# Homologous chromosomes move and rapidly initiate contact at the sites of double-strand breaks in genes in $G_0$-phase human cells

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**W**e recently reported that homologous chromosomes make contact at the sites of double-strand breaks (DSBs) induced by ionizing radiation (IR) and the restriction endonuclease I-PpoI in $G_0/G_1$-phase somatic human cells. The contact involves short segments of homologous chromosomes and is centered on a DSB that occurs in a gene; contact does not occur at a DSB in intergenic DNA. Contact between homologous chromosomes is abrogated by inhibition of transcription and requires the kinase activity of ATM, but not DNA-PK. Here, we report additional insights into the mechanism underlying this novel phenomenon. We identify four patterns of homologous chromosome contact, and show that contact between homologous arms, but not centrosomes, is induced by IR. Significantly, we demonstrate that contact is induced by IR in non-proliferating, $G_0$-phase human cells derived from tissue explants. Finally, we show that contact between homologous chromosomes is detectable as early as 5 min after IR. These results point to the existence of a mechanism that rapidly localizes homologous chromosome arms at sites of DSBs in genes in $G_0$-phase human cells.

## Introduction

We recently reported that homologous chromosomes make contact at the sites of double-strand breaks (DSBs) induced by ionizing radiation (IR) or the restriction endonuclease I-PpoI in $G_0/G_1$-phase human cells.[1] Using multi-color 3D-fluorescence in situ hybridization (FISH) with chromosome arm paints or gene-specific probes and high-resolution confocal microscopy, we identified frequent contact between homologous arms or homologous genes residing on several different chromosomes following the induction of DSBs. In contrast to homologous chromosome pairing that occurs in meiosis, which involves multiple interstitial interactions along the entire length of homologs,[2] the observed contact in somatic cells involves a very limited section of the chromosome surrounding the site of DSB.[1] This phenomenon was identified in both primary epithelial thyroid cells and fibroblasts suggesting that it likely to be a common event in human somatic cells.

We further showed that DSB-induced contact between homologous chromosomes was restricted to genes and required active transcription and the kinase activity of ATM.[1] Not all regions of homologous chromosomes made contact following the induction of a DSB. While the induction of DSBs in genes using the restriction endonuclease I-PpoI caused contact between short segments of homologous chromosomes surrounding the restriction site, the induction of DSBs in intergenic DNA regions did not cause contact between homologous chromosomes. I-PpoI-induced contact between homologous chromosomes was abrogated when RNA polymerase was inhibited with
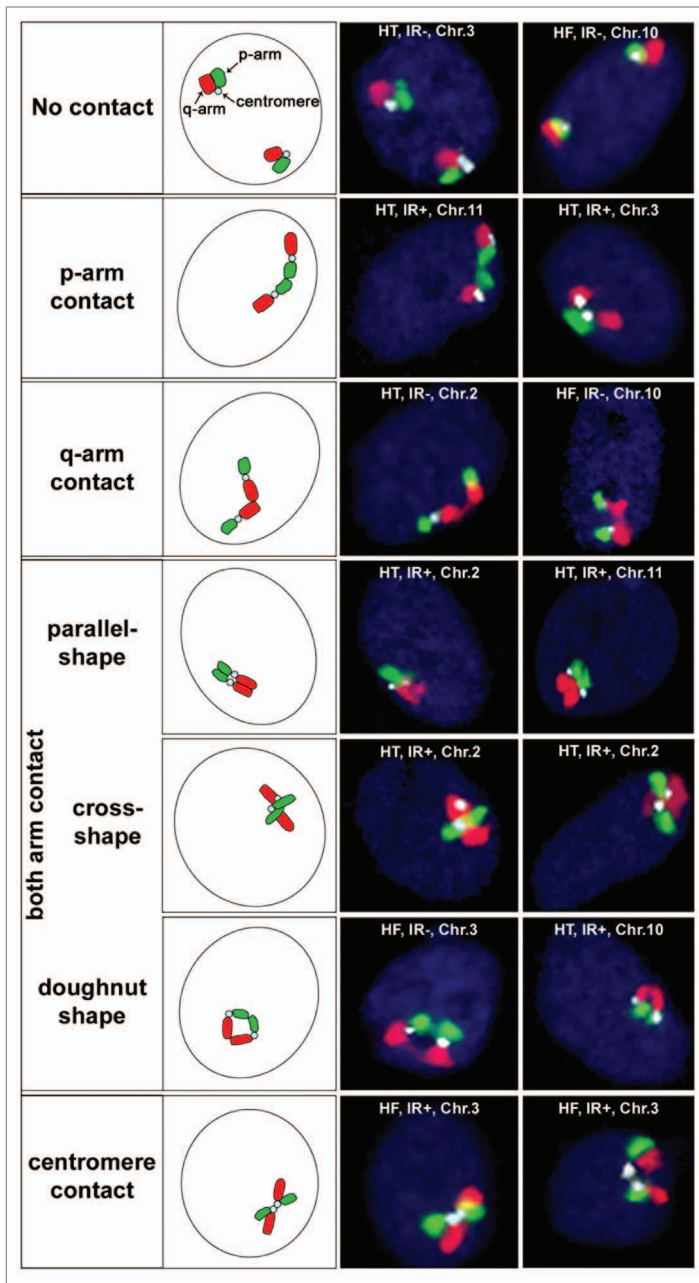
**Figure 1.** Patterns of contact between homologous chromosomes in $G_0/G_1$ human cells. 3D-FISH of cultured human thyroid cells (HT) and human fibroblasts (HF) with p-arm paint (green), q-arm paint (red) and centromeric probe (light blue). Left panel is a schematic representation of each pattern of contact. IR, ionizing radiation; Chr, chromosome.

centromeres, is frequently induced by IR. Second, we show that contact between homologous chromosomes is induced by IR in non-proliferating, $G_0$-phase human cells derived from tissue explants. Third, we show that contact between homologous chromosomes is detectable as early as 5 min after IR.

## Results

**Four patterns of homologous chromosome contact are observed in untreated and irradiated cells.** Multicolor 3D-FISH and confocal microscopy identified four patterns of homologous chromosome contact in $G_0/G_1$-phase cells: (1) contact between two homologous p arms; (2) contact between two homologous q arms; (3) contact between homologous p arms and q arms, i.e., p:p and q:q contact; and (4) contact between homologous centromeres (**Fig. 1**). Contact between centromeres was observed far less frequently than contacts between homologous chromosome arms. In contacts involving a single arm of each homologous chromosome (either p-arm or q-arm contact), significant separation was typically observed between the centromeres, and the opposite arms frequently extended away from each other. Contact between both arms (p:p and q:q contact) presented in three recurrent conformations: parallel arrangement of both homologs, cross-shape conformation and doughnut-shape conformation (**Fig. 1**). The parallel arrangement resulted in the most extended contact between homologous chromosome territories, allowing for interactions between multiple reciprocal regions scattered along both p and q arms. The cross-shape pattern involved interaction between the pericentromeric regions of both arms, while the telomeric parts were repelled from each other. In contrast, the doughnut-shape pattern led to the interaction of the telomeric regions of the homologous p- and q-arms, while the remaining reciprocal regions of the homologous chromosome were located on the largest possible separation from each other.

These patterns of contact were observed with different frequencies in untreated and irradiated cells (**Table 1**). In untreated cells, contact between single arms (p-arms or q-arms) was almost twice as frequent as

either actinomycin D or α-amanitin at all five gene loci studied. Finally, we determined that contact required the kinase activity of ATM but not DNA-PK, which is consistent with an associated homologous recombination repair rather than non-homologous end-joining (NHEJ) mechanism of DSB repair. These findings provided the first documentation of a common transcription-related and ATM

kinase-dependent mechanism that induces contact between allelic regions of homologous chromosomes at sites of DSBs in human somatic cells.

Here, we report additional insights into the mechanism that leads to contact between homologous chromosomes in $G_0/G_1$-phase human cells. First, we show that contact between either the q-arms or both the p- and q-arms, but not the

contact between both arms of homologous chromosomes. Exposure to IR induced contact between both chromosome arms more frequently than contact between single arms. Indeed, in thyroid cells, exposure to 5 Gy IR increased contact between both chromosome arms 2.9-fold and contact between single arms 1.4-fold ($p < 10^{-7}$). In fibroblasts, IR increased contact between both chromosome arms 2.2-fold and contact between single arms 1.5-fold ($p = 0.02$). As a result, in cells exposed to IR, contact between both arms of homologous chromosomes became the most common pattern of contact (**Table 1**). Centromere-only contact and centromere co-localization in all other patterns of contact was rarely seen in untreated and irradiated cells, suggesting that contact between homologous chromosomes does not require for interaction of centromeres.

**Contact between homologous chromosomes is observed in non-proliferating, $G_0$-phase human cells.** In our initial study, contact between homologous chromosomes was observed in primary cultured human cells that were largely in $G_0/G_1$-phase.[1] The occurrence of contact in $G_0/G_1$-phase cells was confirmed by a lack of immunoreactivity for PCNA (a marker of S-phase cells) and cyclin A (a marker of S- and $G_2$-phase cells).[1]

Here, we confirm and expand this finding by showing that contact between homologous chromosomes occurs in $G_0$-phase human cells from thyroid tissue explants. Adult human thyroid cells are known to have an exceedingly low proliferation rate ($\leq 0.1\%$) and are, therefore, among the most enriched $G_0$-phase population of human epithelial cells that can be obtained.[3] The tissue explants used in this study were obtained directly from surgically removed normal tissue fragments adjacent to a benign thyroid nodule and had a Ki-67 labeling index of $0.09 \pm 0.03\%$, confirming that they were $G_0$-phase cells. To confirm that the cells in these tissue explants were viable and could induce a DNA damage response after IR, we performed immunofluorescence for γH2AX and 53BP1.[4] Robust formation of both γH2AX and 53BP1 foci was observed 15 min after IR (**Fig. 2A**). Using 3D-FISH and confocal microscopy, contact between homologous chromosome arms was readily
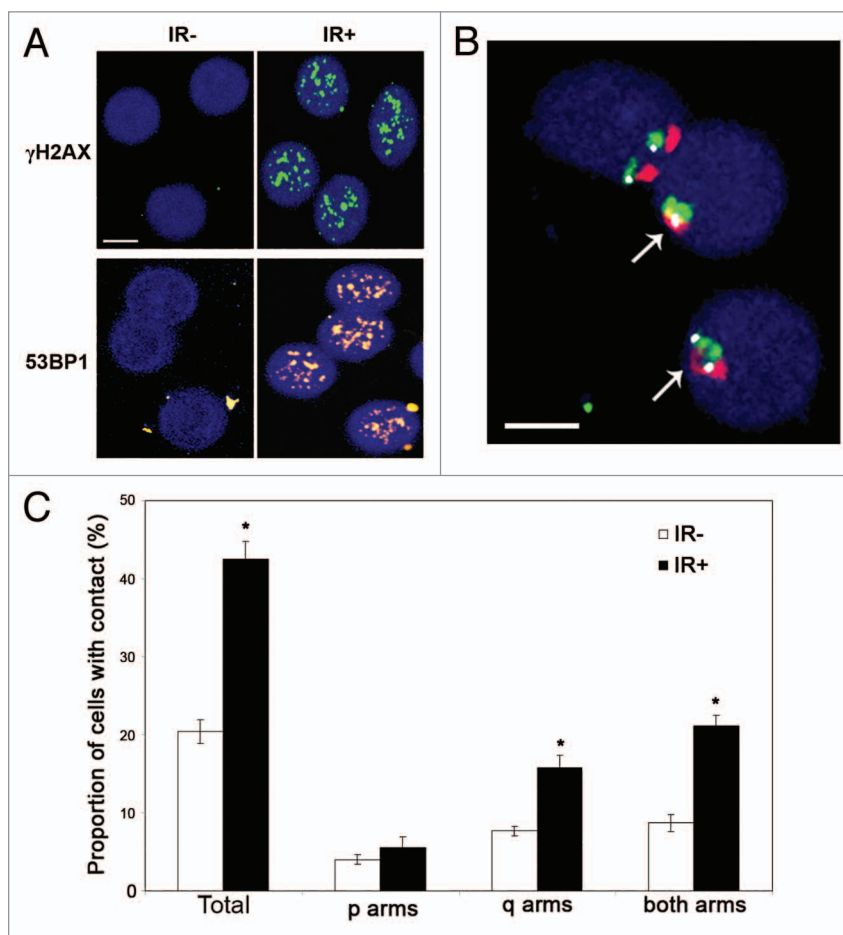
identified in these ex-vivo cells, and the frequency of contact was increased after IR (**Fig. 2B and C**). Exposure to 5 Gy IR increased contact between both chromosome arms 2.4-fold and contact between single arms 1.8-fold, similar to the levels of increase observed in primary cell cultures.

This finding conclusively demonstrates that homologous chromosome contact occurs in $G_0$-phase cells in human tissues and is not a phenomenon associated with cell propagation in culture.

**Contact between homologous chromosomes is detectable as early as 5 min**



**Figure 2.** Homologous chromosome contact in $G_0$-phase cells. (**A**) Formation of γH2AX foci and 53BP1 nuclear foci in cells from human thyroid tissue explants 15 min after exposure to ionizing radiation (IR) as detected by immunofluorescence. (**B**) 3D-FISH on ex-vivo cells showing contact between chromosome 3 homologs in two of the three nuclei (arrows). The middle nucleus shows a parallel-shape contact of both arms, while the lower nucleus shows a doughnut-shape contact of both arms. (**C**) Frequency of contact between homologous chromosomes in cell from human thyroid tissue explants before and 15 min after IR. Results of the analysis of two chromosomes (chr. 3 and chr. 11) are combined. Statistically significant difference between untreated and IR-treated cells is denoted with an asterisk.

**Table 1.** Frequency of various types of arm-specific contact between homologous chromosomes in untreated primary human cells and 15 min after exposure to ionizing radiation (IR)

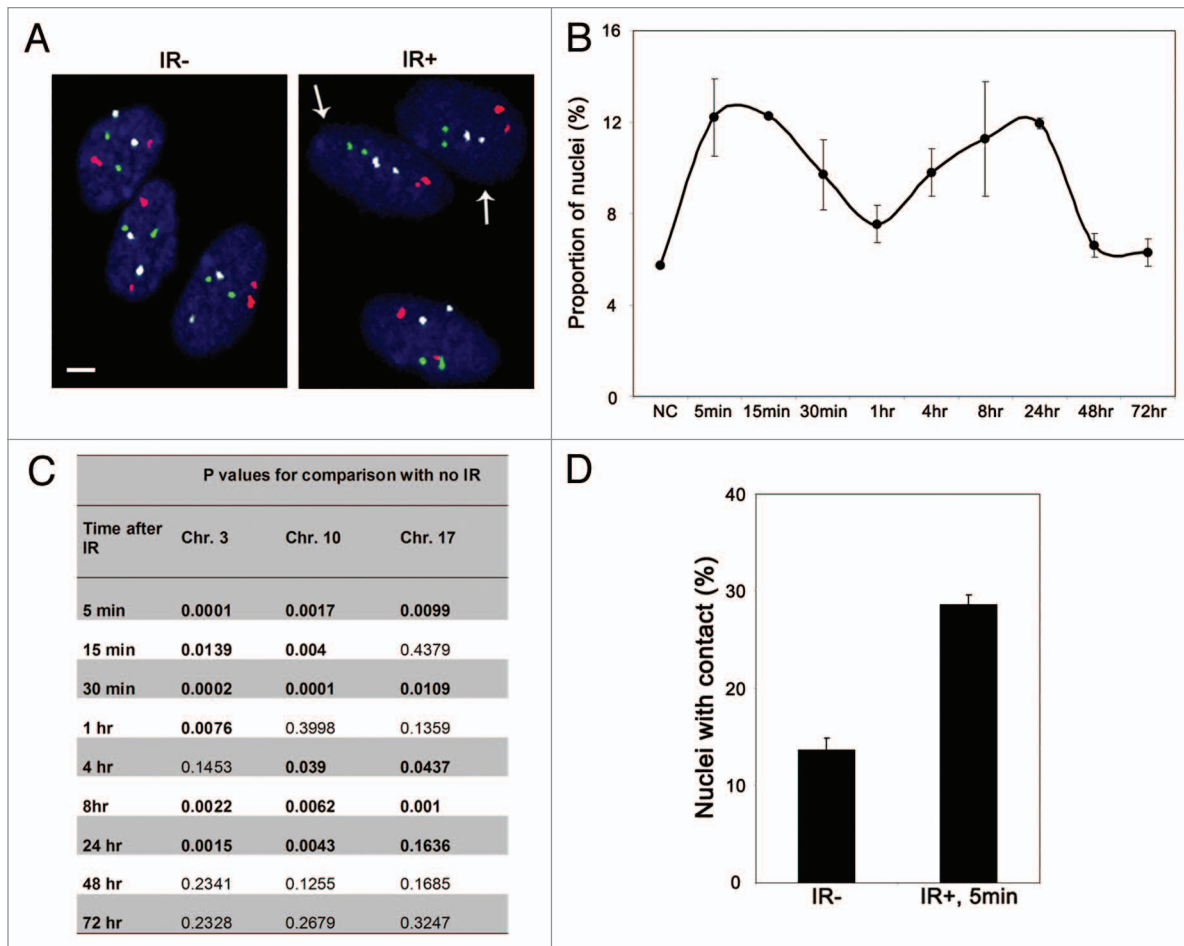| | Thyroid epithelial cells | | Fibroblasts | |
|---|---|---|---|---|
| | IR− | IR+ | IR− | IR+ |
| **p-arm contact** | 5.39% | 5.38% | 3.42% | 3.31% |
| **q-arm contact** | 6.01% | 11.36% | 8.59% | 11.73% |
| **both arm contact** | 7.23% | 21.48% | 8.78% | 15.07% |
| **centromere contact** | 0.3% | 0.1% | 0.2% | 0.3% |

**Figure 3.** Kinetics of homologous chromosome contact after IR. (**A**) FISH on primary cultures of human thyroid cells showing location of centromeres for chromosomes 3, 10 and 17 labeled in red, green and light blue, respectively. Untreated cells (IR−) have random positioning of centromeres of homologous chromosomes. After exposure to ionizing radiation (IR+), two cells (arrows) show at least two sets of centromeres arranged in a paired fashion and separated by ≤ 3.25 μm. (**B**) Change in the proportion of nuclei with at least two of homologous chromosome centromeres paired and separated by ≤ 3.25 μm. (**C**) P values for comparison of cumulative frequency distributions of distances between centromeres of homologous chromosomes at different time points after IR as compared with untreated cells assessed by the two-sample Kolmogorov-Smirnov test. P values that are significant are shown in bold font. (**D**) Frequency of arm-specific contact between chromosome 3 homologs in untreated cells and 5 min after IR.

**after IR.** We investigated the kinetics of homologous chromosome interaction by measuring the distances between the centromeres of chromosomes 3, 10 and 17 at various time intervals after 5 Gy IR. The approach was based on the assumption that in order to interact at the limited sub-chromosomal regions, homologous chromosomes must travel toward each other, which should result in the reduction of distances between homologous centromeres within the nucleus. This was indeed observed after IR as the increase in the proportion of nuclei, in which at least two of the three pairs of homologous centromeres were compartmentalized and separated by a distance of ≤ 3.25 μm (**Fig. 3A**). The distance of 3.25 μm

is the mean distance between homologous centromeres in those nuclei where contact between homologous chromosome arms was observed. The proportion of nuclei in which at least two pairs of the homologous centromeres were within ≤ 3.25 μm increased 2.1-fold at 5 min after IR and fluctuated over the following 24 h in a bimodal manner before returning to the background level by 48–72 h (**Fig. 3B**). Similar findings were obtained by analyzing the change in the entire distribution of distances between centromeres of each of the three homologous chromosomes measured in 200 nuclei in two experimental repeats. This analysis revealed that distances between centromeres of homologous chromosomes were significantly

reduced for all three chromosomes at 5 min, 30 min, 8 h and 24 h after IR (p values are shown in **Fig. 3B**).

To confirm that the reduced distances between centromeres observed 5 min after IR corresponded to the increased contact between homologous chromosomes, we directly analyzed the frequency of arm-specific contact 5 min after cell irradiation. Using FISH with arm-specific paints for chromosome 3, we observed a significant increase in arm-specific contact 5 min after IR (**Fig. 3D**).

## Discussion

Taken together, our recent studies,[1] and the observations presented here,

provide the first demonstration that contact between homologous chromosomal regions is induced by DSBs in genes in human $G_0$- and $G_1$-phase cells. While our findings do not demonstrate that the contact is involved in DSB repair, the fact that contact is centered on a DSB and requires ATM kinase activity raises this possibility. It is well established that DSBs are repaired via two principal repair pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR).[5,6] NHEJ seals the break by re-joining free DNA ends after minimal processing and can result in the deletion or insertion of one or more nucleotides at the site of joining. HR requires a homologous DNA sequence that is copied for the error-free restoration of the broken DNA sequence. In vertebrates, NHEJ and HR are believed to be associated with specific phases of the cell cycle. NHEJ is believed to be the primary mechanism of DSB repair in $G_0/G_1$-phase cells, when no obvious homologous template is available, whereas HR is thought to be the primary mechanism of DSB repair during the late-S- and $G_2$-phases, when a sister chromatid may be used as a template for repair.[6-8] Our observations allow for several additional possibilities. First, in $G_0$- and $G_1$-phase cells, a homologous chromosome may serve as a template for DSB repair. Second, NHEJ and HR may be differentially associated with the repair of DSBs located in different DNA regions, i.e., intergenic regions and genes. In $G_0/G_1$-phase cells, HR mediated by contact between homologous chromosomes would allow error-free repair of the relatively small number of DSBs that arise in genes, which could be a highly significant mechanism that prevents mutations in coding regions of DNA that are critical for cell viability and function.

Our results clearly show that machinery capable of identifying homologous chromosomes regions and co-localizing them within the $G_0/G_1$-phase nucleus exists in human somatic cells. Mammalian cells are known to have the ability to recombine a chromosomal locus with a homologous DNA molecule that is introduced into the nucleus,[9-11] indicating that cells have a mechanism that uses sequence homology to bring otherwise distant DNA molecules into contact. In fact, these so-called targeted recombination events are more frequent when the chromosomal target sustains a DSB.[12,13] Although the molecular mechanisms responsible for the homology search remain unknown, it is reasonable to propose that factors that govern gene targeting also play a role in break-induced chromosome contact. Based on our data and findings reported by others, it is likely that the mechanisms of homology recognition are transcription-based. Indeed, recruitment of some DNA repair factors such as 53BP1 to the sites of DNA damage has been previously shown to require direct interaction with RNA molecules,[14] and homologous chromosomes pairing in meiosis may involve RNA:RNA base pairing.[15] Therefore, there is at least a theoretical possibility that sequence-specific RNA molecules may be involved in both the search for homology and the co-localization of homologous gene loci in somatic cells. Our results presented here suggest that although the co-localization of homologous chromosomes at the sites of breaks involves the movement of the entire chromosome territories, it does not require physical contact between homologous centromeres and therefore may be mediated by factors in the vicinity of the DSB.

While the molecular mechanisms through which homologous chromosomes interact remain to be discovered, our data provide the first documentation of an ATM kinase-dependent mechanism that rapidly induces contact between homologous chromosomes at the sites of DSBs in genes that are being transcribed $G_0/G_1$-phase human cells. We suggest that such mechanisms may be significant for genome stability and human health.

## Materials and Methods

**Primary cell cultures, tissue explants and irradiation.** Primary cultures of human thyroid cells were established as previously described.[1] For tissue explants, the freshly excised normal human thyroid tissues adjacent to a benign thyroid nodule were collected in DMEM, cut into 5 mm fragments and assayed either untreated or after exposure to 5 Gy of γ-irradiation from a cesium-137 source at a dose rate of 301.66 cGy/min. Each experiment was performed using cells/tissues from two different donors.

**Cell fixation, fluorescence in situ hybridization (FISH), immunofluorescence (IF) and immunohistochemistry (IHC).** For 3D analysis, primary cultures of cells were prepared as described before.[1] For 2D analysis, cells on coverslips were fixed by standard methanol-acetic acid (MAA) treatment without hypotonic solution. For tissue explants, touch preparations were made on positively charged slides and subjected to 3D-fixation.[1] Pre-labeled centromeric enumeration probes were also purchased from Abbott laboratories. Chromosome arm paints were obtained from Metasystems. Slide pretreatment and hybridization was performed as previously described.[16] For γH2AX and 53BP1 IF, cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Anti-γH2AX antibody (#05-636, Upstate Biotechnology) and anti-53BP1 antibody (#ab21083, Abcam) were used. For Ki-67 proliferation index, frozen sections of tissue explants were obtained, stained with Ki-67 antibody and scored as described elsewhere.[3]

**Image acquisition and analysis.** Confocal microscopy was performed using a Leica SP5 TCS 4D confocal laser scanning fluorescence microscope and image stacks were reconstructed using the Volocity software as previously described.[1] The analysis of spatial contact between chromosome arms was performed using the intensity-based image segmentation technique.[17] 3D measurement of distances between centromeric probe pairs was performed by selecting the image gravity centers corresponding to the signal by navigating through the image stack. For the 2D analysis of MAA-fixed cells, the maximum intensity projection image was used to obtain the distance measurements between centromere signals.

**Analysis of kinetics using CFDs and estimation of contact frequency in 2D.** Distances between centromeres of homologous chromosomes were measured in 2D in two individuals (200 cells each) and combined for the analysis. Cumulative frequency distributions (CFDs) of distances were built by plotting the values on the X axis and the proportions of all data points

that were smaller than this measurement on the Y axis. CFDs were compared using the two-sample Kolmogorov-Smirnov test.[18] A mean 2D distance between centromeres of homologous chromosomes 3, 10 and 17 was calculated based on direct measurements in those nuclei that showed arm-specific contact on 3D analysis and was found to be 3.25 μm.

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