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LETTER TO THE EDITOR

Lagging chromosomes entrapped in micronuclei are not 'lost' by cells

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Dear Editor,

In the literature, chromosomes lagging during anaphase and entrapped in micronuclei are considered to be a sign of genetic material loss and closely associated with aneuploidy formation [1-4]. However, some of these assumptions were based on analysis of fixed cells, and the actual behaviors of lagging chromosomes and micronuclei in live cells are poorly characterized [5]. To avoid the shortcomings associated with fixed samples, we previously labeled all the chromosomes with fluorescent protein for live cell imaging and have provided preliminary data on the formations of lagging chromosomes, micronuclei and their effect on the fate of cells [6, 7]. Unfortunately, labeling of all chromosomes prevents us from precisely tracking the fates of lagging chromosomes and micronuclei [7]. To address these issues, new experimental strategies are required.

In this study, colorectal cancer HCT116 cells were used to examine the fates of cells containing lagging chromosomes or micronuclei. We generated derivative clones with all the chromosomes labeled with red fluorescent protein (by expression of the mCherry-human histone 2B fusion gene) and one chromosome labeled with green fluorescent protein (GFP), which is achieved by integrating the *Escherichia coli* Lac operator (LacO) sequence into a single chromosomal locus in cells expressing the GFP-Lac repressor (LacI) fusion gene (Supplementary information, Data S1). Thus, the localization and dynamics of the single GFP-tagged chromosomes in every phase of cell cycle could be monitored in live cells (Figure 1Aa). Three clones that contain one copy of the GFP-labeled chromosome (4, 6 or 15) were chosen for further analysis. They displayed a karyotype, cell fates and cell cycle duration indistinguishable from the parental HCT116 cells (Supplementary information, Figures S1, S2 and Table S1).

Merotelic kinetochore (single kinetochore attaches to microtubules from two spindle poles) has been proposed to be the major mechanism underlying the spontaneous formation of lagging chromosomes, which can be remarkably enhanced in cells released from nocodazole treatment [8]. To examine the fates of lagging chromosomes, we utilized nocodazole treatment to increase the incidence of the GFP-tagged laggards (Supplementary information, Figure S3) and employed live cell imaging to track their fates (Supplementary information, Figure S4). We found that vast majority of the lagging chromosomes (84.62%-93.33%) eventually moved to the correct side of spindle. They often ended up as micronuclei in the daughter cells. Infrequently (6.67%-15.38%), the laggards ultimately moved to the incorrect side and produced a trisomic daughter cell with the laggard in micronucleus and a monosomic cell. Consistently, it has been indicated that micronucleus was more likely to reside in the correct cells [9]. Thus, most lagging chromosomes do not directly produce aneuploid progenies, but are entrapped in the micronuclei of correct daughter cells.

Given that the abnormal chromosome number often causes a proliferative disadvantage [10], we next explored whether chromosomes entrapped in micronuclei can also affect cell proliferation. To resolve this issue, we used long-term live cell imaging to estimate the proliferation potential of the cells containing GFP-tagged micronuclei generated from nocodazole-induced segregation error (Figure 1A and 1B, Supplementary information, Figure S5).

Nocodazole-treated mitotic cells were collected by shake-off and washed out. Cells were then plated for 8 h to permit the completion of mitosis prior to 24 h of time-lapse imaging. According to the descendents generated from anaphase cells harboring GFP-tagged laggards (Supplementary information, Figure S4), four types of daughter cells were tracked: (i) cells containing one GFP-tagged chromosome in nuclei (Figure 1Aa); (ii) or in micronuclei (Figure 1Ab, left-side cell); (iii) cells containing two GFP-tagged chromosomes, one each in the main nucleus and in the micronucleus (Figure 1Ac, right-side cell); and (iv) cells lacking the GFP-tagged chromosomes (Figure 1Ac, left-side cell).

For the untreated cells, only those containing one GFP-tagged chromosome in nuclei (scenario i) were ob-

served, of which 96.00%-97.00% entered mitosis during the 24 h of tracking (Figure 1B and Supplementary information, Figure S5). However, for the daughter cells generated from mitotic cells that released from nocodazole treatment, the frequencies (scenario i) of entering mitosis were decreased to 70.79%-76.30%, with the remaining cells undergoing cell cycle arrest or apoptosis (Figure 1B and Supplementary information, Figure S5). These can be explained by the fact that missegregation of unlabeled chromosomes causes a p53-induced proliferative disadvantage [10]. Interestingly, similar frequencies were observed in cells containing one GFP-tagged chromosome in micronuclei (scenario ii), with 67.65%-73.33% of the micronucleus-bearing cells entering mitosis (Figure 1Ab and 1B, Supplementary information, Figure S5). These results argue that chromosomes entrapped in micronuclei do not substantially affect the proliferation of the cells containing them. Conversely, if the cells gained (scenario iii) or lost a copy of chromosomes (scenario iv), their proliferation were significantly compromised with 57.89%-91.67% of the cells undergoing cell cycle arrest or apoptosis (Figure 1Ac and 1B, Supplementary information, Figure S5). In addition, loss of a chromosome (scenario iv) is more detrimental to cells than gain of a chromosome (scenario iii) (Figure 1B and Supplementary information, Figure S5). Therefore, gain or loss of chromosomes causes a proliferative disadvantage, but chromosomes entrapped in micronuclei *per se* do not impair the proliferation of the cells containing them.

These results provide ample evidence that maintaining the integrity of chromosome composition of a cell is crucial for cell survival and proliferation, no matter whether a chromosome is present in the main nucleus or micronucleus. Thus, chromosomes entrapped in micronuclei are not ignored by their host cells regarding pathways involved in cell fate determination. Previously, we have found that cells with micronuclei are more likely to die or undergo cell cycle arrest when compared with the micronucleus-free counterparts [7]. Combining the present findings with the previous ones, it is possible that such detrimental effects were only exerted by micronuclei originated from missegregated or damaged chromosomes but not by micronuclei generated from correctly segregated chromosomes.

Finally, we examined the destinies of micronuclei in cycling cells. Possible scenarios for the fates of the micronuclei include: (1) expulsion from the cell [3]; (2) apoptosis independent of the cell [2]; (3) removal by autophagy [4]; (4) retention within the cytoplasm as an extra-nuclear entity despite cell division [6]; or (5) reincorporation into the main nucleus [9]. The first four scenarios would cause genetic material loss from the cell. However, some of these putative destinies of the micronuclei were confusing because the results were based on analysis of fixed cells and it was difficult to verify in live cells with all chromosomes labeled. For example, although micronuclei might disappear in cells, it was hard to determine whether they were degraded, eliminated or overlaid with the main nuclei [7]. Fortunately, using the cells with a single chromosome labeled with GFP, the micronuclei harboring the labeled chromosomes can be followed as distinct fluorescent dots during cell division, and thus the destinies of micronuclei can be determined.

We scrutinized the behaviors of micronuclei containing GFP-labeled chromatins following nocodazole treatment (Figure 1C and 1D). After analyzing time-lapse videos of 182 micronuclei each with a GFP dot in 182 cells, we did not observe micronuclei expulsion from the cells or degradation *in situ*. These imply that the genetic materials in micronuclei are not lost by the cells. Importantly, when micronucleus-bearing cells entered mitosis, almost all of the GFP-labeled chromatins encapsulated in micronuclei (97.03%, or 98/101) were able to condense, congregate onto metaphase plate, separate and segregate into two daughter nuclei normally as the chromosomes in the main nuclei, regardless of the micronuclei in the correct (Figure 1Ab and 1Ca) or incorrect (Figure 1Cb) cells. Subsequently, such divisions produced two daughter cells without micronuclei (summarized in Figure 1D). Only 3 out of 101 GFP-tagged micronuclei remained as an independent entity during cell divisions (Figure 1Cc and 1D). The chromatins in these micronuclei did not appear to replicate because the GFP dots did not double after division. Given that the genetic material of these micronuclei did not join the chromosomal mass of the main nuclei during mitosis, we can speculate that these micronuclei lack a functional centromere. Indeed, both anaphase laggards and micronuclei composed of acentric chromosome fragments were occasionally observed at a similar frequency (Supplementary information, Figure S6).

Collectively, these results demonstrate that the chromosomes encapsulated in micronuclei behave normally as those in the main nuclei during cell division, and subsequently lead to the formation of cells without micronuclei. Therefore, the commonly used "loss" as a synonym for micronucleation is not accurate [1], at least in this type of micronuclei (originating from an intact chromosome), as the micronuclei appear to be functional. However, it is noteworthy that micronuclei can also originate from extrachromosomal element, namely, double minute (DM) in some cell lines. The origins and destinies of the DM-type micronuclei are far different from the chromosome-type micronuclei, and the DM-type micronuclei
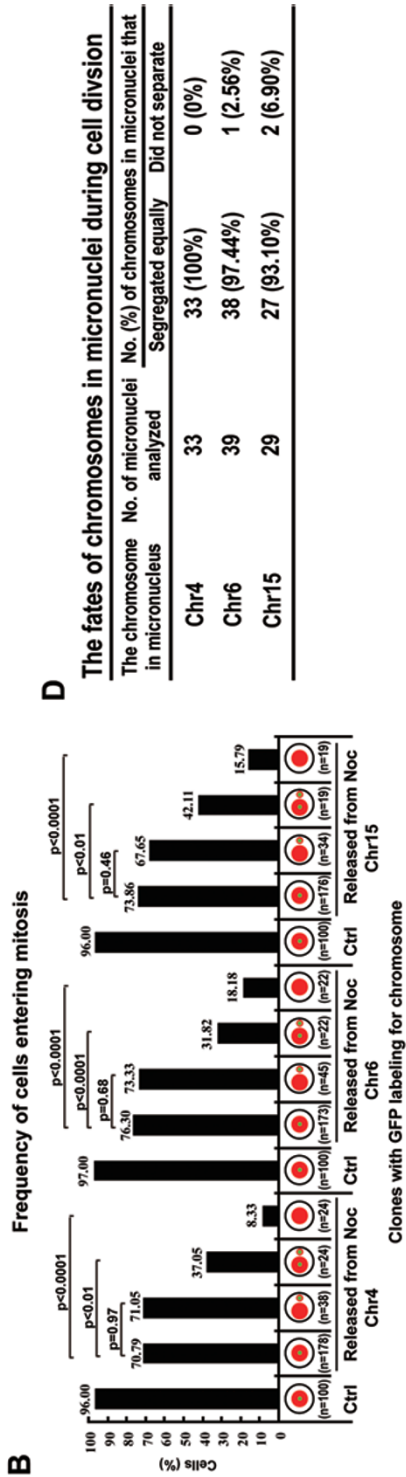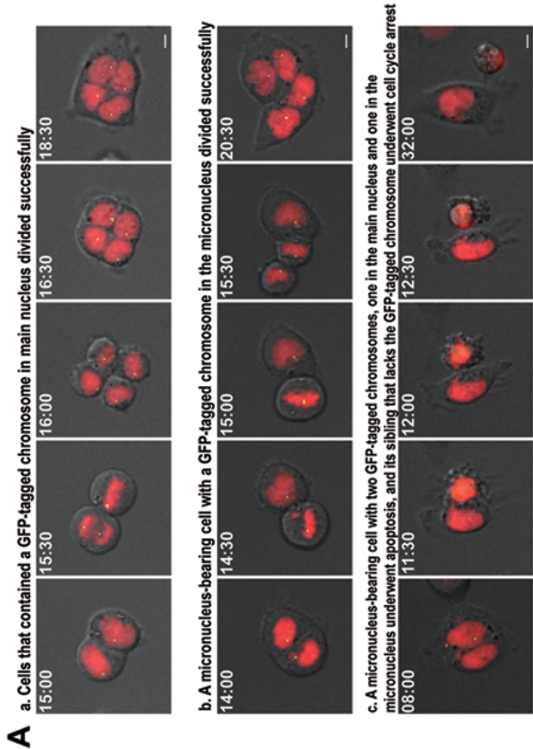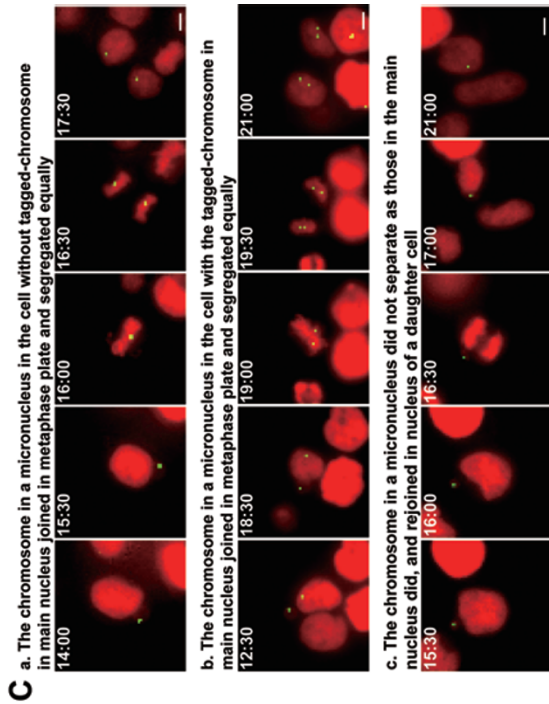
Figure 1 (A) The fates of cells containing GFP-tagged chromosomes in main nuclei or in micronuclei. Mitotic cells receiving nocodazole treatment were shaked off, washed in PBS, plated at low density and cultured for 8 h to allow the completion of mitosis prior to 24 h of time-lapse imaging. Plating at low density rendered the two daughter cells to remain adjacent, which facilitated the tracking with time-lapse microscope. Representative serial images showing that **a** Cells with a GFP-tagged chromosome in main nuclei divided successfully; **b** A micronucleus-bearing cell with one GFP-tagged chromosome in the micronucleus divided successfully; **c** A micronucleus-bearing cell with two GFP-tagged chromosomes, one in the main nucleus and one in the micronucleus underwent apoptosis, and its sibling cell that lacks the GFP-tagged chromosome underwent cell cycle arrest. Time is presented as h:min after mitotic shake-off. Scale bars: 5 μm. **(B)** Comparison of the frequencies of cells entering mitosis between different types of cells in untreated (Ctrl) and nocodazole-released (Noc) groups (*P*-values are from chi-square test; *n*: the number of cells analyzed). The fates of every paired daughter cell are summarized in Supplementary information, Figure S5. **(C)** The behavior of GFP-tagged chromosomes in micronuclei during cell division. Representative serial images showing that the GFP-tagged chromosomes in a micronucleus in a correct cell **(a)** or in an incorrect cell **(b)** segregated normally; **c** The GFP-tagged chromosomes in a micronucleus did not separate during mitosis and rejoined in nucleus of a daughter cell after division. Time is presented as h:min after mitotic shake-off. Scale bars: 5 μm. **(D)** Summarization of the fates of the GFP-tagged chromosomes in micronuclei during cell division.

can be extruded from the cells via cytoplasmic membrane blebbing [3].

In conclusion, our results directly demonstrate that most of the lagging whole chromosomes do not induce aneuploidy but reside in the correct daughter cells as micronuclei. The chromosomes entrapped in micronuclei do not substantially affect the proliferation of the host cells. They behave normally as those in main nuclei during cell division. Thus, the lagging chromosomes entrapped in micronuclei are not 'lost' by cells. Although these findings were obtained from one cell line, they were shared among three clones with a different chromosome labeled in each clone, indicating that the observed phenomena were generally applicable to different chromosomes. Further studies on whether these findings are applicable to other cells and exist *in vivo* as well are still required.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)