Horizontally transferred cell-free chromatin particles function as miniature "predatory" genomes and vehicles for transposable elements within host cells

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Abstract

We previously reported that cell-free chromatin particles (cfChPs) - chromosomal fragments released from the billions of dying cells - that circulate in human blood are horizontally transferred to healthy cells with biological effects. However, the underlying mechanism and function of these effects remained unclear. We treated NIH3T3 mouse fibroblasts cells with cfChPs isolated from human serum and using chromatin fibre fluorography, cytogenetic analysis, immuno-fluorescence and fluorescent *in situ* hybridisation investigated the intracellular activities of cfChPs. We found that the internalised cfChPs containing disparate DNA sequences had randomly combined to form complex concatemers some of which were ostensibly multi-mega base pairs in size. The concatemers performed many functions attributable to the nuclear genome. They could replicate and synthesize numerous proteins which appeared as highly complex fusion proteins. The concatemers harboured human LINE-1 and *Alu* elements and increased their copy number with time in culture. These findings lead us to hypothesise that a cell simultaneously harbours two genome forms that function autonomously: one that is inherited (hereditary genome) and numerous others that are acquired (miniature predatory genomes). The presence of miniature predatory genomes has evolutionary implications given their ability to generate a plethora of novel proteins and to serve as vehicles for transposable elements.

Introduction

Horizontal gene transfer (HGT) plays an important role in the adaptation of microorganisms to changing environments and in evolutionary processes [1]. However, defining the evolutionary role of HGT in mammals has remained a challenge. Nonetheless, experimental studies on HGT have offered insight into the regulations and functions of exogenously introduced genes and facilitating the design of drug-resistant cells, transgenic plants, and animals [2-4]. Indeed, gene therapy is founded on the principle of HGT, and several studies have indicated that oncogenes can be horizontally transferred across mammalian cells [5-9]. These observations support the hypothesis that HGT can occur naturally in mammalian cells and contributes to evolutionary processes.

Several hundred billion to trillion cells die in the body every day [10,11] and release chromosomal fragments in the form of cell-free chromatin particles (cfChPs), which enter the extracellular compartments, including the circulation [12]. We previously reported that cfChPs circulating in the blood and those that are released locally from dying cells are readily internalised by living cells through horizontal transfer followed by their genomic integration [13,14]. The genomic integration of cfChPs was confirmed by whole-genome sequencing and fluorescence *in situ* hybridisation (FISH) analysis, as well as by the detection of multiple human *Alu* elements in the recipient cells. Cellular uptake of cfChPs has several biological ramifications such as induction of DNA damage and the activation of inflammatory and apoptotic pathways [13,14]. Given the high turnover of cells, it can be assumed that all cells in the body continually internalise horizontally transferred cfChPs throughout their lifespan, leading to somatic mosaicism, which may be associated

with ageing, chronic diseases, and cancer [15]. However, the mechanisms and processes governing the intracellular consequences of horizontally transferred cfChPs remain unclear.

In this study, we investigated the intracellular processes and functions that regulate the various biological activities of cfChPs. We selected NIH3T3 mouse fibroblast cells for our experiments as we have extensive experience using this cell line for studies relating to cellular transformation; and these cells have also been widely used in various studies relating to oncogene discovery [16,17]. In our previous study [13], we investigated the relative biological activities and functions of healthy and cancerous cfChPs. We had found that cfChPs isolated from cancer patients had significantly greater activity than those isolated from healthy individuals with respect to all parameters examined. We had also reported that cfChPs released from dying cells can horizontally transfer themselves into healthy bystander cells leading to DNA damage and inflammation [14]. Therefore, in the present study, we treated NIH3T3 cells with cfChPs isolated from the sera of both cancer patients and healthy individuals, as well as with those released from hypoxia induced dying MDA-MB-231 human breast cancer cells. The cells were serially passaged following cfChPs treatment. Using chromatin fibre fluorography, cytogenetic analysis, and treatment with appropriate antibodies and fluorescent *in situ* hybridisation (FISH) probes, we analysed the intracellular dynamics and processes that regulate the functional properties of the internalised cfChPs. Our findings described in this article provide novel insights and research directions to obtain a new perspective about the process of evolution. They also suggest that horizontal gene transfer occurs in mammalian cells on a massive scale via the medium of “self” cfChPs that have undergone extensive and complex modifications, resulting in their behaviour as “foreign” genetic elements.

Results

Our results are described under three separate headings depending on whether NIH3T3 cells were treated with cfChPs isolated from sera of cancer patients or healthy individuals or with cfChPs released from hypoxia induced dying MDA-MB-231 breast cancer cells.

Experiments using cfChPs from cancer patients

cfChPs are rapidly and abundantly taken up by NIH3T3 mouse fibroblast cells

A representative electron microscopy image of the cfChPs isolated from the pooled sera of cancer patients is provided in Supplementary Fig. S1. Examination of NIH3T3 mouse fibroblasts treated with dually fluorescently labelled isolated cfChPs confirmed our earlier observation **[13]** that cfChPs can readily and rapidly horizontally transfer into healthy cells and accumulate in both their cytoplasm and nuclei by 6 h (Fig. 1A). The fluorescent particles appeared prominent, ostensibly owing to the amalgamation of multiple cfChPs to form large concatemers upon their cellular entry (described in detail below). Chromatin fibres prepared from similarly treated NIH3T3 cells revealed the presence of numerous dually labelled cfChPs of varying sizes, which were present in both the cytoplasm and overlapped with mouse DNA (Fig. 1B). Since chromatin fibres are derived from interphase cells, we further investigated whether the internalised concatemers could also be detected in metaphase cells. Metaphase spreads prepared from cfChPs-treated NIH3T3

cells at 6 h revealed many dually labelled cfChPs associated with the chromosomes (Fig. 1C).

Fig. 1

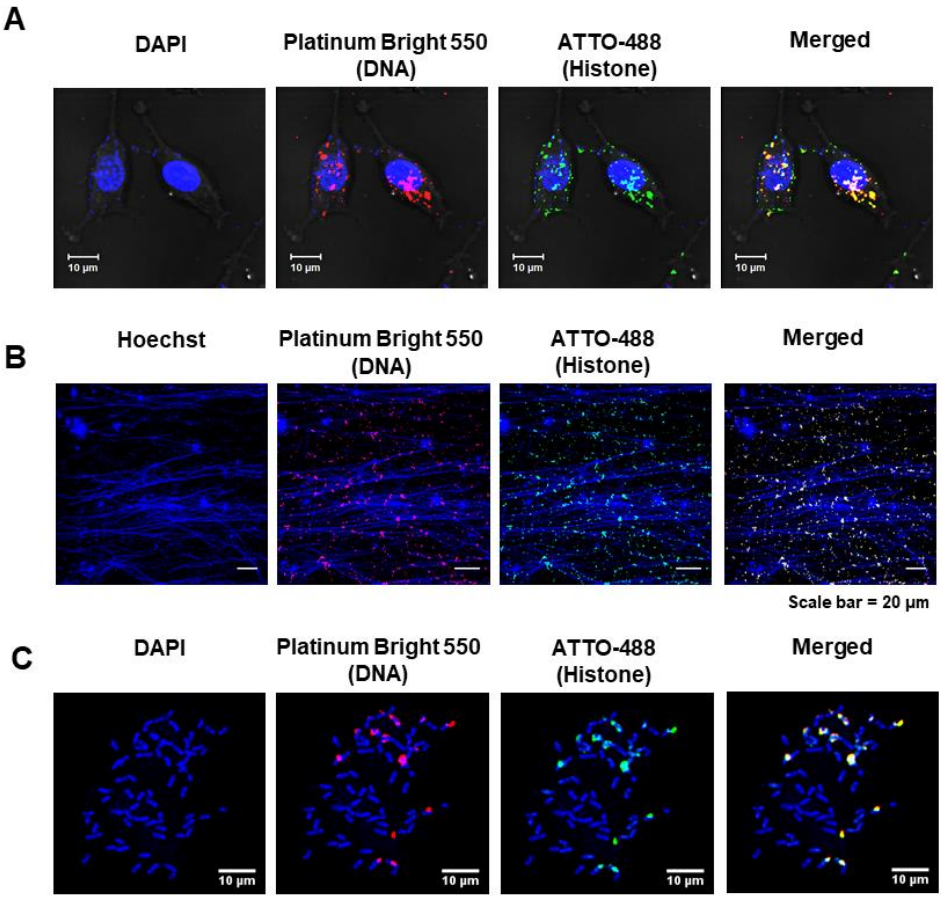


Fig. 1. Abundant uptake of cfChPs by NIH3T3 cells at 6 h. The DNA and histones of cfChPs (10 ng) were dually labelled with Platinum Bright 550 and ATTO-488 respectively and applied to NIH3T3 cells. **A.** Confocal microscopy images of cfChP-treated cells at 6 h showing many dually labelled fluorescent signals in the cytoplasm and nuclei. **B.** Fluorescence microscopy images of chromatin fibres prepared from similarly treated cells at 6 h showing numerous dually labelled fluorescent signals of varying sizes in the cytoplasm and in association with the chromatin fibres. **C.** Fluorescence microscopy images of metaphase spreads prepared from cfChPs-treated cells at 6 h showing multiple dually labelled fluorescent signals associated with the chromosomes.

cfChPs randomly combine to form concatemers

As suspected, FISH analysis of chromatin fibres and metaphase spreads prepared from cells in continuous passage using many different pairs of human chromosome-specific DNA probes revealed their co-localisation to generate dually labelled fluorescent signals (Fig. 2). Since all combinations of chromosome specific probes that we examined showed co-localizing signals, we concluded that concatemers represented highly complex amalgam of DNA fragments derived from different chromosomes with disparate and unrelated DNA sequences that were randomly linked to form complex genomic fragments of variable sizes. Some of the concatemers appeared to be markedly amplified, which is evident from images presented in Figure 2A (first and second panels) wherein concatemers representing chromosome 4 and 16 and chromosome 10 and 18 respectively are strikingly prominent. Even the smallest cfChPs appeared as concatemers (Fig. 2B). As the threshold of detection of a FISH signal is on the order of 100–200 kb **[18]**, even the smallest cfChPs can be assumed to be of a similar size range. Surprisingly, we detected a fluorescence signal of chromosome 4 co-localising with that of centromeres and of chromosome 20 co-localising with the signal of telomeres, suggesting the formation of chaotic chromosomal amalgams (Fig. 2A, panels 4&5). Perhaps the ultimate evidence of the chaotic nature of the concatemers comes from our detection of co-localising signals of telomere and centromere (Fig. 2A, panel 6). The centromeric signal seen in Fig. 2A, panel 4 is strikingly prominent, suggesting that the concatemers had not only incorporated centromeric DNA sequences within their folds but had also undergone large-scale amplification. Results of quantitative analysis of the degree of co-localisation of fluorescent signals generated upon treatment with FISH probes specific to different chromosome pairs are given in Supplementary Figure S2 A. The extent of co-localization varied between 8% and 18%. Concatamerisation could also be detected in metaphase

spreads prepared from serially passaged cfChPs-treated NIH3T3 cells (Fig. 2C). Control experiments performed on NIH3T3 cells that had not been exposed to cfChPs did not react with human specific probes against chromosome 4 and chromosome 16 (Supplementary Fig. 3A).

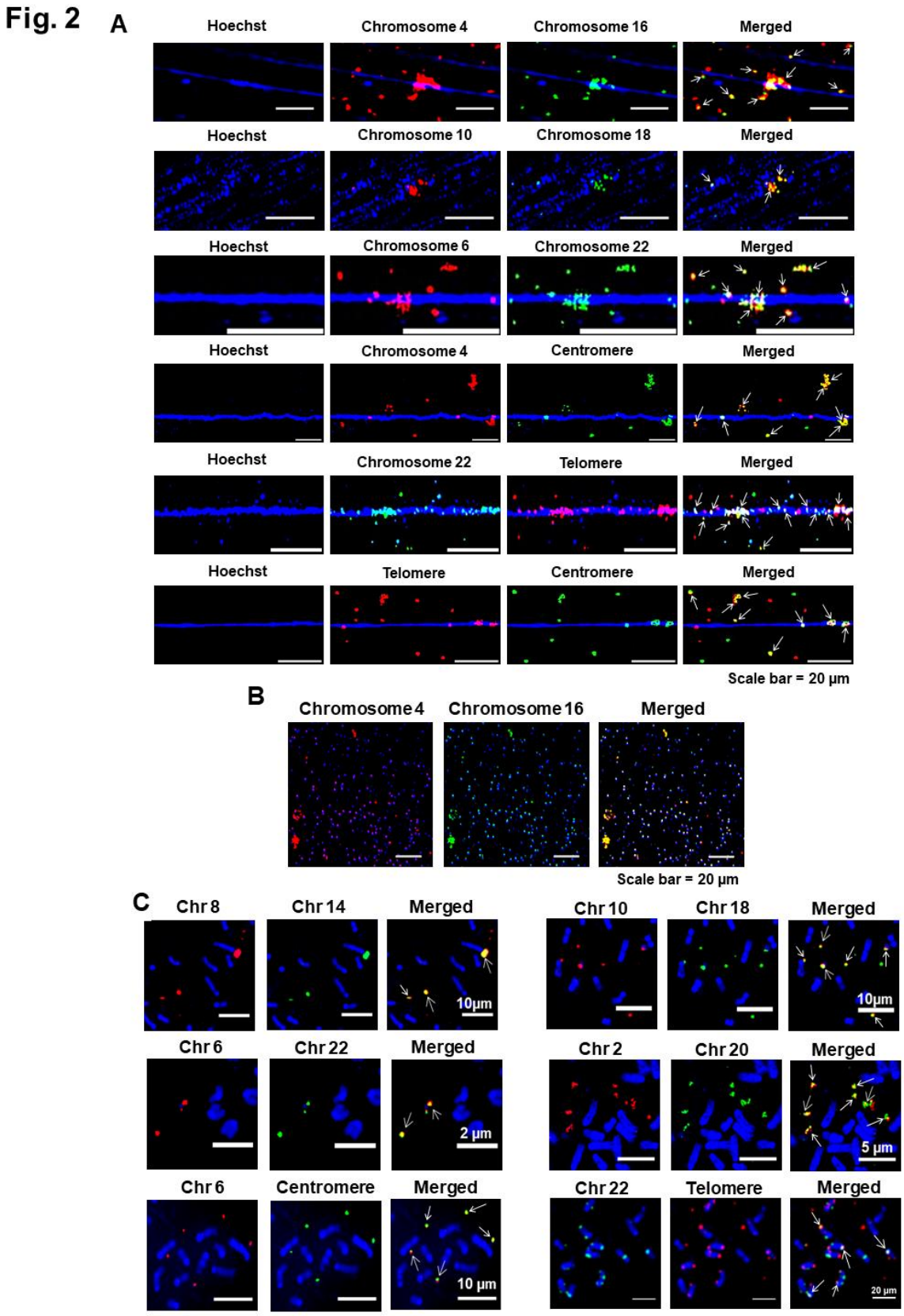


Fig. 2. cfChPs combine to form concatemers of variable sizes. A. FISH analysis of chromatin

fibres prepared from cfChP-treated NIH3T3 cells in continuous passage using different combinations of human chromosome-specific DNA probes, including probes specific for the human centromere and telomere, revealing co-localised fluorescent signals (arrows). **B.** Small cfChPs also show co-localised signals suggesting that they too comprised of concatemers **C.** Similar co-localising signals are shown on metaphase spreads. Co-localised signals are marked with arrows.

Concatemers exhibit variable spatial relationships with mouse DNA

Immuno-FISH analysis using an antibody against histone H4 and a human whole genomic FISH probe on chromatin fibres prepared from cfChPs-treated NIH3T3 cells in continuous passage revealed numerous dually labelled cfChPs that exhibited extensive structural and size variability, as well as remarkably variable spatial relationships with mouse chromatin fibres (Fig. 3A). Many cfChPs were detected in the cytoplasm or aligned with long stretches of mouse DNA, while others exhibited unusual conformations or were dangling from the mouse DNA. A similar peculiar conformation was found in clone D5 which had been developed several years earlier by treatment of NIH3T3 cells with cfChPs isolated from sera of cancer patients and which had undergone numerous passages and multiple freeze–thaw cycles **[13]** (Fig. 3B). This finding suggests that concatemers once formed are not eliminated or degraded can remain within the cell for long periods. The fluorescent signals of the concatemers were also strikingly prominent, suggesting that they greatly exceeded the threshold of detection of FISH signals (100–200 kb) **[18]**.

Fig. 3

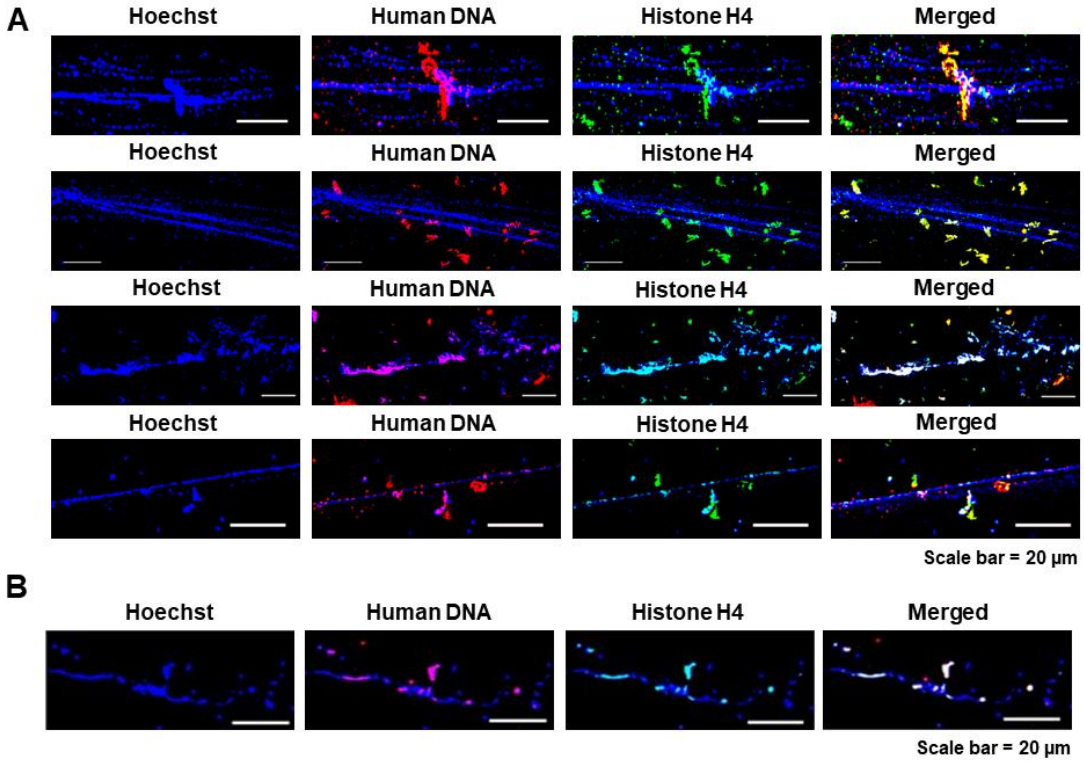


Fig. 3. Variable spatial relationships of concatemers with mouse chromatin fibres. A. Immuno-FISH analysis of chromatin fibres prepared from cfChPs-treated NIH3T3 cells in continuous passages using an antibody against histone H4 and a human whole-genome FISH probe. **B.** Immuno-FISH analysis of chromatin fibres prepared from clone D5 showing that human concatemers persisted even after numerous passages and freeze–thaw cycles.

Concatemers synthesise DNA polymerase and can potentially replicate

Immuno-FISH analysis of pulse-labelled cells using an anti-BrdU antibody and a human genomic DNA probe revealed co-localisation of the fluorescent signals, indicating that the concatemers were actively synthesising DNA (Fig. 4A, upper panel). Moreover, immuno-FISH experiments revealed that the concatemers expressed human DNA polymerase γ (Fig. 4A, middle panel), while dual immunofluorescence staining using antibodies against BrdU and human specific DNA polymerase γ revealed co-localised signals, indicating that

the concatemers had the potential to autonomously replicate themselves (Fig. 4A, lower panel). Results of quantitative analysis of the degree of co-localisation of fluorescent signals of human DNA and BrdU; human DNA and human DNA polymerase γ; and BrdU and human DNA polymerase γ are given in Supplementary Figure S2 B. The extent of co-localization varied between 18% and 27%. These findings were confirmed on metaphase preparations from serially passaged cfChP-treated NIH3T3 cells wherein we detected multiple co-localising signals of human DNA and BrdU, human DNA and human DNA polymerase γ and BrdU and human DNA polymerase γ in the extra-chromosomal spaces (Fig. 4B). Control experiments performed on NIH3T3 cells that had not been exposed to cfChPs did not react with either to the human DNA probe or the antibody against human DNA polymerase γ (Supplementary Fig. 3B).

Fig. 4

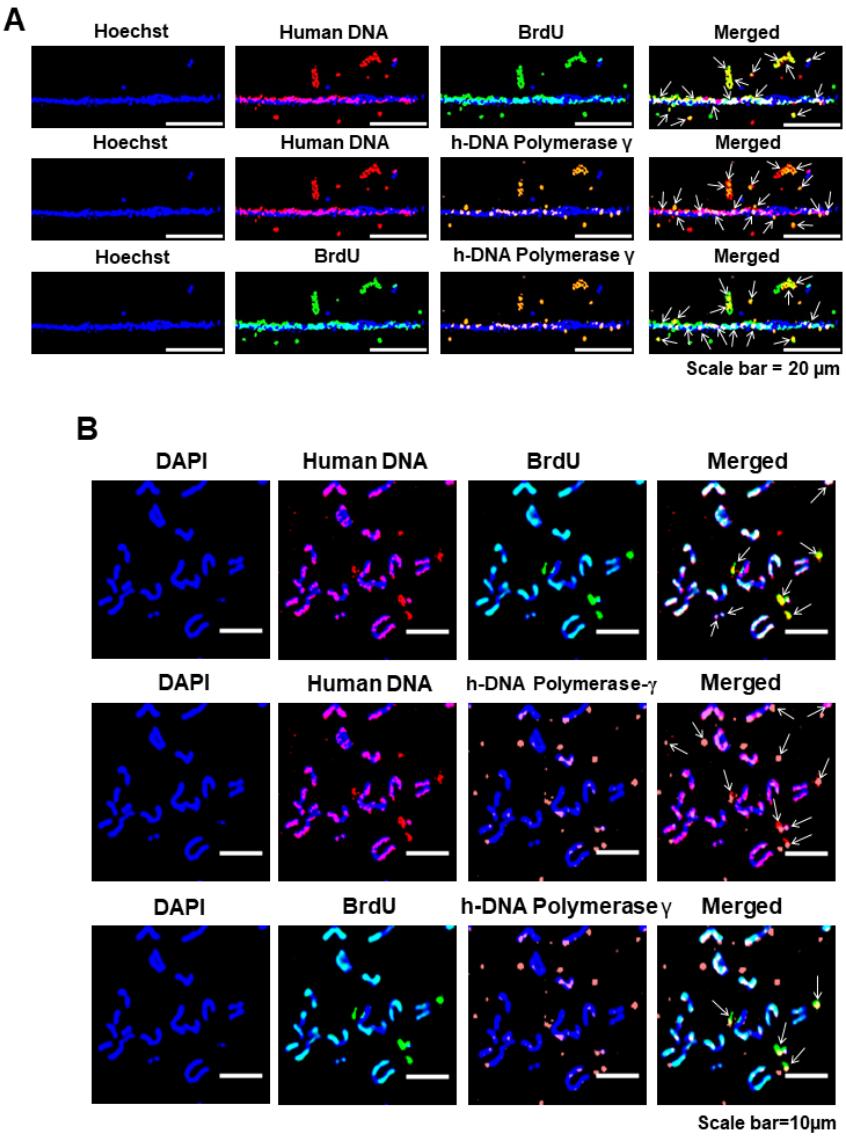


Fig. 4. The concatemers synthesise DNA and express human DNA polymerase γ. A. Chromatin fibres were prepared from NIH3T3 cells in continuous passage, which were pulse-labelled for 24 h with BrdU. Immuno-FISH experiments performed using antibodies against BrdU, human-specific DNA polymerase γ, and a human whole-genome FISH probe show co-localised signals of human DNA and BrdU, human DNA and DNA polymerase γ, and BrdU and DNA polymerase γ. Co-localised signals are marked with arrows. **B.** Similar co-localising signals are shown on metaphase spreads. Co-localised signals are marked with arrows.

Given the above results that the concatemers expressed DNA polymerase, we evaluated their potential for self-replication. Analysis of 10 metaphase spreads prepared at

progressively increasing passage numbers showed a progressive increase in the number of human FISH signals per chromosome which reached a maximum at passage 198. The increase in FISH signals per chromosome between passage 2 and passage 198 was 5 fold (Fig. 5). It should be noted that this analysis is restricted to the proliferative capacity of concatemers that were associated with the chromosomes and did not take into account the replicative potential of those that are present in the cytoplasm.

Fig. 5

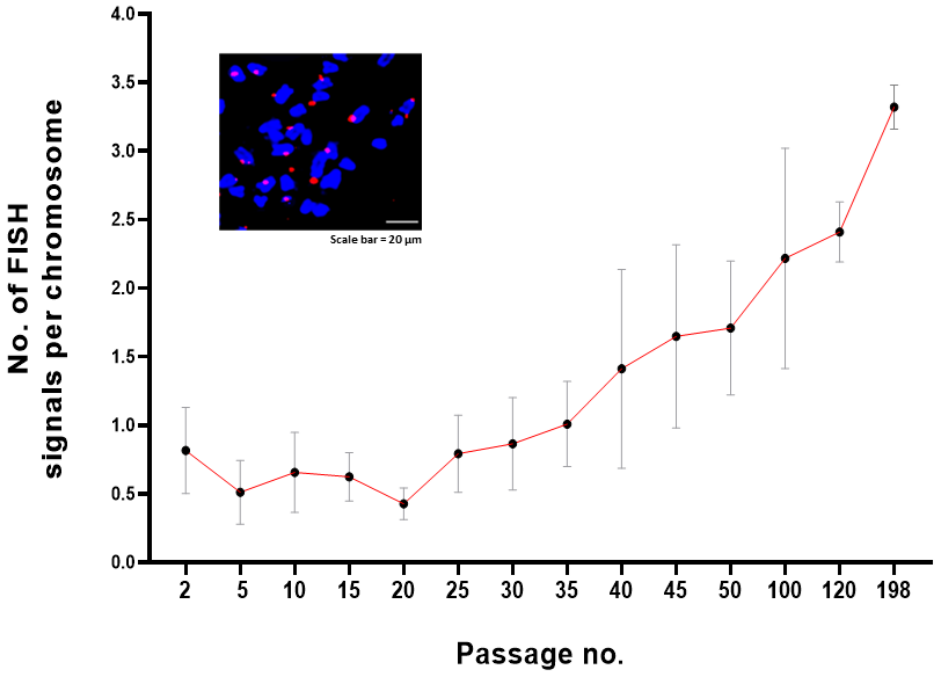


Fig. 5. Concatemers increase their copy number in the mouse genome over time. Metaphase spreads were prepared from cells at progressively increasing passages and stained with a human-specific FISH probe. The mean ± standard error of the number of human FISH signals per chromosome was determined after analysing 10 metaphase spreads at each time point. A progressive increase in concatemer signals per chromosomes is evident with increasing passage number (analysis of variance for linear trend $p < 0.0001$.). The inset shows a representative partial metaphase image of DNA FISH positive signals on chromosomes. Blue and red signals represent DAPI and human DNA, respectively.

Concatemers are composed of open chromatin

Next, we examined the epigenetic constitution of concatemers using antibodies against the histone markers representing trimethylation of histone H3 at lysine 4 (H3K4me3) and trimethylation of histone H3 at lysine 9 (H3K9me3), indicating active gene promoters and regions associated with long-term repression respectively [19]. Chromatin fibres prepared from cfChPs-treated NIH3T3 cells in continuous passage were dually immune-stained with antibodies targeting H3K4me3 and H3K9me3 antibodies and the number of concatemers reacting with the two antibodies was counted. We found that the majority of the concatemers either reacted exclusively with antibodies against H3K4me3 (72%) or were hybrids of H3K4me3 and H3K9me3 (17%). Only 11% of the concatemers reacted exclusively with H3K9me3 (Fig. 6). Taken together, these data indicate that most concatemers largely contained nucleosome-depleted regions and could bind to protein factors that facilitate gene transcription [20] and DNA replication [21].

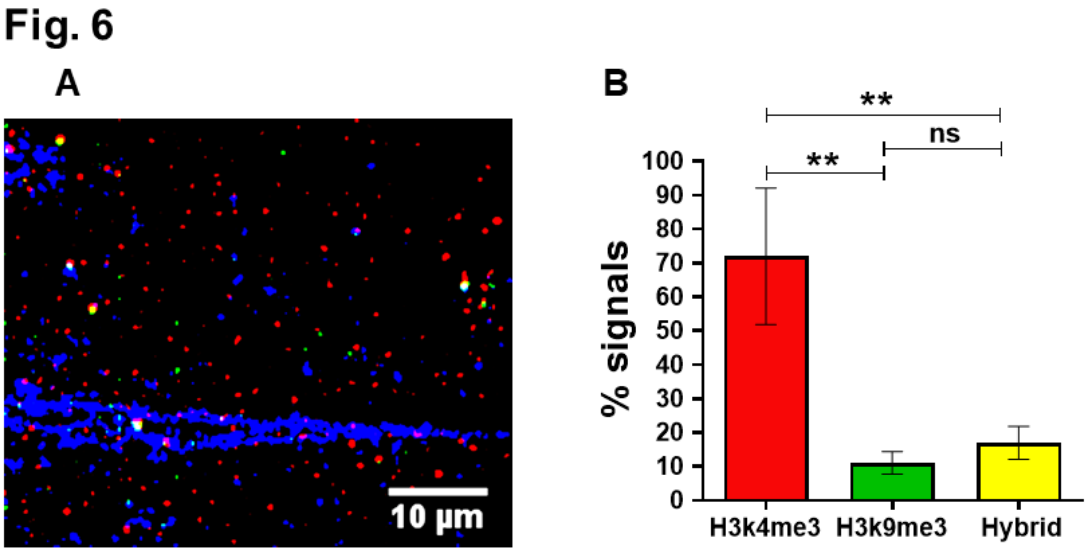


Fig. 6. Concatemers are largely comprised of open chromatin. A. Representative image of a chromatin fibre preparation immunostained with antibodies targeting H3k4me3 (red) and H3k9me3 (green); hybrid concatemers are represented by co-localised yellow signals. The chromatin fibres are stained with DAPI emitting blue fluorescence. **B.** Histogram showing quantitative estimates of H3k4me3, H3k9me3, and hybrid histones after counting 1000

fluorescent signals. The histograms represent mean ± SEM values. Statistical analysis was performed using two-tailed Student's *t*-test, ** $p < 0.01$; ns = not significant.

Concatemers can synthesise RNA

As the above findings suggested that concatemers are potentially capable of active transcription, we investigated their potential for RNA synthesis. Using an assay kit which detects global RNA transcription, we detected abundant RNA in the cytoplasm of cfChP-treated passaged cells, which was absent in the control NIH3T3 cells (Fig. 7A). As RNA synthesis is normally restricted to the nucleus, the detection of RNA in the cytoplasm indicated that DNA contained within the concatemers were undergoing active transcription. Treatment of cells with actinomycin D or maintaining the cells at low temperature abolished RNA synthesis confirming that RNA synthesis is dependent on active cellular metabolism. These findings were confirmed in experiments involving chromatin fibres wherein DNA and RNA signals were found to co-localise, indicating that the satellite concatemers were supporting RNA synthesis (Fig. 7B).

Fig. 7

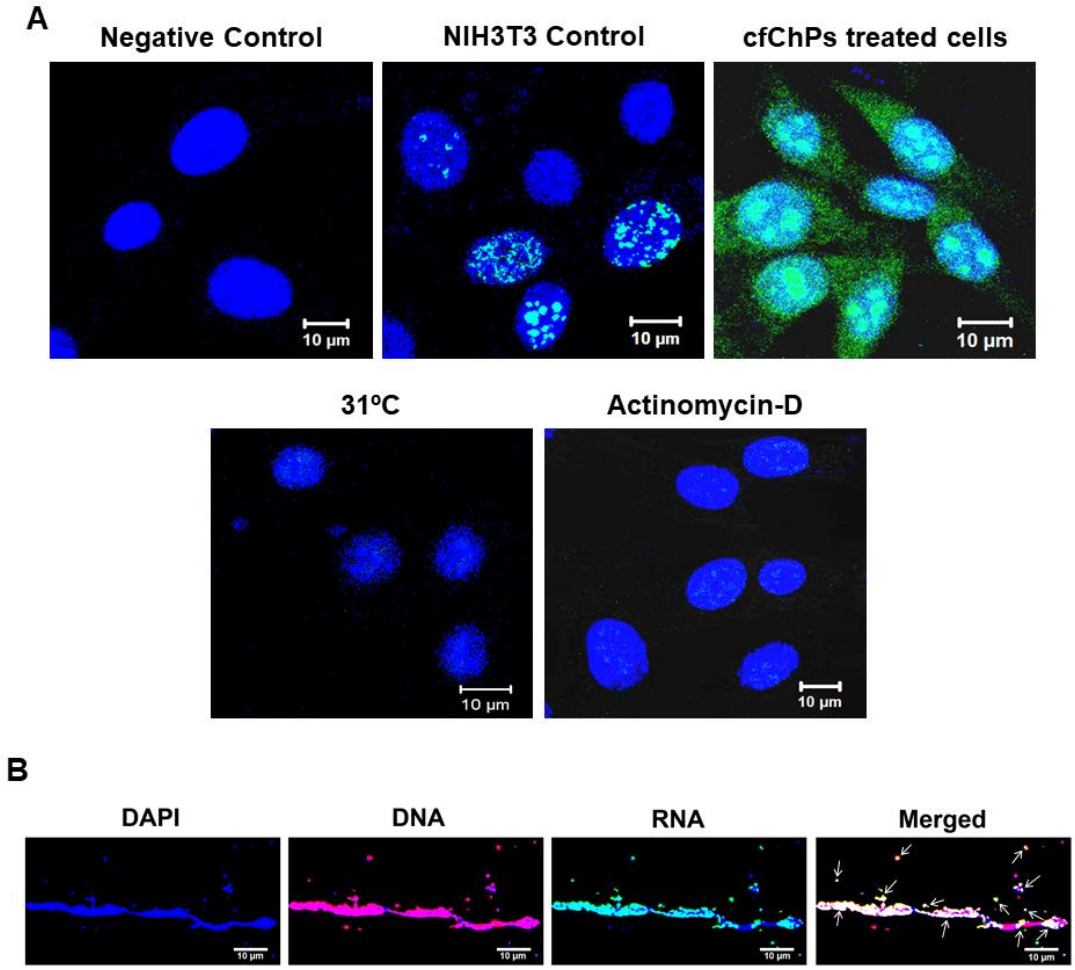


Fig. 7. cfChP-treated NIH3T3 cells synthesise RNA which is dependent on active cellular metabolism. A. RNA synthesis by cfChP-treated cells in continuous passage and control NIH3T3 cells with and without treatment of Actinomycin D (0.0005 µg/mL) or maintenance at 31°C. **B.** Chromatin fibres immunostained with antibodies against DNA and used in the RNA synthesis assay. Co-localised DNA and RNA signals are marked with arrows.

Concatemers can synthesise their own protein synthetic machinery

The extensive presence of RNA in the cytoplasm led us to investigate whether the passaged cells transcribed any specific RNAs. Dual FISH analysis using probes against

human DNA (to detect concatemers) and human ribosomal RNA on chromatin fibres prepared from serially passaged cfChP-treated cells revealed strictly co-localised signals, indicating that the concatemers synthesised ribosomal RNA (Fig. 8A, upper panel). Immuno-FISH analysis revealed co-localised fluorescent signals generated by a human-specific DNA probe and antibodies against human specific RNA polymerase III and ribosomal protein (Fig. 8A, middle and lower panels, respectively). These findings indicated that concatemers can autonomously of synthesise their own critical components of the protein synthetic machinery. Results of quantitative analysis of the degree of co-localisation of fluorescent signals of human DNA and human ribosomal RNA; human DNA and RNA polymerase III; and human DNA and ribosomal protein are given in Supplementary Figure S2 C. The extent of co-localization varied between 17% and 29%. We confirmed these findings in metaphase preparations from serially passaged cells for all three components of the protein synthetic machinery described above (Fig. 8B). Control experiments performed on NIH3T3 cells that had not been exposed to cfChPs did not react with the human specific probe against DNA, ribosomal RNA and the human specific antibodies against RNA polymerase III and ribosomal protein (Supplementary Fig. 3C).

Fig. 8

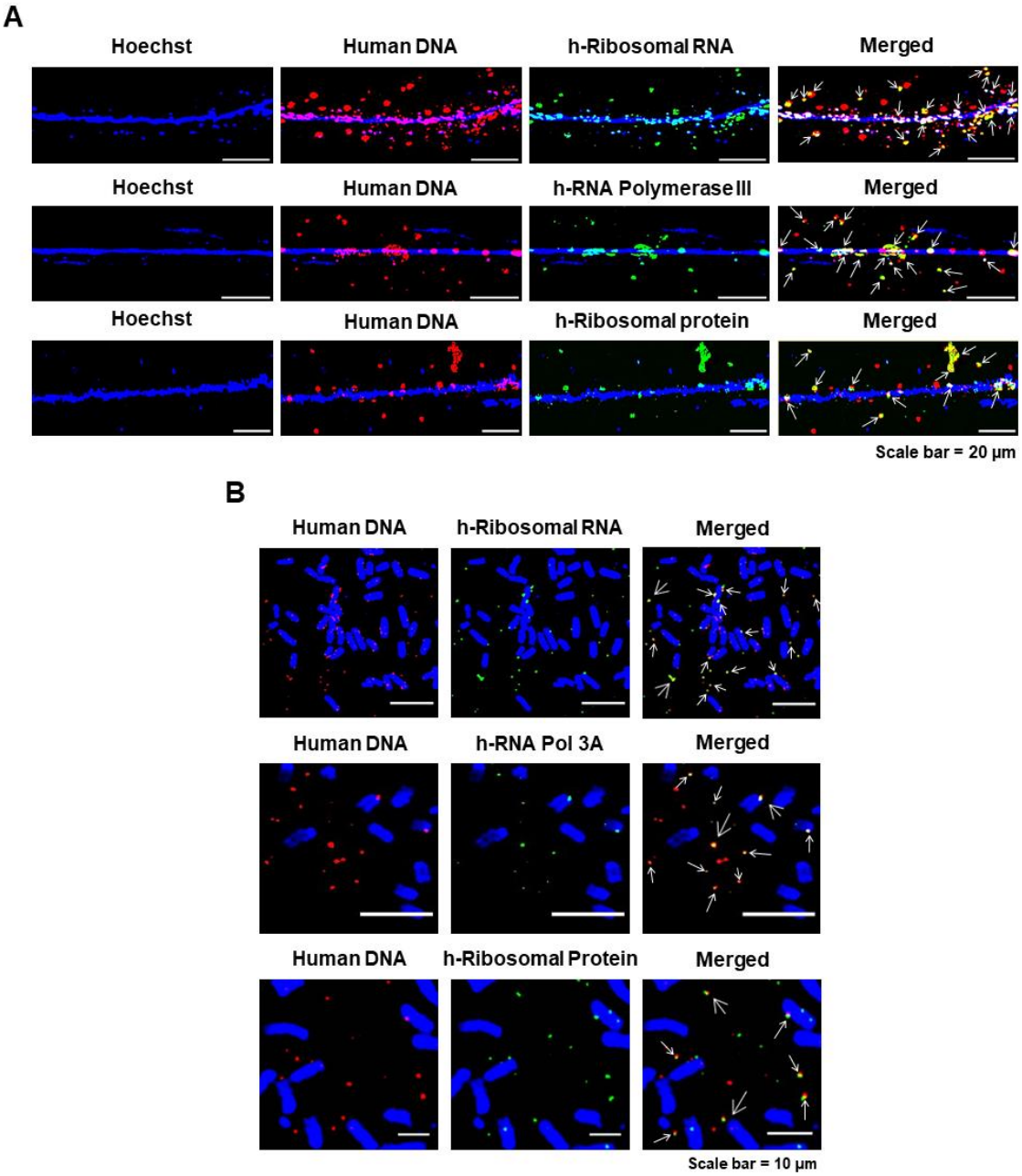
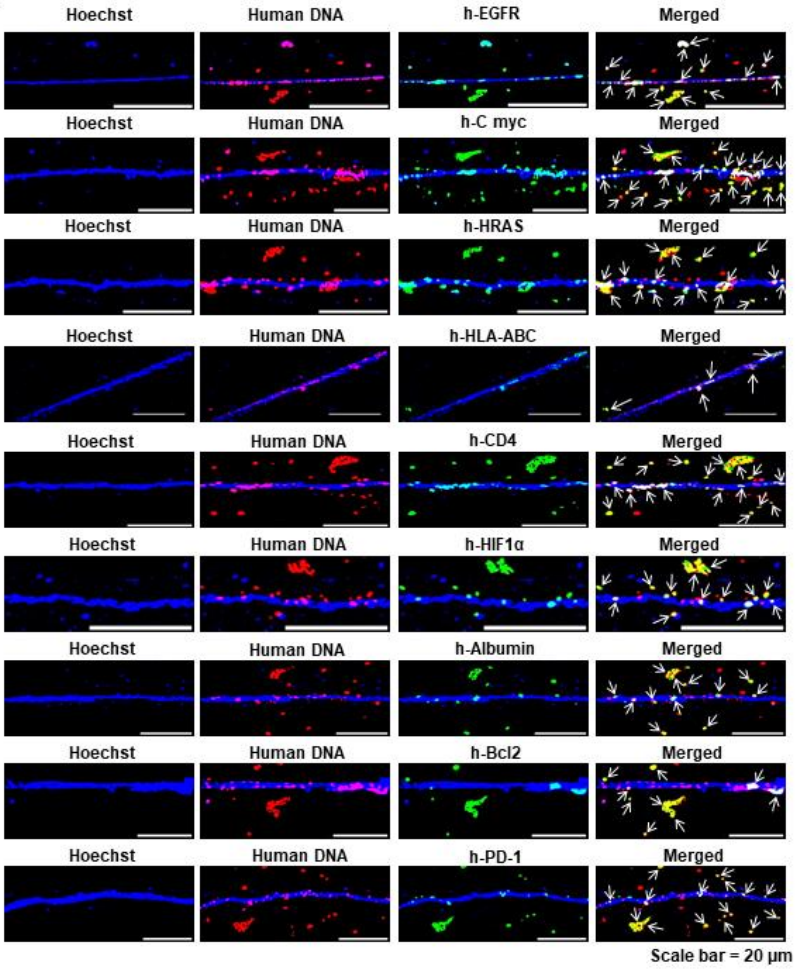


Fig. 8. Concatemers synthesise their own protein synthetic machinery. Dual-FISH analysis of chromatin fibres **(A)** and metaphase spreads **(B)** prepared from cfChP-treated NIH3T3 cells in continuous passage using probes against human DNA and human-specific ribosomal RNA along with immuno-FISH experiments using a human-specific DNA probe and antibodies against human-specific RNA polymerase III and ribosomal protein. Co-localised fluorescent signals are marked with arrows (middle and lower panels).

Concatemers synthesise diverse human proteins in mouse cells

Having confirmed that concatemers are capable of synthesising RNA and assembling their own protein synthetic machinery, we next investigated whether they are capable of autonomously synthesising proteins. We found that the concatemers were capable of synthesising a wide array of proteins that we examined which included both oncogenic and non-oncogenic proteins (Fig. 9A). Significantly, the newly synthesised proteins consistently remained associated with the concatemers of origin (marked by human DNA signals). The implication of this finding is addressed in the discussion section. It should be noted that many of the proteins, such as the oncogenes h-EGFR, h-C myc and h-HRAS as well as the anti-apoptotic protein h-Bcl2 and the immune check-point inhibitor h-PD1 appear highly over-expressed. Results of quantitative analysis of the degree of co-localisation of fluorescent signals of human DNA and of various human proteins are given in Supplementary Figure S2 D. The extent of co-localization varied between 17% and 30%. We further confirmed these findings in metaphase preparations from serially passaged cfChPs-treated NIH3T3 cells (Fig. 9B). We also conducted extensive control experiments on NIH3T3 cells that had not been exposed to cfChPs using a variety of human specific antibodies and found that none of them showed any positive fluorescent signals (Supplementary Fig. 3D).

Fig. 9 A


Hoechst
Human DNA
h-EGFR
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h-HLA-ABC
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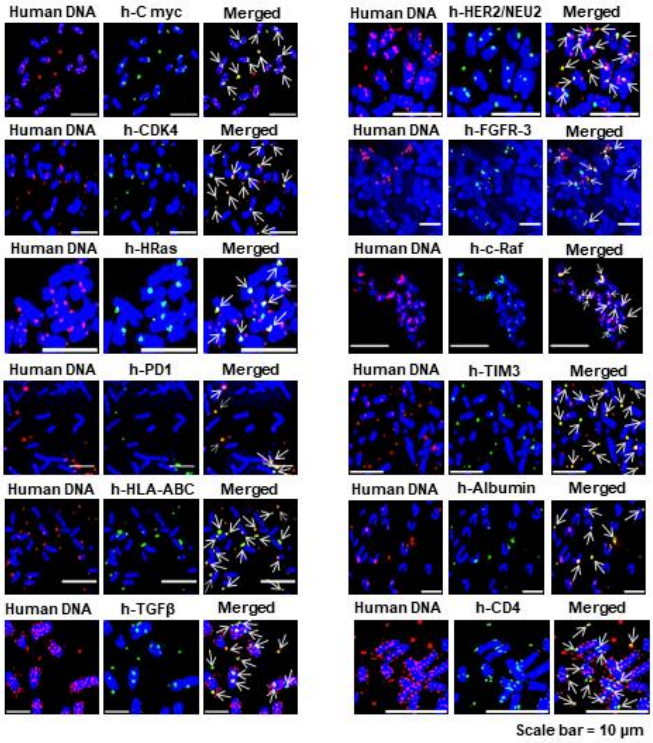
B


Human DNA
h-C myc
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Human DNA
h-c-Raf
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h-PD1
Merged
Human DNA
h-TIM3
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Human DNA
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h-Albumin
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h-CD4
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Fig. 9. Concatemers synthesise various human proteins. Immuno-FISH analysis using antibodies against various human proteins and a human whole-genome FISH probe on chromatin fibres **(A)** and metaphase spreads **(B)** prepared from cfChPs-treated NIH3T3 cells in continuous passage. Co-localised signals of human DNA and various proteins are marked with arrows.

Concatemers synthesise complex fusion proteins

Because concatemers are amalgams of multiple cfChPs containing disparate DNA sequences, we investigated the nature of the proteins that they synthesized. We found that the fluorescent signals of two antibodies against diverse unrelated proteins were frequently co-localised, indicating that the proteins synthesised by the concatemers were fusion proteins with potentially novel functions (Fig. 10). Fusion proteins were detected both on chromatin fibres and metaphase preparations. Results of quantitative analysis of the degree of co-localisation of fluorescent signals indicative of fusion proteins detected by the various antibody pairs are shown in Figure 10A. The extent of co-localised signals ranged between 8% and 36% (Supplementary Figure S2 E).

Fig. 10

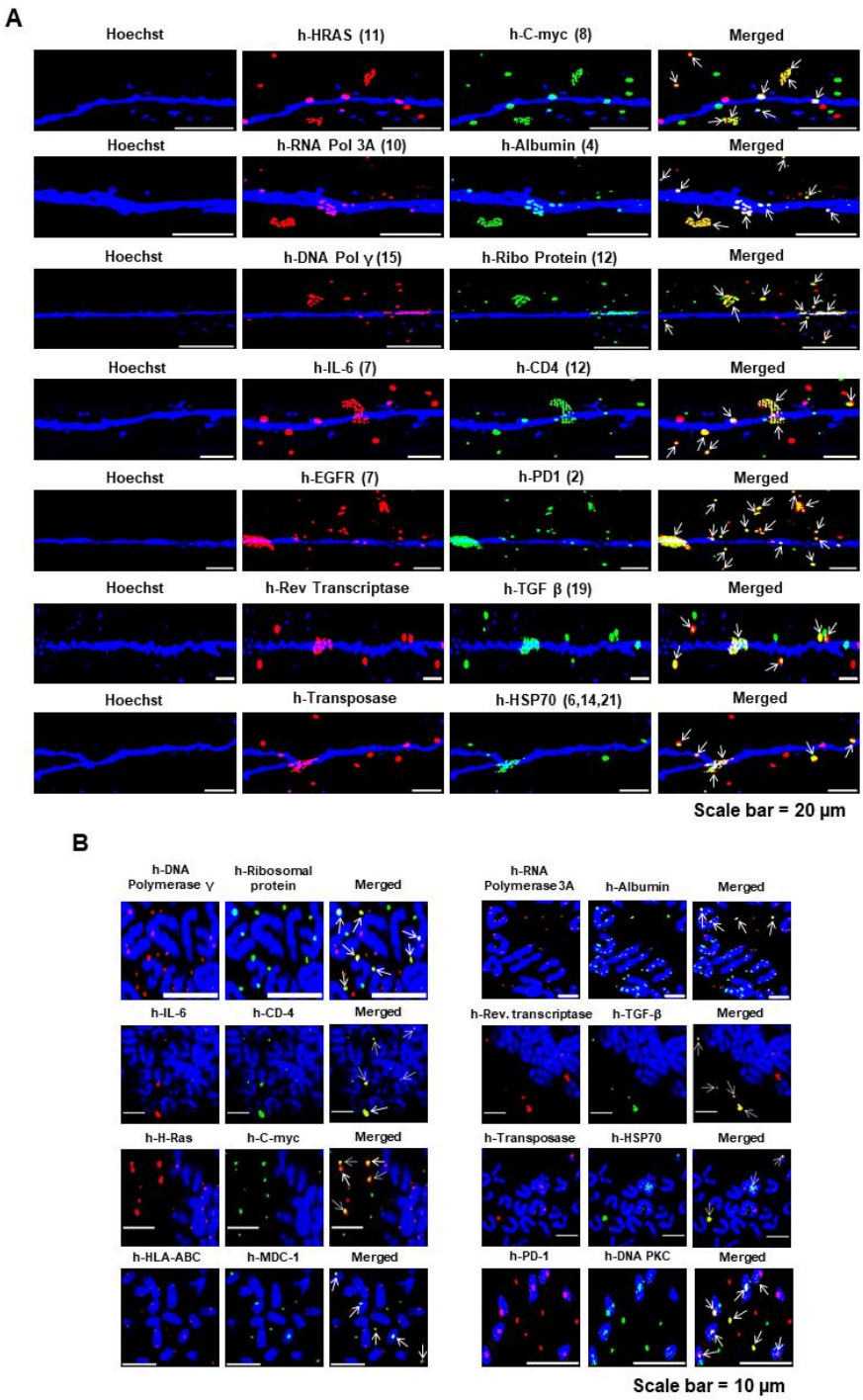

Fig. 10. Proteins synthesised by concatemers are fusion proteins. Dual immunofluorescence analysis using antibody pairs targeting diverse human-specific proteins in chromatin fibres **(A)** and metaphase spreads **(B)** from cfChPs-treated NIH3T3 cells in continuous passage showing frequent co-localised signals indicative of fusion proteins (arrows). The numbers given in brackets in **A** indicate the chromosomal location of the genes that correspond to the proteins.

Concatemers are vehicles of transposable elements

Among the fusion proteins described above, we identified reverse transcriptase and transposase fused with transforming growth factor-β and heat shock protein 70, respectively, suggesting that the concatemers might harbour the transposable elements long interspersed nucleotide element 1 (LINE-1) and *Alu*. Dual-FISH experiments using a human DNA probe and probes targeting human LINE-1 and *Alu* showed that many of the DNA signals had co-localised indicating that the concatemers contained DNA sequences of retro-transposable elements (Fig. 11A). Results of quantitative analysis of the degree of co-localisation of fluorescent FISH signals of human DNA and human LINE-1 and Alu on chromatin fibres are given in Supplementary Figure S2 F. The extent of co-localization was 31% and 25% for LINE-1 and Alu respectively. Similar co-localizing signals were also seen on metaphase preparations (Fig. 11B). The fact that both LINE-1 and *Alu* signals could be clearly detected by FISH analysis indicated that they were extensively amplified, given that LINE-1 and Alu are approximately 6000 bp and 300 bp in size, respectively. Control experiments performed on NIH3T3 cells that had not been exposed to cfChPs using human LINE-1 and Alu probes did not reveal any positive signals (Supplementary Fig. 3E)

Fig. 11

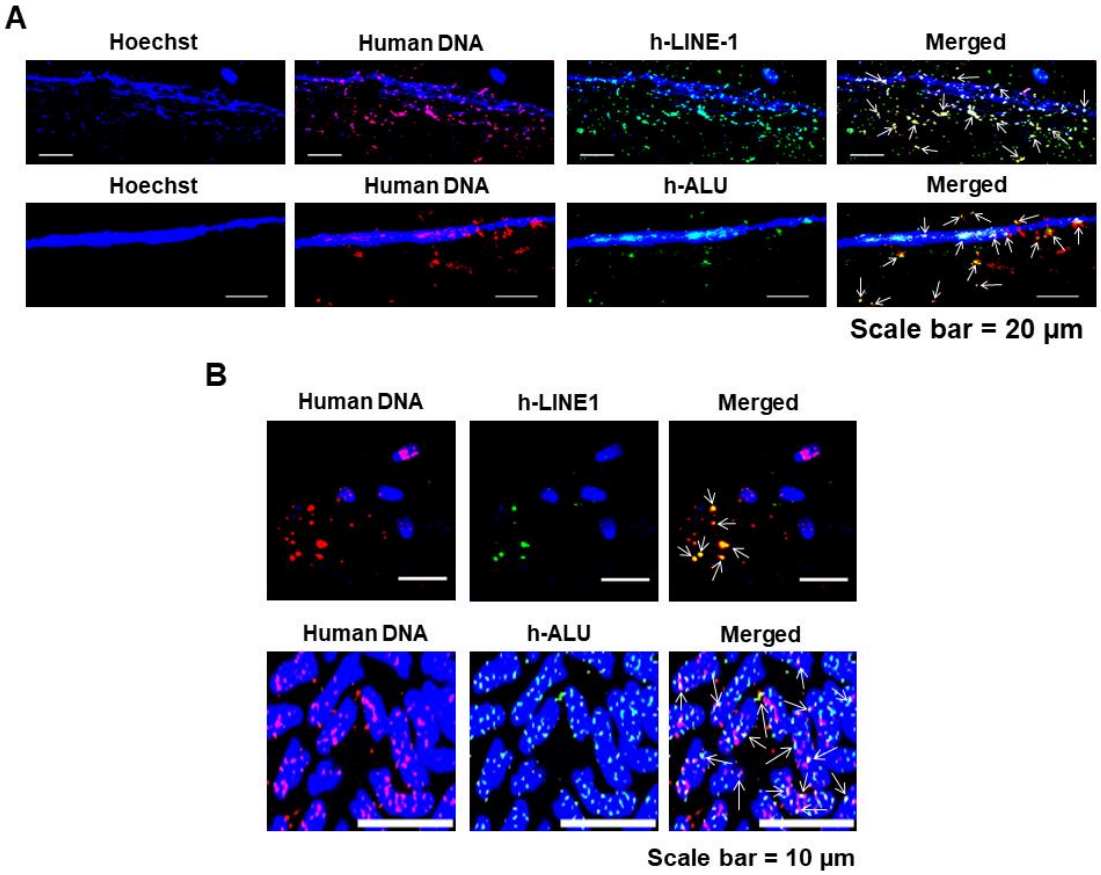

Fig. 11. Concatemers harbour transposable elements. Dual-FISH analysis of chromatin fibres (**A)** and metaphase spreads **(B)** prepared from cfChPs-treated NIH3T3 cells in continuous passage using probes against human LINE-1 or human *Alu* and human genomic DNA. Co-localised signals are marked with arrows.

LINE-1 and *Alu* elements are associated with reverse transcriptase, transposase, and DNA polymerase

Immuno-FISH analysis using antibodies against human reverse transcriptase and transposase and FISH probes against LINE-1 and *Alu* revealed co-localised signals, indicating that reverse transcriptase and transposase were associated with DNA sequences corresponding to LINE-1 and Alu (Fig. 12A and B). Such an association could potentially allow these sequences to translocate from one position in the mouse genome

to another. Results of quantitative analysis of the degree of co-localisation on chromatin fibres of fluorescent FISH signals of human LINE-1 and human reverse transcriptase and transposase; and Alu and human reverse transcriptase and transposase are given in Supplementary Figure S2 G. The extent of co-localization varied between 21% and 30%. Control experiments performed on NIH3T3 cells that had not been exposed to cfChPs using human specific antibodies against reverse transcriptase and transposase and FISH probes against h-LINE-1 and h-ALU did not reveal any positive signals (Supplementary Fig. 3F).

Fig. 12

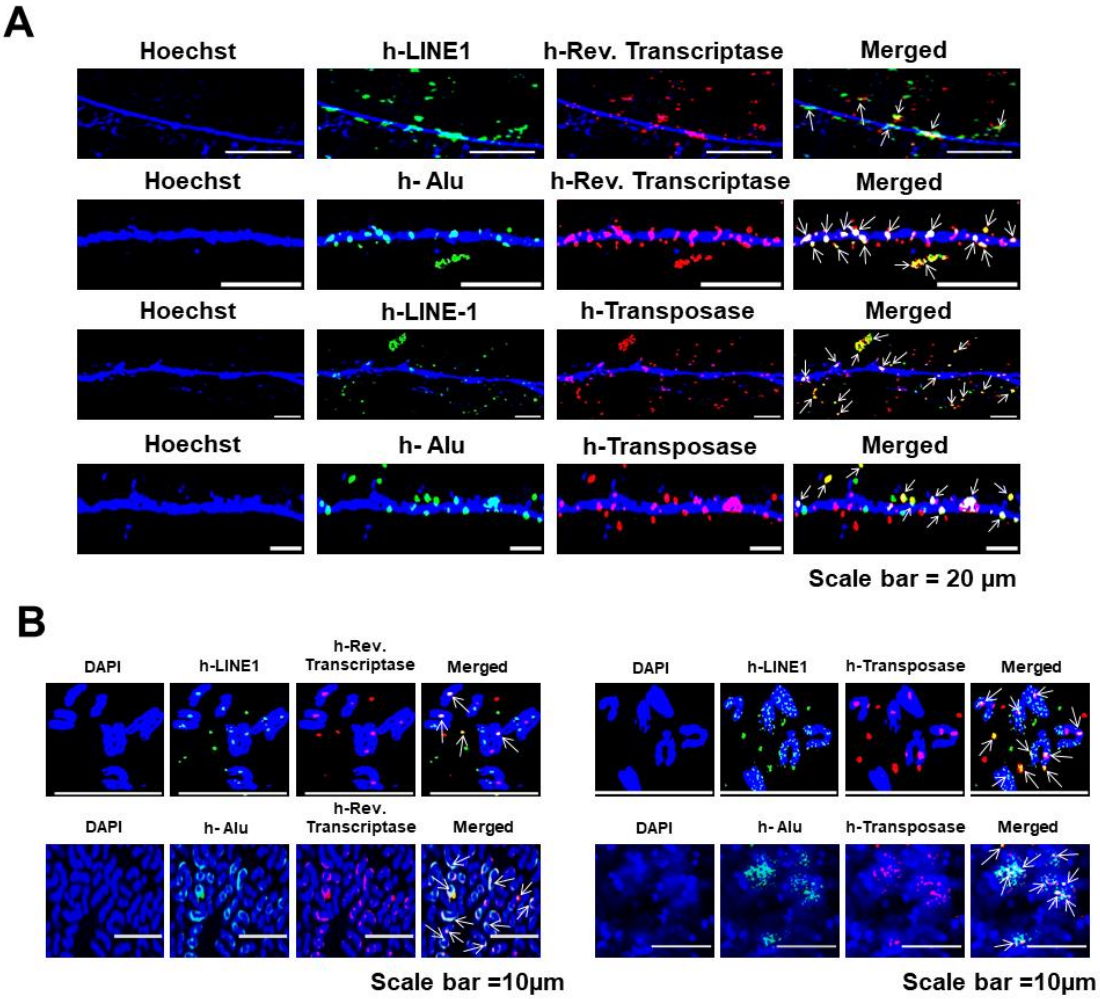

Fig. 12. Concatemers are associated with reverse transcriptase and transposase. Immuno-FISH analysis of chromatin fibres **(A)** and metaphase spreads**(B)** prepared from cfChPs-treated NIH3T3 cells in continuous passage using a human *Alu* or LINE-1 probe and antibodies against human reverse transcriptase and transposase. Co-localised signals in all four experiments are marked with arrows.

We next investigated the ability of these transposable elements to replicate. Immuno-FISH experiments using antibodies against human DNA polymerase γ and FISH probes against LINE-1 and *Alu* showed co-localised signals, indicating that LINE-1 and *Alu* were

associated with DNA polymerase, suggesting that they might be capable of replication and potential amplification (Fig. 13A and B). This possibility was confirmed in cells in continuous passage that had been pulse-labelled with BrdU wherein immuno-FISH analysis showed co-localised signals for antibodies against BrdU and FISH probes for LINE-1 and *Alu*. This indicated that the DNA of both the transposable elements had the potential to replicate (Fig. 13A and B). Results of quantitative analysis of the degree of co-localisation of fluorescent FISH signals of human LINE-1 and human DNA polymerase and BrdU; and Alu and human DNA polymerase and BrdU are given in Supplementary Figure S2 H. The extent of co-localization varied between 14% and 28%.

Fig. 13

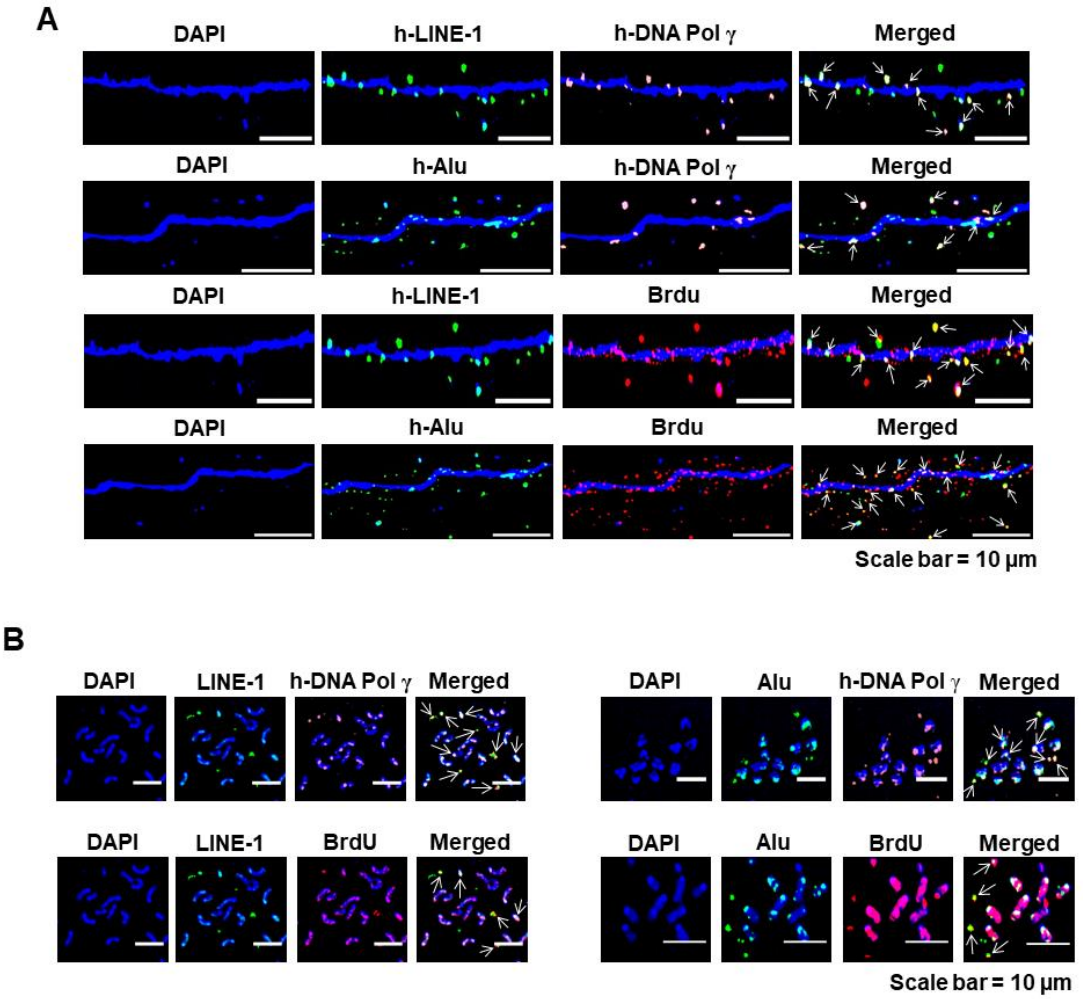

Fig. 13. LINE-1 and *Alu* elements are associated with DNA polymerase and can actively synthesise DNA. (A) Immuno-FISH images of cfChP-treated cells in continuous passage showing co-localising signals of LINE-1 (green) and Alu (green) and DNA polymerase γ (orange) and BrdU (red) on chromatin fibres. **(B)** Similar co-localising signals are shown on metaphase spreads. Co-localised signals are marked with arrows.

LINE-1 and *Alu* elements increase their copy numbers in the mouse cell genome

Given the above findings, we were curious to determine whether LINE-1 and *Alu* elements could physically increase their copy numbers within the mouse genome. Analysis of 15 metaphase spreads (Fig. 14, inset) showed that the numbers of human LINE-1 and *Alu*

signals per chromosome increased steadily between passages 2 and 198, representing a nearly 10-fold increase in both cases (Fig. 14). These data indicated that LINE-1 and *Alu* elements progressively increased their copy numbers by retrotransposition over time in the mouse genome. It should be noted that this analysis is restricted to determining the proliferative capacity of LINE-1 and *Alu* elements that are associated with chromosomes and does not take into account the replicative potential of those that are present in the cytoplasm.

Fig. 14

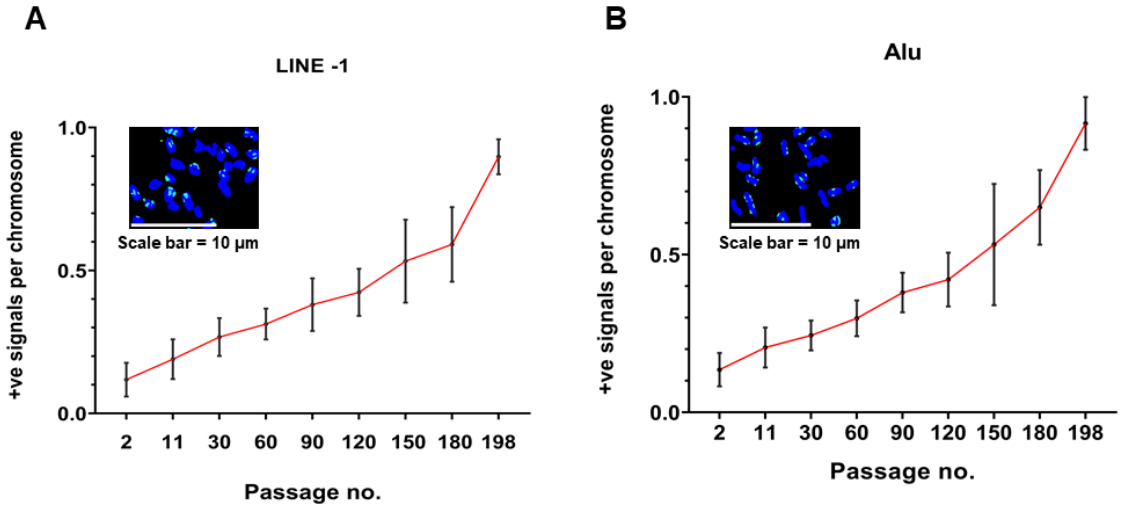

Fig. 14. LINE-1 and *Alu* elements proliferate to increase their copy numbers in the mouse cell genome. The data represent the mean ± standard error values of the number of human LINE-1 and *Alu* FISH signals per chromosome in successive passages after analysing 15 metaphases at each passage. Analysis of variance for linear trend, $p < 0.0001$ for both LINE-1 and Alu. The inset is a representative partial metaphase image showing positive staining for LINE-1 and *Alu*; the blue signals are those of DAPI and red signals represent human LINE-1 and *Alu* detected by FISH.

Experiments using cfChPs isolated from healthy individuals

The experiments described above were performed with NIH3T3 cells that had been treated with cfChPs isolated from the sera of cancer patients and passaged continuously. We simultaneously performed similar experiments with NIH3T3 cells that had been treated with cfChPs isolated from the sera of healthy individuals. We found that cfChPs from healthy individuals were also readily internalised by NIH3T3 cells wherein they behaved in a manner similar to that of cfChPs derived from the cancer patients (Supplementary Fig. S4).

Experiments using cfChPs released from hypoxia-induced dying MDA-MB-231 cells

Finally, given our previous finding that cfChPs spontaneously released from dying cancer cells were readily internalised by healthy cells **[14]**, we further investigated the function of cfChPs released from dying MDA-MB-231 breast cancer cells. We observed that cfChPs (≤ 400 nm) that were released from hypoxia-induced dying MDA-MB-231 cells could reciprocate all the intracellular activities/functions in chromatin fibres prepared from serially passaged NIH3T3 cells that were observed using cfChPs isolated from serum (Supplementary Fig. S5).

Discussion

The present study is founded on our earlier reports that cfChPs released from the billions of cells that die in the body everyday can horizontally transfer themselves to healthy cells resulting in activation of multiple biological processes **[13,14]**. Herein we have reported several additional findings which can be summarised as follows. We found that the internalised cfChPs containing disparate DNA sequences had randomly combined to form

highly complex concatemers of variable sizes many of which were ostensibly multi-mega base pairs in size. The concatemers exhibited variable and bizarre spatial relationships with the host cell DNA with many remaining in the cytoplasm and others being directly associated with chromosomal DNA. The concatemers were ostensibly not eliminated or degraded and retained indefinitely within the cell. They exhibited the potential to perform many functions attributable to the nuclear genome including the ability to replicate and to synthesise RNA, RNA polymerase, ribosomal RNA, ribosomal proteins, and numerous other human proteins in the mouse cells which manifested as exceedingly complex multi-peptide fusion proteins which were often highly overexpressed. Further analysis demonstrated that the concatemers harboured human LINE-1 and *Alu* elements which increased their copy numbers with time. The concatemers were also associated with the enzymes reverse transcriptase and transposase, suggesting their potential to rearrange themselves within the mouse genome. Taken together, these findings lead to the conclusion that horizontal transfer of cfChPs from dying cells to living cells have wide ranging biological consequences.

Conventionally, HGT refers to the transfer of genetic material from one organism to another. Although HGT occurs extensively in microorganisms, it has thus far been difficult to define in mammals. Our results suggest that HGT occurs in mammalian cells on a massive scale via cfChPs released from the billions of host cells that die on a daily basis. We previously proposed that these cfChPs released from dying cells function as a new class of mobile genetic elements (MGEs) that act intra-corporeally and transfer themselves horizontally to the organism's self-cells **[22]**. Although cfChPs (acting as MGEs) comprise fragments of the host's own cellular genomes, they undergo extensive and complex modifications following cellular apoptosis to such an extent that they act as

"foreign" genetic elements. Thus, we hypothesise that evolutionary changes in mammals are brought about not necessarily by foreign genes transferred from another organism but rather by the host's own genomic fragments that have been extensively modified to act as foreign genetic elements. However, for cfChPs to have evolutionary relevance in mammals, they need to be horizontally transferred to the germ line. The latter is not unlikely given the high turnover of daily cell death and release of cfChPs in the blood circulation which could carry them to all cells and tissues of the body including the germ line.

It has been proposed that extreme environmental stress plays a critical role in the adaptation and evolution of species **[23–25]**. We previously hypothesised **[26]** that environmental stress may lead to organism death via apoptosis or similar processes **[27,28]**, resulting in the generation of cfChP-like DNA–protein complexes. In particular, we demonstrated that while high-molecular-weight DNA is incapable of entering foreign cells, once the DNA is reduced to cfChP-sized fragments by sonication, it can readily undergo horizontal transfer regardless of species or even kingdom boundaries **[26]**. Thus, fragmented cfChP-like particles of human origin and those from bacteria and plants can readily enter mouse cells, and those from humans, bacteria, and plants can be transferred into bacteria. These findings together with those reported herein may provide new research directions linking cfChP-like DNA–protein complexes in the evolution of species in general **[26]**.

Our present detection of the unusual intracellular characteristics and functions of cfChPs was facilitated by two factors. First, our trans-species model comprised mouse recipient cells and cfChPs derived from human serum. This allowed us to track the activities of internalised human cfChPs within mouse cells using appropriate human-specific FISH

probes and antibodies. Second, the chromatin fibre technique facilitated the detection of spatial relationships between internalised cfChPs and mouse interphase DNA. One of our significant findings was that disparate cfChPs rapidly linked to form concatemers of variable sizes, some of which were ostensibly multi-megabase pairs in size. The formation of concatemers involves the random compaction of numerous unrelated DNA sequences, thereby creating extensively heterogeneous, complex, and novel genetic elements. The concatemers could apparently perform autonomous functions, many of which are attributable to the nuclear genome of the host cell. For example, they expressed DNA polymerase, which catalysed the synthesis of DNA and the replication of concatemers, leading to an increase in their copy number within host cells. Moreover, we demonstrated that the concatemers synthesised RNA, RNA polymerase, ribosomal RNA, and ribosomal proteins, thereby creating their own autonomous protein synthesis machinery. Concatemers, comprising open chromatin, were capable of the indiscriminate transcription of DNA via RNA polymerase, and the RNA thus generated was translated into proteins with the help of their own ribosomal RNA and ribosomal proteins. The numerous human proteins generated manifested as novel, unique, and multicomponent fusion proteins. The frequent co-localisation of disparate pairs of unrelated antibody probes that we observed suggest that the fusion proteins are likely to be highly complex proteins comprising multiple peptides derived from the variable DNA components that constitute concatemers. We found that all seven pairs of unrelated antibodies that we examined generated co-localising protein signals in around 20% of cases. If all 20000 known proteins were to also co-localise to a similar extent, the complexity of the fusion proteins (and of the corresponding concatemers) can be envisioned as gargantuan. Importantly, the fusion proteins remained associated with the concatemers. This

suggested that, although concatemers contained critical components of the protein synthetic machinery, they were devoid of the machinery required for protein sorting. Consequently, the newly synthesised proteins were not transported to the appropriate destinations within the cell. However, it remains unclear whether newly synthesised proteins are continuously degraded and regenerated in a cyclic manner, a speculation that will require future research for clarification. Nonetheless, and taken together, the above unique functions and activities of the concatemers lead us to propose that once cfChPs are horizontally transferred into a new host cell, they commence to function as autonomous “predatory” genomes.

We further showed that concatemers represented human LINE-1 and *Alu* elements based on the observation of DNA replication and their association with reverse transcriptases and transposases. These enzymes, acting in unison, ostensibly allowed the transposable elements carried by the concatemers the possibility of rearrangement and of *de novo* integration into the mouse cell genome. Based on these results, we hypothesise that transposable elements are not inherent to the cell but are acquired from the exterior from other dying cells via the horizontal transfer of cfChPs. Confirming this hypothesis may help resolve the considerable uncertainty that exists relating to the origin of these elements **[29,30]**.

Importantly, although the majority of the horizontally transferred cfChPs were likely to have been derived from the extended non-coding regions of the apoptotic cells from which they were derived, we found them to be capable of synthesising a wide variety of proteins within the new host cells. The cataclysmic event of cellular apoptosis apparently activated normally transcriptionally silent sequences that are confined to large sections of the genome, which could account for our finding. These sequences, now transcriptionally

active, when horizontally transferred via cfChPs to foreign cells amalgamated with each other to form complex concatemers to generate multi-peptide and novel fusion proteins.

Despite numerous attempts, the DNA counterparts of the fusion proteins were not amenable to detection by conventional reverse transcription-quantitative polymerase chain reaction. Given the complex and chaotic amalgams of disparate DNA fragments that comprise the concatemers, the primers apparently could not find the appropriate DNA sequences to bind to. Advanced sequencing technologies may help to define the complex assembly of the DNA sequences that comprise the concatemers. Likewise, multiple attempts to detect the components of the fusion proteins by Western blotting were also not successful. The results of our studies using liquid chromatography with tandem mass spectrometry to delineate the composition of the fusion proteins will be published in the future.

The presence of extrachromosomal DNA (ecDNA) has been described in plants **[31]**, yeast **[32]**, *Caenorhabditis elegans* **[33]**, *Drosophila* **[34]**, mice, and humans **[35,36]**. Recently, interest in the role of ecDNA in cancer has been renewed. In particular, ecDNA can be a source of extreme gene amplification enabling a cell to harbour multiple copies of oncogenes and can potentially promote tumour heterogeneity and therapy resistance **[37–39]**. The results of the present study suggest the possibility that the ecDNAs detected in human cancers may represent concatemers formed following the amalgamation of multiple unrelated cfChPs and which have been acquired from the exterior.

Our study leaves many unanswered questions, and our findings will require to be validated using more rigorous technologies in future research. For example, we did not determine the nature and size of the concatemers, which would require their isolation and

sequencing. We also did not ascertain whether the concatemers that were found overlapping the chromatin fibres had integrated into the mouse genome. We did not characterise the numerous fusion proteins; which are currently being addressed in our planned studies. We did not quantify the extent to which the fusion proteins amplify. Our results also leave the unanswered question as to the relevance our findings to the extra-chromosomal DNA that have been detected in human tumours.

As such, our results should be considered as representing an expansive theory, wherein the activities of the horizontally transferred cfChPs are governed by unknown biological mechanisms that are distinct from those that govern the nuclear genome. For example, the detection of co-localising signals of centromeres and telomeres within the same concatemer leaves the perplexing question as to what mechanism might guide the concatameres to replicate? We believe that an understanding of these unusual biological functions and their governing mechanisms could offer a new perspective on the complex process of natural evolution.

In summary, the present findings lead to a new hypothesis that posits that a cell harbours two forms of a genome that apparently function independently of each other: one that is inherited (hereditary genome) and numerous others that are acquired (predatory genomes). Predatory genomes may perform evolutionary functions by acting as vehicles of transposable elements and generating a plethora of novel proteins. Our results suggest that the biological life of the genome is not a co-terminus with the death of a cell, but rather that the genome is recycled in a fragmented and extensively modified incarnation to perform new functions in distant destinations as foreign genetic elements. This

suggests that a cell's propensity to internalise cfChPs represents a natural process to modify the hereditary genome, which in turn promotes evolutionary change.

Materials and Methods

Ethics approval

This study was approved by the Institutional Ethics Committee (IEC) of the Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre for the collection of blood (10 mL) from cancer patients and healthy individuals for the isolation of cfChPs (approval no. 900520). A formal informed consent form that was approved by the IEC was signed by each participant.

Isolation of cfChPs from human serum

cfChPs were isolated from the sera of five cancer patients and five healthy volunteers using the protocol described in our previous study **[13]**. The age, sex, and tumour types of the participants are given in Supplementary Table S1. Sera from the five donors from each group were pooled for isolation of cfChPs. The concentrations of cfChPs in the isolates are expressed in terms of their DNA content, as estimated using the Pico Green quantitation assay **[13]**.

Cell lines and culture

NIH3T3 mouse fibroblasts were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% bovine calf serum and grown at 37°C in 35-mm culture

dishes in an atmosphere of 95% air, 5% CO_2, and 100% humidity. The cells (10×10^4) were treated with 10 ng of cfChPs isolated from the serum of cancer patients or healthy individuals and maintained in continuous culture by passaging every fourth day. In some experiments, the cells were treated with cfChPs after dual fluorescent labelling of their DNA and histones (see below).

Collection of conditioned medium containing cfChPs released from dying cancer cells

In addition to cfChPs isolated from the cancer patients and healthy individuals, we also investigated the effects of treating NIH3T3 cells with cfChPs that had been released into the conditioned medium from hypoxia-induced dying human breast cancer cells. In brief, MDA-MB 231 breast cancer cells (ATCC) were seeded in a six-well culture plate at a density of 2×10^5 cells/well on ThinCert® Cell Culture Inserts (pore size 400 nm) containing 1.5 mL of DMEM and incubated at 37°C for 24 h. The conditioned medium was obtained using a dual-chamber technique. The six-well plate with ThinCert® Inserts was then placed in a hypoxia chamber containing 1% O_2 to induce hypoxic cell death. After 48 h, the plate was taken out of the hypoxia chamber and 700 µL of DMEM was added to the lower chamber beneath the ThinCert® Inserts. The plate was then incubated for a further 48 h at 37°C in normoxic conditions to allow cfChPs with a size of less than 400 nm to seep into the medium in the lower chamber. The medium in the lower chamber (~1 mL) was collected and added to 10×10^4 NIH3T3 cells.

Fluorescent dual labelling of cfChPs

The DNA and histones of cfChPs were fluorescently dually labelled with Platinum Bright™ 550 Red Nucleic Acid Labelling Kit (Kreatech Diagnostics, Cat # GLK-004) and ATTO

488 NHS-ester (ATTO-TEC GmbH, Cat # AD488-35), respectively, according to our previously reported protocol [13]. NIH3T3 cells were treated with 10 ng of dually labelled cfChPs for 6 h and then examined under a confocal microscope (Carl-Zeiss, GmbH, Germany).

Preparation of chromatin fibres

Chromatin fibres were prepared from interphase NIH3T3 cells according to previously described methods [40,41] with minor modifications. Although the inventers of this technique had described the fibres as DNA fibres, when stained with histone H4 antibody, we found them to comprise of chromatin (Supplementary Fig. 6). Two microlitres of the cell suspension was spotted at one end of a glass slide and semi-evaporated for 10 min at room temperature (~25°C). The spotted cells were incubated with 7 μL of lysis buffer (0.1% sodium dodecyl sulphate in phosphate-buffered serum) for 2 min with gentle shaking, and the slides were t tilted at an angle of 15°–25° to allow the cell lysate containing chromatin fibres to roll down along the slide surface. The slides were fixed with chilled methanol for 10 min and processed for immunofluorescence and FISH analyses.

Metaphase spread preparation

Metaphase spreads from NIH3T3 cells in continuous culture were prepared as described previously [13]. The slides were processed for immunofluorescence and FISH analyses.

Immunofluorescence and FISH

The expression of various proteins in the cells was evaluated using indirect immunofluorescence, and the presence of human DNA in chromatin fibres and

metaphase spreads was detected using FISH, as described previously [13]. In brief, the immune-stained chromatin fibres were imaged at 400× magnification and all images were analysed using an Applied Spectral Bio-imaging System (Applied Spectral Imaging, Israel). To detect DNA synthesis, we pulse-labelled the cells in continuous passage with bromodeoxyuridine (BrdU) for 24 h. The BrdU-labelled cells were then used for the preparation of chromatin fibres and metaphase spreads, as described above. Sources and other details of the antibodies and FISH probes used in this study are provided in Supplementary Table S2.

Detection of RNA synthesis

RNA synthesis in cfChP-treated cells in continuous passage was detected using an Abcam assay kit (Cambridge, UK; Catalogue No. ab228561) as per the manufacturer's protocol. With the use of metabolic tagging with 5-ethynyluridine (EU), this assay offers a reliable method for fluorescent staining of newly produced RNA. Click chemistry facilitates viewing. In some experiments, the cells were treated with actinomycin D (0.0005 µg/mL) for 24 h or grown at low temperature (31°C) for 24 h.

Statistical analysis

For statistical analysis, Student's t-test was applied to compare the composition of histones using GraphPad version 8, and analysis of variance for linear trend was applied to compare FISH signals using SPSS version 25.

Data availability

All data generated during this study are included in this published article and supplementary information.

Ethics Statement

The Institutional Ethics Committee (IEC) provided approval to collect blood samples from cancer patients and healthy participants. Consent forms approved by the IEC (Approval no. 900520) were used to obtain signed informed consent.

Author contributions

SB, SS, RL, SP, RJ, LK, and NKK performed the experiments; GVR supervised the experiments and analysed the data; IM conceptualized and designed the project, was responsible for overall supervision and acquisition of funding, interpreted the data, wrote the manuscript, and approved the final draft.

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Conflict of interests

The authors declare no competing interests.

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