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

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Abstract

Background:

Horizontal gene transfer is known to drive adaptation and evolutionary processes in microorganisms; however, its precise evolutionary role in mammals is not known. 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 into healthy cells, leading to DNA damage and activation of apoptotic pathways. However, the underlying mechanism and function of these processes remained unclear.

Method:

We treated NIH3T3 mouse fibroblasts with cfChPs isolated from the serum of cancer patients and healthy individuals and serially passaged the cells. Using DNA fibre fluorography, cytogenetic analysis, and treatment with appropriate antibodies and fluorescent *in situ* hybridisation probes we investigated the intracellular activities of cfChPs.

Results:

The internalised cfChPs containing disparate DNA sequences had randomly combined to form concatamers of variable sizes some of which were ostensibly multi-mega base pairs in size. Many concatamers remained in the cytoplasm, whereas others were directly associated with chromosomal DNA. The concatamers exhibited the potential to perform many functions attributable to the nuclear genome in mouse cells, including the ability to replicate and to synthesise RNA, DNA polymerase, RNA polymerase, ribosomal RNA, ribosomal proteins, and numerous other human proteins manifesting as

complex multi-peptide fusion proteins. Further analysis demonstrated that the concatamers harboured human long interspersed nuclear element-1 and short *Alu* elements that increased their copy number with time. The concatamers were also associated with the enzymes reverse transcriptase and transposase, indicating their potential to rearrange themselves within the mouse genome.

Conclusion:

These findings lead us to hypothesise that a cell simultaneously harbours two genome forms that can function autonomously: one that is inherited (hereditary genome) and numerous others that are acquired (miniature predatory genomes). The existence 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. Finally, our results 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.

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 been a challenge. Nonetheless, studies on HGT have offered insight into the functions and regulation of exogenously introduced genes, while 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 occurs 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. This process has numerous biological ramifications such as inducing 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 fibroblasts for cfChP treatment 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, intracellular activities, and functions of healthy and cancerous cfChPs. We found that cfChPs isolated from cancer patients had significantly greater activity than those isolated from healthy individuals with respect to all parameters examined. Therefore, in the present study, we treated NIH3T3 cells with cfChPs isolated from the sera of both cancer patients and healthy individuals and then serially passaged the cells. Using DNA fibre fluorography, cytogenetic analysis, and treatment with appropriate antibodies and FISH probes, we further analysed the intracellular dynamics and processes that regulate the functional properties of the internalised cfChPs. Our findings provide novel insights and research directions to obtain a new perspective about the process of evolution.

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 DNA fibres

DNA fibres were prepared from interphase NIH3T3 cells according to previously described methods [18,19] with minor modifications. 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 DNA 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 DNA fibres and metaphase spreads was detected using FISH, as described previously [13]. In brief, the immune-stained DNA 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 DNA 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. 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.

Results

Experiments using cfChPs from cancer patients

cfChPs are rapidly and abundantly taken up by mouse fibroblasts

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 large and prominent,

potentially owing to the combination of multiple cfChPs to form concatamers (described in detail below). DNA 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 DNA fibres are derived from interphase cells, we further investigated whether the internalised concatamers 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 chromosomes (Fig. 1C).

Fig. 1

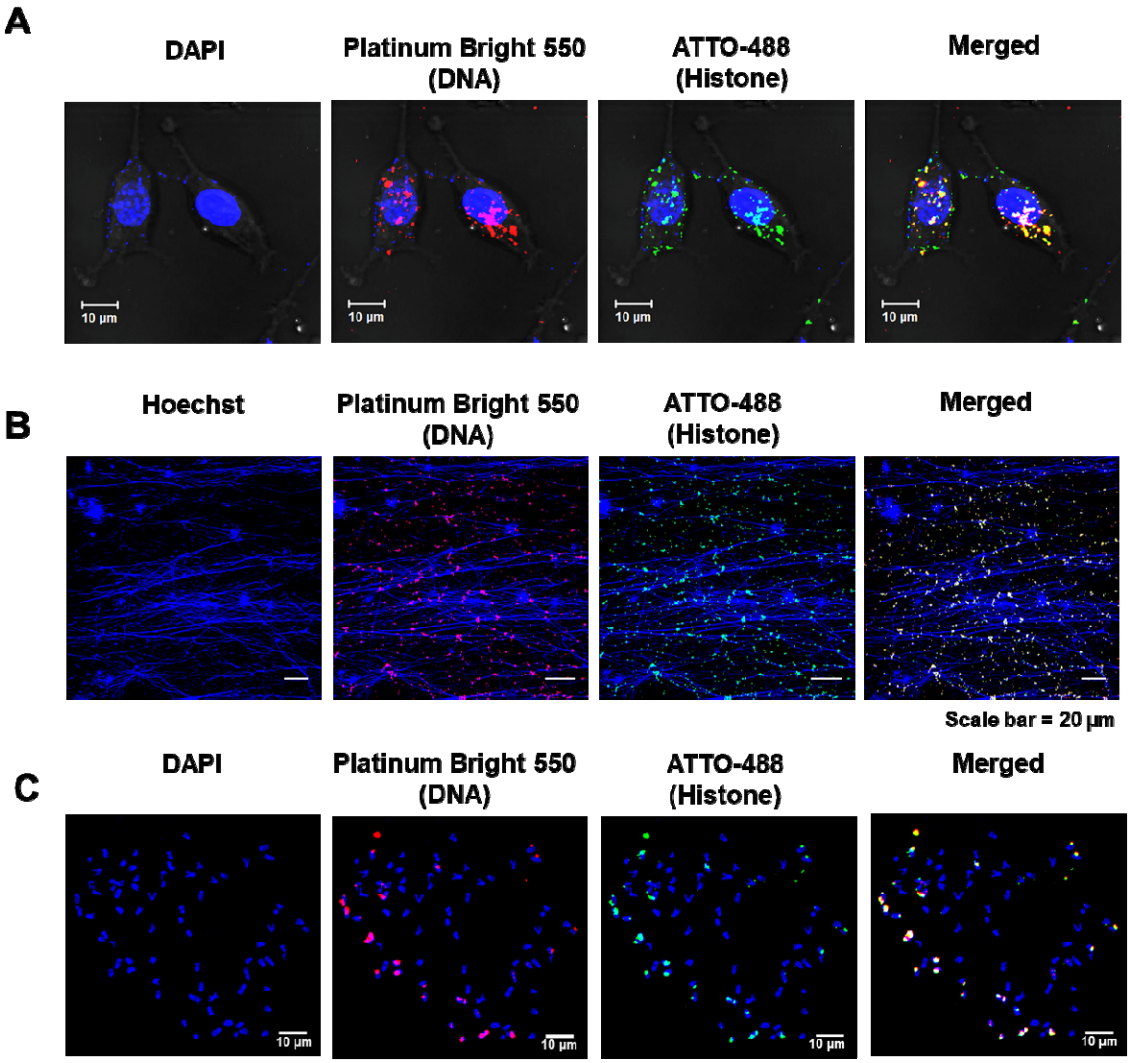
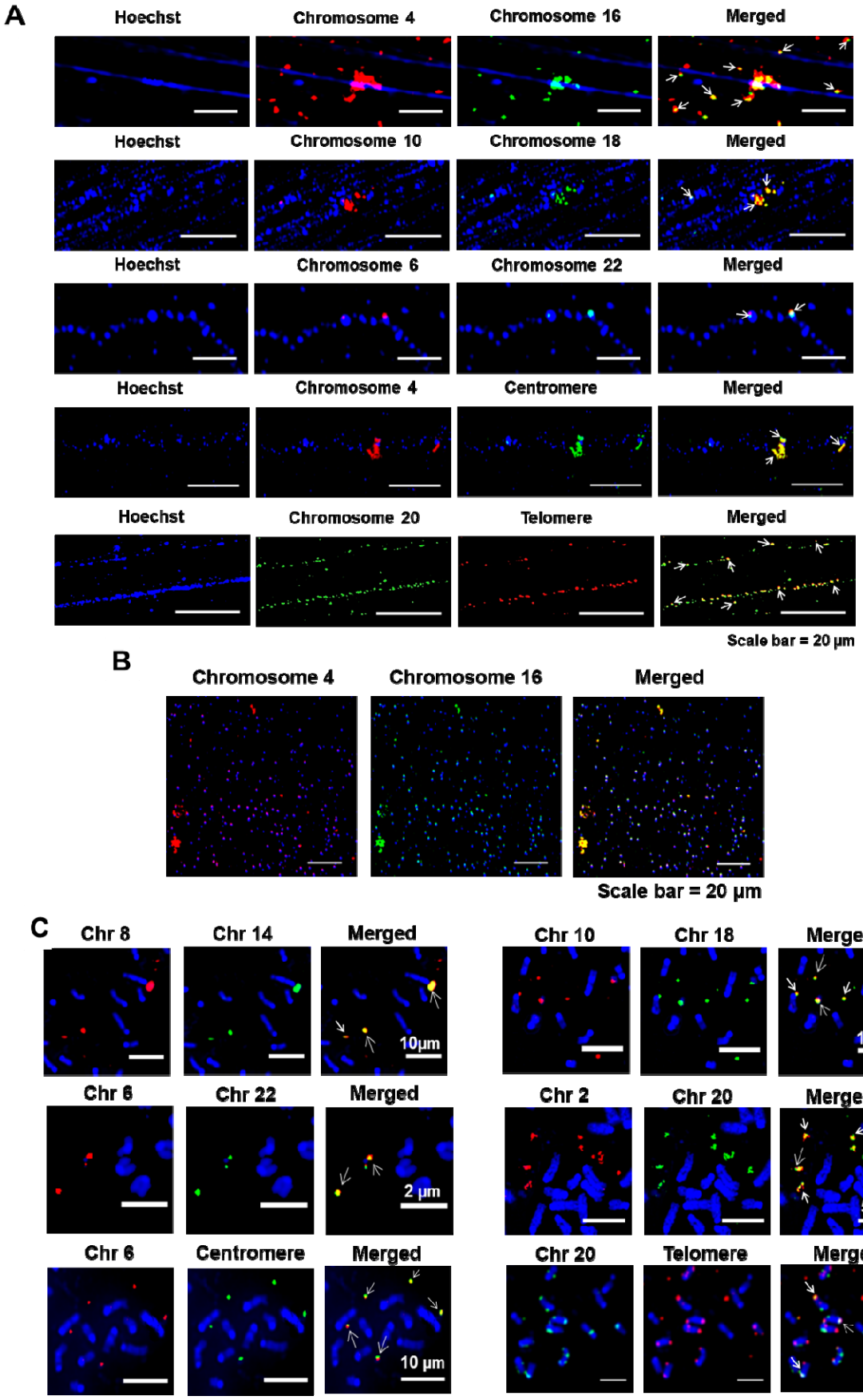


Fig. 1. Abundant uptake of cfChPs by NIH3T3 cells at 6 h. The DNA and histones of cfChPs (10 ng) were dually labelled with Platinum Bright 550 and ATTO-488, respectively, and applied to NIH3T3 cells. **A.** Confocal microscopy images of cfChP-treated cells at 6 h showing numerous dually labelled fluorescent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

cfChPs combine to form concatamers

As suspected, analysis of DNA fibres and metaphase spreads prepared from cells in continuous passage using different pairs of human chromosome-specific DNA probes showed their co-localisation to generate dually labelled fluorescent signals (Fig. 2A). This result indicated that multiple disparate intracellular cfChPs were linked to form concatamers of widely variable sizes, which were found even for the smallest cfChPs (Fig. 2B). Because the threshold of detection of a FISH signal is on the order of 100–200 kb **[20]**, even the smallest cfChPs can be assumed to be of a similar size range. This implies that many of the larger concatamers could be multi-megabase pairs in length and that the DNA contained within them has been extensively amplified. The co-localisation of various combinations of chromosome-specific signals suggested that the concatamers represented a complex amalgam of variable DNA fragments from different chromosomes with disparate and unrelated DNA sequences that were randomly linked to form complex genomic fragments of variable sizes. 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, bottom panels). The centromeric signal was prominent, suggesting that the concatamers had not only incorporated centromeric DNA sequences within their folds but had also undergone large-scale amplification (Fig. 2A, bottom panel). Such concatamerisation could also be detected in metaphase spreads prepared from serially passaged cfChPs-treated NIH3T3 cells (Fig. 2C).

Fig. 2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Chromosome 20
Telomere
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Chromosome 4
Chromosome 16
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Chr 10
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10µm
Chr 6
Chr 22
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Chr 2
Chr 20
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5 µm
Chr 6
Centromere
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10 µm
Chr 20
Telomere
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Fig. 2. cfChPs combine to form concatamers of varying sizes. A. FISH analysis of DNA fibres prepared from cfChP-treated NIH3T3 cells in continuous passage using different combinations of human chromosome-specific probes, including probes specific for the human centromere and telomere, revealing co-localised fluorescent signals (arrows). **B.** Small cfChPs show co-localised signals, suggesting that they also comprise concatemers **C.** FISH analysis of metaphase spreads prepared from cfChP-treated NIH3T3 cells in continuous passage using different combinations of human chromosome-specific probes, including probes specific for human centromeres and telomeres, revealing co-localised fluorescent signals (arrows).

Concatamers exhibit variable spatial relationships with mouse DNA

Immuno-FISH analysis using an antibody against histone H4 and a human-specific FISH probe on DNA 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 DNA fibres (Fig. 3A). Although many cfChPs were detected in the cytoplasm or aligned with long stretches of mouse DNA, others exhibited unusual conformations or were dangling from the mouse DNA. A similar peculiar conformation was found in clone D5, which had been prepared several years earlier and had undergone numerous passages and multiple freeze–thaw cycles **[13]** (Fig. 3B). The fluorescent cfChP signals were strikingly prominent, suggesting that they markedly exceeded the threshold of detection of FISH signals (100–200 kb) **[20]**. Metaphase preparations from treated and serially passaged cells exhibited the presence of many fluorescent cfChP signals in extra-chromosomal spaces (Fig. 3C).

Fig. 3

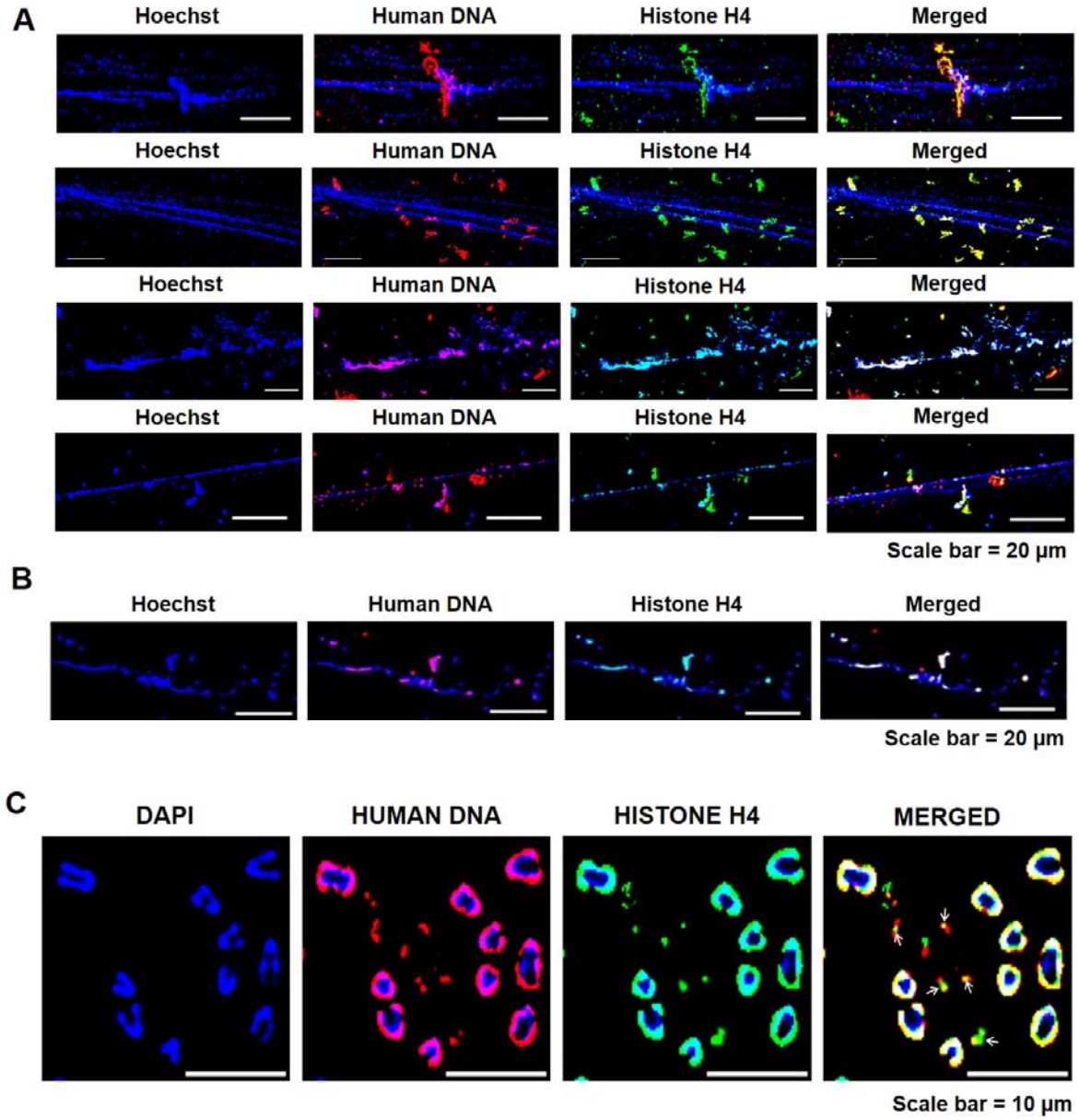

Fig. 3. Variable spatial relationships of concatamers with mouse DNA fibres and metaphase spreads. A. Immuno-FISH analysis of DNA fibres prepared from cfChPs-treated NIH3T3 cells in continuous passages using an antibody against histone H4 and a human whole-genome FISH probe. **B.** Immuno-FISH analysis of DNA fibres prepared from clone D5 showing that human concatamers persisted even after numerous passages and freeze–thaw cycles. **C.** Spatial relationship of concatamers with mouse chromosomes on metaphase spreads. Co-localised DNA and histone signals are marked with arrows.

Concatamers synthesise DNA polymerase and have replication potential

Immuno-FISH analysis of pulse-labelled cells using an anti-BrdU antibody and a human genomic DNA probe revealed the co-localisation of fluorescent signals, indicating that the concatamers were actively synthesising DNA (Fig. 4A, upper panel). Moreover, immuno-FISH experiments revealed that the concatamers expressed human DNA polymerase γ (Fig. 4A, middle panel). Dual immunofluorescence staining using antibodies against BrdU and DNA polymerase γ revealed co-localised signals, indicating that the concatamers not only have the potential to synthesise DNA but also to autonomously replicate themselves (Fig. 4A, lower panel). These findings were confirmed in metaphase preparations from serially passaged cfChP-treated NIH3T3 cells (Fig. 4B).

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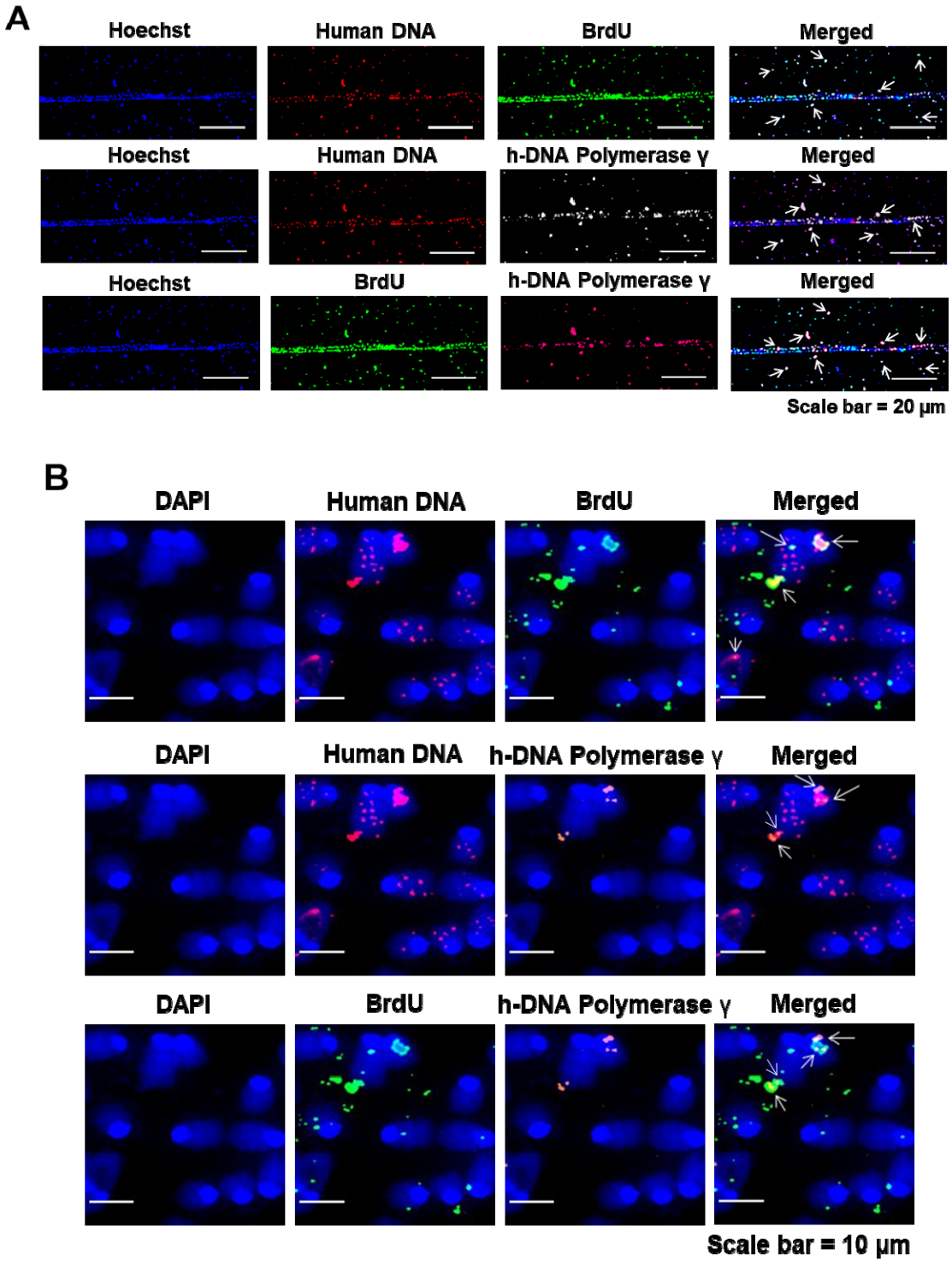
Fig. 4


A
Hoechst
Human DNA
BrdU
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h-DNA Polymerase γ
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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 = 10 μm

Fig. 4. Concatamers synthesise DNA and express human DNA polymerase γ. A. DNA 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.** Immuno-FISH experiments on metaphase spreads from cfChPs-treated NIH3T3 cells showing co-localised signals of human DNA and BrdU, human DNA and human DNA polymerase γ, and BrdU and DNA polymerase γ. Co-localised signals are marked with arrows.

Given the above results showing that the concatamers can express DNA polymerase, we next evaluated their potential for self-replication. Analysis of 10 metaphase preparations showed an increase in the number of human FISH signals per chromosome after passage 20, which steadily increased thereafter until passage 198, representing a four-fold increase in human DNA signals per chromosome (Fig. 5). It should be noted that this analysis is restricted to determining the proliferative capacity of concatamers that are associated with chromosomes and does not take into account the replicative potential of those that are present in the cytoplasm.

Fig. 5

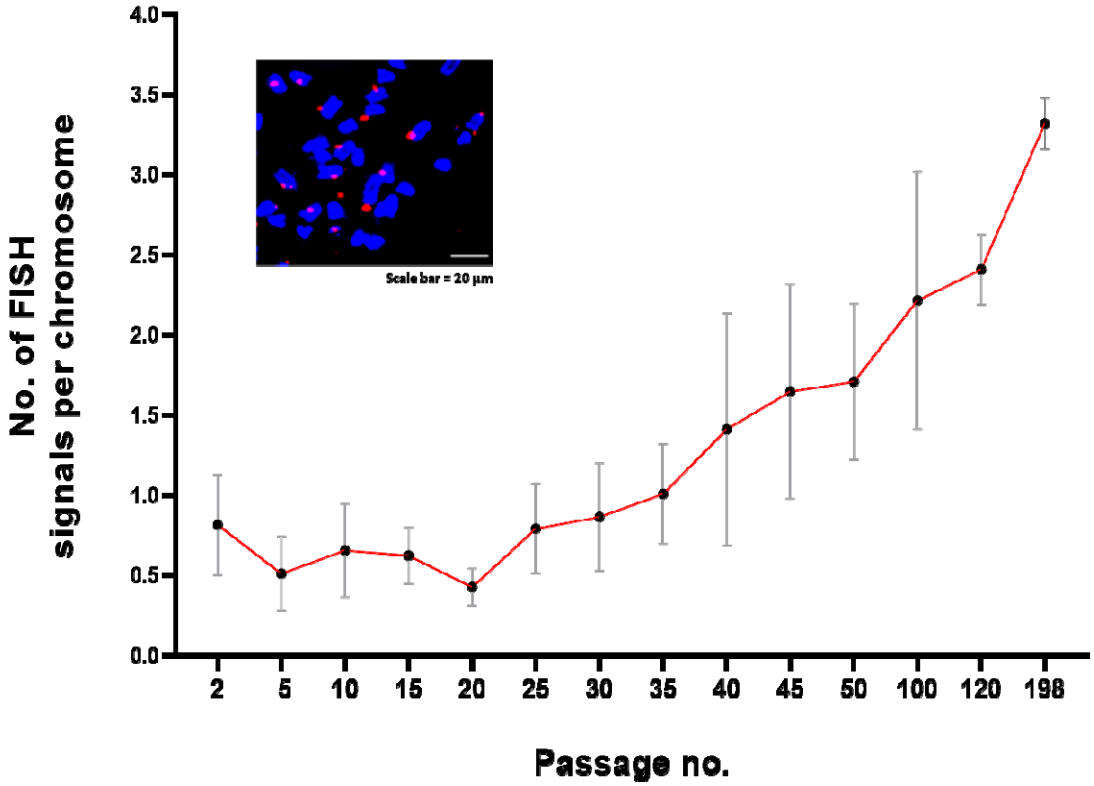


Fig. 5. Concatamers increase their copy number in the mouse genome over time. Metaphase spreads were prepared from cells at successive passages, stained with a human-specific FISH probe, and the mean ± standard error of the number of human FISH signals per chromosome was determined after analysing 10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

Concatamers are composed of open chromatin

Next, we examined the epigenetic constitution of concatamers 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 **[21]**. DNA fibres prepared from cfChPs-treated NIH3T3 cells in continuous passage were dually

immunostained with antibodies targeting H3K4me3 and H3K9me3 and the number of concatamers reacting with the two antibodies was counted. We found that the majority of the concatamers either reacted exclusively with antibodies against H3K4me3 (72%) or were hybrids of H3K4me3 and H3K9me3 (17%). Only 11% of the concatamers reacted exclusively with H3K9me3 (Fig. 6). Taken together, these data indicate that most concatamers contain nucleosome-depleted regions and can bind to protein factors that facilitate gene transcription [22] and DNA replication [23].

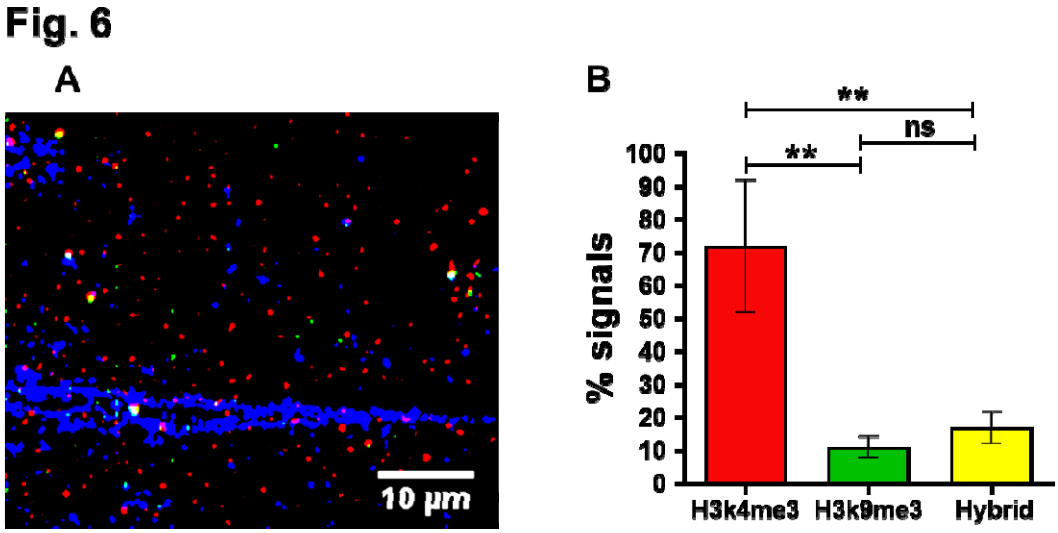


Fig. 6. Concatamers are largely comprised of open chromatin. A. Representative image of a DNA fibre preparation immunostained with antibodies targeting H3k4me3 (red) and H3k9me3 (green); hybrid concatamers are represented by co-localised yellow signals. The DNA fibres are stained with DAPI emitting blue 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.

Concatamers can synthesise RNA

As the above findings suggested that concatamers are potentially capable of active transcription, we further investigated their potential for RNA synthesis. Abundant RNA was detected in the cytoplasm of cfChP-treated passaged cells, which was absent in

the control NIH3T3 cells (Fig. 7A). Because RNA synthesis is normally restricted to the nucleus, the detection of RNA in the cytoplasm indicated that DNA within the concatamers underwent active transcription. Treatment of cells with actinomycin D or maintenance at low temperature completely abolished RNA synthesis. These findings confirmed that RNA synthesis is dependent on active cellular metabolism. Similar findings were obtained in experiments involving DNA fibres wherein DNA and RNA signals were found to be co-localised, indicating that the satellite concatemers were supporting RNA synthesis (Fig. 7B).

Fig. 7

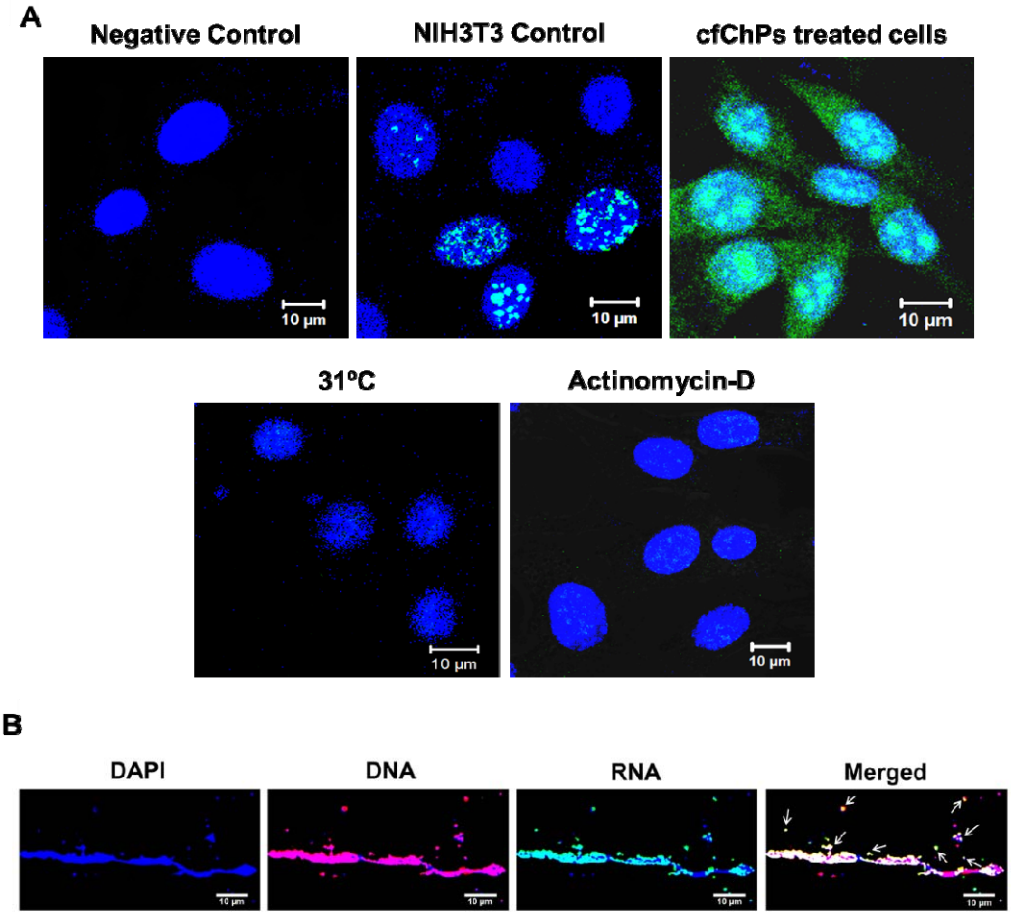


Fig. 7. cfChP-treated NIH3T3 cells synthesise RNA, which is abolished by treatment with Actinomycin D or maintenance at low temperature. A. RNA synthesis by cfChP-treated cells in continuous culture and control NIH3T3 cells with and without treatment of Actinomycin D (0.0005 µg/mL) or when maintained at 31°C. **B.** DNA 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 concatamers) and human ribosomal RNA on DNA fibres prepared from serially passaged cfChP-treated cells revealed strictly co-localised signals, indicating that the concatamers synthesised ribosomal RNA (Fig. 8A, upper

panel). Immuno-FISH analysis revealed co-localised fluorescent signals for human-specific DNA probes and antibodies against human RNA polymerase 3A and human ribosomal proteins (Fig. 8A, middle and lower panels, respectively). These findings indicated that concatamers are autonomously capable of synthesising critical components of the protein synthetic machinery. We confirmed these findings in metaphase preparations from serially passaged cells for all three components of the protein synthetic machinery described above (Fig. 8B).

Fig. 8

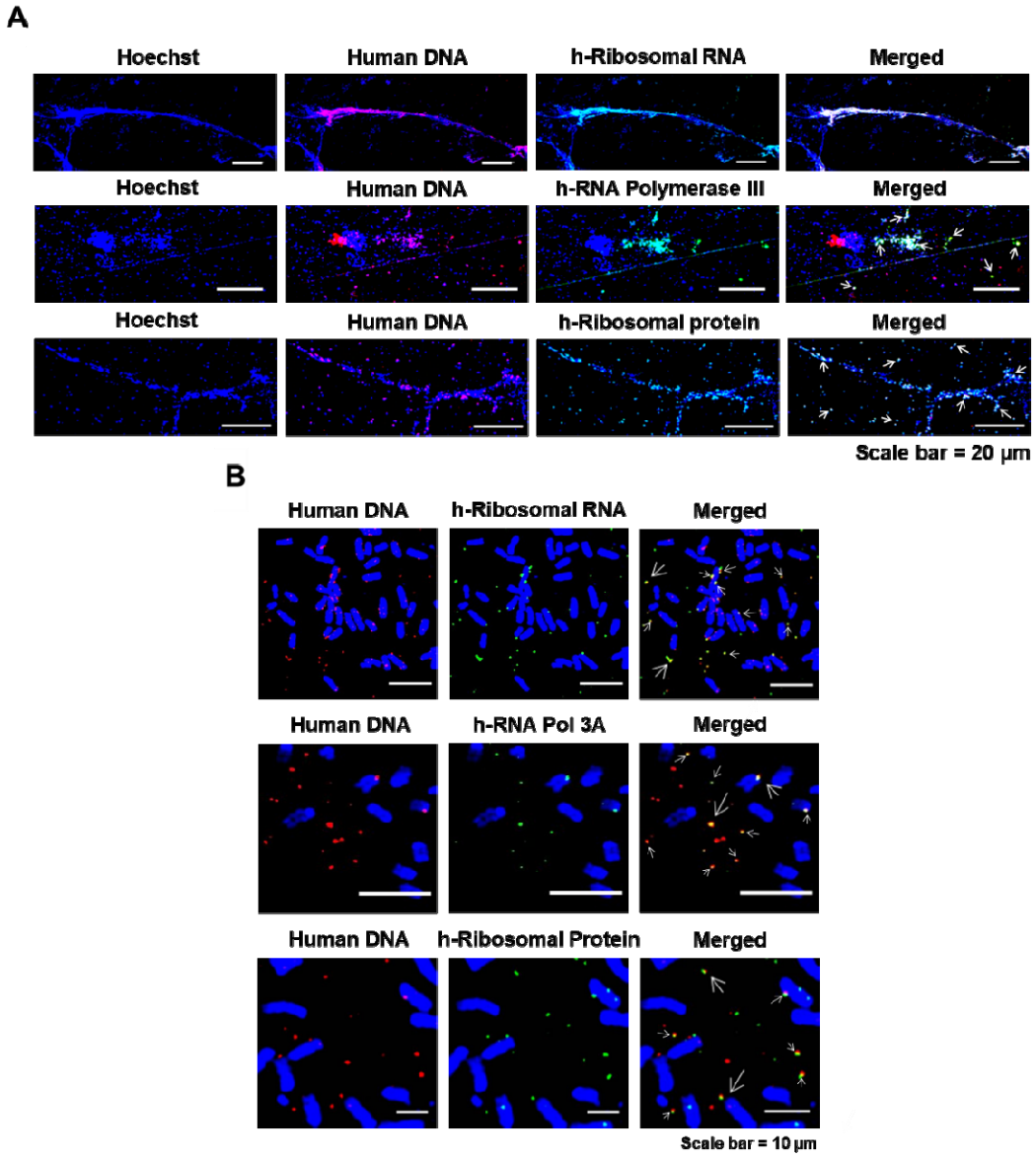
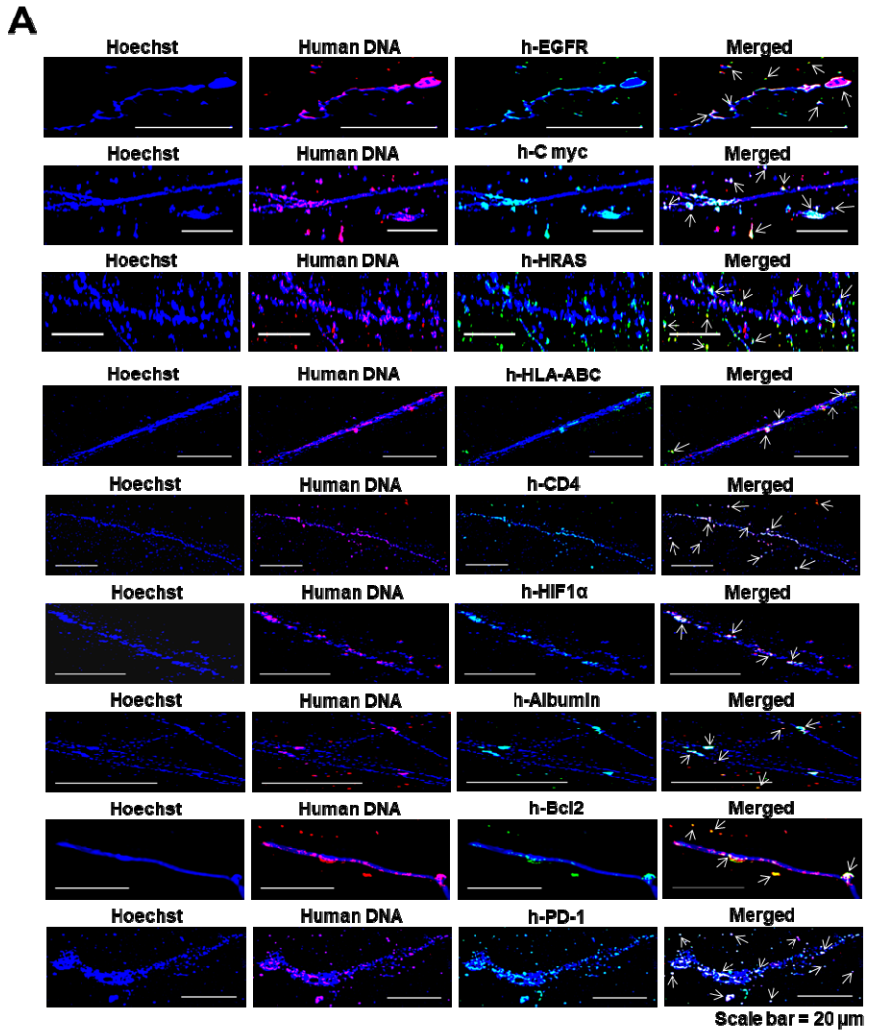


Fig. 8. Concatamers synthesise their own protein synthetic machinery. Dual-FISH analysis of DNA fibres **(A)** and metaphase spreads **(B)** prepared from cfChP-treated NIH3T3 cells in continuous passage using probes against human DNA and human-specific ribosomal RNA along with immuno-FISH experiments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

Concatamers synthesise diverse human proteins in mouse cells

Having confirmed that concatamers 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 concatamers were capable of synthesising all forms of the wide array of proteins that we examined including oncogenic and diverse non-oncogenic proteins (Fig. 9A). Significantly, the newly synthesised proteins consistently remained associated with the concatemers of origin (marked by human DNA signals). We further confirmed these findings in metaphase preparations from serially passaged cfChPs-treated NIH3T3 cells (Fig. 9B). We also performed extensive control experiments using immuno-FISH to detect human DNA and/or proteins in control NIH3T3 cells that had not been treated with cfChPs. Neither the DNA FISH probe nor antibodies against multiple proteins detected human DNA or proteins in mouse cells (Supplementary Fig. S2).

Fig. 9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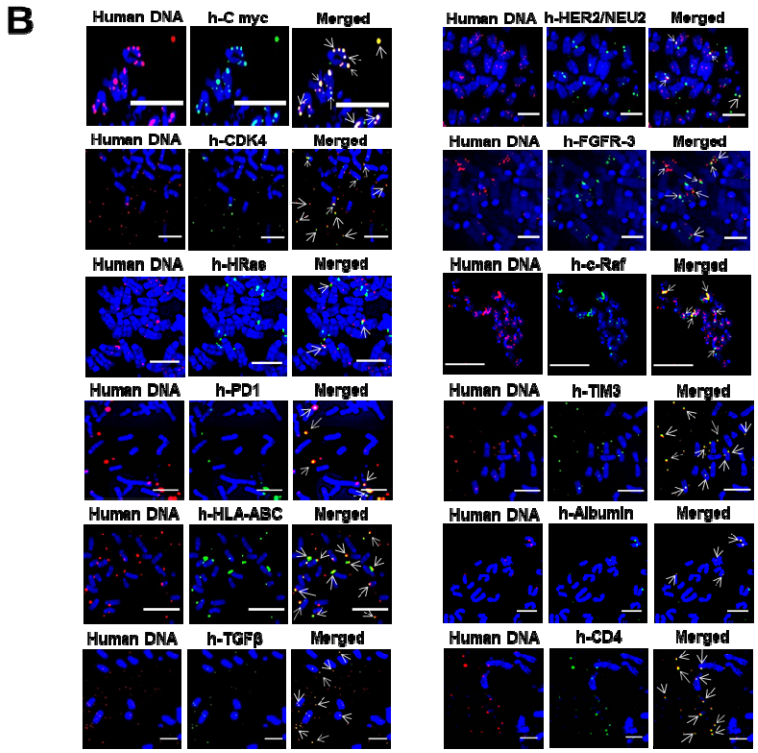
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Merged
Human DNA
h-c-Raf
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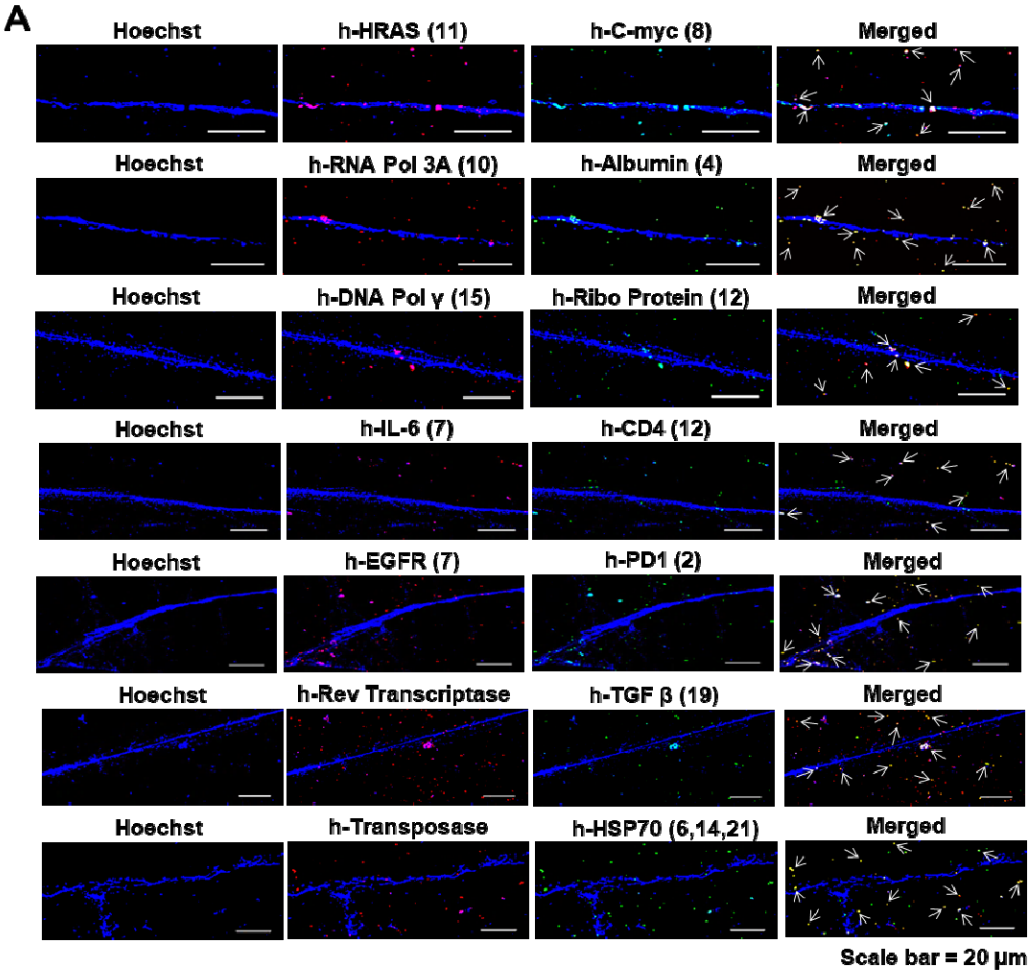
Human DNA
h-PD1
Merged
Human DNA
h-TIM3
Merged
Human DNA
h-HLA-ABC
Merged
Human DNA
h-Albumin
Merged
Human DNA
h-TGFβ
Merged
Human DNA
h-CD4
Merged

Fig. 9. Concatamers synthesise various human proteins. Immuno-FISH analysis using antibodies against various human proteins and a human whole-genome FISH probe for **(A)** DNA fibres and **(B)** metaphase spreads prepared from cfChPs-treated NIH3T3 cells in continuous passage. Co-localised signals of human DNA and various proteins are marked with arrows.

Concatamers synthesise complex fusion proteins

Because concatamers are amalgams of multiple disparate cfChPs, we investigated whether the proteins they synthesised were fusion proteins. The fluorescent signals of two antibodies against diverse unrelated proteins were frequently co-localised, indicating that the proteins synthesised by the concatamers were indeed fusion proteins with potentially novel functions (Fig. 10). Fusion proteins were detected both on DNA fibres and metaphase preparations. Quantification of the extent of co-localisation indicative of fusion proteins in the antibody pairs is shown in Figure 10A. The extent of co-localised signals ranged between 7% and 35% (Supplementary Figure S3).

Fig. 10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h-C-myc (8)
Merged
Hoechst
h-RNA Pol 3A (10)
h-Albumin (4)
Merged
Hoechst
h-DNA Pol γ (15)
h-Ribo Protein (12)
Merged
Hoechst
h-IL-6 (7)
h-CD4 (12)
Merged
Hoechst
h-EGFR (7)
h-PD1 (2)
Merged
Hoechst
h-Rev Transcriptase
h-TGF β (19)
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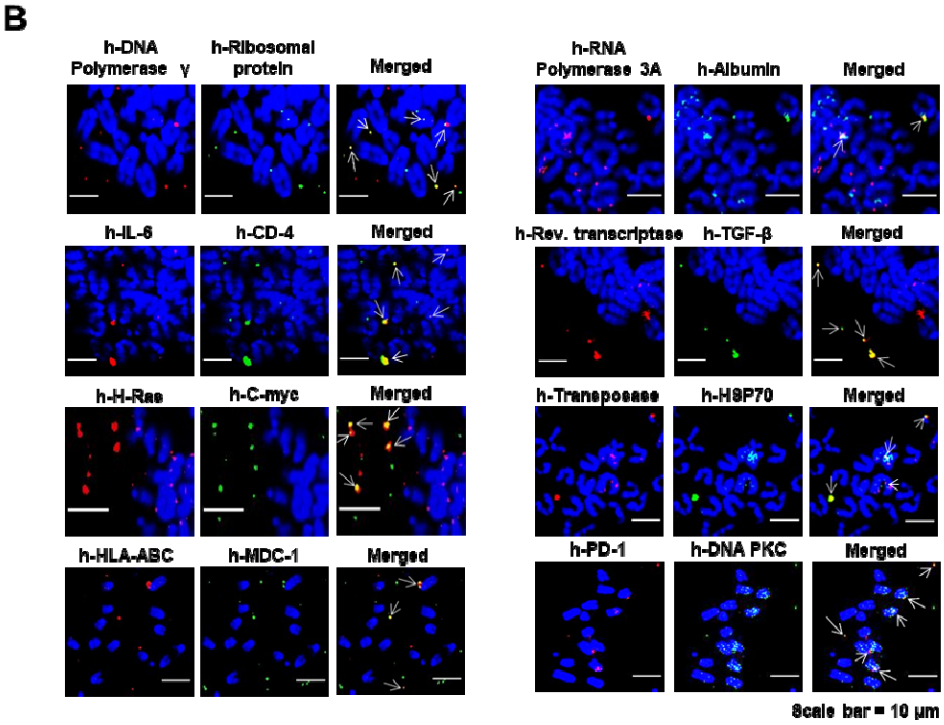
Hoechst
h-Transposase
h-HSP70 (6,14,21)
Merged
Scale bar = 20 μm


B
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Merged
h-Rev. transcriptase
h-TGF-β
Merged
h-H-Ras
h-C-myc
Merged
h-Transposase
h-HSP70
Merged
h-HLA-ABC
h-MDC-1
Merged
h-PD-1
h-DNA PKC
Merged
Scale bar = 10 μm

Fig. 10. Proteins synthesised by concatamers are fusion proteins. Dual immunofluorescence analysis using antibody pairs targeting diverse human-specific proteins in (**A)** DNA fibres and **(B)** metaphase spreads 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.

Concatamers are vehicles of transposable elements capable of autonomous replication

Among the fusion proteins, we identified reverse transcriptase and transposases fused with transforming growth factor-β and heat shock protein 70, respectively, suggesting that the concatamers 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 the vast majority of DNA signals were co-localised. This finding was confirmed for both DNA fibres (Fig. 11A) and metaphase preparations (Fig. 11B), indicating that the concatamers did indeed contain retro-transposable elements. The fact that both LINE-1 and *Alu* signals could be clearly detected by FISH analysis suggested that they were extensively amplified, given that LINE-1 and *Alu* are approximately 6000 bp and 300 bp in size, respectively.

Fig. 11

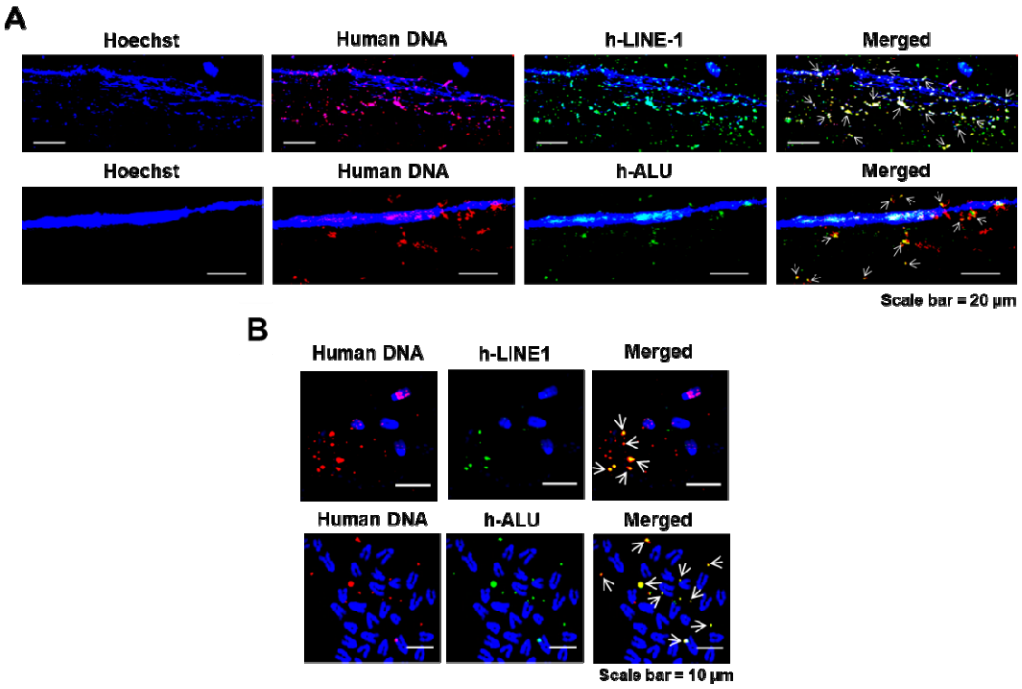

Fig. 11. Concatamers include transposable elements. Dual-FISH analysis of (**A)** DNA fibres and **(B)** metaphase spreads prepared from cfChPs-treated NIH3T3 cells in continuous passage using probes against human LINE-1 or human *Alu* and human genomic DNA. Co-localised signals are marked with arrows.

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

Immuno-FISH analysis using antibodies against 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.

Fig. 12

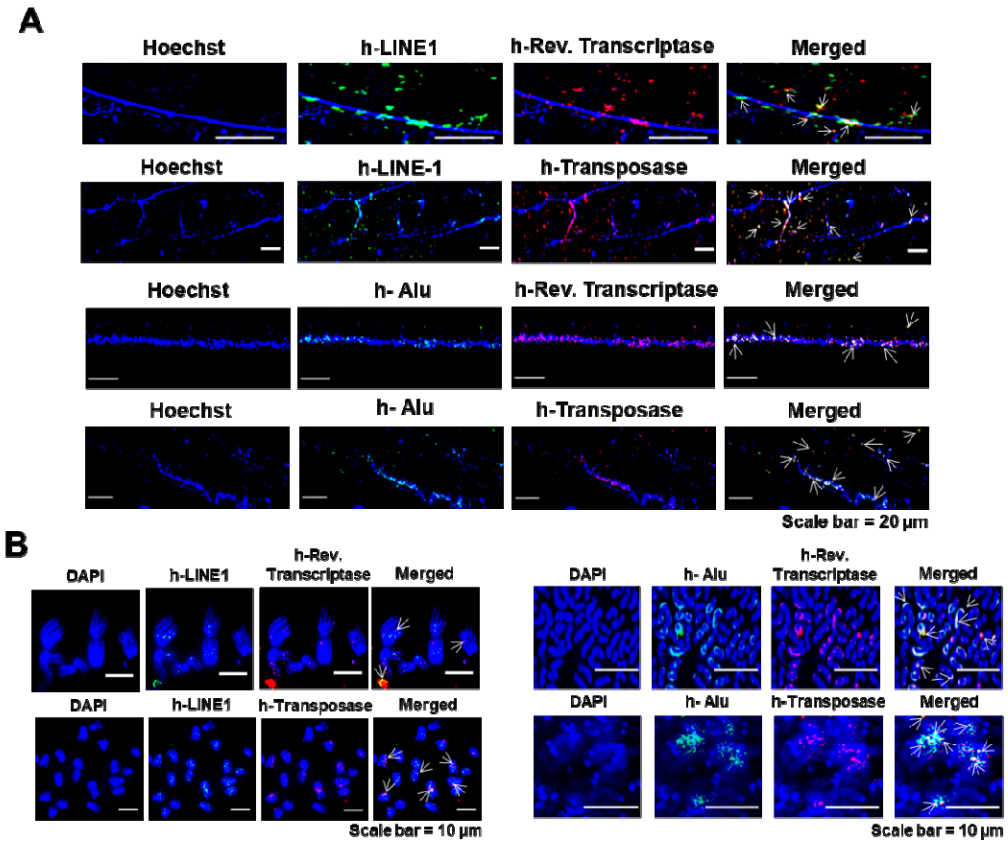

Fig. 12. Concatamers are associated with reverse transcriptase and transposase. Immuno-FISH analysis of **(A)** DNA fibres and **(B)** metaphase spreads prepared from cfChPs-treated NIH3T3 cells in continuous passage using a human *Alu* or LINE-1 probe and antibodies against reverse transcriptase and 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. Immuno-FISH

analysis showed co-localised signals for antibodies against BrdU and LINE-1 or *Alu* FISH probes, demonstrating that the DNA of both transposable elements was actively being replicated (Fig. 13A and B).

Fig. 13

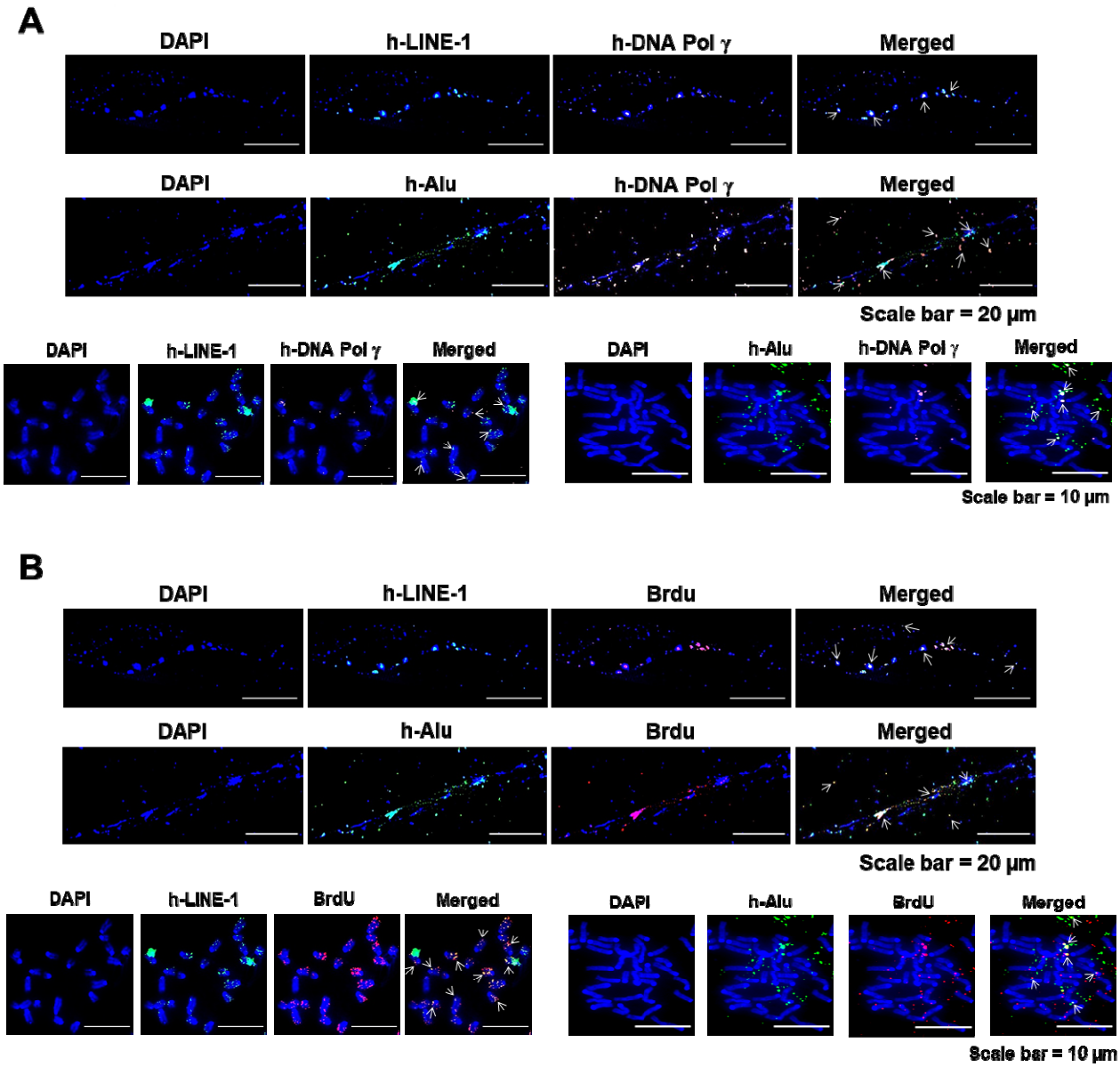

Fig. 13. LINE-1 and *Alu* elements are associated with DNA polymerase and can actively synthesise DNA. (A) Immuno-FISH images of cfChP-treated cells in continuous passage showing co-localising signals of LINE-1 and *Alu* (green) and DNA polymerase γ (orange) on both DNA fibres and metaphase spreads.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

signals of LINE-1 and *Alu* (green) and an antibody targeting BrdU (red) on both DNA fibres and 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 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 10-fold increase in both cases (Fig. 14). These data indicated that LINE-1 and *Alu* elements progressively increased their copy numbers over time in the mouse genome by retrotransposition. 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.

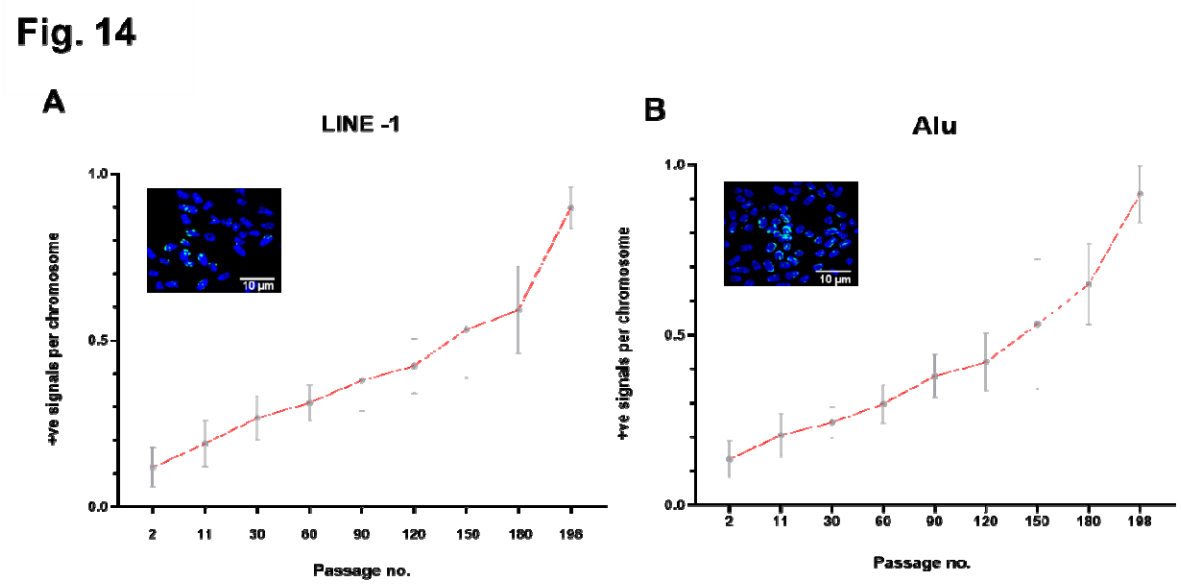

Fig. 14. LINE-1 and *Alu* elements proliferate to increase their copy numbers in the mouse genome. The data represent the mean ± standard error values of the number of human LINE-1 and *Alu* FISH signals per chromosome in successive passages after analysing 15 metaphases at each passage. Analysis of variance for linear trend, $p < 0.0001$ for both LINE-1 and Alu. The inset shows an image of 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 the same 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 DNA fibres prepared from serially passaged NIH3T3 cells that were observed using cfChPs isolated from serum (Supplementary Fig. S5).

Discussion

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 [24]. 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. The horizontally transferred MGEs express a plethora of novel fusion proteins, and by acting as transposable elements, have the potential to bring about evolutionary changes in the "self" organism. It is necessary to point out that the frequent co-localisation of various unrelated antibody probes that we observed suggested that the fusion proteins are likely to be much more complex proteins

comprising multiple peptides derived from the variable DNA components that constitute concatamers. 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 modified to act as foreign genetic elements.

It has been proposed that extreme environmental stress plays a critical role in the adaptation and evolution of species **[25–27]**. We previously hypothesised **[28]** that environmental stress may lead to organism death via apoptosis or similar processes **[29,30]**, 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 **[28]**. Thus, fragmented cfChP-like particles of human origin and those from bacteria and plants can readily enter mouse cells, while those from humans, bacteria, and plants can likewise 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 **[28]**.

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 DNA 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 concatamers 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 concatamers synthesised RNA, RNA polymerase, ribosomal RNA, and ribosomal proteins, thereby creating their own autonomous protein synthesis machinery. Concatamers, 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, which are products of the complex and heterogeneous DNA sequences of concatamers. Importantly, the fusion proteins remained associated with the concatamers. This suggests that although concatamers 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.

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, apparently allow transposable elements the possibility of *de novo* integration carried by extrachromosomal concatamers. Based on these results, we hypothesised that transposable elements are acquired 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 transposable elements **[31,32]**.

Importantly, although the vast majority of the cfChPs were apparently derived from the non-coding regions of the donor cells, we found that they were capable of synthesising a wide variety of proteins within the new host cells. One possible explanation for this finding might be that the cataclysmic events surrounding cellular apoptosis expose hidden coding sequences that are normally sequestered within the silent regions of the genome. These hidden sequences are then horizontally transferred, via cfChPs, into foreign cells where they amalgamate with each other with unrelated DNA sequences to form complex concatamers. Once transcribed and translated, these complex concatamers generate multi-peptide and novel fusion proteins.

Despite numerous attempts, the components of the fusion proteins were not amenable to detection by conventional reverse transcription-quantitative polymerase chain reaction or western blotting. Multiple experiments using these techniques consistently failed to detect the relevant proteins. We are currently analysing the proteins using liquid chromatography with tandem mass spectrometry, the results of which will be published in the near future.

The presence of extrachromosomal DNA (ecDNA) has been described in plants **[33]**, yeast **[34]**, *Caenorhabditis elegans* **[35]**, *Drosophila* **[36]**, mice, and humans **[37,38]**.

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 **[39–41]**. The present study suggests the possibility that the ecDNAs detected in cancer cells represent concatamers formed as a result of the fusion of numerous unrelated cfChPs derived from the surrounding dying cancer cells. Indeed, we previously reported that cfChPs from dying cancer cells can readily enter bystander cells **[14]**, a finding that was confirmed in our present study using conditioned medium containing cfChPs from hypoxia-induced dying MDA-MB-231 cells.

Our study has several limitations that need to be addressed and our findings will need to be validated in future research. First, we did not determine the sizes of the different concatamers, which would require isolation and sequencing each concatamer. Second, we did not ascertain whether the concatamers that were found overlapping the DNA fibres had integrated into the mouse genome.Third, we did not characterise the numerous fusion proteins; we are currently addressing this aspect in our planned studies using liquid chromatography-tandem mass spectrometry. Fourth, wedid not quantify the extent to which the fusion proteins amplify. As such, our results should be considered as representing an expansive theory, wherein horizontally transferred cfChPs are governed by unknown biological mechanisms that are distinct from those that govern the nuclear genome. We believe that unusual biological functions and 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 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.

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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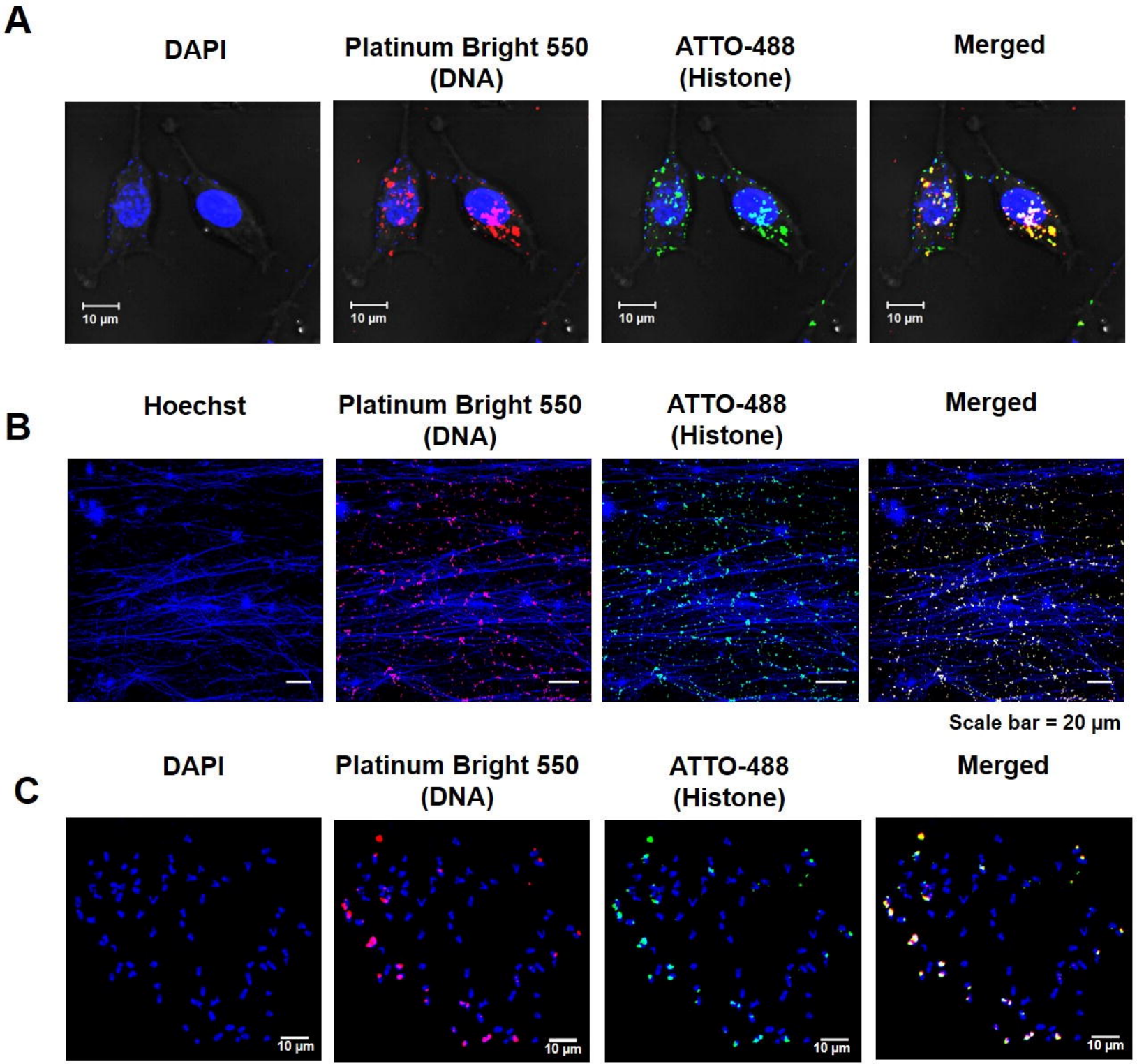
Fig. 1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10 µm

Fig. 2

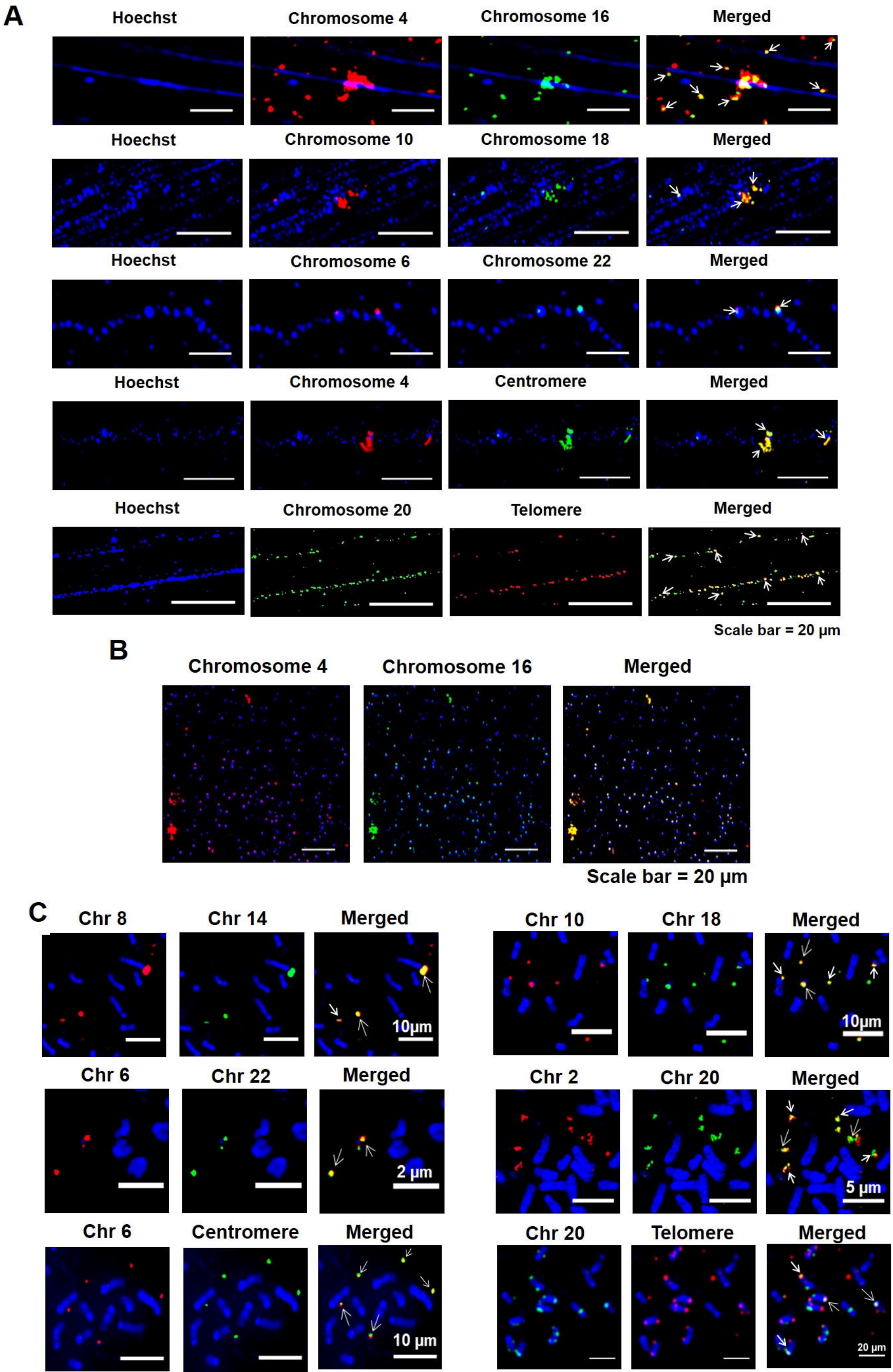
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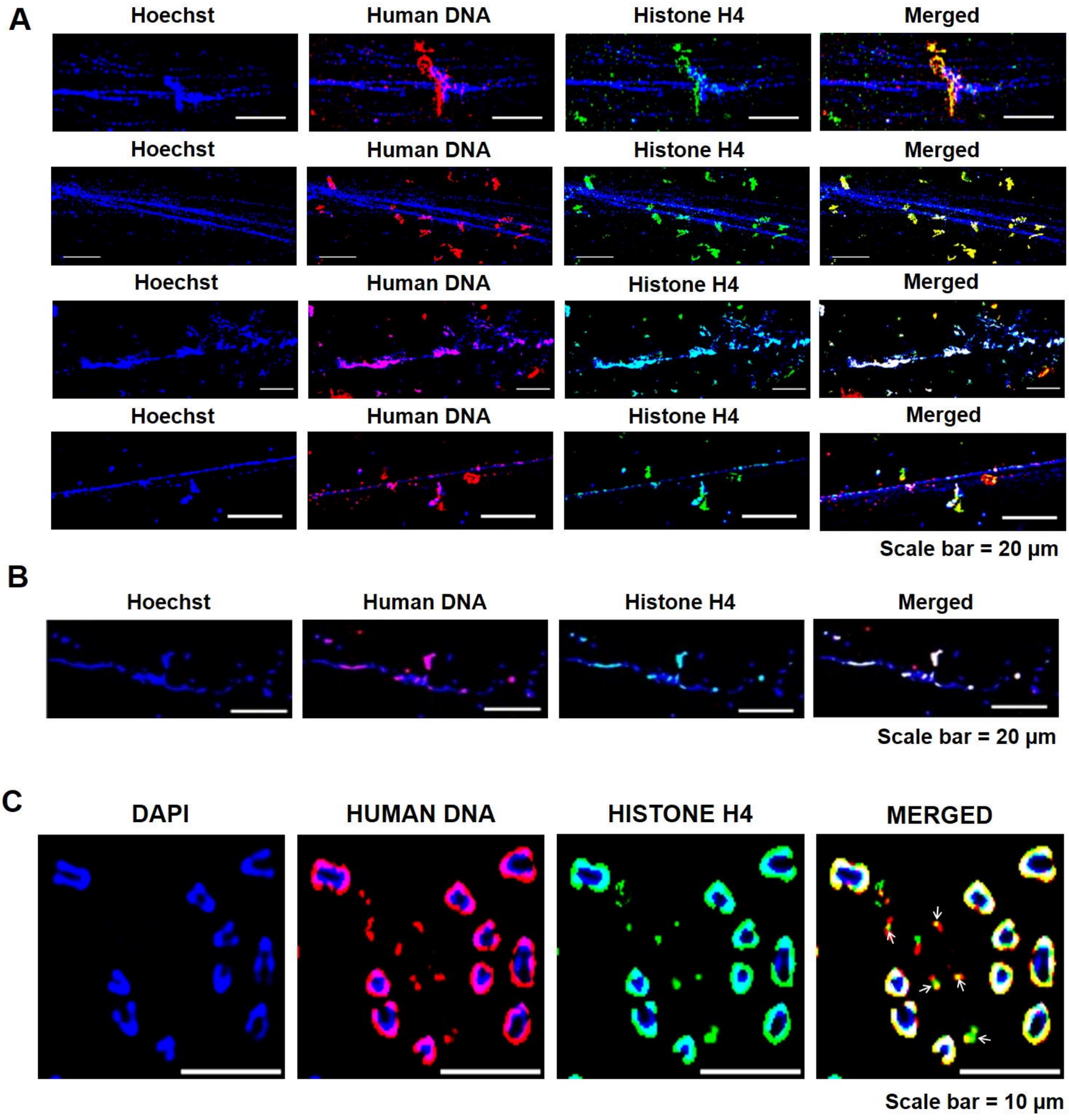
Fig. 3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B
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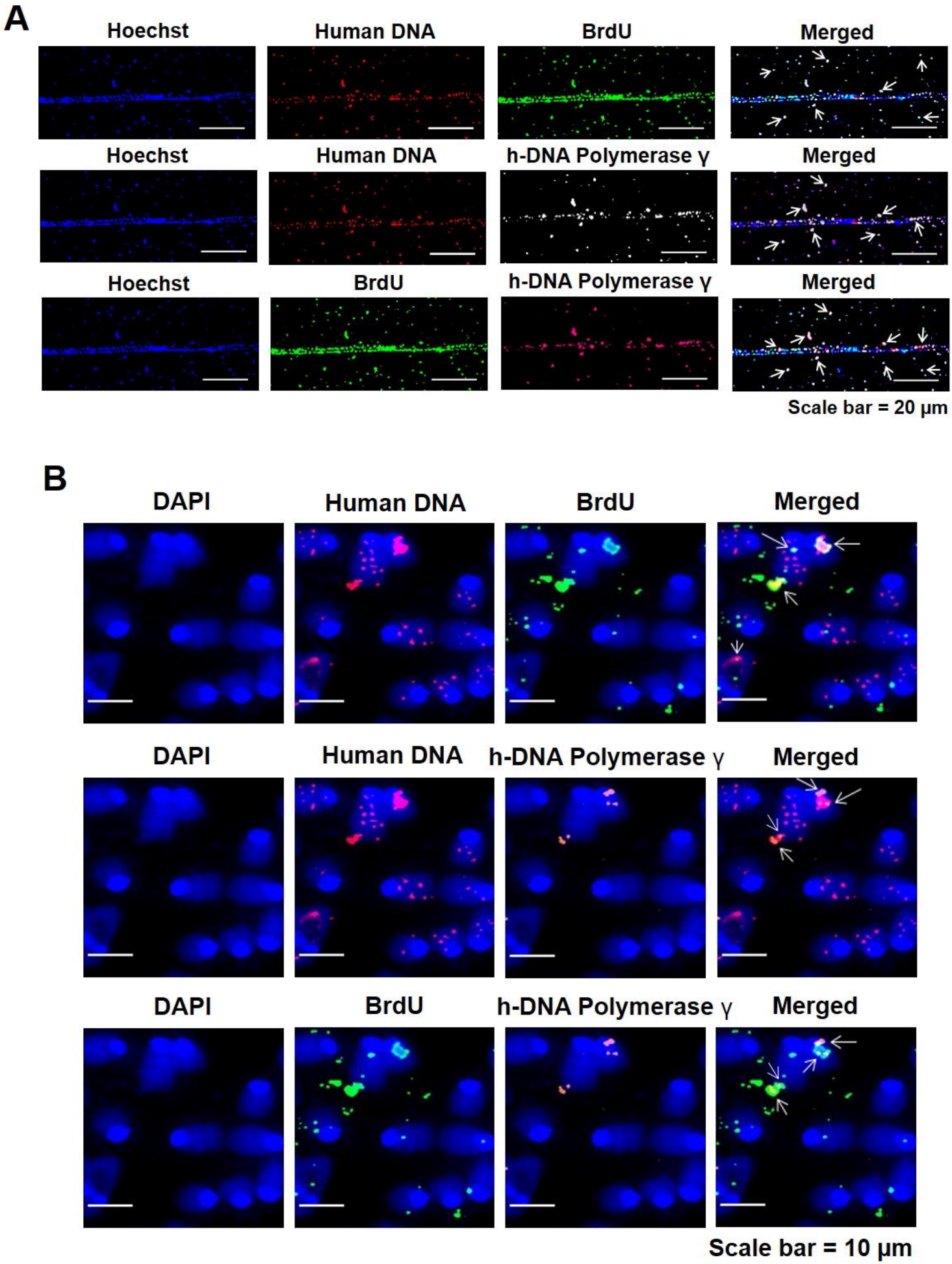
Fig. 4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h-DNA Polymerase γ
Merged
Scale bar = 20 μm
B
DAPI
Human DNA
BrdU
Merged
DAPI
Human DNA
h-DNA Polymerase γ
Merged
DAPI
BrdU
h-DNA Polymerase γ
Merged
Scale bar = 10 μm

Fig. 5

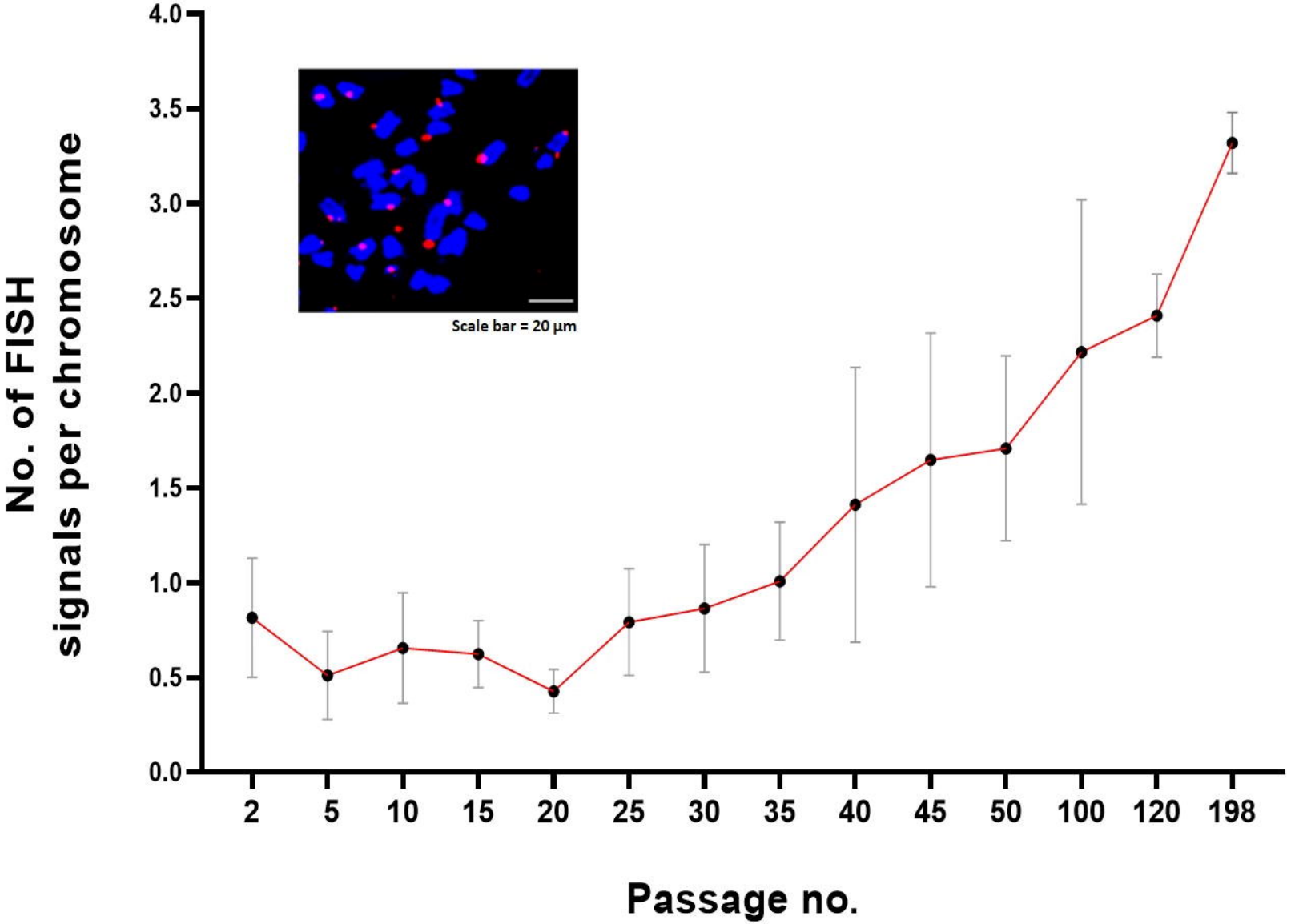
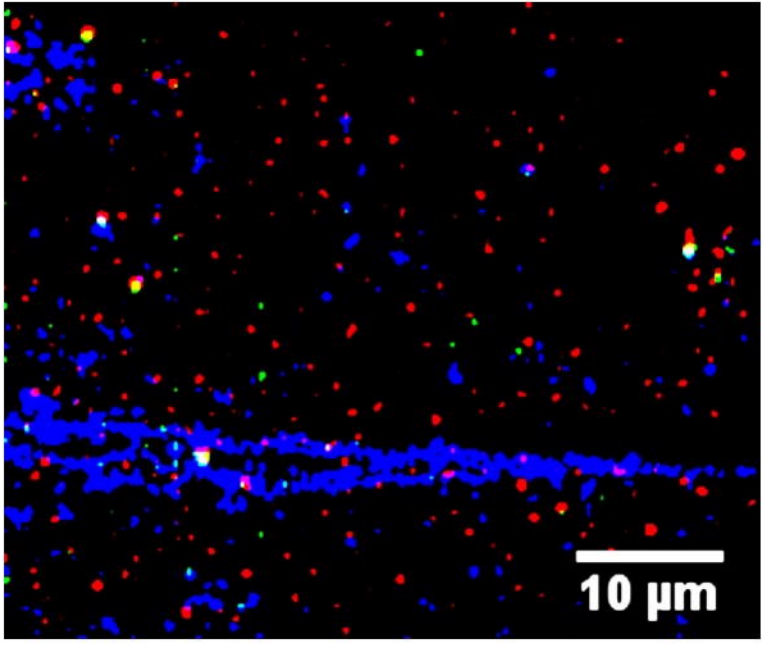

No. of FISH signals per chromosome
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Scale bar = 20 µm
2
5
10
15
20
25
30
35
40
45
50
100
120
198
Passage no.

Fig. 6

A


10 µm

B

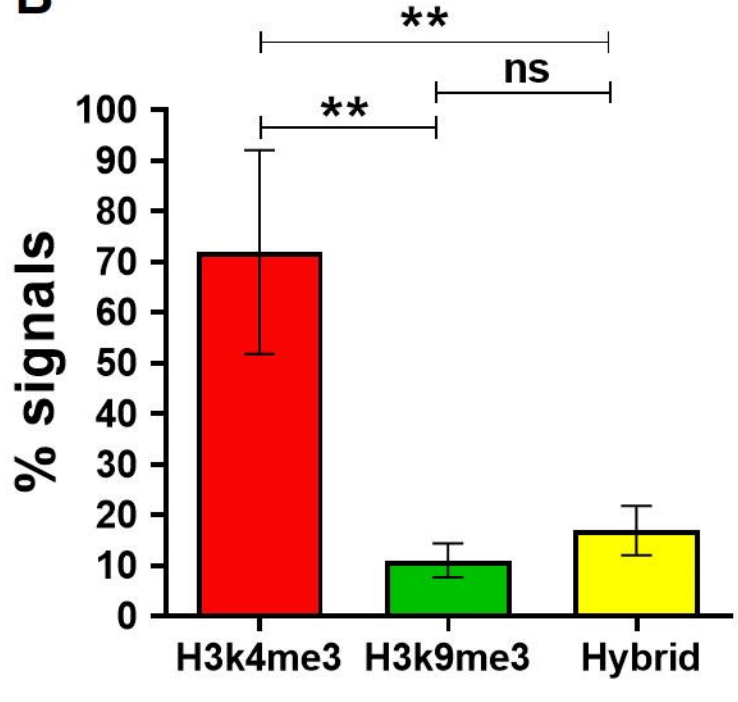

**
ns
**
% signals
100
90
80
70
60
50
40
30
20
10
0
H3k4me3
H3k9me3
Hybrid

Fig. 7

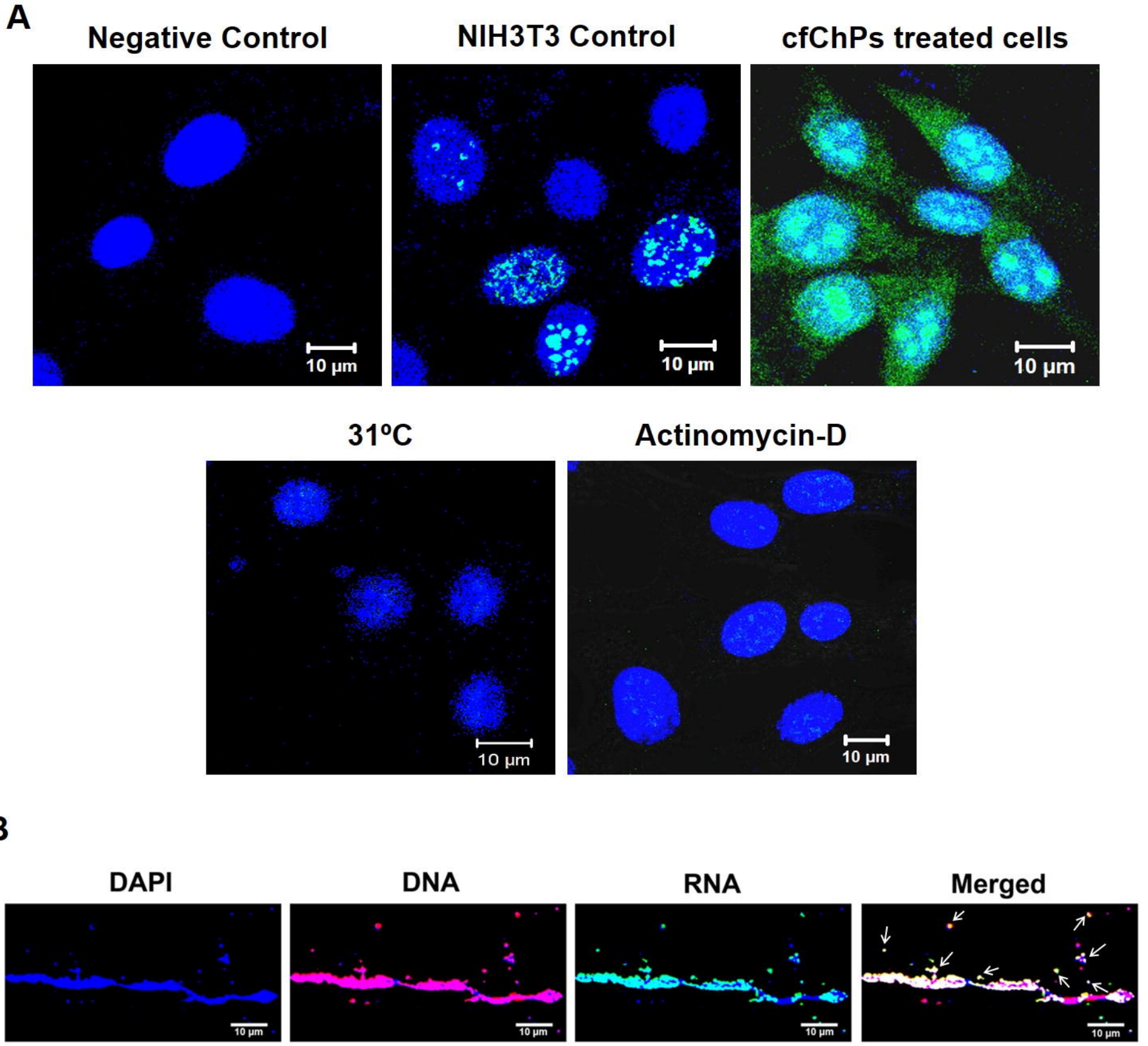

A
Negative Control
NIH3T3 Control
cfChPs treated cells
10 µm
10 µm
10 µm
31ºC
Actinomycin-D
10 µm
10 µm
B
DAPI
DNA
RNA
Merged
10 µm
10 µm
10 µm
10 µm

Fig. 8

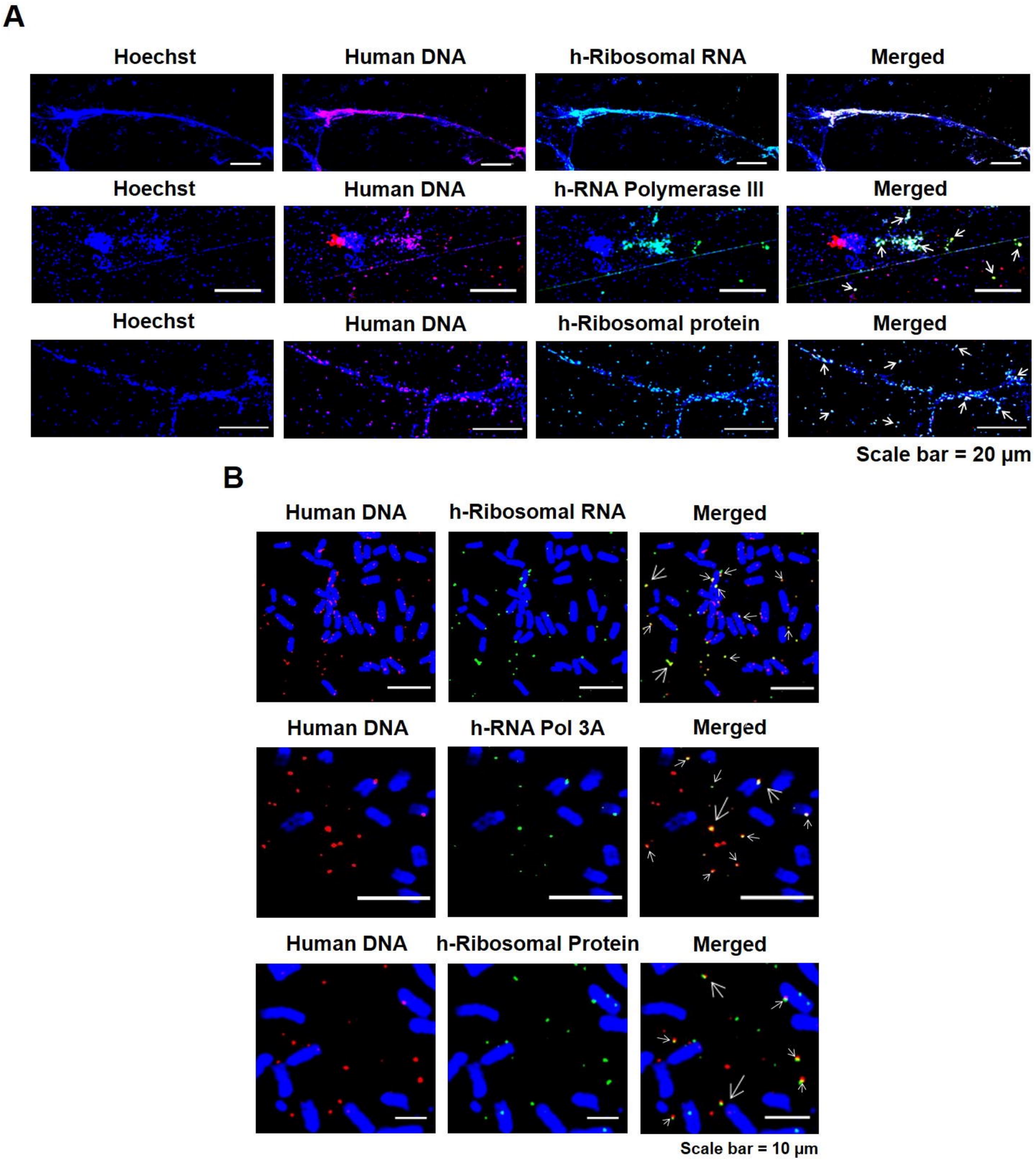
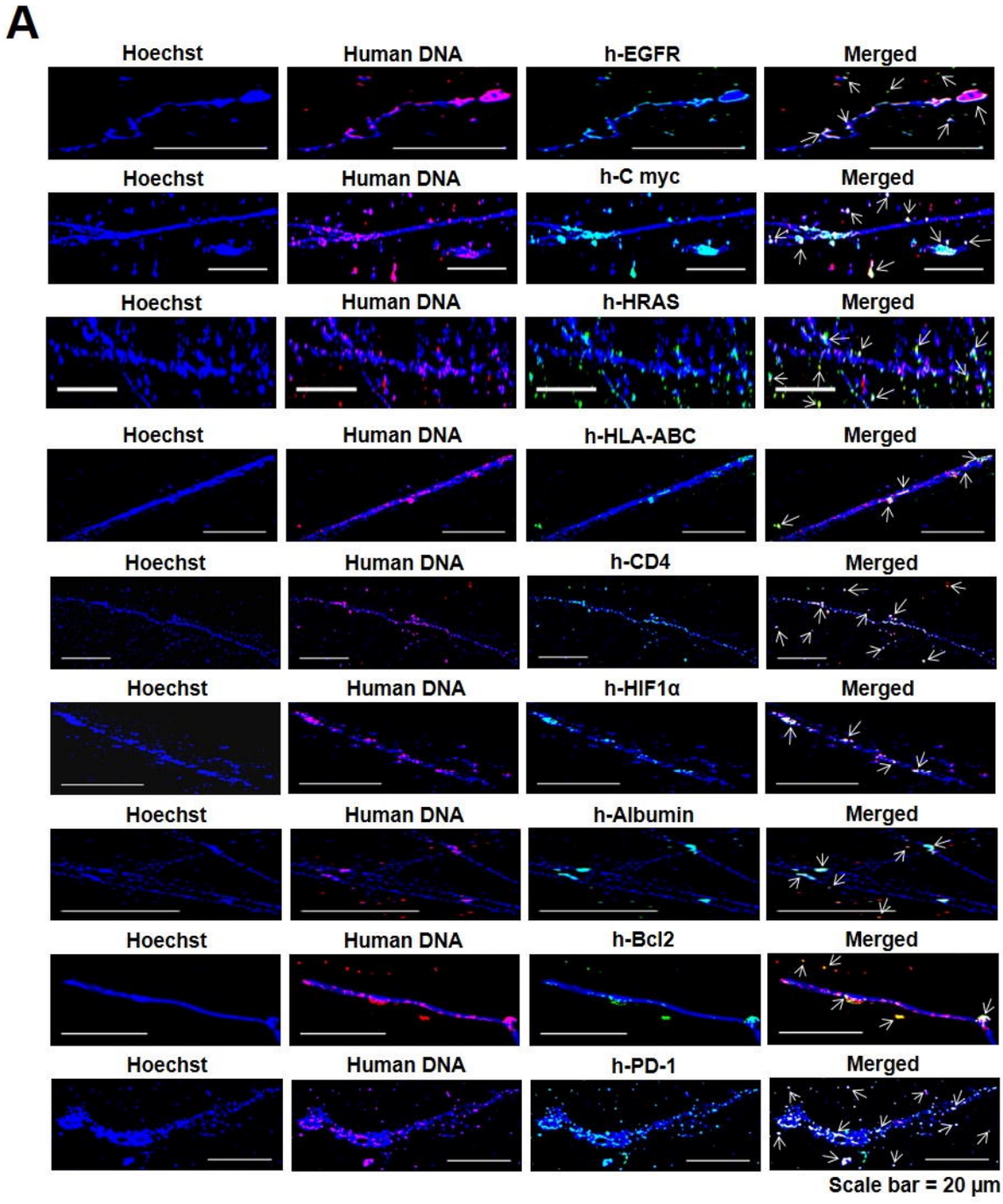

A
Hoechst
Human DNA
h-Ribosomal RNA
Merged
Hoechst
Human DNA
h-RNA Polymerase III
Merged
Hoechst
Human DNA
h-Ribosomal protein
Merged
Scale bar = 20 μm
B
Human DNA
h-Ribosomal RNA
Merged
Human DNA
h-RNA Pol 3A
Merged
Human DNA
h-Ribosomal Protein
Merged
Scale bar = 10 μm

Fig. 9


A
Hoechst
Human DNA
h-EGFR
Merged
Hoechst
Human DNA
h-C myc
Merged
Hoechst
Human DNA
h-HRAS
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

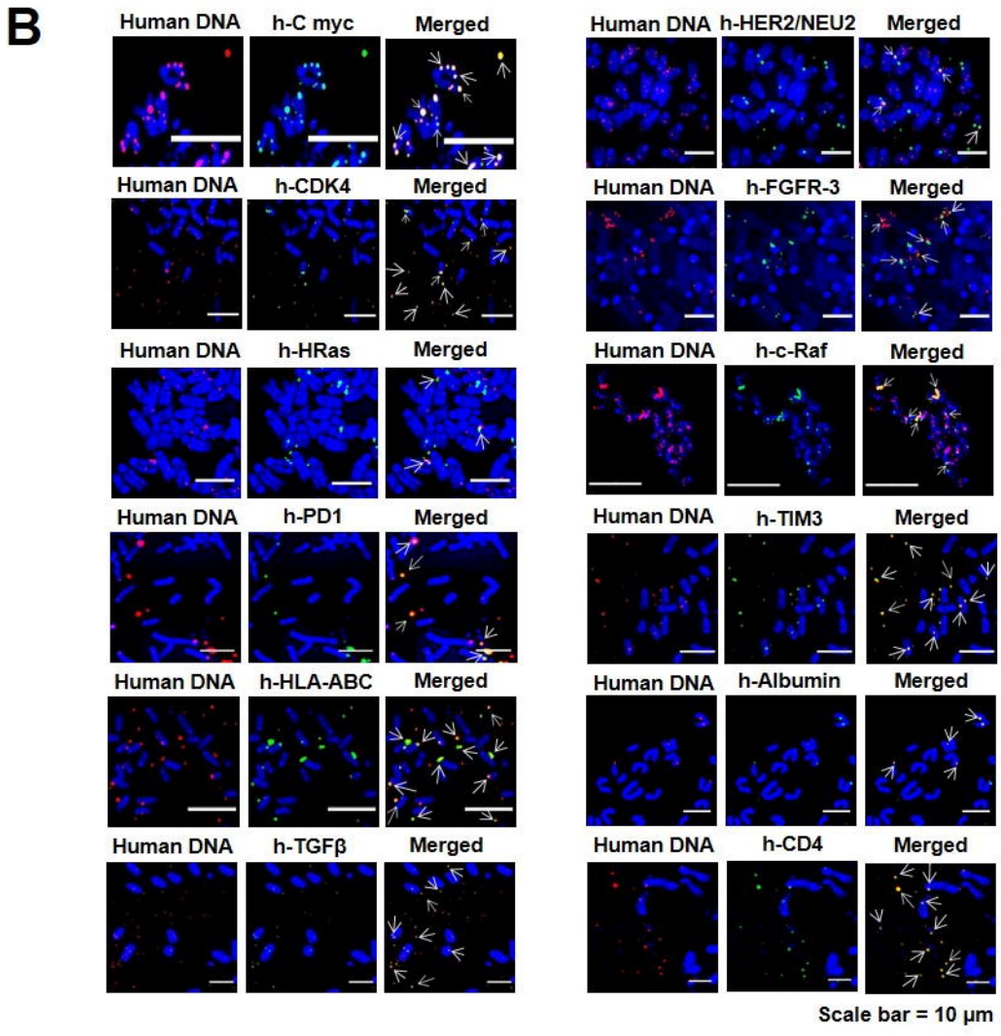
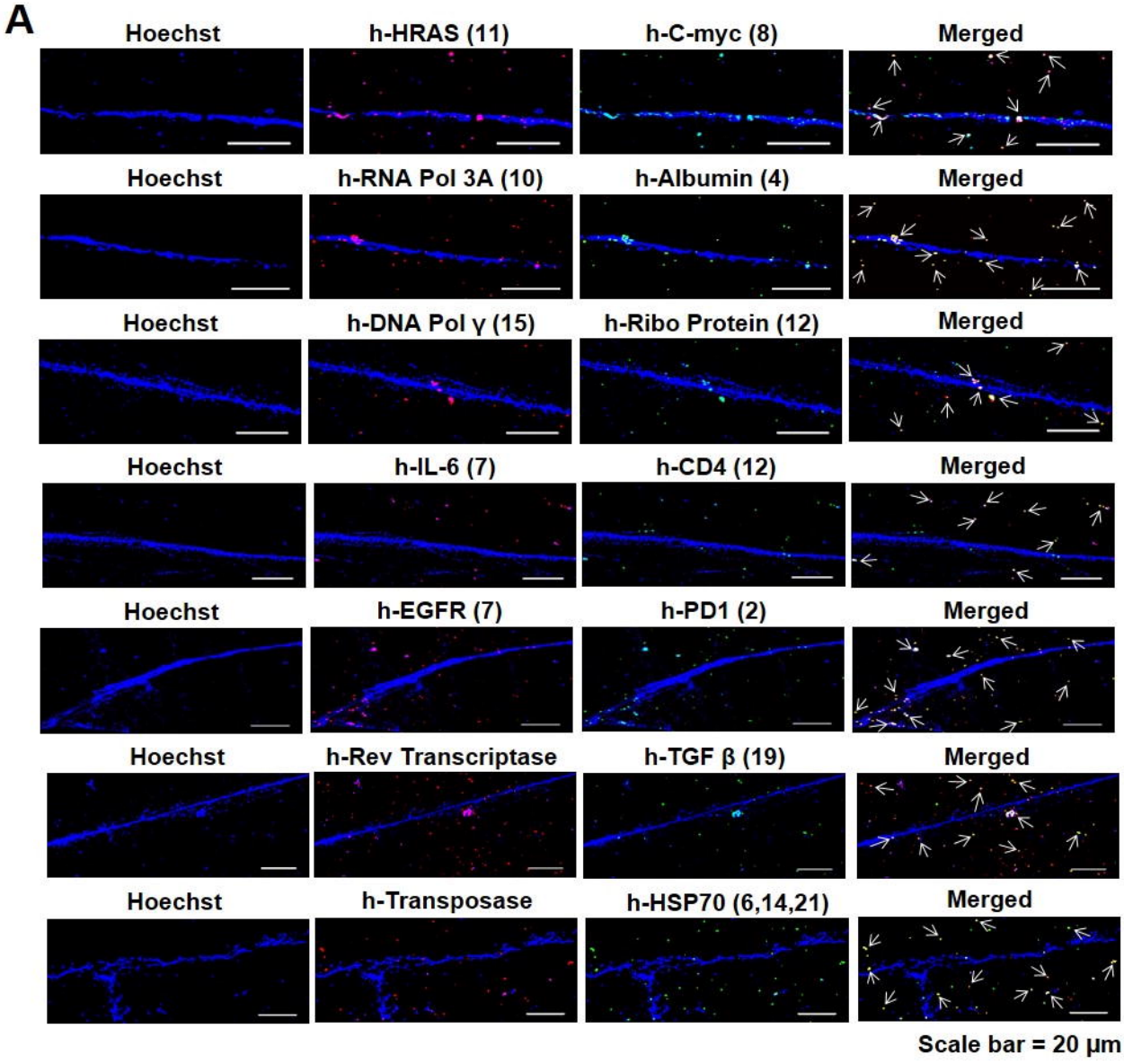

B
Human DNA
h-C myc
Merged
Human DNA
h-HER2/NEU2
Merged
Human DNA
h-CDK4
Merged
Human DNA
h-FGFR-3
Merged
Human DNA
h-HRas
Merged
Human DNA
h-c-Raf
Merged
Human DNA
h-PD1
Merged
Human DNA
h-TIM3
Merged
Human DNA
h-HLA-ABC
Merged
Human DNA
h-Albumin
Merged
Human DNA
h-TGFβ
Merged
Human DNA
h-CD4
Merged
Scale bar = 10 μm

Fig. 10


A
Hoechst
h-HRAS (11)
h-C-myc (8)
Merged
Hoechst
h-RNA Pol 3A (10)
h-Albumin (4)
Merged
Hoechst
h-DNA Pol γ (15)
h-Ribo Protein (12)
Merged
Hoechst
h-IL-6 (7)
h-CD4 (12)
Merged
Hoechst
h-EGFR (7)
h-PD1 (2)
Merged
Hoechst
h-Rev Transcriptase
h-TGF β (19)
Merged
Hoechst
h-Transposase
h-HSP70 (6,14,21)
Merged
Scale bar = 20 μm

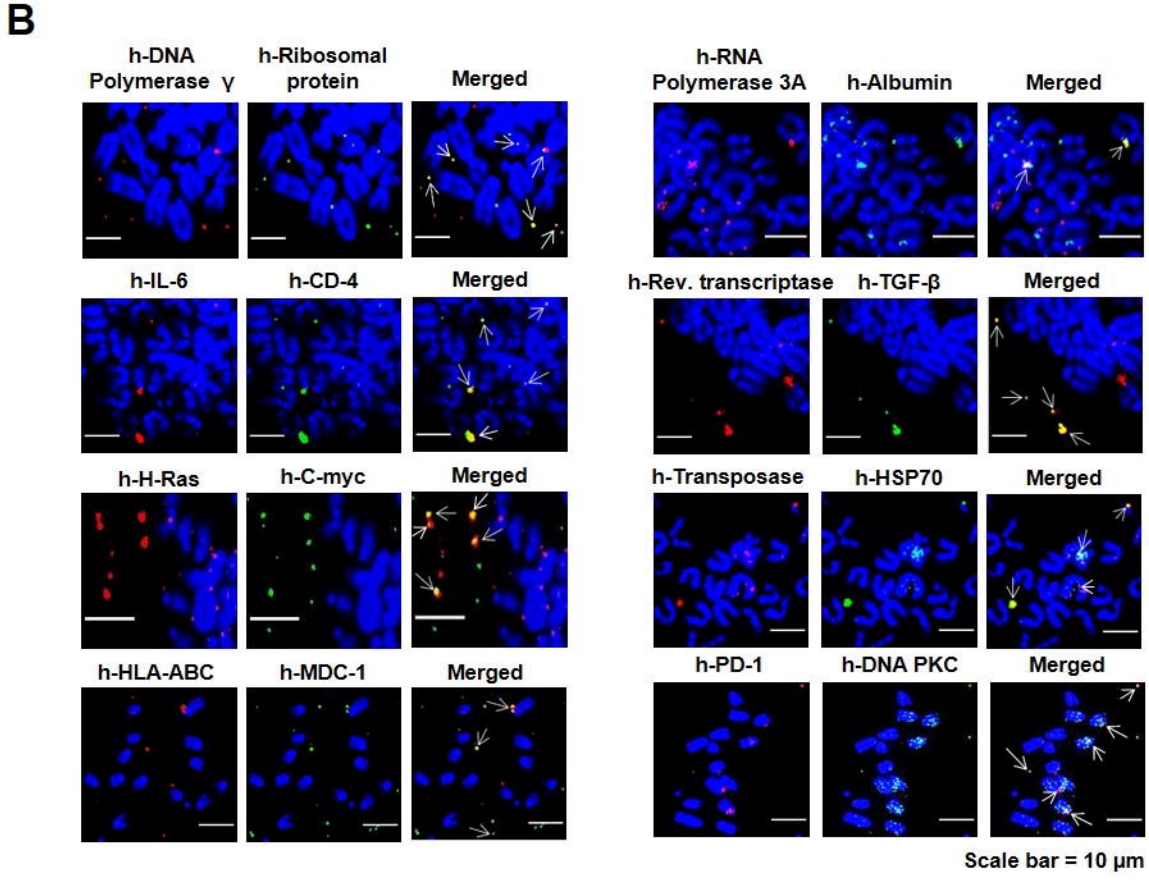
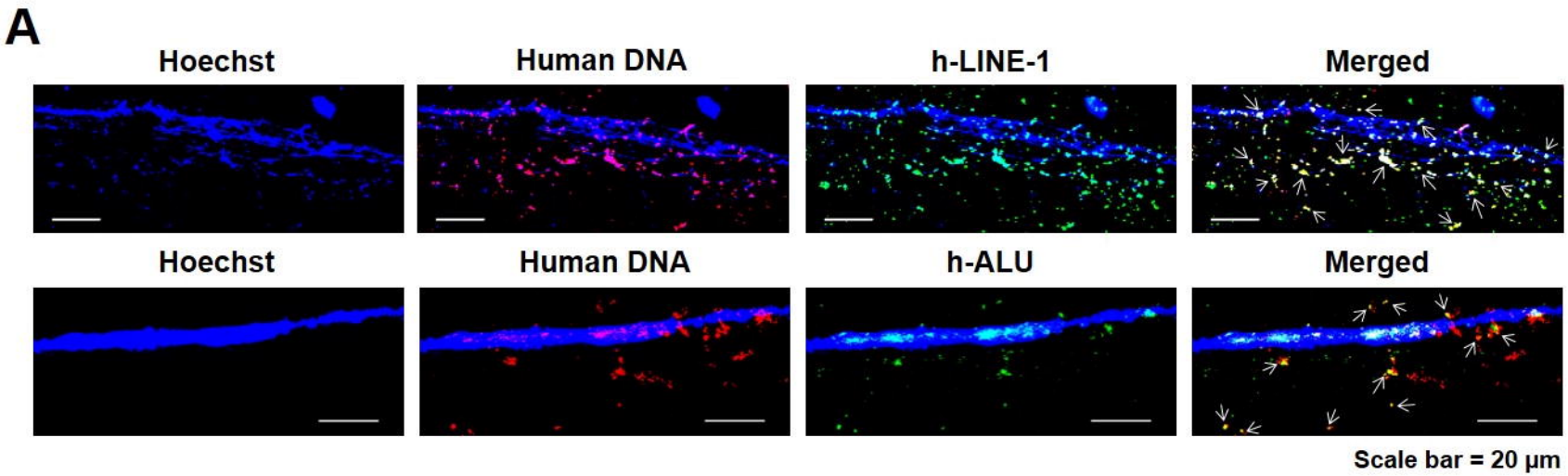

B
h-DNA Polymerase γ
h-Ribosomal protein
Merged
h-RNA Polymerase 3A
h-Albumin
Merged
h-IL-6
h-CD-4
Merged
h-Rev. transcriptase
h-TGF-β
Merged
h-H-Ras
h-C-myc
Merged
h-Transposase
h-HSP70
Merged
h-HLA-ABC
h-MDC-1
Merged
h-PD-1
h-DNA PKC
Merged
Scale bar = 10 μm

Fig. 11


A
Hoechst
Human DNA
h-LINE-1
Merged
Hoechst
Human DNA
h-ALU
Merged
Scale bar = 20 μm

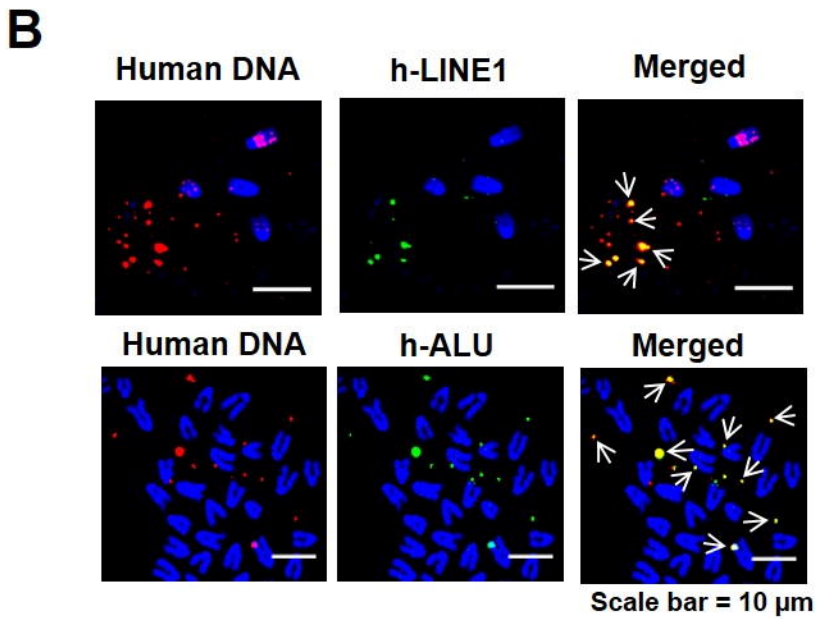

B
Human DNA
h-LINE1
Merged
Human DNA
h-ALU
Merged
Scale bar = 10 μm

Fig. 12

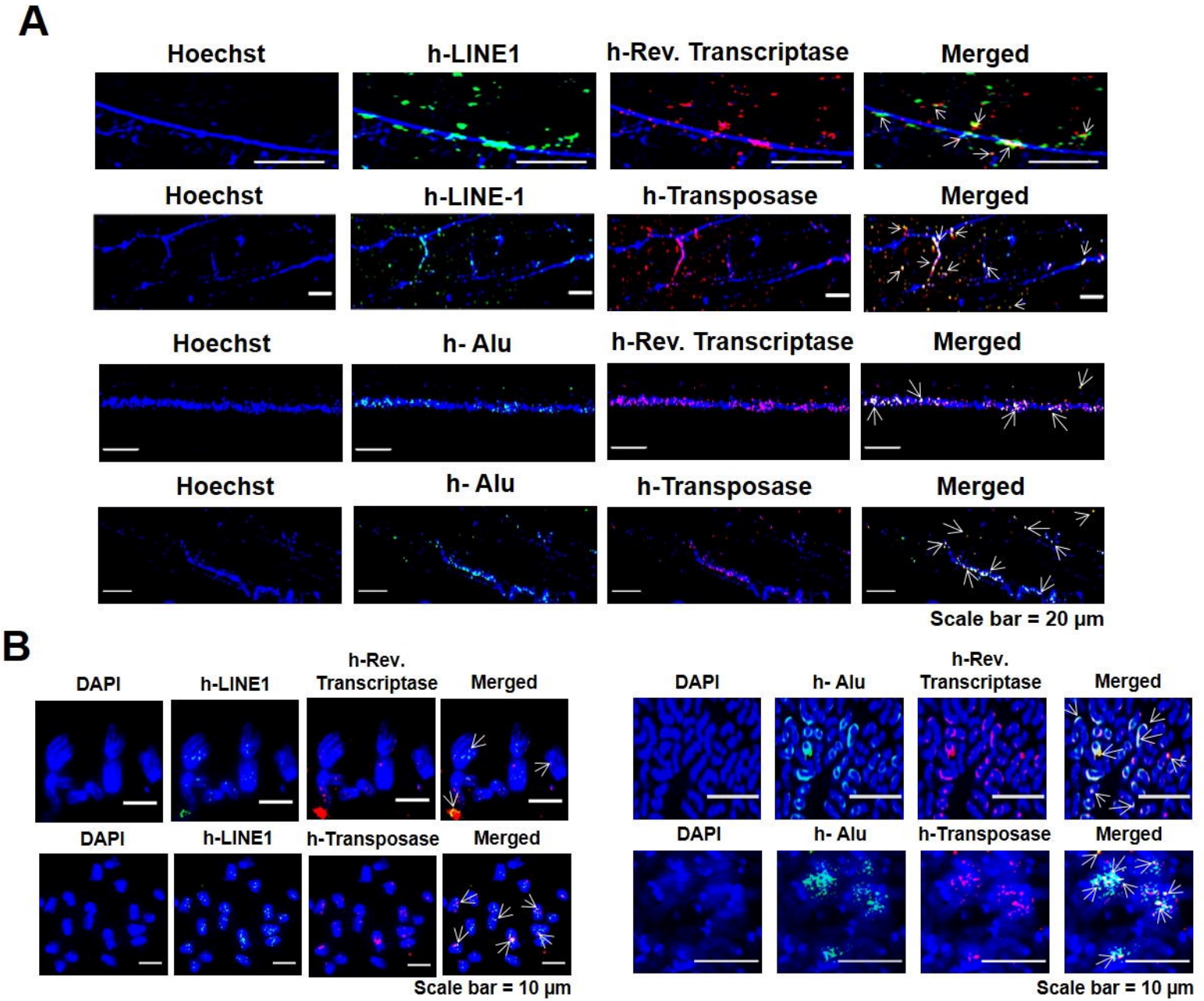

A
Hoechst
h-LINE1
h-Rev. Transcriptase
Merged
Hoechst
h-LINE-1
h-Transposase
Merged
Hoechst
h- Alu
h-Rev. Transcriptase
Merged
Hoechst
h- Alu
h-Transposase
Merged
Scale bar = 20 μm
B
DAPI
h-LINE1
h-Rev. Transcriptase
Merged
DAPI
h-LINE1
h-Transposase
Merged
Scale bar = 10 μm
DAPI
h- Alu
h-Rev. Transcriptase
Merged
DAPI
h- Alu
h-Transposase
Merged
Scale bar = 10 μm

Fig. 13

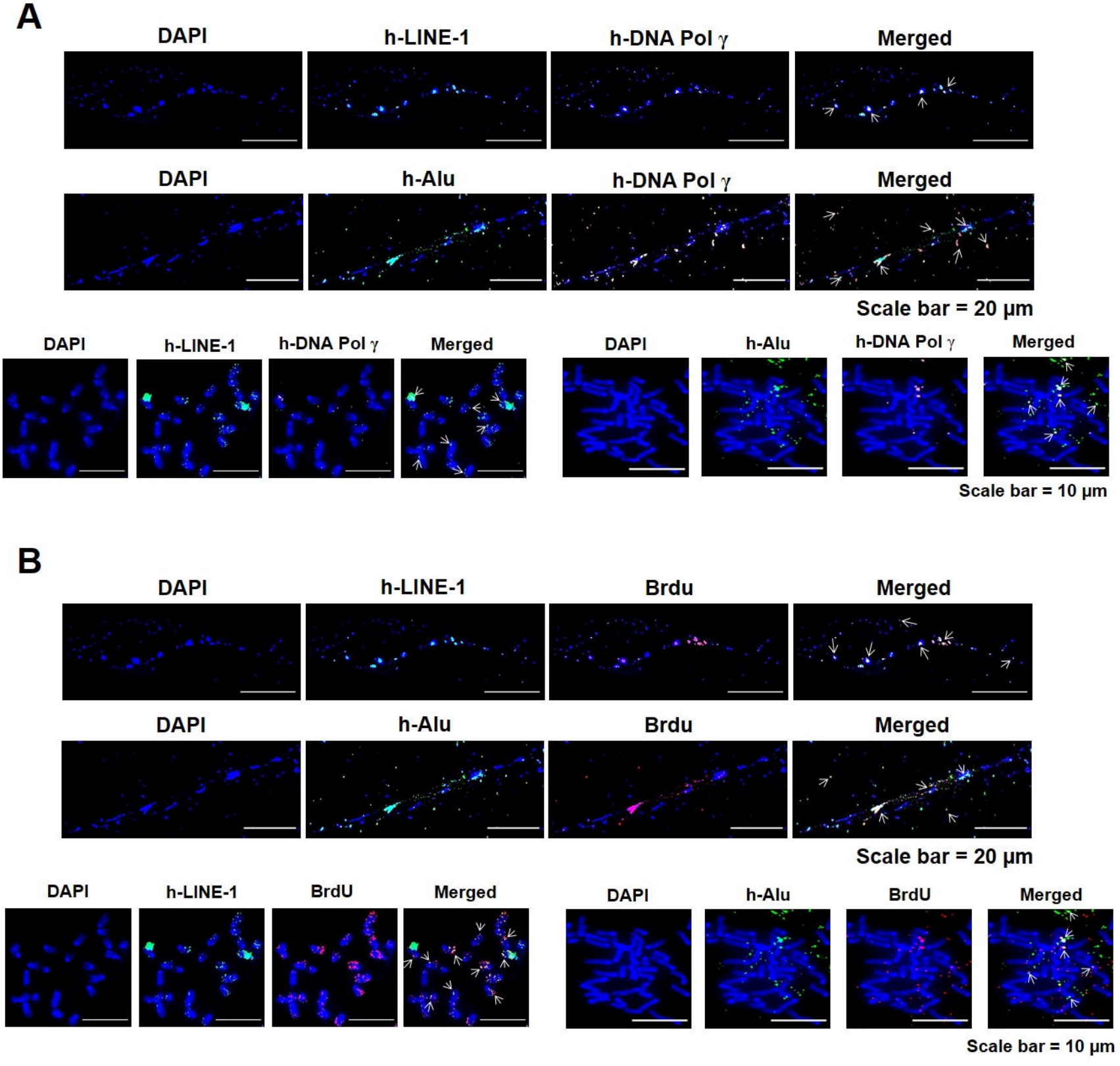
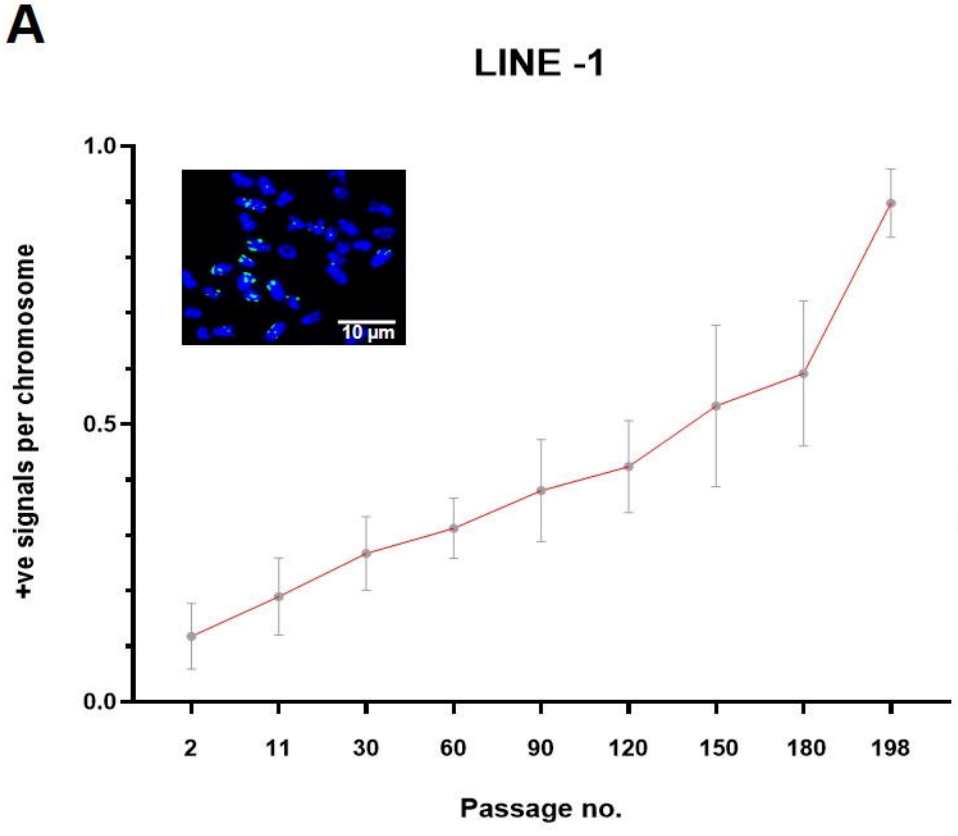
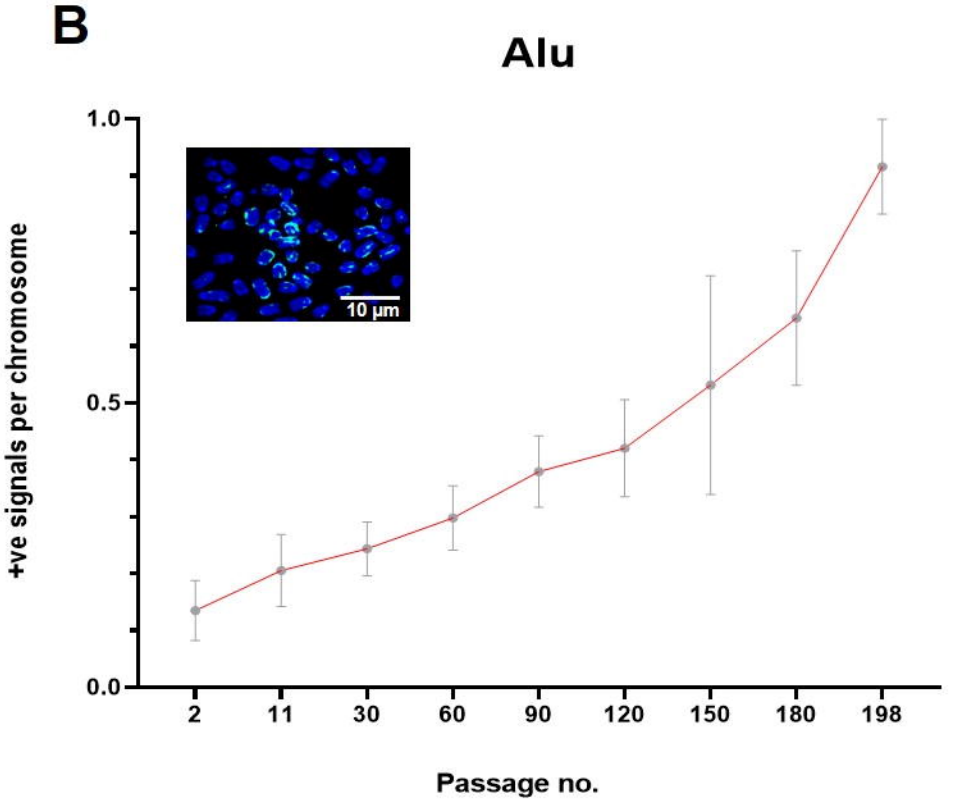

A
DAPI
h-LINE-1
h-DNA Pol γ
Merged
DAPI
h-Alu
h-DNA Pol γ
Merged
Scale bar = 20 μm
DAPI
h-LINE-1
h-DNA Pol γ
Merged
DAPI
h-Alu
h-DNA Pol γ
Merged
Scale bar = 10 μm
B
DAPI
h-LINE-1
Brdu
Merged
DAPI
h-Alu
Brdu
Merged
Scale bar = 20 μm
DAPI
h-LINE-1
BrdU
Merged
DAPI
h-Alu
BrdU
Merged
Scale bar = 10 μm

Fig. 14


A
LINE -1
10 µm
+ve signals per chromosome
1.0
0.5
0.0
2
11
30
60
90
120
150
180
198
Passage no.


B
Alu
10 µm
+ve signals per chromosome
1.0
0.5
0.0
2
11
30
60
90
120
150
180
198
Passage no.