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

Soumita Banerjee[1,2,†], Soniya Shende[1,2,†], Laxmi Kata[1,2,†], Relestina Lopes[1,2,†], Swathika Praveen[1,2], Ruchi Joshi[1,2], Naveen Kumar Khare[1,2], Gorantla V Raghuram[1,2], Snehal Shabrish[1,2], Indraneel Mittra[1,2*]

[1]Translational Research Laboratory
Advanced Centre for Treatment, Research and Education in Cancer
Tata Memorial Centre
Kharghar, Navi Mumbai 410210, India

[2]Homi Bhabha National Institute, Anushakti Nagar, Mumbai 400094, India

[*] Author for correspondence: Indraneel Mittra; imittra@actrec.gov.in

[†] These authors contributed equally to the project.

Short title: Cell-free chromatin particles are messengers of evolutionary change

Keywords: Evolution of species, Horizontal gene transfer, Cell-free chromatin, Concatemer, Transposable elements, Predatory genome, Extra-chromosomal DNA, Cancer, Natural selection

Abstract

Horizontal gene transfer (HGT) plays an important evolutionary role in prokaryotes, but its role in mammals is poorly defined. 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 serially passaged the cells. The intracellular activities of cfChPs were analysed using chromatin fibre fluorography, cytogenetic analysis, immuno-fluorescence and fluorescent *in situ* hybridisation. We discovered that the internalised cfChPs comprising of widely disparate DNA sequences had randomly combined to form complex concatemers some of which were ostensibly multi-mega base pairs in size. The concatemers exhibited variable and bizarre spatial relationships with the host cell interphase DNA with many remaining in the cytoplasm and others aligning themselves with the mouse chromosomal DNA. The concatemers performed many functions attributable to the nuclear genome. They could replicate, synthesise RNA, RNA polymerase, ribosomal RNA, ribosomal proteins, and numerous other human proteins within the mouse cells which manifested as complex multi-peptide fusion proteins. The concatemers harboured human LINE-1 and *Alu* elements which markedly amplified themselves and increased their copy number with time in culture and exhibited the potential to rearrange themselves within the mouse genome. 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 (predatory genomes). The presence of predatory genomes has evolutionary

implications given their ability to generate a plethora of novel proteins and to serve as vehicles for transposable elements. Finally, our results suggest that HGT occurs in mammalian cells on a massive scale via the medium of cfChPs that have undergone extensive and complex modifications resulting in their behaviour as “foreign” genetic elements.

Video Abstract

https://drive.google.com/file/d/1SHNBwUhZPlgRlLFtt-dmnxQef0PsrA3F/view?usp=sharing

Introduction

Horizontal gene transfer (HGT) plays an important role in the adaptation of microorganisms to changing environments and in evolutionary processes (Bushman, 2002). 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 facilitated the design of drug-resistant cells, transgenic plants, and animals (Hofmann et al., 2004; Nagler et al., 2011; Puonti-Kaerlas et al., 1990). Indeed, gene therapy is founded on the principle of HGT; and several studies have indicated that oncogenes can be horizontally transferred across mammalian cells (Anker et al., 1994; Bergsmedh et al., 2001; Dvořáková et al., 2013; García-Olmo et al., 2010; Trejo-Becerril et al., 2012). These observations support the hypothesis that HGT may occur naturally in mammalian cells and contributes to evolutionary and potentially oncogenic processes.

Several hundred billion to trillion cells die in the human body every day (Fliedner et al., 2002; Sender and Milo, 2021) and release chromosomal fragments in the form of cell-free chromatin particles (cfChPs), which enter the extracellular compartments, including the circulation (Holdenrieder and Stieber, 2009). 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 association with the host cell genome (Mittra et al., 2015, 2017). The latter 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 mouse recipient cells. Cellular uptake of cfChPs has several biological ramifications such as induction of DNA damage and the

activation of inflammatory and apoptotic pathways (Mittra et al., 2015, 2017). 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 (Raghuram et al., 2019). 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 (Parada et al., 1982 & Aaronson et al., 1982). In our previous study (Mittra et al., 2015), we investigated the relative biological activities and functions of healthy and cancerous cfChPs. We found that cfChPs isolated from cancer patients had significantly greater activity in terms of DNA damage and activation of apoptotic pathways than those isolated from healthy individuals. We had also reported that cfChPs released from dying cells can horizontally transfer themselves into healthy bystander cells leading to DNA damage and inflammation (Mittra et al., 2017). 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.

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 that were released from hypoxia induced dying MDA-MB-231 breast cancer cells. Most experiments were performed more than once with reproducible results.

1. Experiments using cfChPs from cancer patients

1.1 cfChPs are rapidly and abundantly internalised by NIH3T3 mouse fibroblast cells

We isolated cfChPs from the sera of cancer patients by a protocol described by us earlier (Mittra et al., 2015). A representative electron microscopy image of the cfChPs is given in Supplementary Fig. S1. In order to track the intracellular activities of cfChPs, we fluorescently dually labelled the isolated cfChPs in their DNA with Platinum Bright Red 550 and in their histones with ATTO-488 and applied them (10ng) to NIH3T3 mouse fibroblast cells. A representative image of isolated cfChPs that had been fluorescently dually labelled and used to treat the NIH3T3 cells is given in Fig. 1a. Examination of NIH3T3 mouse fibroblasts treated with the dually fluorescently labelled cfChPs confirmed our earlier observation (Mittra et al., 2015) that cfChPs can readily and rapidly horizontally transfer themselves to healthy cells via phagocytosis (Mittra et al., 2017) to accumulate in their cytoplasm and nuclei by 6 h (Fig. 1b). The fluorescent cfChPs appeared prominent, ostensibly owing to the amalgamation of multiple cfChPs to form large concatemers following their cellular entry (described in detail later). Chromatin fibres

prepared from similarly treated NIH3T3 cells at 6h revealed the presence of numerous dually labelled cfChPs of varying sizes, which were present in both the cytoplasm and overlapping with mouse chromatin fibres (Fig. 1c). 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, some of which were associated with the chromosomes while others were present in extrachromosomal spaces (Fig. 1d).

Fig. 1

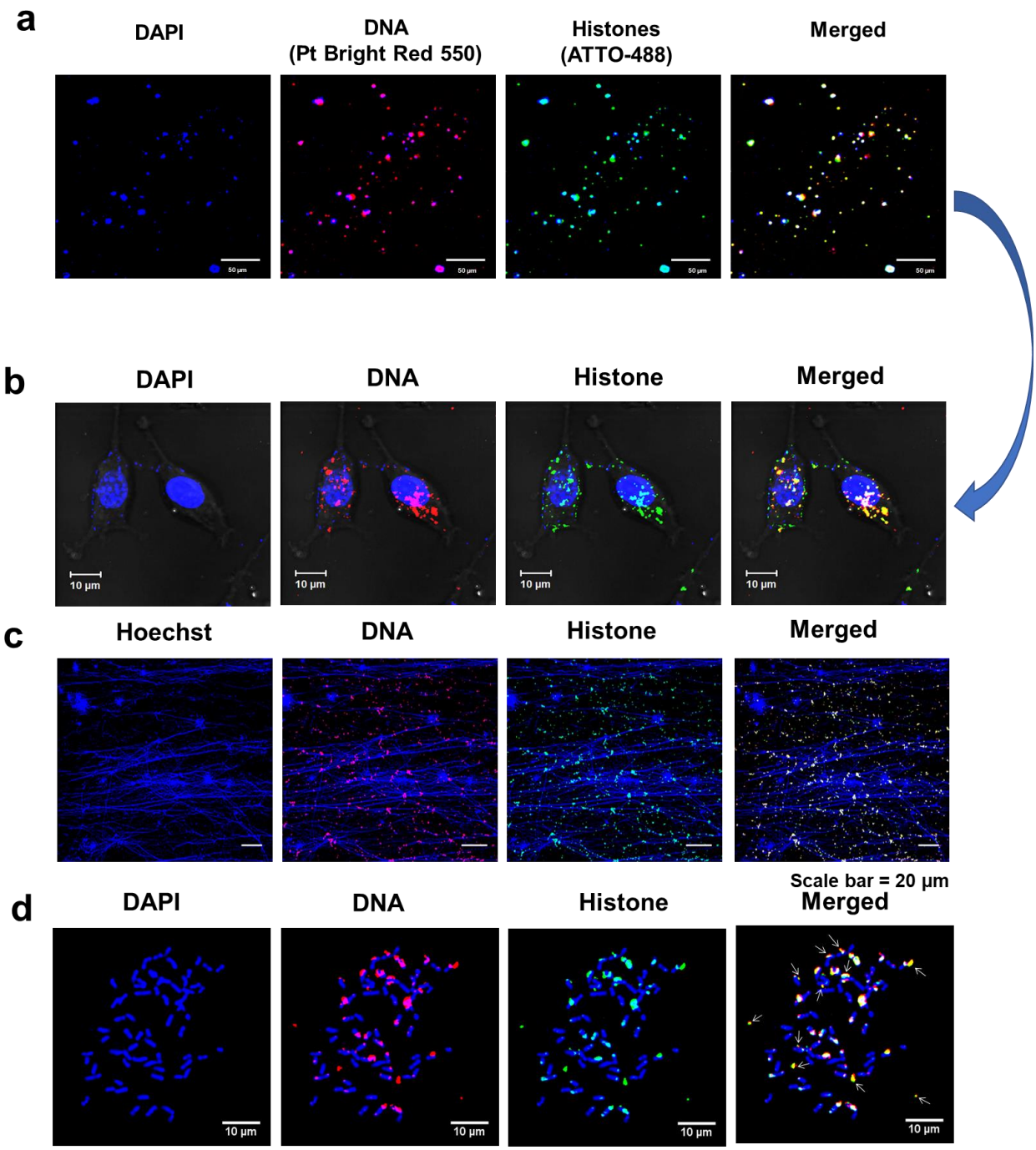


Fig. 1. Abundant uptake of cfChPs by NIH3T3 cells at 6 h. The DNA and histones of cfChPs were dually fluorescently labelled with Platinum Bright 550 and ATTO-488 respectively and applied (10ng) to NIH3T3 cells. **a.** Representative images of dually-labelled cfChPs prior to their application to NIH3T3 cells. **b.** Confocal microscopy images of cfChPs-treated cells at 6 h showing many dually labelled fluorescent signals in the cytoplasm and nuclei. **c.** 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. **d.** Fluorescence microscopy images of metaphase spreads prepared from cfChPs-treated cells at 6 h showing multiple dually labelled fluorescent signals which are either associated with the chromosomes or are present in extrachromosomal spaces. The latter are marked with arrows.

1.2 cfChPs randomly combine to form complex concatemers

We had earlier hypothesised that when the cfChPs are horizontally transferred to another cell, the latter perceives the dsDNA breaks present in their two ends as damaged 'self' DNA, and in an attempt to repair the 'perceived damage', activates proteins of the DDR pathway which links-up multiple disparate cfChPs as a part of the repair process leading to the formation of concatemers of variable sizes (Mittra et al., 2015). To test this hypothesis, we performed FISH analysis using multiple different pairs of human chromosome-specific FISH probes on chromatin fibres and metaphase spreads prepared from cfChPs treated NIH3T3 cells that were in continuous passage. Since these chromosome specific probes were custom synthesized, we ensured that the probes that we used were human specific and did not cross-react with mouse chromosomes (Supplementary Fig. S2a and S2b). We detected frequent co-localisation of red and green fluorescent signals suggesting that unrelated chromosomal fragments containing disparate DNA sequences had randomly amalgamated with each other to form highly complex concatemers (Fig. 2). Some of the concatemers appeared strikingly prominent, which is particularly evident from images presented in Figure 2a. This suggested that the components of the concatemer had been markedly amplified. Even the small fluorescent signals shown in Figure 2b appeared as concatemers. As the analytical resolution of a FISH signal under a fluorescent microscope is in the range of 100–200 Kb (Cui et al., 2016) even the small concatemers can be assumed to be at least of a similar size range. Surprisingly, we detected the fluorescence signals of chromosome 4 to co-localise with

those of centromeres and of chromosome 20 to co-localise with those of telomeres. This finding highlighted the extent of genetic complexity and chaotic nature of the concatemers (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 telomeres and centromeres (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 (171bp in size) within their folds but that they had 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 Fig. S3a. The extent of co-localization ranged between 8% and 18%. Concatemerisation could also be detected in metaphase spreads prepared from serially passaged cfChPs-treated NIH3T3 cells (Fig. 2c). Negative control experiments performed on native NIH3T3 cells that had not been exposed to cfChPs treatment did not react with human specific FISH probes against chromosome 4 and chromosome 16 which were tested (Supplementary Fig. S4a).

Fig. 2

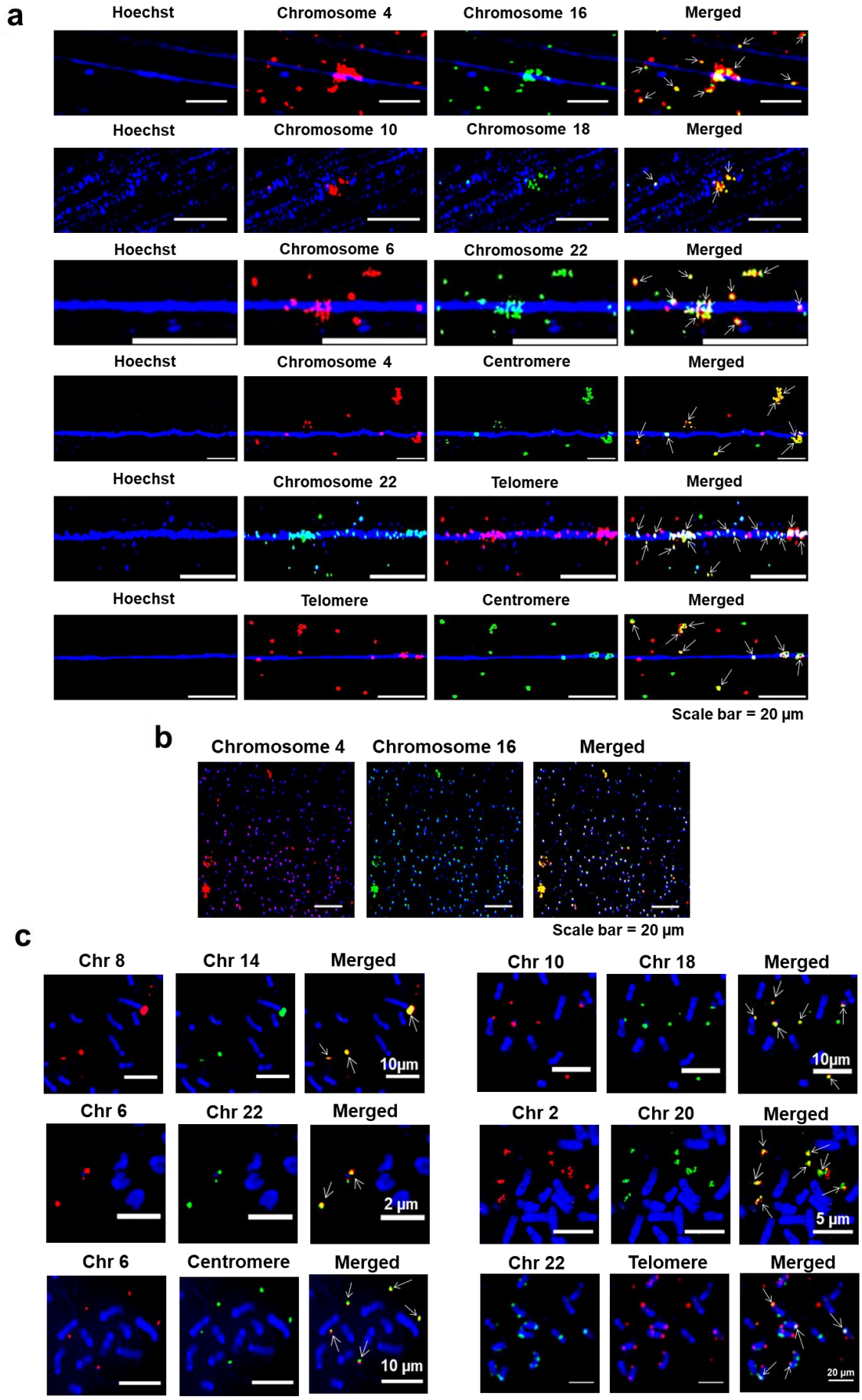

a
Hoechst
Chromosome 4
Chromosome 16
Merged
Hoechst
Chromosome 10
Chromosome 18
Merged
Hoechst
Chromosome 6
Chromosome 22
Merged
Hoechst
Chromosome 4
Centromere
Merged
Hoechst
Chromosome 22
Telomere
Merged
Hoechst
Telomere
Centromere
Merged
Scale bar = 20 μm
b
Chromosome 4
Chromosome 16
Merged
Scale bar = 20 μm
c
Chr 8
Chr 14
Merged
10μm
Chr 10
Chr 18
Merged
10μm
Chr 6
Chr 22
Merged
2 μm
Chr 2
Chr 20
Merged
5 μm
Chr 6
Centromere
Merged
10 μm
Chr 22
Telomere
Merged
20 μm

Fig. 2. Internalised cfChPs combine to form complex concatemers. a. FISH analysis of chromatin fibres prepared from cfChPs-treated NIH3T3 cells in continuous passage using different combinations of human chromosome-specific FISH 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 are comprised of concatemers **c.** Similar co-localising signals are shown on metaphase spreads. Co-localised signals are marked with arrows.

1.3 Concatemers exhibit variable spatial relationships with mouse chromatin fibres

We next undertook 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. We detected numerous dually labelled signals representing concatemers which exhibited extensive structural and size variability, as well as remarkably variable spatial relationships with mouse chromatin fibres (Fig. 3a). Many concatemers were detected in the cytoplasm or to have aligned with long stretches of mouse DNA, while others exhibited unusual conformations or were seen to be 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 (Mittra et al., 2015) (Fig. 3b). This finding suggested that concatemers once formed are not eliminated or degraded and 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) (Cui et al., 2016). Control experiments were performed to confirm that the whole genomic DNA FISH probe used was human specific and did not cross-react with mouse (Supplementary Fig. S13).

Fig. 3

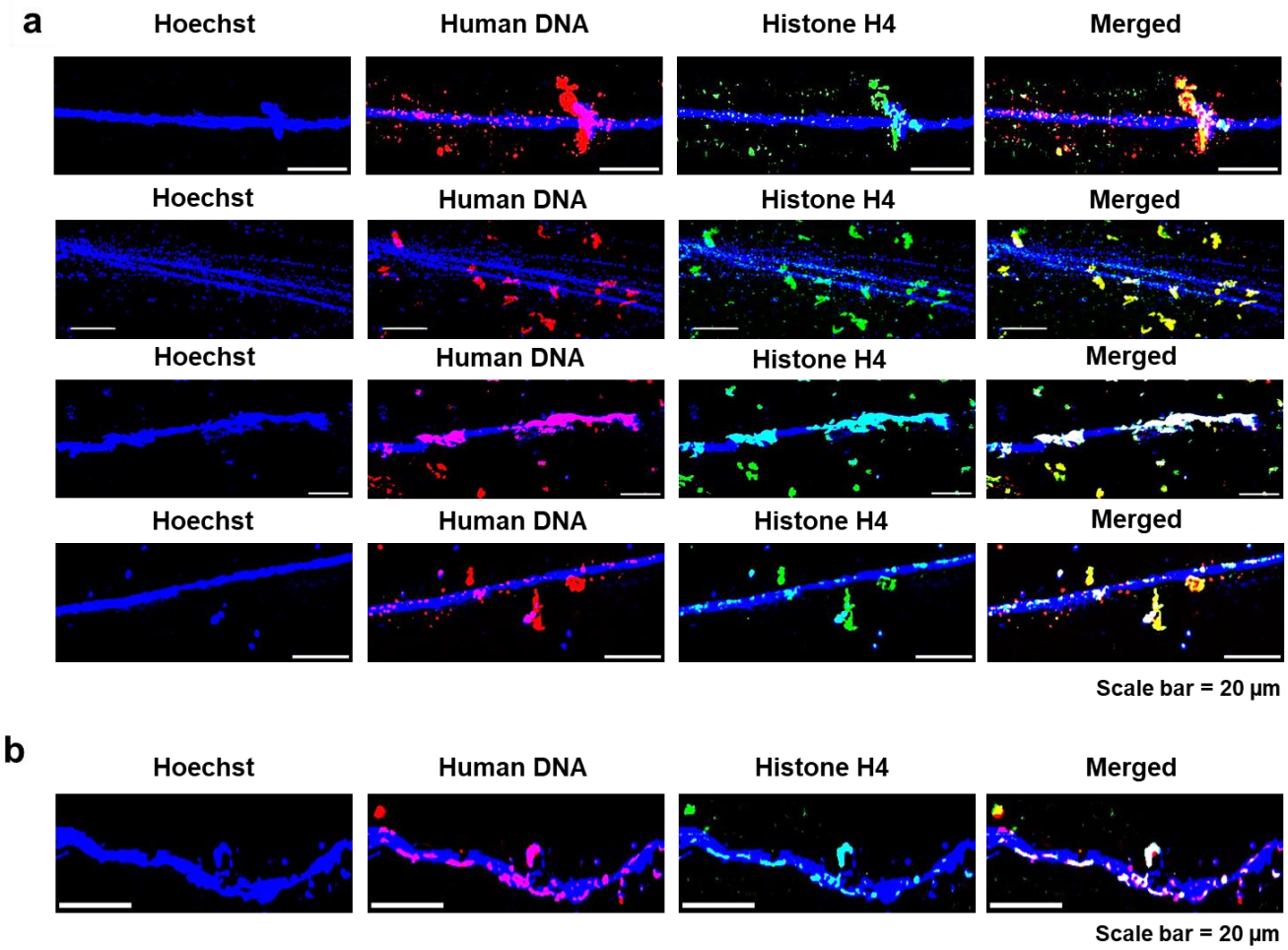


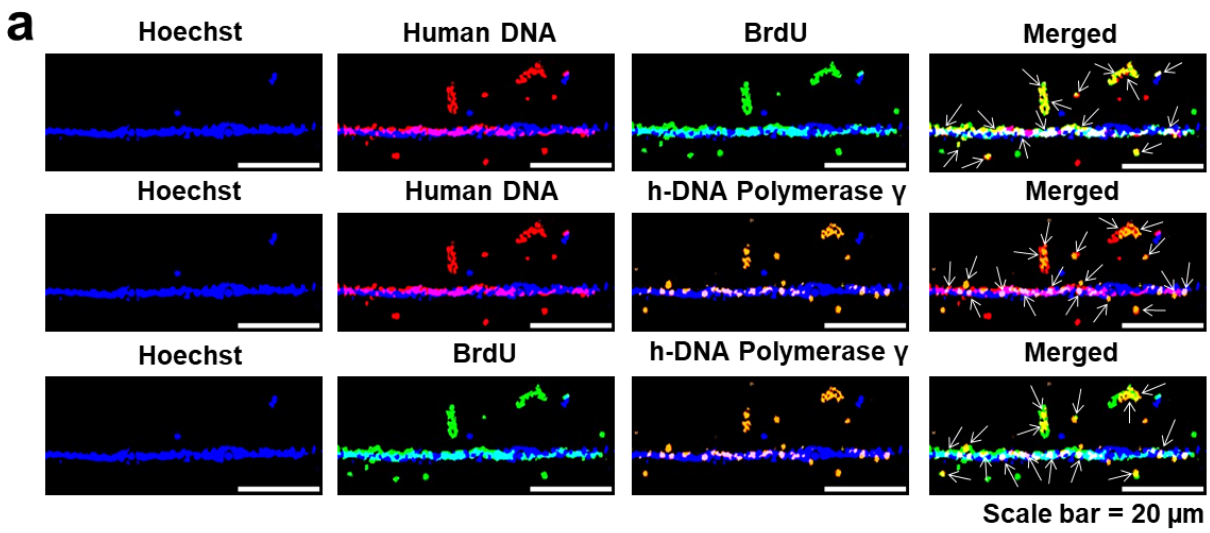
Fig. 3. Variable spatial relationships of concatemers with mouse chromatin fibres. a. Immuno-FISH analysis of chromatin fibres prepared from cfChPs-treated NIH3T3 cells in continuous passages using an antibody against histone H4 and a human whole-genome FISH probe. **b.** Immuno-FISH analysis of chromatin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

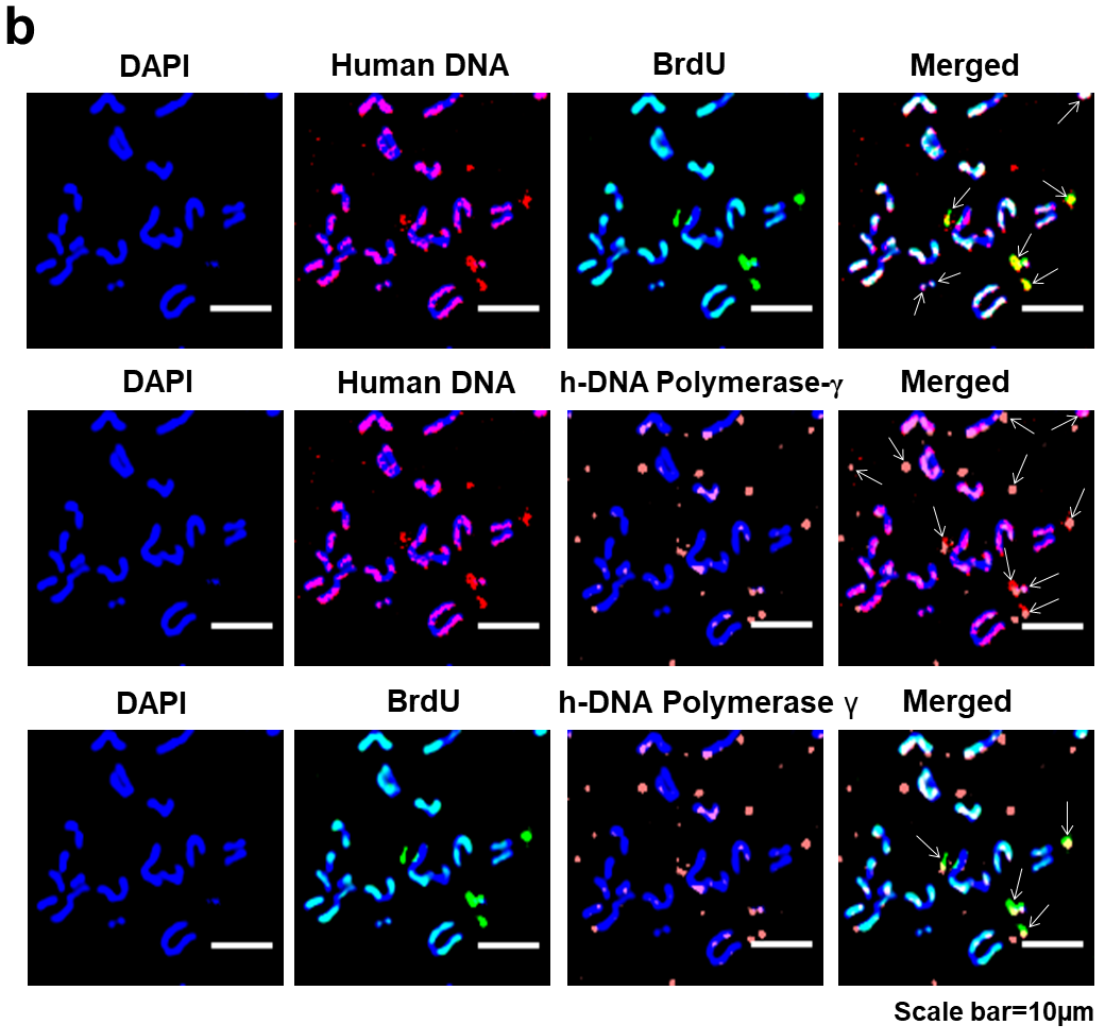
1.4 Concatemers synthesise DNA polymerase and can replicate

Cells in continuous passage were pulse-labelled with BrdU followed by immuno-FISH analysis using an antibody against BrdU and a human genomic DNA probe. The analysis revealed co-localisation of the fluorescent signals of BrdU and DNA, indicating that the concatemers were actively synthesising DNA (Fig. 4a, upper panel). Immuno-FISH

experiments further 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). Since human DNA polymerases is well conserved in mouse and across mammals, we ensured that the antibodies against DNA Polymerase γ was human specific and did not cross-react with mouse (Supplementary Fig. S5). 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 Fig. S3b. The extent of co-localization ranged 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 extrachromosomal spaces (Fig. 4b). Negative control experiments performed on native NIH3T3 cells that had not been exposed to cfChPs treatment did not react with either to the human genomic DNA probe or the antibody against human DNA polymerase γ (Supplementary Fig. S4b).

Fig. 4


a
Hoechst
Human DNA
BrdU
Merged
Hoechst
Human DNA
h-DNA Polymerase γ
Merged
Hoechst
BrdU
h-DNA Polymerase γ
Merged
Scale bar = 20 μm


b
DAPI
Human DNA
BrdU
Merged
DAPI
Human DNA
h-DNA Polymerase-γ
Merged
DAPI
BrdU
h-DNA Polymerase γ
Merged
Scale bar=10μm

247

Fig. 4. The concatemers synthesise DNA and express human DNA polymerase γ. a. Chromatin fibres were prepared from NIH3T3 cells in continuous passage were pulse-labelled for 24 h with BrdU (10µm). Immuno-FISH experiments 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 which co-localised with BrdU signals, we investigated their potential for self-replication. Ten metaphase spreads were prepared from cells in each successively increasing passage and the number of human FISH signals (representing concatemers) per chromosome was determined. The result showed that human FISH signals on mouse chromosomes increased progressively such that the number of signals at passage 198 was 4.07 times higher than those in passage 2 (Fig. 5a). 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. We also did a similar exercise to investigate whether the concatemers could amplify themselves with time by estimating the mean fluorescent intensity (MFI) per chromosome. We found that the increase in MFI between passage 2 and 198 was 237.2 fold (Fig. 5b).

Fig. 5

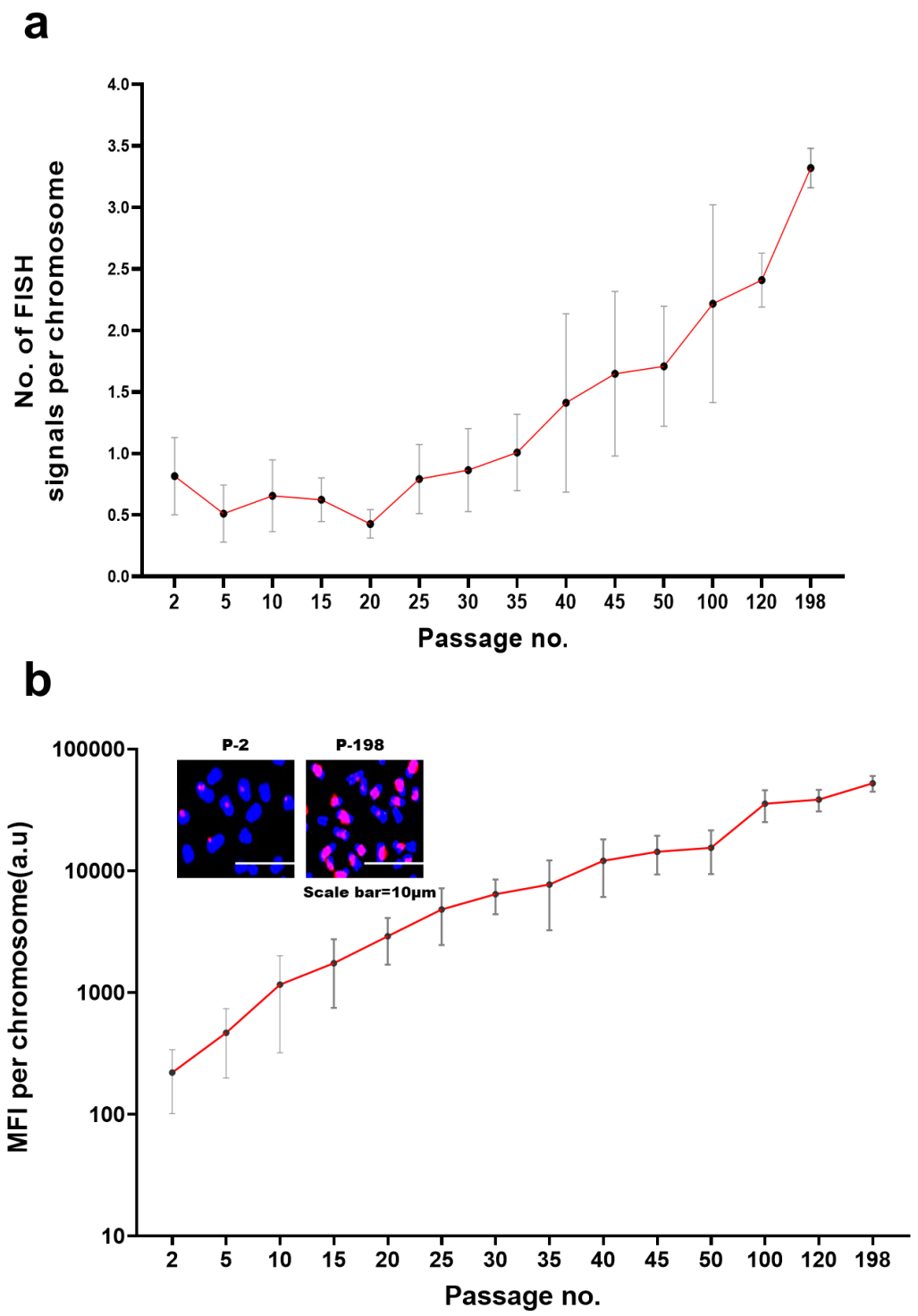


Fig. 5. Concatemers proliferate and amplify themselves within the mouse genome over time. Metaphase spreads were prepared from cells in each progressively increasing passage and probed with a human-specific DNA FISH probe. **a.** The total number of human DNA FISH signals on the DAPI

stained chromosomes was counted, and the mean number of human FISH signals per chromosome was calculated after analysing 10 metaphase spreads at each passage. A progressive increase in mean FISH signals per chromosomes is evident with increasing passage number (analysis of variance for linear trend $p < 0.0001$). The mean number of human FISH signals per chromosome between passage no. 2 and passage no. 198 increased by a factor of 4.07. **b.** a similar exercise was done as above except that mean fluorescent intensity (MFI) of human FISH signals per chromosome, indicative of amplification, was determined. A progressive increase in mean MFI per chromosomes is evident with increasing passage number (analysis of variance for linear trend $p < 0.0001$). The MFI per chromosome increased by a factor of 237.19 between passage no. 2 and passage no. 198. The insets represent partial metaphase images showing human DNA signals on the chromosomes. Blue and red signals represent DAPI and human DNA, respectively.

1.5 Concatemers are composed of open chromatin

We next examined the epigenetic constitution of the 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), indicative of regions associated with active gene promoters and regions associated with long-term repression respectively (Barski et al., 2007). Chromatin fibres prepared from cfChPs-treated NIH3T3 cells in continuous passage were dually immune-stained with antibodies targeting H3K4me3 and H3K9me3, and the number of concatemers that reacted with the antibodies was counted. We found that the vast majority of the concatemers either reacted exclusively with antibodies against H3K4me3 (open chromatin) or were hybrids of H3K4me3 and H3K9me3, with only a small fraction of reacting exclusively with H3K9me3 (Fig. 6). The figure also shows that the concatemers contained open chromatin irrespective of whether the regions of the host mouse DNA reacted with antibodies against H3K4me3 or H3K9me3. Taken together, these data indicated that concatemers primarily contained nucleosome-depleted regions and could bind to protein factors that facilitate gene transcription (Thomas et al., 2011) and DNA replication (MacAlpine et al., 2010).

Fig. 6

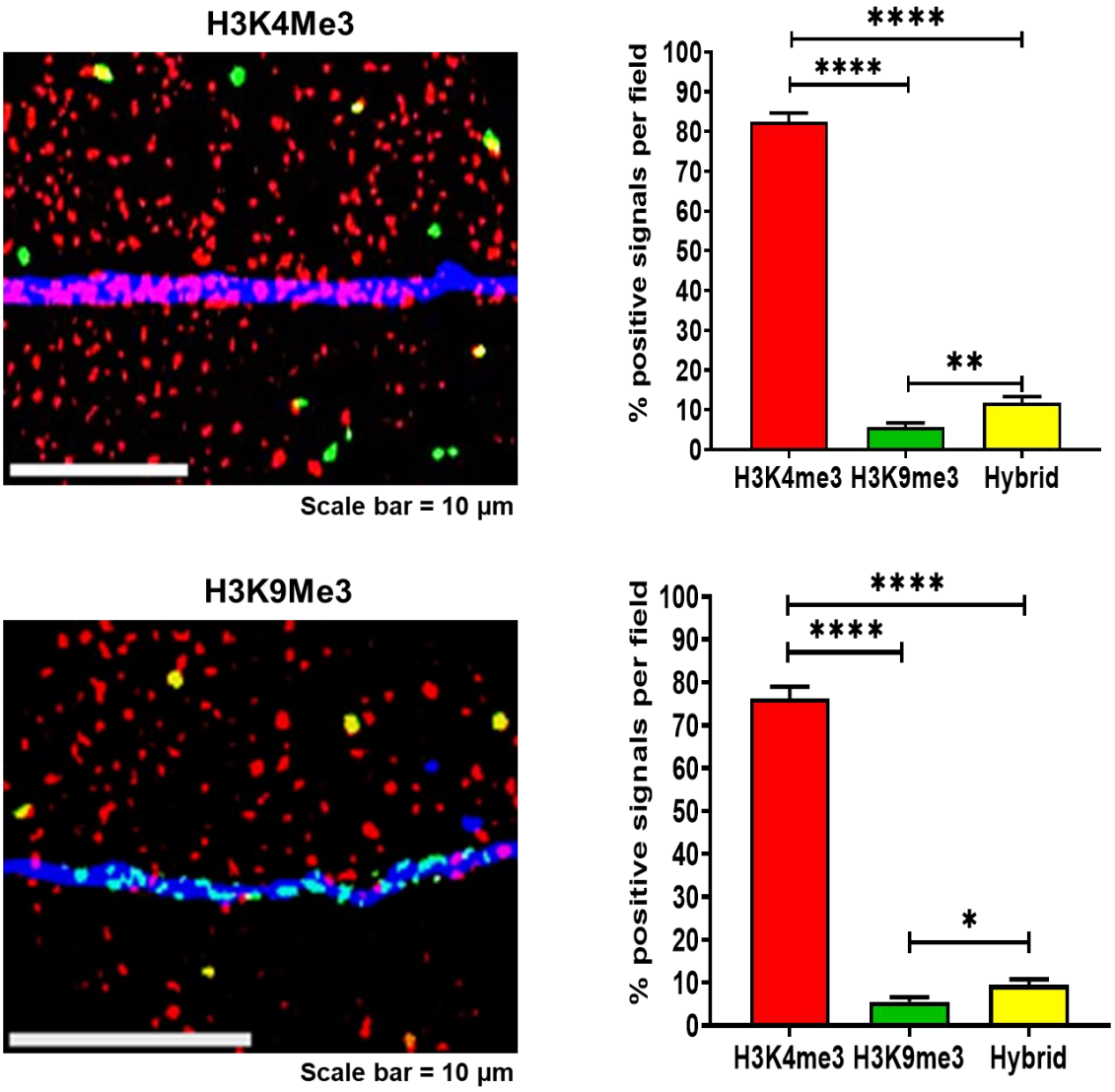


Fig. 6. Concatemers largely comprise of open chromatin irrespective of the epigenetic constitution of the mouse DNA. Representative images of chromatin fibres immunostained with antibodies against H3k4me3 (red) and H3k9me3 (green). The hybrid concatemers are represented by co-localised yellow signals. The host mouse DNA in the upper image is seen to react with H3k4me3 antibody representing open chromatin while the host mouse DNA segment in the lower image is comprised of heterochromatin and seen to react with antibody against H3k9me3. Histograms represent quantitative estimates of H3k4me3, H3k9me3, and hybrid histones after counting 100 fluorescent signals. The values are expressed as mean ± SEM values. Statistical analysis was performed using two-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$.

1.6 Concatemers can synthesise RNA

As the above findings suggested that concatemers are largely composed of open chromatin and 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. 7). 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 the cfChPs treated passaged cells with actinomycin D or maintaining the cells at low temperature (31ºC) abolished RNA synthesis. These data indicated that the concatemers were actively involved in RNA synthesis which is dependent on active cellular metabolism.

Fig. 7

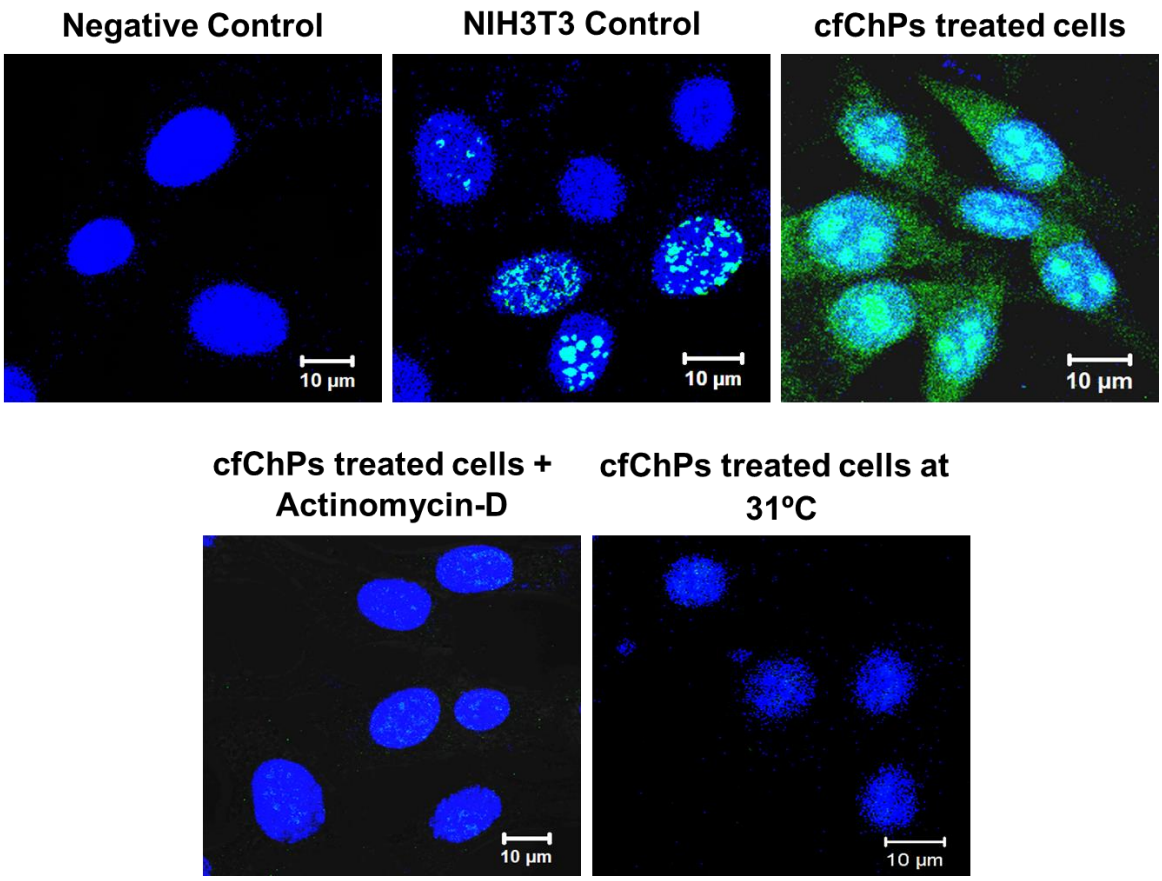


Fig. 7. Concatemers synthesise RNA which is dependent on active cellular metabolism. Images showing cytoplasmic RNA synthesis by cfChP-treated cells in continuous passage which is absent in control NIH3T3 cells. Treatment of cfChP-treated cells with Actinomycin D (0.0005 µg/mL) or maintenance at low temperature (31°C) abolishes RNA synthesis indicating that the latter is dependent on active cellular metabolism.

1.7 Concatemers synthesise their own protein synthetic machinery

The ability of the concatemers to synthesise RNA led us to investigate whether they were involved in protein synthesis. We looked for three critical components of protein synthetic machinery viz. ribosomal RNA, RNA polymerase and ribosomal protein. For detection of ribosomal RNA we undertook dual-FISH using a human genomic FISH probe (to detect the concatemers) and a FISH probe against human ribosomal RNA. We ensured that the

latter FISH probe was specific to human and did not cross-react with mouse (Supplementary Fig. S6, upper panel). Dual FISH analysis on chromatin fibres prepared from serially passaged cfChP-treated cells revealed strictly co-localised signals, indicating that the concatemers were synthesising ribosomal RNA (Fig. 8a, upper panel). We next investigated whether the concatemers could synthesise the other two components of the protein synthetic machinery viz. RNA polymerase and ribosomal protein after ensuring that the antibodies against them were specific to humans (Supplementary Fig. S6, middle and lower panels). Immuno-FISH analysis revealed co-localised fluorescent signals generated by a human-specific genomic DNA probe and antibodies against RNA polymerase III and ribosomal protein (Fig. 8a, middle and lower panels, respectively). These findings indicated that concatemers could autonomously synthesise 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 Fig. S3c. The extent of co-localization ranged between 17% and 29%. We confirmed these findings in metaphase preparations from serially passaged cells for all three components of the protein synthetic machinery mentioned above (Fig. 8b). Negative control experiments performed on native NIH3T3 cells that had not been exposed to cfChPs treatment did not react with the human specific probes against DNA, ribosomal RNA and the human specific antibodies against RNA polymerase III and ribosomal protein (Supplementary Fig. S4c).

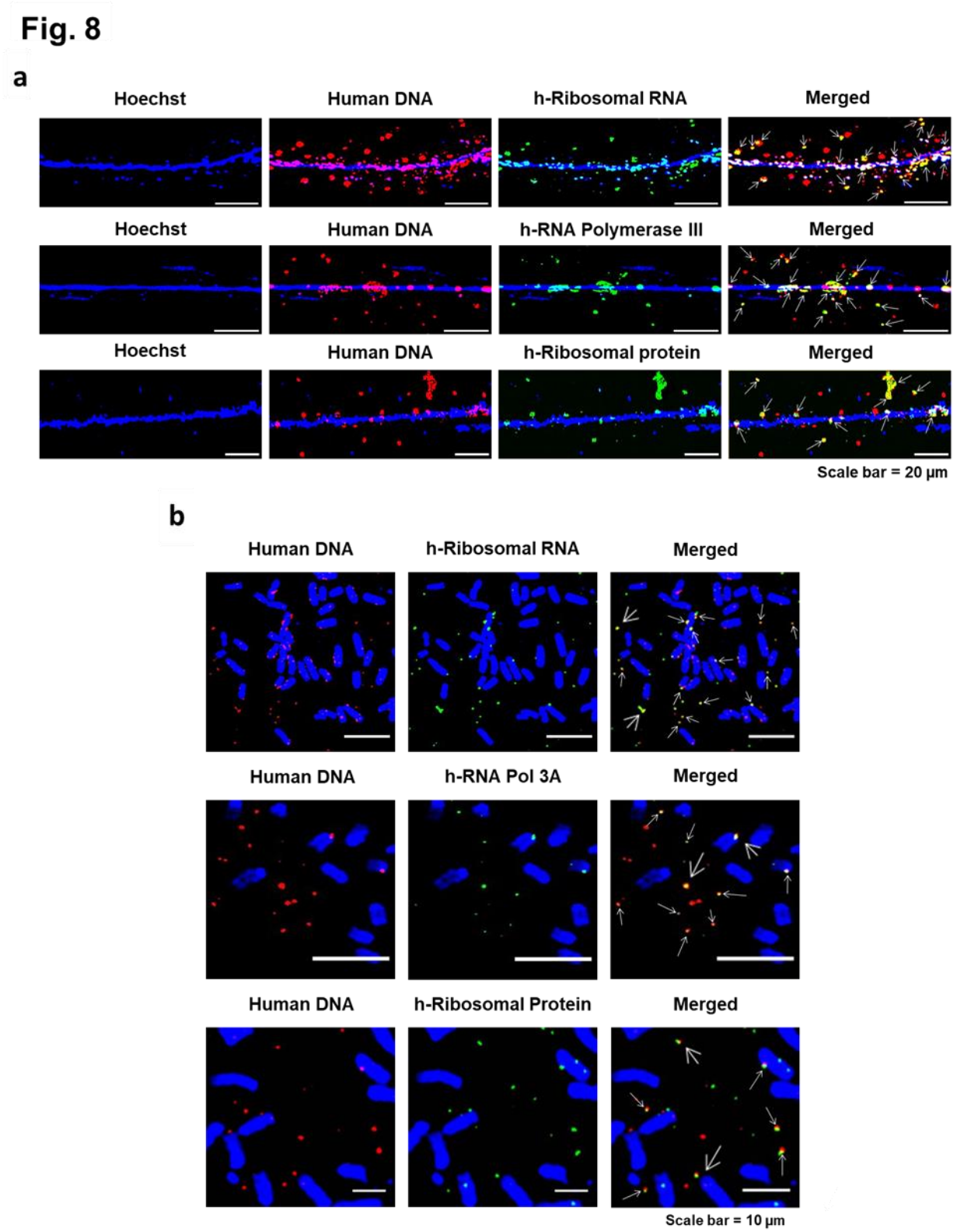


Fig. 8. Concatemers synthesise their own protein synthetic machinery. Chromatin fibres **(a)** and metaphase spreads **(b)** were prepared from cfChPs treated cells in continuous passage. Dual-FISH experiments were performed using a human specific genomic DNA probe and a probe against human specific ribosomal RNA, and immune-FISH experiments using a human specific genomic DNA probe

and antibodies against human specific RNA polymerase III and human specific ribosomal protein. Co-localised fluorescent signals of DNA and the components of the above protein synthetic machinery are marked with arrows.

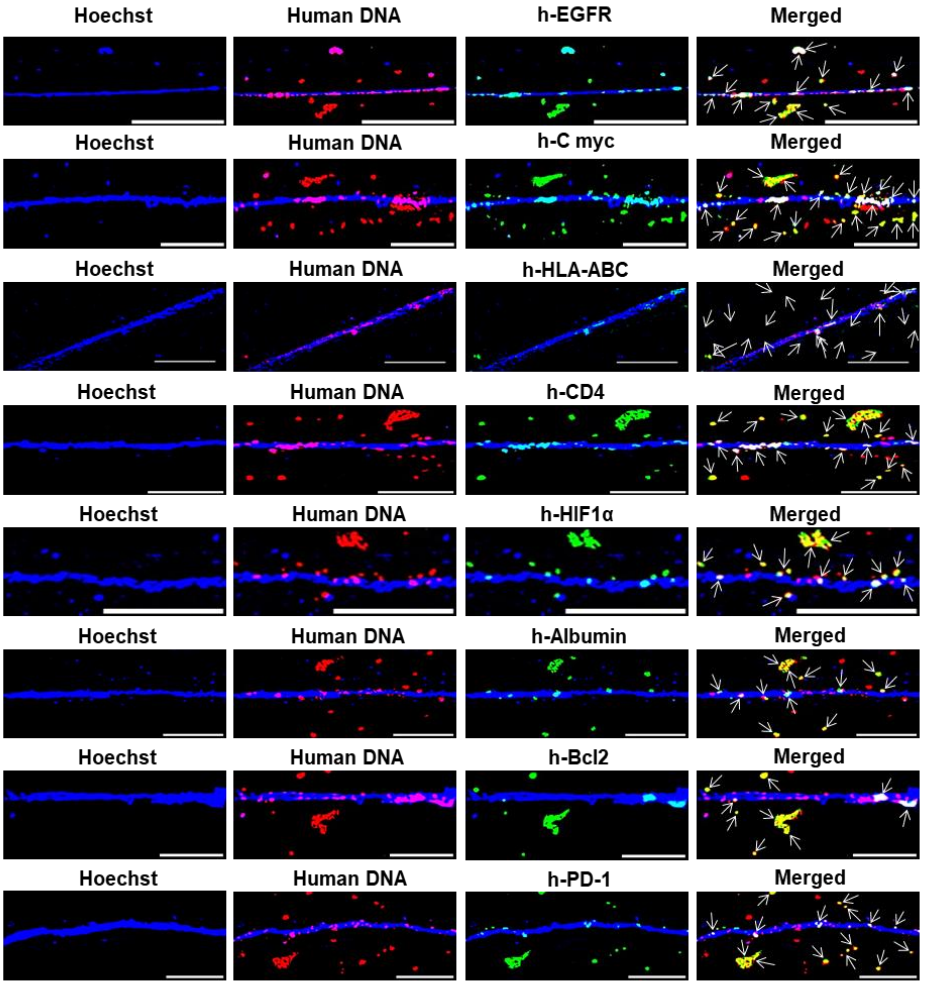
1.8 Concatemers synthesise a variety of human proteins in mouse cells

Having confirmed that concatemers are capable of synthesising RNA and assembling their own protein synthetic machinery, we went on to investigate whether they were capable of autonomously synthesising proteins. We conducted immune-FISH experiments using a human specific genomic DNA probe (to detect the concatemers) and antibodies against various proteins. We found that the fluorescent signals of the various proteins frequently co-localized with those of human DNA suggesting that the concatemers were capable of synthesizing proteins (Fig. 9a). Significantly, the newly synthesised proteins consistently remained associated with the concatemers of their origin (identified by fluorescent human DNA signals). This finding suggested that, although the concatemers contained the critical components of a protein synthetic machinery, they apparently lacked the machinery required for protein sorting. It should be noted that all the proteins that we detected seemed to be over-expressed, confirming that the gene segments corresponding to the proteins within the concatemers were amplified. 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 Fig. S3d. 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 native NIH3T3 cells that had not been exposed to cfChPs treatment using a variety of human specific antibodies and found that none of them showed any positive fluorescent signals (Supplementary Fig. S4d). The above results indicated that the disparate DNA sequences that comprise the

concatemers are transcribed and translated to generate proteins corresponding to the diverse DNA sequences that they contain apparently using their own protein synthetic machinery. Results of control experiments confirming the human specificity of the antibodies against all the proteins described above are given in Supplementary Fig. S7.

400

Fig. 9


a
Hoechst
Human DNA
h-EGFR
Merged
Hoechst
Human DNA
h-C myc
Merged
Hoechst
Human DNA
h-HLA-ABC
Merged
Hoechst
Human DNA
h-CD4
Merged
Hoechst
Human DNA
h-HIF1α
Merged
Hoechst
Human DNA
h-Albumin
Merged
Hoechst
Human DNA
h-Bcl2
Merged
Hoechst
Human DNA
h-PD-1
Merged
Scale bar = 20 μm

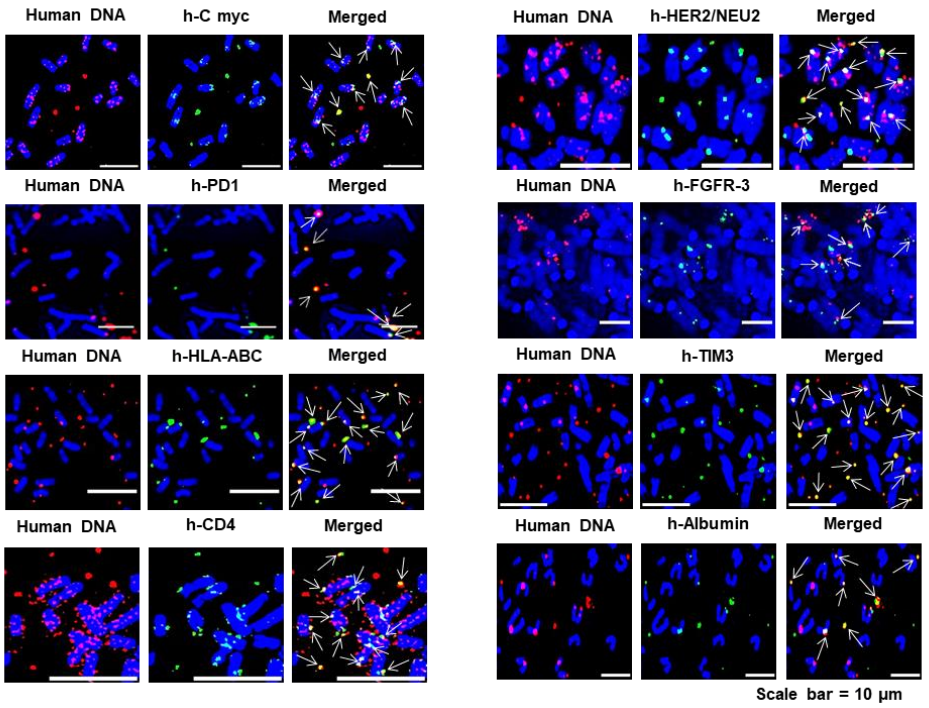
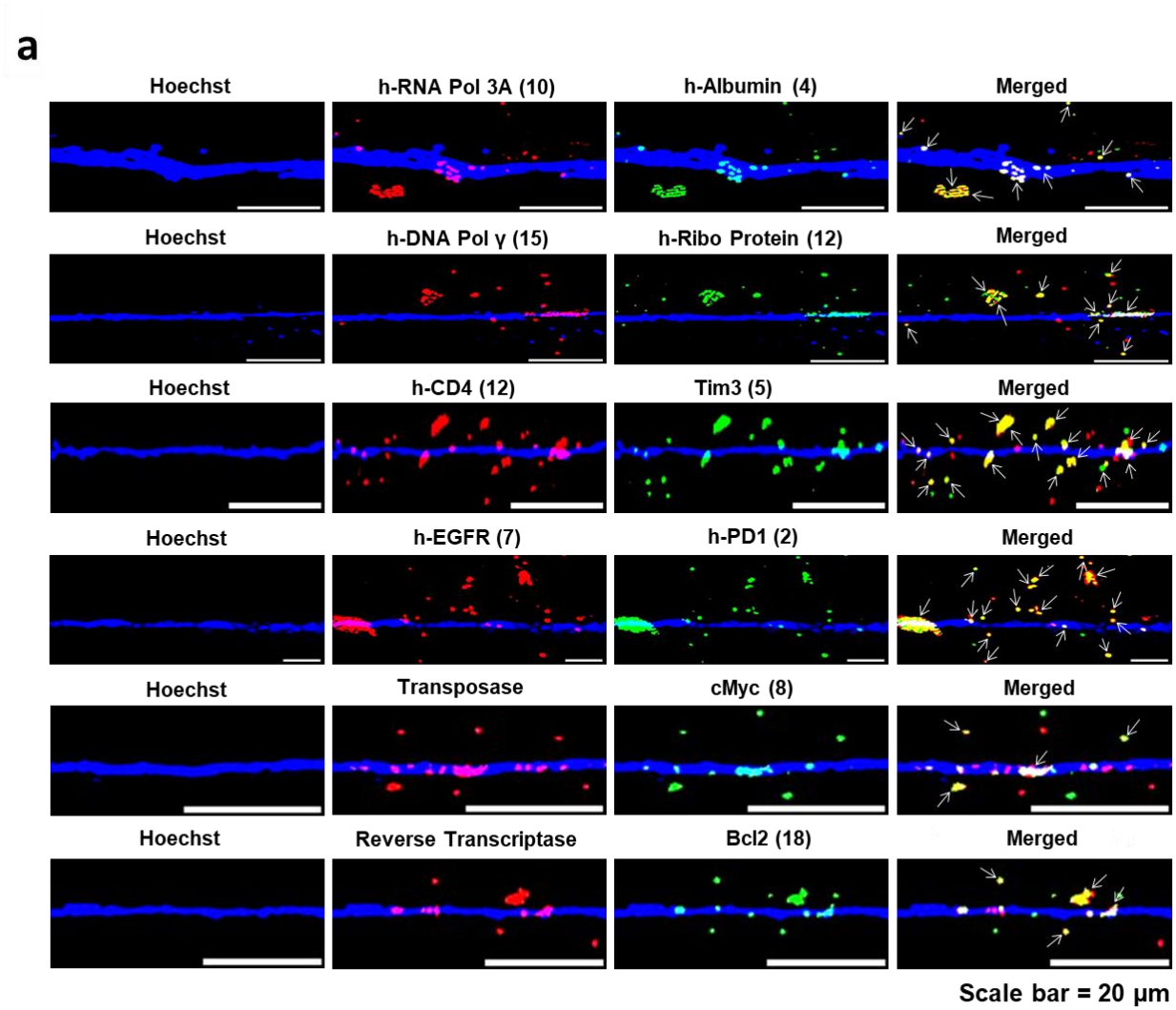

b
Human DNA
h-C myc
Merged
Human DNA
h-HER2/NEU2
Merged
Human DNA
h-PD1
Merged
Human DNA
h-FGFR-3
Merged
Human DNA
h-HLA-ABC
Merged
Human DNA
h-TIM3
Merged
Human DNA
h-CD4
Merged
Human DNA
h-Albumin
Merged
Scale bar = 10 μm

Fig. 9. Concatemers synthesise a variety of 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 cells in continuous passage show co-localizing signals. The co-localizing signals of human DNA and various proteins are marked with arrows.

1.9 The proteins that concatemers synthesize are complex fusion proteins

As the concatemers are formed as a result of amalgamation of widely disparate DNA sequences, we investigated whether the proteins that they synthesized might be fusion proteins. Dual-immunofluorescence experiments using pairs of antibodies against proteins, the corresponding genes of which were located on different chromosomes, revealed that the fluorescent signals frequently co-localised. This finding indicated 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 are shown in Figure 10a. The extent of co-localising signals ranged between 8% and 36% (Supplementary Fig. S3e). Results of control experiments confirming the human specificity of the antibodies against the fusion proteins not included in Supplementary Fig. S7 are given in Supplementary Fig. S8.

422 **Fig. 10**


a
Hoechst
h-RNA Pol 3A (10)
h-Albumin (4)
Merged
Hoechst
h-DNA Pol γ (15)
h-Ribo Protein (12)
Merged
Hoechst
h-CD4 (12)
Tim3 (5)
Merged
Hoechst
h-EGFR (7)
h-PD1 (2)
Merged
Hoechst
Transposase
cMyc (8)
Merged
Hoechst
Reverse Transcriptase
Bcl2 (18)
Merged
Scale bar = 20 μm

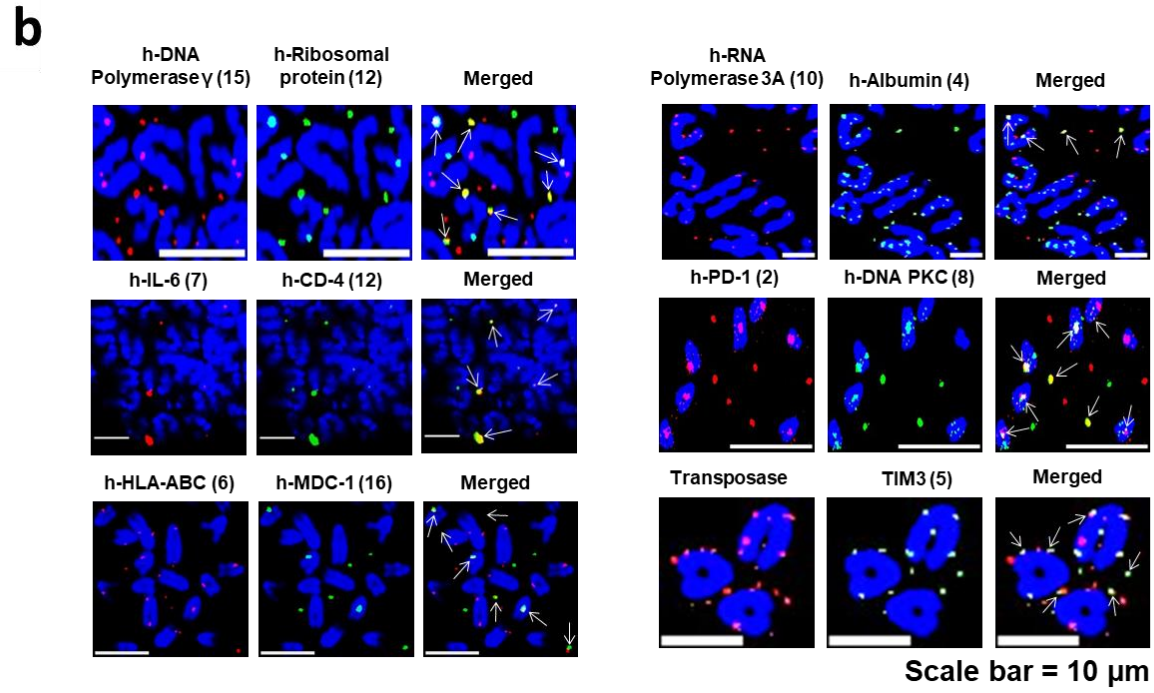

b
h-DNA Polymerase γ (15)
h-Ribosomal protein (12)
Merged
h-RNA Polymerase 3A (10)
h-Albumin (4)
Merged
h-IL-6 (7)
h-CD-4 (12)
Merged
h-PD-1 (2)
h-DNA PKC (8)
Merged
h-HLA-ABC (6)
h-MDC-1 (16)
Merged
Transposase
TIM3 (5)
Merged
Scale bar = 10 μm

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)** prepared from cfChPs-treated NIH3T3 cells in continuous passage. Results show frequent co-localised signals indicative of fusion proteins (arrows). The numbers given in the parenthesis indicate the chromosomal location of the genes that correspond to the proteins.

1.10 Concatemers are vehicles for transposable elements

Among the fusion proteins shown in Figure 10 we accidently found reverse transcriptase and transposase to co-localise with Bcl2 and cMyc, respectively. This finding raised the possibility that the concatemers might harbour gene components related to transposable elements. We used human LINE-1 and human *Alu* DNA probes which had been custom synthesised (Supplementary Table S2). Nonetheless, we checked for their specificity to ensure that they reacted only to human and not to mouse transposable elements (Supplementary Fig. S9). Dual-FISH experiments using a human genomic DNA probe and those targeting human LINE-1 and *Alu* showed that many of the fluorescent signals had co-localised indicating that the concatemers harboured 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 Fig. S3f. 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. The issue of amplification of the transposable elements is discussed in detail under section 1.12. Control experiments performed on native NIH3T3 cells that had not been exposed to cfChPs treatment using human LINE-1 and Alu probes did not reveal any positive signals (Supplementary Fig. S4e)

Fig. 11

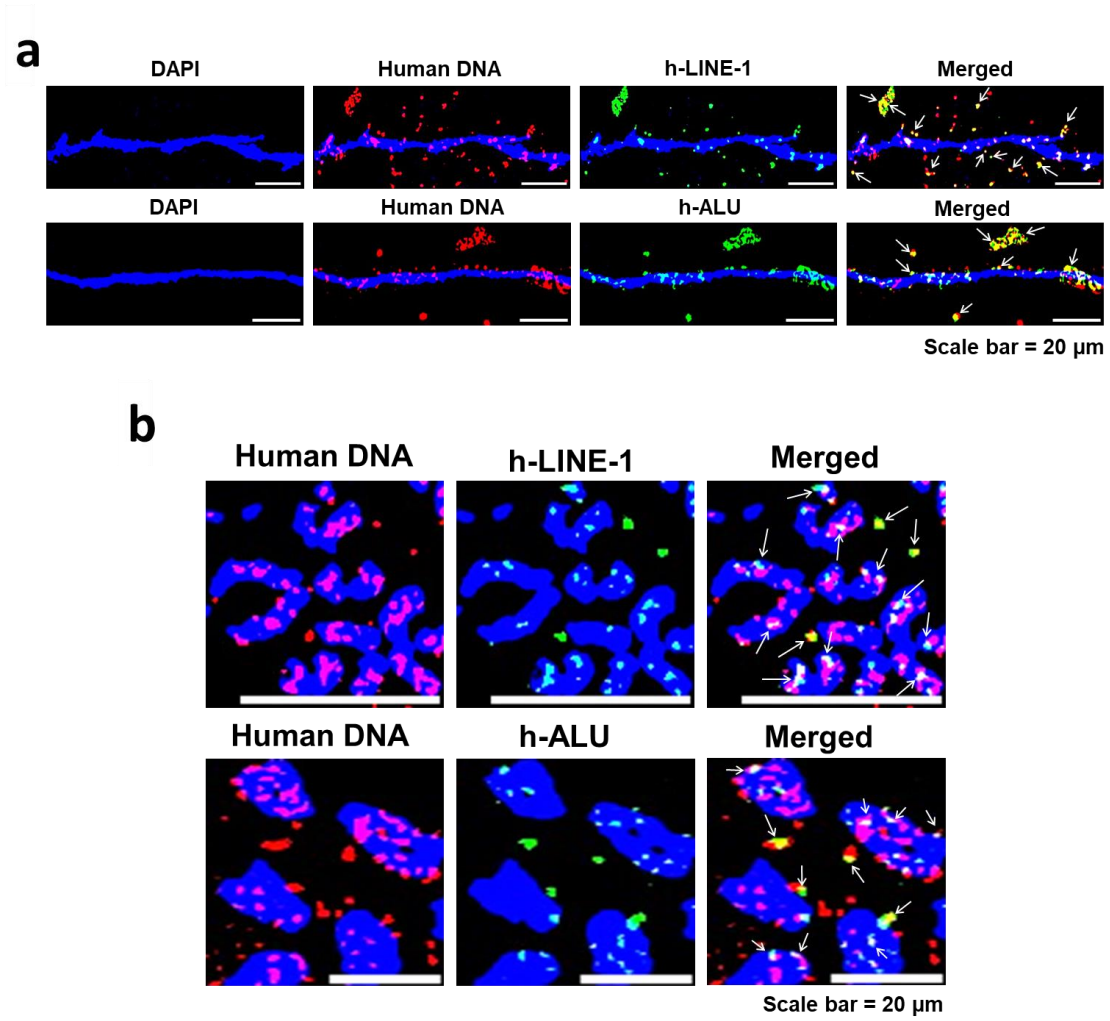


Fig. 11. Concatemers harbour transposable elements. Dual-FISH analysis on chromatin fibres (**a)** and metaphase spreads **(b)** prepared from cfChPs-treated NIH3T3 cells in continuous passage using a human specific genomic DNA probe and those against human LINE-1 or human *Alu* show co-localised signals (marked with arrows).

1.11 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 the enzymes reverse transcriptase and transposase were frequently associated with DNA sequences corresponding to LINE-1 and Alu (Fig. 12a and b). Such

an association could potentially allow the transposable elements to re-arrange themselves on the mouse cell genome by translocating themselves from one location 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 Fig. S3g. The extent of co-localization ranged between 21% and 30%. Control experiments performed on native NIH3T3 cells that had not been exposed to cfChPs treatment 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. S4f).

Fig. 12

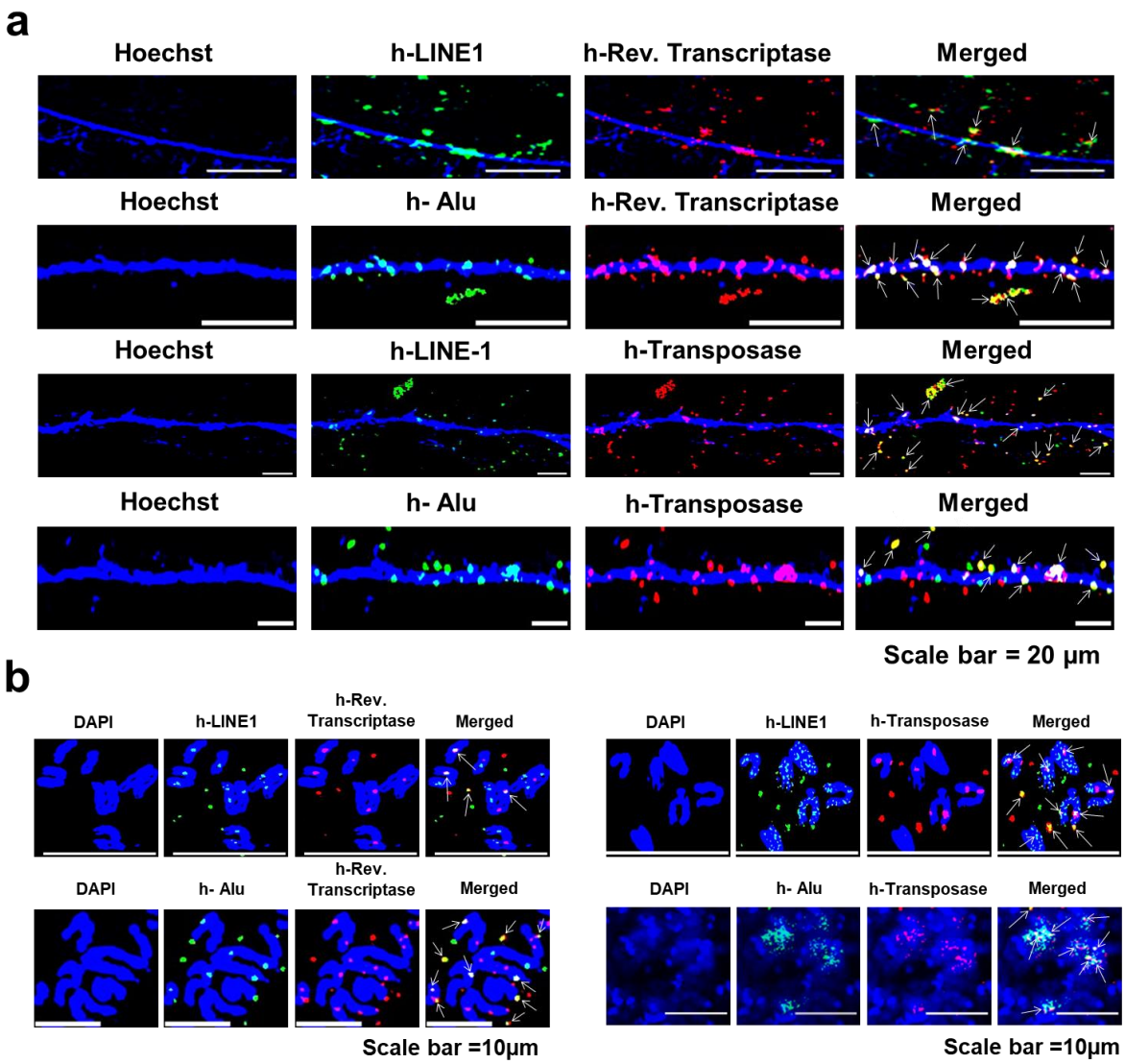


Fig. 12. LINE-1 and Alu sequences are associated with reverse transcriptase and transposase. Immuno-FISH analysis of chromatin fibres **(a)** and metaphase spreads**(b)** prepared from cfChPs-treated NIH3T3 cells in continuous passage using human *Alu* or LINE-1 probes and antibodies against human reverse transcriptase and human transposase show co-localising signals (marked with arrows).

As transposable elements are known to increase their copy number with time (Li et al., 2013), we were curious to find out whether the LINE-1 and *Alu* elements would proliferate and increase their copy number with progressively increasing passages. Immuno-FISH

experiments using antibodies against human DNA polymerase γ and FISH probes against LINE-1 and *Alu* detected co-localised signals, indicating that LINE-1 and *Alu* elements were associated with DNA polymerase, raising the possibility that they may have the potential to proliferate (Fig. 13a and b). This possibility was supported by the finding that LINE-1 and *Alu* elements could synthesize DNA. Immuno-FISH analysis of cells in continuous passage that had been pulse-labelled with BrdU showed co-localised signals generated by antibodies against BrdU and FISH probes against LINE-1 and *Alu*. Taken together these findings supported the idea that the DNA of both the transposable elements had the potential to replicate. 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 Fig. S3h. The extent of co-localization varied between 14% and 28%.

Fig. 13

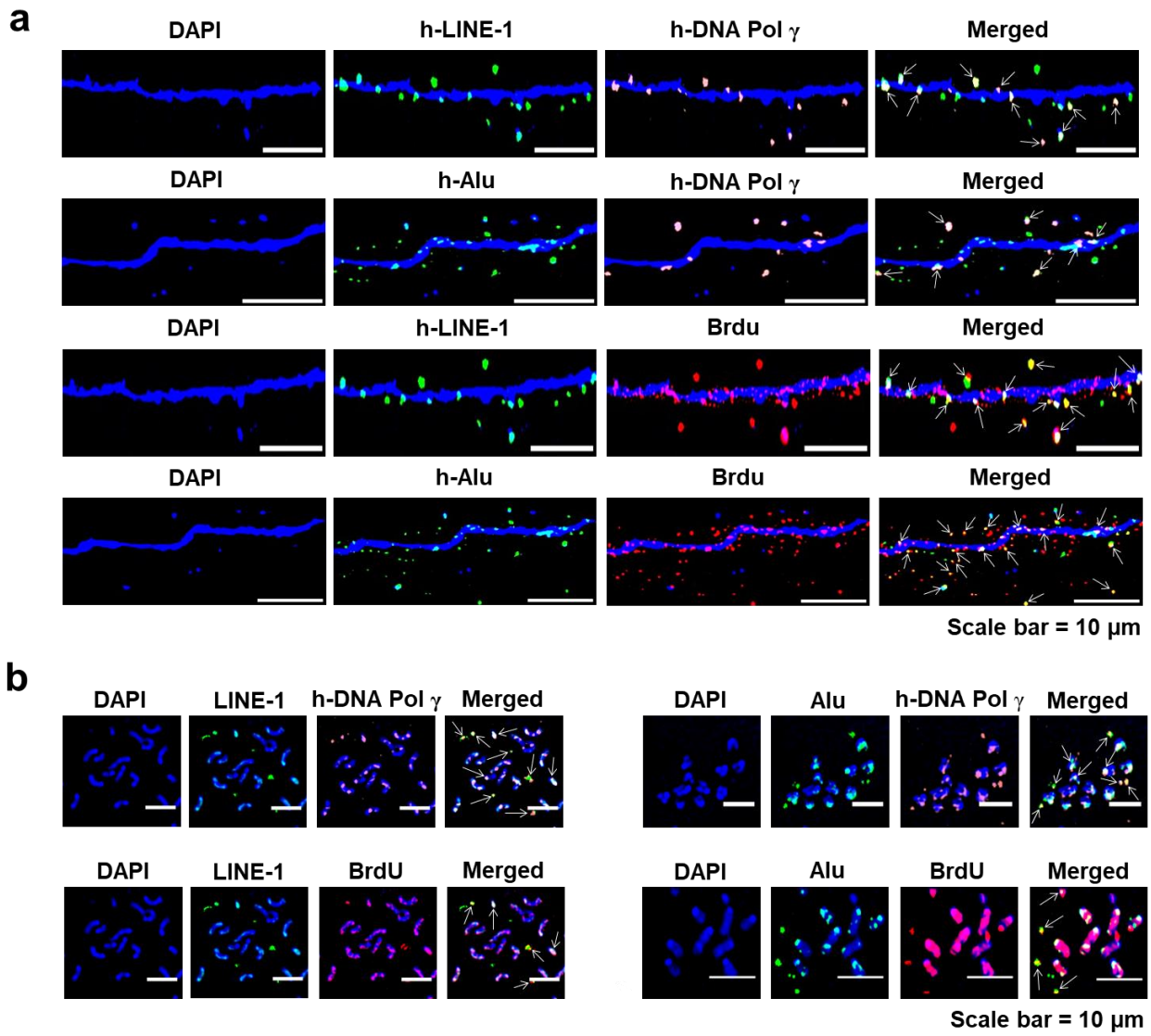


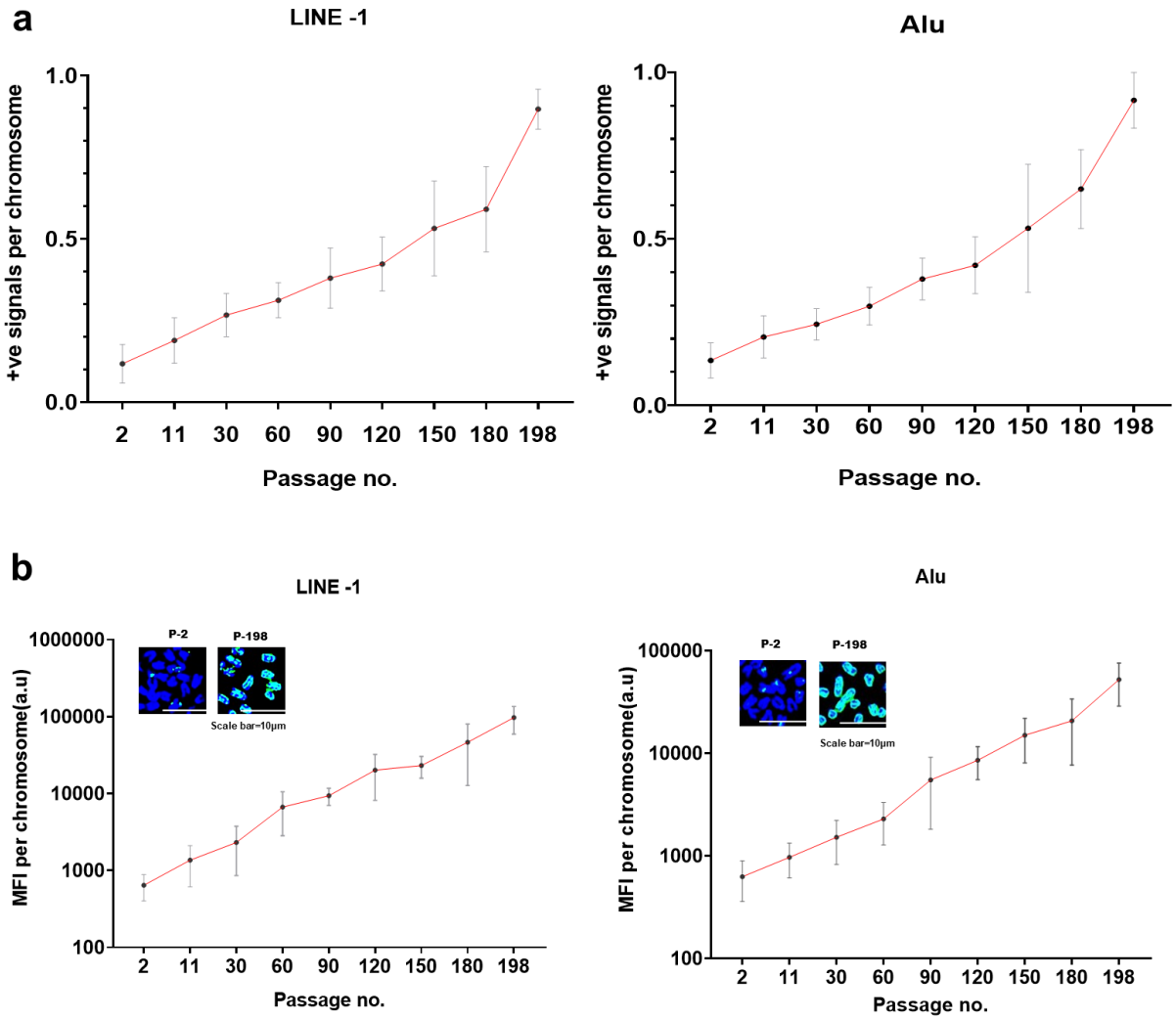
Fig. 13. LINE-1 and *Alu* elements are associated with DNA polymerase and can actively synthesise DNA. (a) Immuno-FISH images of cfChPs-treated cells in continuous passage showing co-localising signals of LINE-1 (green) and Alu (green) and DNA polymerase γ (orange) and BrdU (red) on chromatin fibres.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

1.12 LINE-1 and *Alu* elements increase their copy number and amplify themselves with time

Given the above findings, we were curious to determine whether LINE-1 and *Alu* elements could indeed replicate and increase their copy numbers within the mouse genome.

Metaphase spreads were prepared from cfChPs treated NIH3T3 cells in continuous passage and probed with FISH probes against LINE-1 and *Alu* elements. Fifteen metaphases were analysed at each passage and the number of LINE-1 and *Alu* fluorescent signals per metaphase were determined (Fig. 14). Our results showed that the numbers of human LINE-1 and *Alu* signals per chromosome increased steadily between passage 2 and 198 resulting in a 7.6-fold increase in copy number in case of LINE-1 and a 6.7-fold increase in case of *Alu* (Fig. 14a). 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 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 elements that are present in the cytoplasm. We also did a similar exercise to investigate whether the concatemers could amplify themselves with time by estimating the mean fluorescent intensity (MFI) of LINE-1 and *Alu* signals per chromosome. We found that the increase in MFI between passage 2 and 198 was 151.3 fold in case of LINE-1 and 83.4 fold in case of *Alu* (Fig. 14b). Taken together these data indicated that the transposable elements could not only proliferate but also extensively amplify themselves thereby becoming increasingly effective in modifying the host genome.

Fig. 14



532

533 **Fig. 14. LINE-1 and *Alu* elements proliferate and amplify themselves within the mouse genome**
534 **over time.** Metaphase spreads were prepared from cells in each progressively increasing passage
535 and probed with a human-specific LINE-1 and *Alu* probes. **a.** The total number of human LINE-1 and
536 *Alu* signals on the DAPI stained chromosomes were counted, and the mean number of LINE-1 and
537 *Alu* signals per chromosome was calculated after analysing 15 metaphase spreads at each passage.
538 A progressively increasing LINE-1 and *Alu* signals per chromosomes is evident with increasing
539 passage number (analysis of variance for linear trend $p < 0.0001$). The number of human LINE-1
540 signals per chromosome increased by a factor of 7.6 between passage no. 2 and passage no. 198,
541 and by a factor of 6.7 in case of *Alu.* **b.** a similar exercise was done as above except that mean
542 fluorescent intensity (MFI) of human LINE-1 and *Alu* per chromosome, indicative of amplification, was
543 determined. A progressively increasing in MFI per chromosomes is evident with increasing passage
544 number (analysis of variance for linear trend $p < 0.0001$). Mean MFI per chromosome increased by a
545 factor of 151.3 between passage no. 2 and passage no. 198 in case of LINE-1, and by a factor of 83.4
546 in case of *Alu.* The insets represent partial metaphase images showing human LINE-1 and Alu signals
547 on the chromosomes. Blue and red signals represent DAPI and human DNA, respectively.

2. 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. S10).

3. 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 (Mittra et al., 2017), we further investigated the intracellular activities of cfChPs released from dying MDA-MB-231 breast cancer cells. Hypoxia-induced cfChPs were collected in 1 ml of culture medium and added to NIH3T3 cells (please see Materials and Methods). We observed that cfChPs that were released from hypoxia-induced dying MDA-MB-231 cells could reciprocate all the intracellular activities and functions that were observed using cfChPs isolated from serum of both cancer patients and healthy individuals (Supplementary Fig. S11).

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 several biological processes (Mittra et al., 2015, 2017). Herein,

we have reported multiple additional activities and biological processes which can be summarised as follows. The internalised cfChPs containing widely disparate DNA sequences randomly combined to form 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 interphase DNA with many remaining in the cytoplasm and others being directly associated with the mouse chromosomal DNA. The concatemers 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. The latter manifested as 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 and extensively amplied themselves 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, the above findings lead us to conclude that the concatemers that form following horizontal transfer of cfChPs act as “predatory” genomes which function independently of the “inherited” genome.

Some of the summary points mentioned above call for further elaboration. For example, our demonstration that the concatemers synthesise ribosomal RNA, RNA polymerase and ribosomal protein suggests that they are capable of creating their own autonomous protein synthesis machinery. The concatemers being composed of open chromatin are apparently able to indiscriminately transcribe their DNA via RNA polymerase; the RNAs thus generated are translated into proteins with the help of their own ribosomal RNA and ribosomal proteins. Significantly, the newly synthesised proteins remained strictly

associated with their concatemers of origin suggesting that, although the concatemers contained the critical components of a protein synthetic machinery, they lacked the machinery required for protein sorting. Taken together, these findings suggest a novel mechanism of protein synthesis which function autonomously without recourse to the cells own protein synthetic machinery. Clearly, such an assertion, which defies existing knowledge, requires critical experimental scrutiny to establish the mechanistic processes that might underlie such a unique biological process. Furthermore, our demonstration that the concatemers act as vehicles of human LINE-1 and *Alu* elements raise the hypothesise that transposable elements are not inherent to the cell, but rather that they 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 surrounds the origin of these elements (Gilbert and Feschotte, 2018; Panaud, 2016). Nonetheless, our results suggest that cfChPs derived from the billions of cells that die in the body daily and are horizontally transferred to living cells may have evolutionary functions by acting as transposable elements.

Our detection of the unusual intracellular characteristics and functions of cfChPs was facilitated by three factors. First, our trans-species model comprising of mouse recipient cells and cfChPs derived from human serum. This allowed us to track the activities of internalised human cfChPs within the mouse cells using appropriate human-specific FISH probes and antibodies. Second, the chromatin fibre technique facilitated the detection of spatial relationships between internalised cfChPs concatemers and the mouse interphase DNA. Third, our use of human specific genomic DNA FISH probes and antibodies which could detect short DNA sequences and small peptides by binding to the corresponding sequences and epitopes within the compacted concatemers. These would not have been

detectable by the standard techniques of molecular biology (discussed in the next paragraph).

Numerous attempts on our part to characterise the concatemers by conventional real time PCR were unsuccessful. Given the complex and chaotic amalgams of disparate DNA fragments that comprise the concatemers, the primer sequences specific to a particular gene could not find the appropriate DNA sequences to bind to. Advanced sequencing technologies may help to define the complex nature of the DNA sequences that comprise the concatemers. Likewise, multiple attempts to detect the components of the fusion proteins by Western blotting proved unsuccessful. Even if an antibody reacted to a peptide component of a fusion protein, the band obtained would not correspond to the molecular weight of the positive control band, and the possibility of it being an artefact could not be excluded. Studies using liquid chromatography with tandem mass spectrometry may help to delineate the composition of the fusion proteins.

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 HGT 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 (Mittra, 2015). Although cfChPs (acting as MGEs) comprise of fragments of the host's own cellular genome, they undergo extensive and complex modifications following cellular apoptosis to such an extent that they act as "foreign" genetic elements when horizontally transferred to the new host cells.

Thus, we hypothesise that evolutionary changes in mammals are brought about not necessarily by foreign genes being 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 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 cells.

It has been proposed that extreme environmental stress plays a critical role in the adaptation and evolution of species (Bijlsma and Loeschcke, 2005; Hoffmann and Parsons, 1997; Nevo, 2011). We previously hypothesised (Raghuram et al., 2017) that extreme environmental stress may lead to organismal death via apoptosis or similar processes (Bayles, 2014; Häcker, 2013), 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, they can readily undergo horizontal transfer to foreign cells regardless of species or even kingdom boundaries (Raghuram et al., 2017). 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 (Raghuram et al., 2017).

The presence of extrachromosomal DNA (ecDNA) has been described in plants (Wong and Wildman, 1972), yeast (Møller et al., 2015), *Caenorhabditis elegans* (Shoura et al.,

2017), *Drosophila* (Stanfield and Lengyel, 1979), mice, and humans (Møller et al., 2018; Shibata et al., 2012). Recently, there has been a resurgence of interest in the role of ecDNA in cancer. In particular, it has been suggested that 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 (Yan et al., 2024). The results of the present study raise the possibility that the ecDNAs detected in human cancers may represent “predatory” genomes formed by the amalgamation of multiple unrelated cfChPs which have been acquired from the surrounding dying cancer cells.

Our study leaves many unanswered questions, and our findings will require to be validated in future research. As such, our results should be seen as representing an expansive theory, wherein the activities of the concatemers formed by amalgamation of the horizontally transferred cfChPs are governed by unknown biological processes that are distinct from those that govern the nuclear genome. We believe that an understanding of these unexplored biological processes could offer a new perspective on the complex process of natural evolution.

In summary, the present findings lead to a novel hypothesis that a cell simultaneously harbours two genome forms that function autonomously: one that is inherited (hereditary genome) and numerous others that are acquired (predatory genomes). The 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 within the body. This

suggests that a cell's propensity to internalise cfChPs represents a natural process to modify the hereditary genome, which in turn may promote 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 sera of five patients suffering from cancer and five healthy volunteers using the protocol described by us earlier (Mittra et al., 2015). Serum from five donors of each group was pooled prior to a cfChPs isolation. The steps of the isolation protocol can be summarized as follows: 1) centrifugation of the pooled serum samples (1 mL) at 700,000g for 16 h at 4°C; 2) treating the pellet obtained with lysis buffer; 3) centrifugation of the lysate at 700,000g for 16 h at 4°C; 4) suspension of the pellet in 1 ml PBS; 5) passing the suspension through an affinity column (ThermoFisher Scientific, USA) containing biotinylated anti-histone H4 antibody (125 µg) bound to 2 mL of Pierce® Streptavidin Plus Ultralink® Resin (ThermoFisher Scientific, USA); 6) elution of the column with 1 mL 0.25 M NaCl; 7) ultra-centrifuging the elute as described above and suspending the pellet containing cfChPs in 1 mL PBS. The presence of cfChPs was confirmed using a nucleosome specific sandwich ELISA kit (Cell Death Detection

ELISA^{PLUS} kit, Roche Diagnostics GmbH, Germany). A representative electron microscopy image of the isolated cfChPs is given in Supplementary Fig. S1. The concentrations of cfChPs in the isolates are expressed in terms of their DNA content, as estimated using the PicoGreen dsDNA quantitation assay (ThermoFisher Scientific, USA) (Mittra et al., 2015). The age, sex, and tumour types of the participants are given in Supplementary Table S1.

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 95% 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.

Collection of conditioned medium containing cfChPs released from dying MDA-MB-231 human breast 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 were released into the conditioned medium from hypoxia-induced dying MDA-MB-231 human breast cancer cells. The protocol for collecting cfChPs from hypoxia induced dying cells has been described by us in detail earlier (Raghuram et al., 2024). The only difference was that in the earlier study we had used dying NIH3T3 cells instead of MDA-MB-231 cells for collecting media containing hypoxia induced cfChPs. In the dual-chamber system used

in these experiments, the pore size of the filter separating the two chambers was 400µm. Consequently, the cfChPs from the dying MDA-MB-231 cells that were collected in the lower chamber were <400µm in size. NIH3T3 cells (1×10^5) were treated with 1 ml of the hypoxic media containing cfChPs.

Fluorescent dual labelling of cfChPs

In some experiments, the DNA and histones of the isolated cfChPs from serum 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 (Mittra et al., 2015). NIH3T3 cells were treated with 10 ng of dually labelled cfChPs for 6 h, washed with PBS x 3 and examined under a confocal microscope (Carl-Zeiss, GmbH, Germany). A representative image of dually labelled cfChPs is given in Fig. 1a.

Preparation of chromatin fibres

Chromatin fibres were prepared from NIH3T3 cells according to previously described methods (Nieminuszczy et al., 2016; Quinet et al., 2017) with minor modifications. Although the inventers of this technique had described the fibres as DNA fibres, when stained with histone H4 antibody, we found the fibres to comprise of chromatin (Supplementary Fig. S12). To prepare the chromatin fibres, 2 µl of the cfChPs treated NIH3T3 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 saline) for 2 min with gentle shaking, and the slides were tilted at an angle of 15°–25° to allow the cell lysate containing

the 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 treated with cfChPs in continuous culture were prepared using a standard protocol (Mittra et al., 2015). The slides were processed for immunofluorescence and FISH analyses.

Human specificity of antibodies and FISH probes

Sources and other details of the antibodies and FISH probes used in this study are provided in Supplementary Table S2. Irrespective of the specifications of the antibodies given in the vendors' data sheets, and the custom synthesised FISH probes, we independently verified their human specificity by undertaking extensive positive and negative control experiments to ensure that all antibodies and FISH probes were human specific. The results of these control experiments are given in Supplementary Figs. S2 and S5 – S9 and S13.

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 (Mittra et al., 2015). Briefly, for immunofluorescence, the chromatin fibres were fixed in 4% paraformaldehyde, blocking in saponin buffer containing 10% normal goat serum followed by immune-staining with appropriate primary and secondary antibodies. In order to

remove unbound antibodies, 1X PBS containing 0.05% Tween20 washes were given x 3 before mounting with VectaShield DAPI for microscopic examination. In case of immunofluorescence on metaphase spreads, the paraformaldehyde fixation step was omitted. For FISH on both chromatin fibres and metaphase spreads, the slides were dehydrated using alcohol series (70%, 80% and 100%) followed by hybridisation overnight with appropriate FISH probes in a humidified chamber at 37ºC. In order to remove the unbound probes, the slides were washed in 0.4X SSC at 70ºC for 1 minute followed by 4X SSCT (4X SSC in 0.05% Tween20) washes at 45ºC for 5 minutes each. The final wash was in 4X SSCT for 2 minutes at room temperature followed by mounting with VectaShield DAPI for microscopic examination. For immuno-FISH experiments, the slides were first stained for immunofluorescence as described above and fixed with 2% paraformaldehyde for 10 minutes followed by FISH procedure as described above. For microscopic examination, immunofluorescence slides were imaged at 400× magnification while FISH slides were analysed at 600× under an Applied Spectral Bio-imaging System (Applied Spectral Imaging, Israel). The immuno-FISH slides were analysed at 600× magnification.

Detection of DNA synthesis

To detect DNA synthesis on chromatin fibres and metaphase spreads, cells were pulse-labelled while in culture with 10 µM bromodeoxyuridine (BrdU) for 24h, washed in PBS x 3, trypsinized and used for preparing chromatin fibres and metaphase spreads. Newly synthesized DNA was detected using anti-BrdU antibody.

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 with the click chemistry facilitates viewing. In some experiments, the cfChPs treated 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

Statistical analysis was done by Student's t-test and by Analysis of variance for linear trend. Both analyses were done using GraphPad version 8.

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

Funding

This study was supported by the Department of Atomic Energy, Government of India, through its grant CTCTMC to the Tata Memorial Centre awarded to IM. The funding

agency had no role in research design, collection, analysis, and interpretation of data, or manuscript writing.

Acknowledgements

We thank Mr. Ashish Pawar for his help in preparing this manuscript. We would also like to thank Editage (www.editage.com) for English language editing support.

Conflict of interests

The authors declare no competing interests.

References

Aaronson SA, Pulciani S & Barbacid M. 1982. T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB-and Harvey-MSV transforming genes. Nature, 298:343-347 doi: 10.1038/298343a0

Anker P, Lyautey J, Lefort F, Lederrey C, Stroun M. 1994. Transformation of NIH/3T3 cells and SW 480 cells displaying K-ras mutation. *C R Acad Sci III* **317**:869–874.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. 2007. High-Resolution Profiling of Histone Methylations in the Human Genome. *Cell* **129**. doi:10.1016/j.cell.2007.05.009

Bayles KW. 2014. Bacterial programmed cell death: making sense of a paradox. *Nat Rev Microbiol* **12**:63–69.

Bergsmedh A, Szeles A, Henriksson M, Bratt A, Folkman MJ, Spetz A-L, Holmgren L. 2001. Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proceedings of the National Academy of Sciences* **98**:6407–6411.

Bijlsma R, Loeschcke V. 2005. Environmental stress, adaptation and evolution: An overviewJournal of Evolutionary Biology. doi:10.1111/j.1420-9101.2005.00962.x

Bushman F. 2002. Lateral DNA transfer. Cold Spring Harbor Laboratory Press.

Cui C, Shu W, Li P. 2016. Fluorescence in situ hybridization: cell-based genetic diagnostic and research applications. *Front Cell Dev Biol* **4**:89.

Dvořáková M, Karafiát V, Pajer P, Kluzáková E, Jarkovská K, Peková S, Krutílková L, Dvořák M. 2013. DNA released by leukemic cells contributes to the disruption of the bone marrow microenvironment. *Oncogene* **32**. doi:10.1038/onc.2012.553

Fliedner TM, Graessle D, Paulsen C, Reimers K. 2002. Structure and function of bone marrow hemopoiesis: mechanisms of response to ionizing radiation exposure. *Cancer Biother Radiopharm* **17**:405–426.

García-Olmo DC, Domínguez C, García-Arranz M, Anker P, Stroun M, García-Verdugo JM, García-Olmo D. 2010. Cell-Free Nucleic Acids Circulating in the Plasma of Colorectal Cancer Patients Induce the Oncogenic Transformation of Susceptible Cultured CellsCirculating DNA Induces Oncogenic Transformation. *Cancer Res* **70**:560–567.

Gilbert C, Feschotte C. 2018. Horizontal acquisition of transposable elements and viral sequences: patterns and consequences. *Curr Opin Genet Dev* **49**:15–24. doi:10.1016/j.gde.2018.02.007

Häcker G. 2013. Is there, and should there be, apoptosis in bacteria? *Microbes Infect*.

Hoffmann AA, Parsons PA. 1997. Extreme environmental change and evolution.

Hofmann A, Zakhartchenko V, Weppert M, Sebald H, Wenigerkind H, Brem G, Wolf E, Pfeifer A. 2004. Generation of transgenic cattle by lentiviral gene transfer into oocytes. *Biol Reprod* **71**. doi:10.1095/biolreprod.104.028472

Holdenrieder S, Stieber P. 2009. Clinical use of circulating nucleosomes. *Crit Rev Clin Lab Sci* **46**:1–24. doi:10.1080/10408360802485875

Li W, Prazak L, Chatterjee N, Grüninger S, Krug L, Theodorou D, Dubnau J. 2013. Activation of transposable elements during aging and neuronal decline in Drosophila. *Nat Neurosci* **16**:529–531. doi:10.1038/nn.3368

MacAlpine HK, Gordân R, Powell SK, Hartemink AJ, MacAlpine DM. 2010. Drosophila ORC localizes to open chromatin and marks sites of cohesin complex loading. *Genome Res* **20**:201–211.

Mittra I. 2015. Circulating nucleic acids: A new class of physiological mobile genetic elements. *F1000Res*. doi:10.12688/f1000research.7095.1

Mittra I, Naveen Kumar K, Gorantla Venkata R, Rohan C, Fatema K, Deepika G, Ashwini G. 2015. Circulating nucleic acids damage DNA of healthy cells by integrating into their genomes. *J Biosci* **40**:91–111.

Mittra I, Samant U, Sharma S, Raghuram G V, Saha T, Tidke P, Pancholi N, Gupta D, Prasannan P, Gaikwad A, Gardi N, Chaubal R, Upadhyay P, Pal K, Rane B, Shaikh A, Salunkhe S, Dutt S, Mishra PK, Khare NK, Nair NK, Dutt A. 2017. Cell-free chromatin from dying cancer cells integrate into genomes of bystander healthy cells to induce DNA damage and inflammation. *Cell Death Discov* **3**:17015. doi:10.1038/cddiscovery.2017.15

Møller HD, Mohiyuddin M, Prada-Luengo I, Sailani MR, Halling JF, Plomgaard P, Maretty L, Hansen AJ, Snyder MP, Pilegaard H, Lam HYK, Regenberg B. 2018. Circular DNA elements of chromosomal origin are common in healthy human somatic tissue. *Nat Commun* **9**. doi:10.1038/s41467-018-03369-8

Møller HD, Parsons L, Jørgensen TS, Botstein D, Regenberg B. 2015. Extrachromosomal circular DNA is common in yeast. *Proc Natl Acad Sci U S A* **112**:E3114-22. doi:10.1073/pnas.1508825112

Nagler C, Hardt C, Zänker KS, Dittmar T. 2011. Co-cultivation of murine BMDCs with 67NR mouse mammary carcinoma cells give rise to highly drug resistant cells. *Cancer Cell Int* **11**. doi:10.1186/1475-2867-11-21

Nevo E. 2011. Evolution under environmental stress at macro-and microscales. *Genome Biol Evol* **3**:1039–1052.

Nieminuszczy J, Schwab RA, Niedzwiedz W. 2016. The DNA fibre technique – tracking helicases at work. *Methods*. doi:10.1016/j.ymeth.2016.04.019

Panaud O. 2016. Horizontal transfers of transposable elements in eukaryotes: the flying genes. *C R Biol* **339**:296–299.

Parada LF, Tabin CJ, Shih C & Weinberg RA. 1982. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. Nature, 297:474-478. doi: 10.1038/297474a0

Puonti-Kaerlas J, Eriksson T, Engström P. 1990. Production of transgenic pea (Pisum sativum L.) plants by Agrobacterium tumefaciens—mediated gene transfer. *Theoretical and Applied Genetics* **80**:246–252.

Quinet A, Carvajal-Maldonado D, Lemacon D, Vindigni A. 2017. DNA fiber analysis: mind the gap! *Methods Enzymol* **591**:55–82.

Raghuram GV, Gupta D, Subramaniam S, Gaikwad A, Khare NK, Nobre M, Nair NK, Mittra I. 2017. Physical shearing imparts biological activity to DNA and ability to transmit itself horizontally across species and kingdom boundaries. *BMC Mol Biol* **18**. doi:10.1186/s12867-017-0098-8

Raghuram G v., Chaudhary S, Johari S, Mittra I. 2019. Illegitimate and repeated genomic integration of cell-free chromatin in the aetiology of somatic mosaicism, ageing, chronic diseases and cancer. *Genes (Basel)*. doi:10.3390/genes10060407

Raghuram GV, Tripathy BK, Avadhani K, Shabrish S, Khare NK, Lopes R, ... & Mittra I. 2024. Cell-free chromatin particles released from dying cells inflict mitochondrial damage and ROS production in living cells. Cell Death Discovery 10, 30. doi: 10.1038/s41420-023-01728-z

Sender R, Milo R. 2021. The distribution of cellular turnover in the human body. *Nat Med* **27**:45–48. doi:10.1038/s41591-020-01182-9

Shibata Y, Kumar P, Layer R, Willcox S, Gagan JR, Griffith JD, Dutta A. 2012. Extrachromosomal microDNAs and chromosomal microdeletions in normal tissues. *Science (1979)* **335**. doi:10.1126/science.1213307

Shoura MJ, Gabdank I, Hansen L, Merker J, Gotlib J, Levene SD, Fire AZ. 2017. Intricate and cell type-specific populations of endogenous circular DNA (eccDNA) in Caenorhabditis elegans and Homo sapiens. *G3: Genes, Genomes, Genetics* **7**. doi:10.1534/g3.117.300141

Stanfield SW, Lengyel JA. 1979. Small circular DNA of Drosophila melanogaster: chromosomal homology and kinetic complexity. *Proc Natl Acad Sci U S A* **76**. doi:10.1073/pnas.76.12.6142

Thomas S, Li X-Y, Sabo PJ, Sandstrom R, Thurman RE, Canfield TK, Giste E, Fisher W, Hammonds A, Celniker SE. 2011. Dynamic reprogramming of chromatin accessibility during Drosophila embryo development. *Genome Biol* **12**:1–17.

Trejo-Becerril C, Pérez-Cárdenas E, Taja-Chayeb L, Anker P, Herrera-Goepfert R, Medina-Velázquez LA, Hidalgo-Miranda A, Pérez-Montiel D, Chávez-Blanco A, Cruz-Velázquez J, Díaz-Chávez J, Gaxiola M, Dueñas-González A. 2012. Cancer Progression Mediated by Horizontal Gene Transfer in an In Vivo Model. *PLoS One* **7**:e52754. doi:10.1371/journal.pone.0052754

Wong FY, Wildman SG. 1972. Simple procedure for isolation of satellite DNA's from tobacco leaves in high yield and demonstration of minicircles. *BBA Section Nucleic Acids And Protein Synthesis* **259**. doi:10.1016/0005-2787(72)90468-6

Yan X, Mischel P, Chang H. 2024. Extrachromosomal DNA in cancer. *Nat Rev Cancer* **24**:261–273. doi:10.1038/s41568-024-00669-8