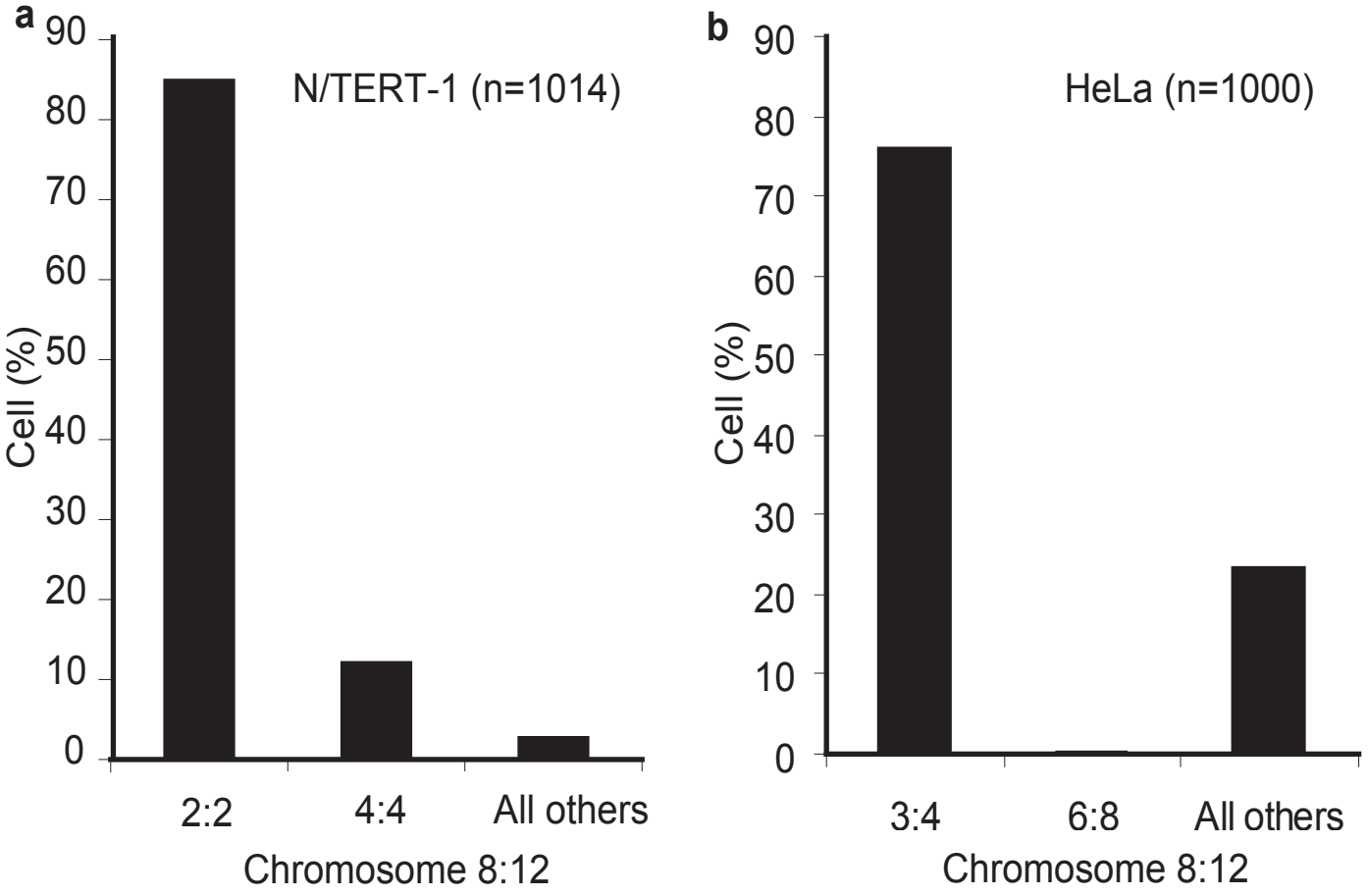
Supplementary Figure 2. Treatments that increase chromosome missegregation also increase binucleation. HeLa cells were transfected with a nonspecific control siRNA or siRNAs that target Mad2 or Separase (100nM) and were fixed 72 hours later and processed by fluorescence *in situ* hybridization using chromosome 8- or 12-specific probes. **a**, Analysis of the rate of chromosome mis-segregation in anaphase/telophase cells. **b**, Analysis of the percentage of binucleated cells in the population. For ICRF-193, asynchronous HeLa cells were treated with 2 μM of ICRF-193 for 20 hours and then processed. Error bars represent standard deviation from multiple independent experiments (**a**,control (3 experiments), Mad2 siRNA (4 experiments), Separase siRNA (1 experiment), ICRF-193 (4 experiments), **b,** control (4 experiments), Mad2 siRNA (8 experiments), Separase siRNA (5 expeirments), ICRF-193 (4 experiments)).



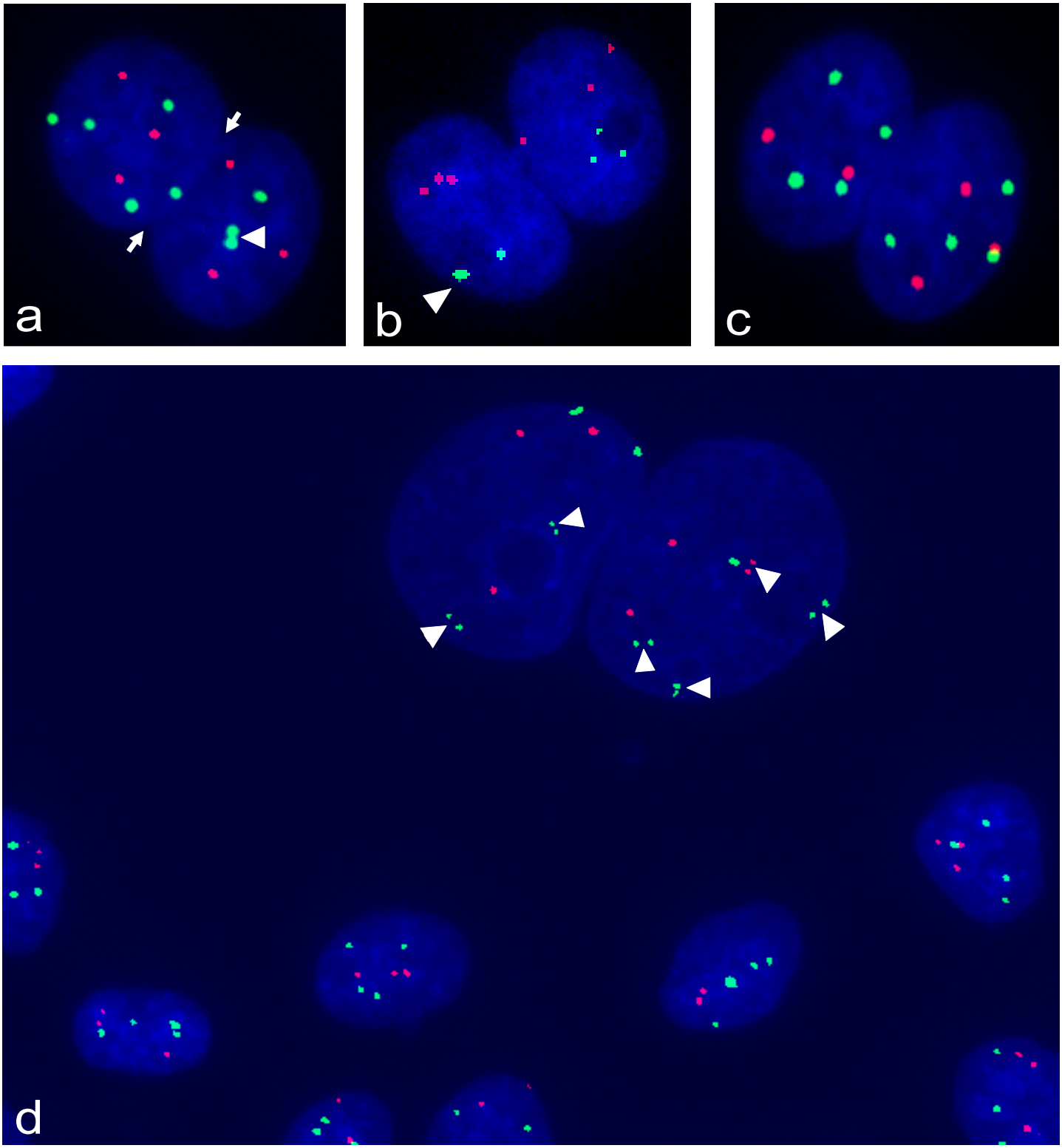
Supplementary Figure 3. Treatments that increase chromosome missegregation also increase binucleation in HeLa cells. For VP16, cells were synchronized with a double thymidine procedure and treated with VP16 (10 μM) 8.5 hours after release. Cells were treated for 2 hours and then VP16 was washed out and cells allowed to recover for 14 hours prior to fixation. For the nocodazole experiments, cells were synchronized by double thymidine block and release and were treated with nocodazole (2 μM) for 3 hours beginning 8.5 hours after release from the thymidine block. Nocodazole was washed out and cells were allowed to recover for 16.5 hours. Control cells were synchronized but did not receive VP16 or nocodazole. Binucleated cells were identified by staining with DAPI and an α-tubulin antibody. Error bars represent standard deviation from the results of two independent experiments for each treatment condition.



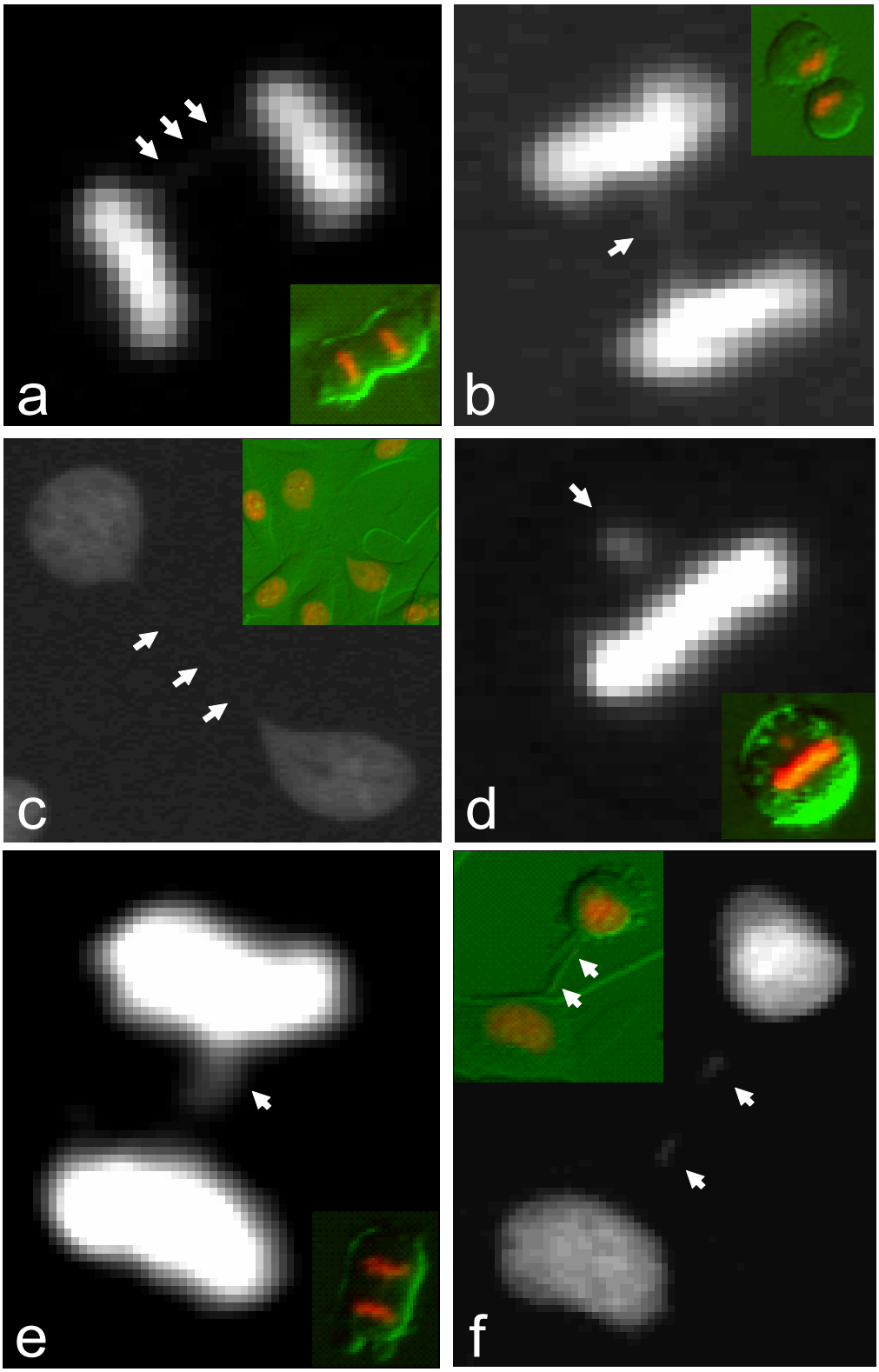
Supplementary Figure 4. Spontaneous binucleation occurs primarily through a normal mitosis followed by regression of the cleavage furrow in HeLa and N/TERT-1 cells. **a-e**, examples of HeLa cells expressing H2B-GFP undergoing normal mitosis (**a**), binucleation with furrow regression (**b**), abnormal mitosis (**c**), cell fusion (**d**) and a binucleated cell dividing with a multipolar mitosis (**e**). Time is shown in hours: minutes. Arrows in (**d**) indicate products of a common division. For corresponding movies, see Supplementary Movies. **f**, Quantitation of experiments performed in both N/TERT-1 and HeLa cells. For HeLa cells that completed cytokinesis, the average time from cytokinesis initiation to abscission was 7.4 hours; for cells that underwent furrow regression, the average time from cytokinesis initiation to furrow regression was 13.6 hours. For N/TERT-1 cells, these times were 46 minutes and 144 mintues, respectively. Error bars are standard deviation. Results are the mean from 4 independent experiments.



Supplementary Figure 5. Ploidy analysis of interphase mononucleated cells in asynchronous cultures as measured by fluorescence *in situ* hybridization using chromosome 8- and 12-specific probes. **a**, In N/TERT-1 cultures, most interphase cells have 2 copies of chromosome 8 and 12; however, a significant fraction contain 4 copies each, representing tetraploid cells. **b**, In HeLa cells, the modal chromosome number is 3 and 4 for chromosomes 8 and 12, respectively. Very few cells show 6 and 8 copies of chromosomes 8 and 12, indicating that a stable 4n interphase population is not present. Instead, HeLa cells show a higher fraction of interphase cells with other non-modal chromosome numbers.



Supplementary Figure 6. Scoring criteria for FISH analysis in HeLa cells. Chromosome 8 centromeric probe is indicated in red; chromosome 12 in green. Examples of cells excluded from the analysis are those with very close or overlapping dots (**a** and **b**, arrowheads), or missing dots (**c**). Only binucleated cells containing an even number of total dots were included in the analysis; each of the preceding cases results in the presence of an odd number of dots and were thus excluded. We also excluded cells that contained dots in an overlapping nuclear region (**a**, arrows). Nuclei that were abnormally large, perhaps due to endoreduplication, were also excluded from the analysis (**d**).



Supplementary Figure 7. Examples of cells showing bridging or lagging chromosomes. These images are derived from time-lapse analysis of HeLa cells expressing H2B-GFP described in Figure 2. Movies were acquired using a 20x objective. Each panel shows a digital enlargement of the H2B-GFP signal; inset shows both GFP (red) and DIC (green) for the corresponding cell. Examples of bridging chromosomes (arrows) occurring between two daughter nuclei in early (**a**) and late telophase (**b**) and in interphase cells (**c**). Example of a cell with a lagging chromosome during prometaphase (**d**), anaphase (**e**), or in an interphase cell (**f**), where the lagging chromosomes are evident in the cytoplasmic bridge.

Supplementary Table 1. Rates of chromosome nondisjunction are similar between parental and their H2B-GFP-expressing derivatives in both HeLa and N/TERT-1 cells.

	% of cells with chromosome 8 nondisjunction	% of cells with chromosome 12 nondisjunction	% of cells with chromosome 8 and 12 nondisjunction	Total cells analyzed
Binucleated HeLa cells				
Parent Line	11.9	19.4	5.6	160
H2BGFP Line	14.3	16.7	8.7	489
Mean/Total	13.7	17.4	8.0	649
Anaphase/telophase HeLa cells				
Parent Line	0.195	0.391	0	1024
H2BGFP Line	0.290	0.386	0	1035
Mean/Total	0.243	0.389	0	2059
Binucleated N/TERT-1 cells				
Parent Line	8.10	8.41	5.30	321
H2BGFP Line	8.89	8.15	2.96	135
Mean/Total	8.33	8.33	4.61	456
Anaphase/Telophase N/TERT-1 cells				
N/TERT-1	0.063	0.063	0	1570
N/TERT-1 H2BGFP	0	0.199	0	503
Mean/Total	0.048	0.096	0	2073

Supplementary Table 2. Chromosome segregation errors in HeLa-H2B-GFP cells that become binucleated by furrow regression.

	Observed Nondisjunctions		Expected Nondisjunctions	
Chromosome	Number	Frequency (%)	Number	Frequency (%)
Chr 6	5	4.3	nd	nd
Chr 8	5	4.3	0.28	0.24
Chr 12	6	5.1	0.45	0.39
Chr 17	3	2.5	nd	nd

FISH was performed on HeLa H2B-GFP cells in three separate experiments after 64, 68, and 88 hours of live imaging. Binucleated cells that arose through a normal mitosis followed by furrow regression as shown in Figure 2a were analyzed for chromosome segregation by FISH. These data are from the same cells described in Figure 2a. A total of 18 out of 116 binucleated cells showed nondisjunction, an overall rate of 16%. One of these cells showed nondisjunction of two chromosomes (8 and 12); the remainder were single nondisjunction events. This table shows the frequency of nondisjunction of each chromosome, and includes the cell that missegregated two chromosomes, yielding a total of 19 nondisjunction events.

In the right hand columns (Expected Nondisjunctions) we have calculated the number of nondisjunction events that would be predicted to occur in this set of cells if binucleation were unrelated to chromosome nondisjunction. This value is obtained by multiplying the overall frequency of nondisjunction in bipolar mitosis (as measured in the experiments described in Fig. 1) by the number of cells studied in this experiment (116). The average rate of nondisjunction in binucleated cells was 4.1% (the average of the rates from chromosomes 6, 8, 12 and 17), whereas the average rate in all mitotic cells was 0.32% (the average of the rates from chromosomes 8 and 12 determined in Fig. 1 and shown under expected nondisjunctions). This analysis indicates that the rate of nondisjunction is elevated at least 10-fold in cells that become binucleated by furrow regression compared to the rates measured in the entire mitotic population, indicating that the processes of nondisjunction and binucleation are tightly coupled.

If the average nondisjunction rate per chromosome is 4.1% in binucleated HeLa cells, then the overall nondisjunction rate is (0.041*23) for all 23 chromosomes, for a total nondisjunction rate of 94%. This value suggests that all binucleated cells have sustained at least one nondisjunction event. nd, not determined.

Supplementary Table 3. Products of division of binucleated cells that formed a multipolar spindle during mitosis.

Daughter Cells Produced	HeLa (43 cells analyzed)		N/TERT-1 (12 cells analyzed)	
	Number of cells	% in total	Number of cells	% in total
3 mononucleated cells	0	0	3	25
2 mononucleated cells	0	0	2	17
1 mononucleated + 1 binucleated cell	0	0	4	33
2 mononucleated + 1 binucleated cell	9	21	1	8
1 mononucleated + 1 trinucleated cell	16	37	0	0
2 binucleated cells	6	14	1	8
1 tetranucleated cell	12	28	1	8

Mitotic results of binucleated cells that formed multipolar spindle detected by time lapse imaging. During long-term time lapse experiments,102 binucleated N/TERT-1 and 78 binucleated HeLa cells were followed. Only 12 out of 102 binucleated Ntert-1 cells (11.8%) underwent multipolar mitosis and generated 28 daughter cells, while 60 out of 78 binucleated HeLa cells (76.9%) followed multipolar mitosis producing 83 daughter cells. Of the latter, only 43 of the 60 cells could be followed long enough to accurately ascertain the products of division.

Supplementary Video Legends

Supplementary Video 1
This movie shows a mononucleated HeLa cell expressing an H2B-GFP fusion protein that undergoes a normal bipolar mitosis. The time lapse covers a period of about 495 min, during which time two new mononucleted cells are generated following normal nuclear division, cytokinesis and midbody abscission. The time from the end of telophase to abscission, as determined by presence of the midbody, is about 245 minutes.Times are shown in hour:min:sec:10^{-6} sec (QuickTime; 378KB). This cell is shown in Supplementary Figure 4a.

Supplementary Video 2
This movie shows the generation of a binucleated cell through normal bipolar mitosis followed by cleavage furrow regression. The time lapse covers a period of about 1,575 min, during which time a binucleted cell is generated following normal nuclear division, normal cytokinesis and regression of the cleavage furrow. The time between the start of telophase until furrow regression is about 254 min. Times are shown in hour:min:sec: 10^{-6} sec (QuickTime; 2,041KB). This cell is shown in Supplementary Figure 4b.

Supplementary Video 3
This movie shows a second example of the generation of a binucleated cell through normal bipolar mitosis followed by cleavage furrow regression. The time lapse covers a period of about 1,575 min, during which time a binucleted cell is generated following normal nuclear division, normal cytokinesis and regression of the cleavage furrow. The time between the end of telophase until furrow regression is about 952 min. Times are shown in hour:min:sec: 10^{-6} sec (QuickTime; 1,224KB).

Supplementary Video 4
This movie shows the generation of a binucleated cell through abnormal mitosis of a mononucleated cell. The time lapse covers a period of about 1,210 min, during which time a binucleted cell is generated following an abnormal nuclear division with all chromosomes moving to one daughter cell. Times are shown in hour:min:sec: 10^{-6} sec (QuickTime; 935KB). This cell is shown in Supplementary Figure 4c.

Supplementary Video 5
This movie shows the generation of a binucleated cell through fusion of two newly generated mononucleated cells. The time lapse covers a period of about 788 min, during which time two linked mononucleted cells enter mitosis and generate two linked mononucleated daughter cells that subsequently fuse. Times are shown in hour:min:sec: 10^{-6} sec (QuickTime; 1,162KB).This cell is shown in Supplementary Figure 4d.

Supplementary Video 6
This movie shows a binucleated cell that divides with a tetrapolar mitosis to produce two binucleated cells. The time lapse covers a period of about 715 min. Times are shown in hour:min:sec: 10^{-6} sec (QuickTime; 1,064KB). This cell is shown in Supplementary Figure 4e.