LETTERS

Chromosome nondisjunction yields tetraploid rather than aneuploid cells in human cell lines

Qinghua Shi[1]† & Randall W. King[1]

Although mutations in cell cycle regulators or spindle proteins can perturb chromosome segregation[1–7], the causes and consequences of spontaneous mitotic chromosome nondisjunction in human cells are not well understood. It has been assumed that nondisjunction of a chromosome during mitosis will yield two aneuploid daughter cells. Here we show that chromosome nondisjunction is tightly coupled to regulation of cytokinesis in human cell lines, such that nondisjunction results in the formation of tetraploid rather than aneuploid cells. We observed that spontaneously arising binucleated cells exhibited chromosome mis-segregation rates up to 166-fold higher than the overall mitotic population. Long-term imaging experiments indicated that most binucleated cells arose through a bipolar mitosis followed by regression of the cleavage furrow hours later. Nondisjunction occurred with high frequency in cells that became binucleated by furrow regression, but not in cells that completed cytokinesis to form two mononucleated cells. Our findings indicate that nondisjunction does not directly yield aneuploid cells, but rather tetraploid cells that may subsequently become aneuploid through further division. The coupling of spontaneous segregation errors to furrow regression provides a potential explanation for the prevalence of hyperdiploid chromosome number and centrosome amplification observed in many cancers[8,9].

Despite pathways such as the spindle checkpoint that promote accurate chromosome segregation[10,11], nondisjunction still occurs with significant frequency in human cells[12,13]. Because chromosome mis-segregation is irreversible, aneuploid cells will accumulate unless mechanisms exist to eliminate them or suppress their proliferation. To identify such mechanisms, we asked whether the accuracy of chromosome segregation is compromised in cells that show altered behaviour in subsequent steps of cell division, such as cytokinesis. We therefore compared the frequency of chromosome mis-segregation in all mitotic cells with the frequency measured in spontaneously arising binucleated cells. We analysed anaphase or telophase cells containing bipolar spindles by fluorescence *in situ* hybridization (FISH) using centromeric probes for chromosomes 8 and 12 (Fig. 1a; see also Supplementary Fig. 1). In diploid human keratinocytes immortalized with telomerase (N/TERT-1; ref. 14), we observed mis-segregation of chromosome 8 and 12 in 0.05% and 0.10% of mitotic cells, respectively (Fig. 1c), comparable to reported rates in human lymphocytes and fibroblasts[12,13]. HeLa cells, which are aneuploid but maintain a stable karyotype[15], showed higher rates of mis-segregation, 0.24% per mitosis for chromosome 8 and 0.39% for chromosome 12 (Fig. 1d). Similar results were obtained in immortalized prostate epithelial (PrEC) cells[16] (see Supplementary Fig. 1).

The frequency of chromosome mis-segregation was found to be much higher in spontaneously arising binucleated cells (Fig. 1b; see also Supplementary Fig. 1). In binucleated N/TERT-1 cells, 8.3% of

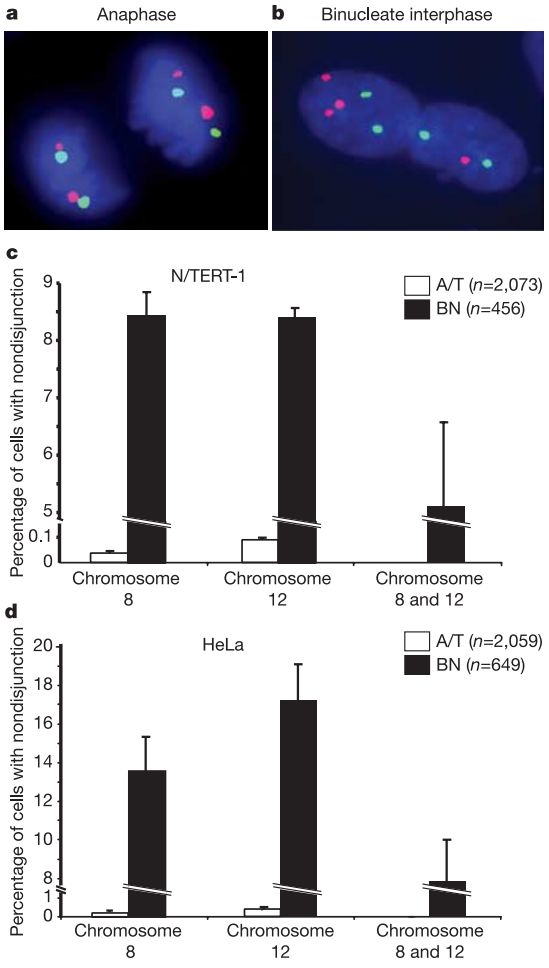

Figure 1 | Chromosome mis-segregation occurs at high frequency in spontaneously arising binucleated cells. a, **b**, Examples of mitotic (**a**) and binucleated interphase (**b**) N/TERT-1 cells analysed by fluorescence *in situ* hybridization using chromosome 8-(red) and 12-(green) specific centromeric probes. Example of normal segregation (**a**) and chromosome 8 nondisjunction in a binucleated cell (**b**). **c**, Quantification of chromosome nondisjunction in binucleated (BN) and anaphase/telophase (A/T) N/TERT-1 cells with normal bipolar spindles. **d**, Quantification of chromosome mis-segregation in HeLa cells (for images see Supplementary Fig. 1). Error bars are one standard deviation, from results of 4 (**c**) or 5 (**d**) independent experiments.

[1]Department of Cell Biology, Harvard Medical School, 240 Longwood Ave, Boston, Massachusetts 02115, USA. †Present address: Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei 230026, China.

cells mis-segregated chromosome 8 or 12 during the preceding mitosis, increases of 166-fold and 83-fold, respectively, compared to the frequencies measured in mitotic cells. In binucleated HeLa cells, the frequency of cells with chromosome mis-segregation was 13.7% for chromosome 8 and 17.4% for chromosome 12, increases of 57-fold and 45-fold, respectively. In binucleated PrEC cells, non-disjunction rates were 14.5% for chromosome 8 and 15.0% for chromosome 12, increases of 48-fold and 50-fold, respectively (see Supplementary Fig. 1). In all cell lines, a fraction of binucleated cells mis-segregated multiple chromosomes. Together these data indicate that spontaneously arising binucleated cells have substantially elevated chromosome mis-segregation rates compared to the overall mitotic population.

Our data suggested that cytokinesis might be inhibited in cells that spontaneously mis-segregate chromosomes during the preceding mitosis. To determine whether treatments that induce chromosome mis-segregation also increase the frequency of binucleation, we treated HeLa cells with short interfering RNAs (siRNAs) or drugs that would reduce the accuracy of chromosome segregation[10]. Reducing expression of the spindle checkpoint protein Mad2 or the protease separase by siRNA increased the rate of chromosome mis-segregation and doubled the frequency of binucleated cells (see Supplementary Fig. 2). Partial inhibition of topoisomerase II with low doses of ICRF-193 (ref. 17) also increased chromosome mis-segregation and binucleation. Similar results were obtained with VP-16 and nocodazole (see Supplementary Fig. 3), supporting the possibility that chromosome segregation errors are functionally coupled to the regulation of cytokinesis.

To determine how spontaneous binucleation occurs, we imaged HeLa and N/TERT-1 cells expressing green fluorescent protein (GFP)-tagged histone 2B (ref. 18). Expression of GFP–histone 2B did not alter rates of chromosome mis-segregation (see Supplementary Table 1). In both cell lines, most binucleated cells (65–75%) arose from mononucleated cells that formed a bipolar spindle and initiated cytokinesis appropriately, but underwent furrow regression at a later time (see Supplementary Fig. 4). The delay between mitosis and furrow regression was long, averaging 13.6 h in HeLa cells, almost twice the average period between mitosis and furrow abscission (7.4 h) in cells that completed cytokinesis. In addition, 12–18% of binucleated cells arose from mononucleated cells that underwent a grossly abnormal mitosis. A small proportion of binucleated cells (3–6%) arose by fusion of two second-generation descendents of the same parent cell. Finally, 12% of binucleated cells arose through division of pre-existing binucleated cells. The genesis of binucleated cells is therefore complex, but the predominant pathway involves regression of the cleavage furrow in cells that undergo an otherwise normal-appearing mitosis.

Abnormal mitosis could potentially explain a high rate of chromo-

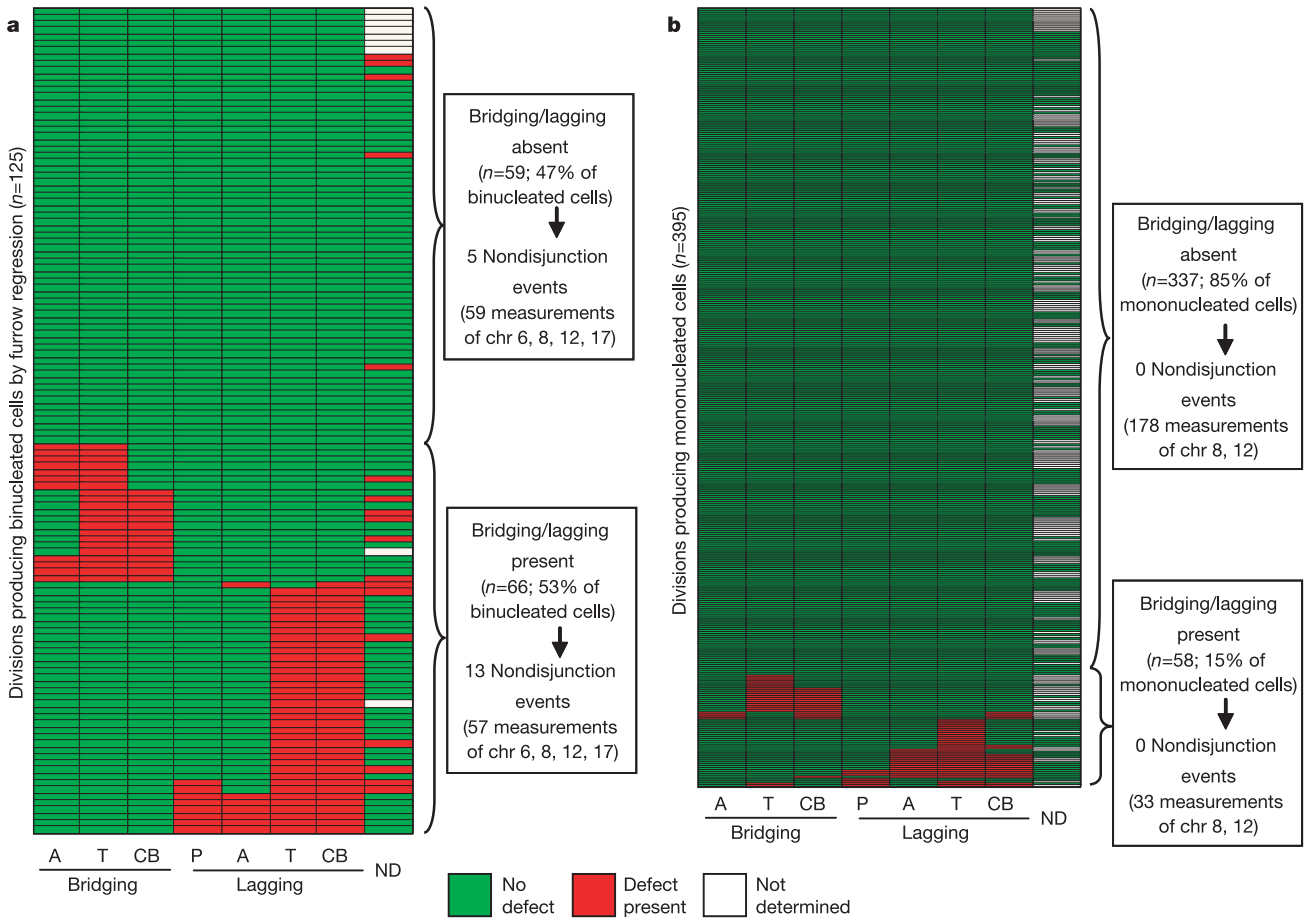

Figure 2 | Nondisjunction occurs with high frequency in cells that become binucleated by furrow regression, but not in cells that complete cytokinesis. HeLa cells expressing GFP–histone 2B were followed by time-lapse imaging and then fixed and analysed for chromosome segregation errors by FISH. **a**, Analysis of mononucleated cells that underwent furrow regression after bipolar mitosis to produce a binucleated cell. Each row represents analysis of a single cell division. Colour indicates whether the cell showed the presence (red) or absence (green) of chromosome bridging or lagging, as determined by inspection of the movie, or nondisjunction, as measured by FISH. **b**, Analysis of mononucleated cells that completed cytokinesis after bipolar mitosis producing two mononucleated cells. The vertical scale of this plot is compressed due to the larger number of cells analysed. Abbreviations are as follows: A, anaphase; T, telophase, CB, interphase period following mitosis when cells are connected by a cytoplasmic bridge; P, prometaphase; ND, nondisjunction; and chr, chromosome.

some mis-segregation in binucleated cells, but only a small fraction of binucleated cells showed gross defects in mitosis. We therefore wanted to determine whether cells that divided with a normal bipolar spindle but became binucleated through furrow regression would also show an elevated rate of chromosome mis-segregation. We followed HeLa GFP–histone 2B cells by time-lapse microscopy, and then fixed the cells to analyse chromosome segregation by FISH. Using probes for chromosomes 6, 8, 12 and 17, we successfully measured chromosome segregation in 116 cells that became binucleated by furrow regression, and identified 18 cells that exhibited nondisjunction (Fig. 2a; see also Supplementary Table 2). In contrast, we detected no nondisjunction events in cells that completed cytokinesis to form two mononucleated cells (Fig. 2b; 211 measurements using probes for chromosomes 8 and 12). The average nondisjunction rate per chromosome in cells that became binucleated by furrow regression was 4.1% (see Supplementary Table 2), over ten times higher than that observed in the entire mitotic population (0.32%). If the rate of chromosome nondisjunction is similar for other chromosomes, then we predict that 94% of binucleated cells would show evidence of nondisjunction if segregation of all chromosomes was measured (see Supplementary Table 2).

These results indicate that regression of the cleavage furrow is tightly coupled to chromosome nondisjunction in human cells. One mechanism that could explain this finding is the presence of chromatin in the cleavage furrow, which has been reported to interfere with the completion of cytokinesis[19]. We therefore examined the time lapse movies from the previous experiments for the presence of chromosome bridging or lagging (CBL) during mitosis, and clustered the cells on this basis (Fig. 2). CBL was frequent in cells that underwent furrow regression (Fig. 2a; 53%), but was also present in a fraction of cells that completed cytokinesis (Fig. 2b; 15%). Although CBL is more frequent in cells that become binucleated, it appears neither necessary nor sufficient to explain binucleation. CBL was present in only half of all binucleated cells, and a significant number of cells completed cytokinesis despite the presence of CBL. We believe that CBL is more frequent in binucleated cells because nondisjunction is more likely to occur in cells with CBL. For example, merotelic kinetochore attachment is a known cause of lagging chromosomes and nondisjunction in mammalian cells[20]. However, merotelic attachment may not always result in nondisjunction if the lagging chromosome is ultimately moved to the correct pole, explaining why chromosome lagging may not be sufficient for binucleation.

If nondisjunction itself, rather than CBL, is coupled tightly to furrow regression, then the rate of nondisjunction in all mitotic cells should be similar to the rate of binucleation by furrow regression. We therefore determined the rate of binucleation by furrow regression in mononucleated cells that divided with a normal bipolar spindle (Fig. 3a, b). We found that the rates of binucleation, 1.9% per division for N/TERT-1 cells and 7.7% for HeLa cells, were very similar to the predicted rates of nondisjunction of all chromosomes during normal bipolar mitosis, that being 1.7% in N/TERT-1 cells and 7.3% in HeLa cells (see Supplementary Material for calculation). Thus, the rate of chromosome nondisjunction in cells with a normal bipolar mitosis is sufficient to account for the rate of binucleation by furrow regression, and a higher rate of chromosome nondisjunction in HeLa cells may explain the correspondingly higher rate of binucleation compared to N/TERT-1 cells.

To explore the potential consequences of binucleation, we compared the fates of mononucleated and binucleated cells by time-lapse imaging. During the period of imaging, we found that 86% of mononucleated N/TERT-1 cells divided, whereas only 40% of binucleated cells did so (Fig. 3c). In contrast, binucleated HeLa cells showed little delay in cell cycle progression compared to their mononucleated counterparts. We also determined whether dividing binucleated cells underwent mitosis with a bipolar or multipolar spindle (Fig. 3d). In binucleated N/TERT-1 cells that entered mitosis, 65% formed a bipolar spindle, with subsequent formation of two mononucleated daughter cells, and 35% formed multipolar spindles. In contrast, 94% of binucleated HeLa cells that entered mitosis

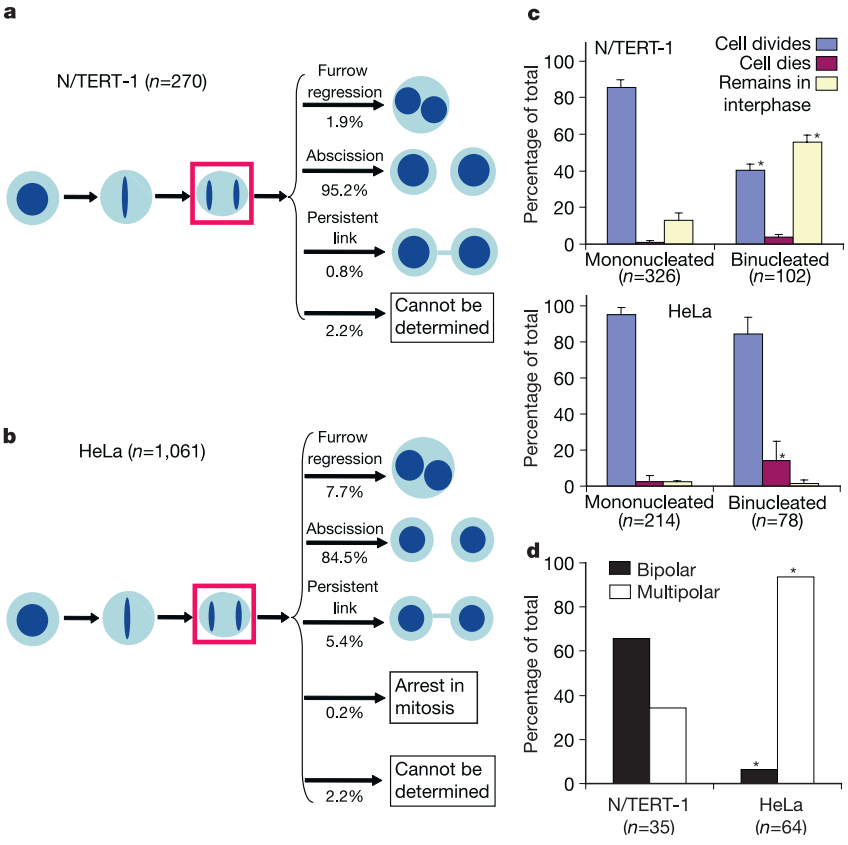

Figure 3 | Fates of mononucleated and binucleated N/TERT-1 and HeLa cells determined by long-term imaging. **a**, **b**, Mononucleated cells that underwent a bipolar mitosis followed by normal cytokinesis initiation were analysed by time-lapse imaging. The red box corresponds to the point at which cells were analysed for chromosome segregation by FISH in Fig. 1 and Supplementary Fig. 1. **c**, Analysis of division and apoptotic frequency of mononucleated and binucleated cells. Error bars are one standard deviation. **d**, Analysis of spindle polarity during mitotic division of binucleated cells. Asterisks indicate $P < 0.001$, $2 \times 2\ \chi^2$ test, comparing frequencies in binucleated and mononucleated cells for both N/TERT-1 and HeLa cells (**c**) or comparing spindle polarity frequencies in binucleated N/TERT-1 and HeLa cells (**d**).

formed multipolar spindles, with only 6% forming a bipolar spindle. The products of the multipolar divisions included mononucleated, binucleated and multinucleated cells (see Supplementary Table 3). These results predict that mononucleated tetraploid cells should be present among N/TERT-1 cells but not HeLa cells. Indeed, measurements of the ploidy of interphase mononucleated N/TERT-1 cells by FISH indicated that 12% of these cells are tetraploid (see Supplementary Fig. 5). In contrast, only 0.2% of interphase mononucleated HeLa cells have a chromosome composition consistent with a 4N DNA content. These data indicate that mononucleated tetraploid cells can arise through a process involving chromosome nondisjunction and furrow regression, followed by a bipolar mitosis in the subsequent division.

Our data indicate that completion of cytokinesis requires accurate chromosome segregation in human cells (Fig. 4). Although the mechanistic basis for this association remains to be elucidated, our findings have significant implications for understanding how chromosome segregation errors are related to changes in ploidy. We propose that single nondisjunction events are tightly coupled to regression of the cleavage furrow, such that nondisjunction results in the formation of one binucleated cell rather than two mononucleated aneuploid cells. As suggested by our analysis of N/TERT-1 cells, cell division may be delayed in spontaneously arising binucleated cells, thus suppressing the accumulation of cells with mis-segregated chromosomes. As cells do not seem to possess a tetraploidy checkpoint[21,22], this delay may be a consequence of nondisjunction rather than tetraploidization. If binucleated cells do divide, they appear to have the capacity to resegregate the errant chromosome in the subsequent mitosis to produce two genetically equivalent tetraploid cells. However, binucleated cells also contain an additional centrosome, which may promote a multipolar mitosis, as was observed in HeLa cells. Thus, depending on the regulation of spindle and centrosome function in binucleated cells, coupling of nondisjunction with cytokinesis may also potentiate chromosome mis-segregation during subsequent rounds of division.

Although loss of spindle checkpoint proteins[23] or amplification of mitotic regulators such as Aurora A[6] may be sufficient to induce chromosome segregation defects and cytokinesis failure, our findings suggest that such mutational events may not be required to initiate changes in ploidy in human cells. Instead, we propose that single stochastic errors in chromosome segregation may be sufficient to double cellular ploidy. The activation of oncogenes or loss of tumour suppressor genes might be necessary at a subsequent step, enabling binucleated cells to proliferate inappropriately, or to divide with a multipolar spindle to yield aneuploid progeny. Such a model might explain the correlation between loss of p53 and expansion of a tetraploid cell population in Barrett's oesophagus[24], a premalignant condition that precedes development of oesophageal cancer. Our findings are consistent with the notion that aneuploid cells may first proceed through a tetraploid state[9,24–27], and that spontaneous segregation errors may be sufficient to initiate a process that ultimately leads to aneuploidy[28]. The tight coupling of nondisjunction with cytokinesis suggests that tetraploid cells may arise more frequently than previously thought, and may explain why single gains or losses of chromosomes appear to be relatively rare in cancers, whereas a pattern of genome duplication followed by chromosome loss seems common[29].

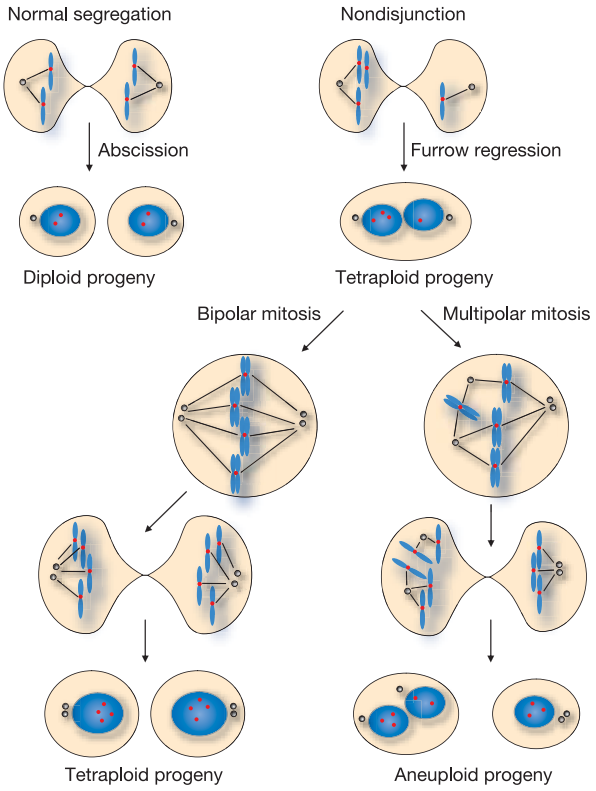

Figure 4 | Model summarizing the relationship of chromosome mis-segregation in the regulation of cytokinesis, and subsequent possible fates of resulting binucleated cells. DNA is blue and red spots represent centromeric FISH probes. Normal segregation (left) is associated with completion of cytokinesis producing two diploid mononucleated daughter cells. Chromosome nondisjunction (right) is associated with furrow regression, producing a binucleated tetraploid cell. If this cell divides, it can proceed through bipolar mitosis to produce two mononucleated tetraploid cells with equivalent genomes. However, if a multipolar spindle is formed, aneuploid progeny are likely to be produced due to high rates of chromosome mis-segregation resulting from multipolar mitosis.

METHODS

Cell lines. N/TERT-1 cells were cultured according to published conditions[14] and were found to have a normal male karyotype. PrEC cells (LH derivative line) were cultured as described[16]. HeLa cells (CCL2 from ATCC) were cultured in DMEM supplemented with 10% fetal calf serum and 1% non-essential amino acids (Invitrogen). HeLa cells had a modal chromosome number of 82, with 4 copies of chromosome 12 and 3 copies of chromosomes 6, 8 and 17. HeLa and N/TERT-1 cells expressing GFP–histone 2B were created as described in Supplementary Methods. Rates of chromosome mis-segregation and binucleation were similar between the parental cell lines and their GFP–histone 2B-expressing derivatives (see Supplementary Table 1).

Fluorescence *in situ* hybridization. Probes were labelled, hybridized and analysed by fluorescence microscopy as described[30] (see Supplementary Methods). For the data presented in Fig. 2 and Supplementary Table 2, coverslips were processed twice using two different sets of probes. Non-overlapping, intact cells were analysed for chromosome mis-segregation. For the data presented in Fig. 1 and Supplementary Fig. 1, mitotic and binucleated cells were analysed from the same regions of the coverglass. For mitotic cells, only anaphase or telophase cells with normal bipolar morphology were analysed. Interphase cells were verified for binuclear status using differential interference contrast microscopy. Binucleated cells were scored only if there was no overlap of the two nuclei or no hybridization signal in the overlap area if the two nuclei overlapped (see Supplementary Fig. 6). Nuclei were scored as having two or more copies of a specific chromosome if the signals of the same colour were of similar size and intensity and separated by a distance of more than half the diameter of the spot. To eliminate artefacts (for example, close, overlapping, missing or split signals; see Supplementary Fig. 6), only cells with an even total number of hybridization signals for every chromosome were scored. If the number of hybridization signals for a specific chromosome in each of the two nuclei of a cell was different, the cell was scored as having nondisjunction. Cells that underwent endomitosis were excluded from analysis based on their large nuclear size compared to neighbouring cells (see Supplementary Fig. 6).

Live cell imaging and analysis. HeLa or N/TERT-1 cells expressing GFP–histone 2B were grown on gridded coverglass bottom dishes (MatTek, Cat No. P35G-1.5-7-C-grid) for 26 h (HeLa, 2×10^5 cells per dish) or 3 d (N/TERT-1, 4×10^4 cells per dish) before imaging. Images were acquired automatically at multiple locations on the coverslip using a Nikon TE2000E inverted microscope fitted with a ×20 Nikon Plan Fluor objective, a linearly-encoded stage (Proscan, Prior)

and a Hamamatsu Orca-ER CCD camera. Fluorescence illumination used a mercury-arc lamp with two neutral density filters (for a total 32-fold reduction in intensity). The microscope was controlled using Simple PCI (Compix). The microscope was housed in a custom-designed 37 °C chamber with a secondary internal chamber that delivered humidified 5% CO_2. Fluorescence and differential interference contrast images were obtained every 15–22.5 min for a period of 28–88 h. Autofocusing was performed every 90 min using the fluorescence channel. Imaging conditions did not adversely affect cell proliferation or viability or increase the rate of binucleation compared to unimaged controls (data not shown).

For the experiment in Fig. 2, HeLa GFP–histone 2B cells were imaged for periods of 64, 68 and 88 h in three separate experiments, and then the coverslips were processed for FISH. Figure 2 illustrates the combined results of the three experiments; comparable results were obtained in each experiment. Cells were analysed if they entered mitosis at least 20 h before the end of imaging. Mononuclear cells that divided with a bipolar mitotic spindle were analysed for the presence of chromosome bridges or lagging chromosomes. We also documented whether cells completed cytokinesis or underwent regression of the cleavage furrow. The cells were scored as having chromosome bridges if the GFP–histone 2B positive nuclear material extended continuously between the two masses of chromosomes in anaphase or telophase, or between two daughter nuclei in the subsequent interphase (see Supplementary Fig. 7). Cells were considered to have lagging chromosomes if GFP–histone 2B-positive chromatin was observed in the midzone in anaphase or telophase, or in the cytoplasmic bridge in interphase, without evidence of chromatin linking the two daughter nuclei. Based on the presence or absence of chromosome lagging or bridging, the cells were then clustered hierarchically using Spotfire (UPGMA method using correlation as similarity measure). Results were displayed in Spotfire as a heat map.

For the experiments in Fig. 3a, b, cells expressing GFP–histone 2B were imaged for periods of 42–48 h (N/TERT-1 cells) and 28–72 h (HeLa cells). For Fig. 3c–e, cells were imaged for periods of 42–52 h (N/TERT-1 cells) or 28–72 h (HeLa cells). Standard deviations were derived from the results from 5 (binucleated N/TERT-1), 2 (mononucleated N/TERT-1), 4 (binucleated HeLa) or 2 (mononucleated HeLa) experiments.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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