A meeting at risk: Unrepaired DSBs go for broke

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ABSTRACT
Translocations are dramatic genomic rearrangements due to aberrant rejoining of distant DNA ends that can trigger cancer onset and progression. Translocations frequently occur in genes, yet the mechanisms underlying their formation remain poorly understood. One potential mechanism involves DNA Double Strand Break mobility and juxtaposition (i.e. clustering), an event that has been intensively debated over the past decade. Using Capture Hi-C, we recently found that DSBs do in fact cluster in human nuclei but only when induced in transcriptionally active genes. Notably, we found that clustering of damaged genes is regulated by cell cycle progression and coincides with damage persistency. Here, we discuss the mechanisms that could sustain clustering and speculate on the functional consequences of this seemingly double edge sword mechanism that may well stand at the heart of translocation biogenesis.

DNA Double-Strand Breaks (DSBs) represent highly detrimental lesions, which are handled by two main repair pathways, namely Non Homologous End Joining (NHEJ) and Homologous Recombination (HR) (for review1). These two pathways not only differ in their mechanisms but also in their accuracy and their availability across the cell cycle. Chromatin, that organizes DNA within the nuclear space in eukaryotes, largely contributes to these repair mechanisms by ensuring 1) DSB signaling and activation of the DNA Damage Response (DDR), and 2) the establishment of a structural context that is compatible with the chosen repair pathway such as, for example, single strand DNA generation, homology search and DSB relocalization within the nucleus (for review2).

To cluster or not cluster: A long lasting debate
It has long been known that chromosomes are mobile within the nuclear space3 and multiple studies have reported that damages to DNA trigger changes in global chromatin mobility. In S.cerevisiae, induction of a single DSB using the I-SceI endonuclease leads to increased motion of all chromosomes.4–6 In higher eukaryotes DNA damaging agents such as cisplatin and hydrogen peroxide elicit repositioning of some chromosome territories from the inner nucleus to the periphery.7,8 However, when in yeast this increase in global chromatin mobility comes together with an increased mobility of the DSB itself,4–6 in higher eukaryotes this has been subjected to controversy. Indeed, chromatin does not reveal significant movement differences whether undamaged or laser UV-light damaged.9 In addition in mouse cells, a DSB induced by I-SceI within a LacO array is submitted to only limited motion within the nucleus10 and similarly, DSBs induced by charged particles, X- and γ-rays were found relatively immobile.9,11,12 However, the 53BP1 foci formed following ionizing radiation (IRIF) display increased movement compared to non-damaged labeled chromatin domains or labeled centromeres,13 indicating that in some instances, damaged DNA can indeed exhibit enhanced movement. In agreement, in some specific contexts, DSBs were found to exhibit large scale mobility. For example, DSB occurring within heterochromatin in Drosophila and murine cells rapidly expel from the center to the periphery of the heterochromatin focus.14–16 Similarly, DSB induced on ribosomal DNA also relocate to the periphery of the nucleolus17–19 and in yeast, persistent DSBs relocate to the nuclear envelope.20,21 Moreover, DSBs generated at subtelomeric loci show large scale...
and directional movements in cells that undergo Alternative Lengthening of Telomere (ALT cells). Notably such large-scale movement can in some instances lead to DSB clustering, i.e. to the physical juxtaposition of multiple DSBs (reviewed in 2,22). In yeast, induction of multiple DSBs using endonucleases produces a single Rad52 focus,24 when in Drosophila, DSBs occurring in heterochromatin also cluster into large foci within the heterochromatin domain before being extruded to the periphery.14 In mammalian cells, some DSBs produced following exposure to irradiation exhibit cycles of association/dissociation9 and can cluster over time when induced by α particles or high doses of ionizing radiation,13,25,26 although this was not observed for damages produced by charged particles.11,12 Importantly, induction of clean DSBs at well-defined positions on the human genome were found to occasionally coalesce when induced by I-SceI,27 by a Zn-nuclease at subtelomeric loci22 and by AsiSI at other endogenous loci.28 In a recent report, we used Capture Hi-C to decipher the properties of those DSBs able to cluster.29

Clustering occurs between DSBs induced in active genes primarily in G1 and coincides with delayed repair

Clustering occurs at DSBs induced in active genes

The DIvA cell line (DSB Inducible via AsiSI) represents a convenient system, where multiple DSBs can be induced at annotated endogenous positions throughout the human genome by the AsiSI restriction enzyme.30 Importantly, although being restricted to euchromatin, AsiSI-induced DSBs locate in intergenic regions and also to a fair extent within or at the immediate vicinity of transcribing genes.31 Previously, we showed by live imaging and high-resolution microscopy that DSBs induced by AsiSI can exhibit clustering.28 However, the molecular identity of those AsiSI-DSBs (i.e. their position on the genome) able to coalesce had remained undetermined until very recently. In our latest study, we determined them using Capture Hi-C, a genomic approach derived from chromosome conformation capture (3C) assays.32 Surprisingly, we unambiguously found that while some DSBs do cluster this was restricted to DSBs induced in or at the vicinity (in promoters) of transcribing genes.29

Clustering occurs in G1

In agreement with a previous report,25 our work revealed that DSB clustering mainly takes place in G1. Notably, chromatin is globally more mobile in G1,33–35 a feature proposed to contribute to the reestablishment of chromosome territories, TADs and chromosome loops in daughter nuclei following exit from mitosis (reviewed in 36). Hence, enhanced chromatin mobility may contribute to DSB clustering in G1 cells.

Clustering coincides with delayed repair

By analyzing repair kinetics throughout the genome using direct in situ Break Labeling, Enrichment on Streptavidin and next generation Sequencing (BLESS), we found that clustering of DSBs induced in active genes coincides with a delayed repair in G1.29 Interestingly, we previously demonstrated that active genes are more prone to be repaired by HR than other genomic locations and that HR usage at active genes is restricted to G2.31 Hence, in line with the increased motion previously observed for persistent or difficult DSBs in many organisms (such as those occurring in heterochromatin or rDNA) (reviewed in 2), DSBs produced in active genes may 1) persist in G1 due to the downregulation of HR (reviewed in 37) and 2) initiate mobility leading to DSB clustering (Fig. 1).

To which extent can we generalize these findings?

AsiSI induced DSBs are particular in that, like for other nucleases (I-SceI, Zn Finger nuclease, Cas9 or HO endonuclease…) they harbor “clean” DNA ends at specific positions which likely undergo several cycles of cleavage. One may thus wonder whether these findings can reveal insights into the behavior of DSBs induced more physiologically in cells. Interestingly, while DSBs were initially thought to marginally occur in somatic cells, many studies have recently established that in fact they arise on a regular basis in normally cycling cells (reviewed in 38). Furthermore, high resolution genomic studies have identified active genes as DSB hotspots.39-45 Several endogenous mechanisms likely account for gene fragility. These include collisions between transcription and replication machineries, replication fork slow-down and stalling, as well as topoisomerase activity as part of the process leading to early responsive gene activation (reviewed in 38). Indeed, Topo IIβ mediated DNA breakage occurs at
paused genes in order to release topological constraints and resume RNA Polymerase II elongation. It has been proposed that impaired resealing of Topo II intermediates would occasionally give rise to DSBs (reviewed in38). This likely accounts, at least in part, for the high DSB incidence observed in active genes. Notably, we previously found that DSBs induced by etoposide (a Topoisomerase II poison) also exhibit clustering.28 Hence, DSB clustering observed at AsiSI-mediated DSBs is also likely to occur at TopoII-mediated DSBs in active genes. Moreover, the G1-forming clusters of damaged genes that we observed following AsiSI induced DSB are very reminiscent of the so-called 53BP1/OPT bodies, proposed to form at common fragile sites (CFS).46-48 CFS are fragile regions of the genome, mainly located in long genes49,50 that exhibit under-replication and endonucleolytic cleavage in late G2/mitosis.51-53 These DSBs form 53BP1 bodies upon entry in the next G1, which remain assembled until the next S phase is reached.47,48

Hence, in agreement with our findings, we would like to bring forward the hypothesis that DSBs occurring at active genes, either through incomplete replication followed by mitotic dependent resolution/breakage, or due to incomplete topoisomerase reaction upon activation of transcriptionally paused genes, are refractory to efficient repair in G1 and cluster together in sub-nuclear structures (Fig. 1), whose function remain enigmatic (see below).

Figure 1. DSBs induced in active genes may persist and cluster in G1, while being repaired by HR in G2. DSBs induced in intergenic/silent genes are primarily repaired by NHEJ throughout the cell cycle. In contrast, DSBs occurring in active genes (for instance following accidental unsealing of Topo II intermediates during transcription elongation or due to broken un-replicated DNA) are refractory for quick NHEJ repair. Physical hindrance by the RNA polymerase II machinery or/and initial processing of DNA ends may account for such suboptimal NHEJ. In S/G2, the availability of HR allows efficient and non-mutagenic repair of these degraded DSBs. In G1, HR is not available, and these DSBs persist and cluster. Clustering may contribute to pause repair at these DSBs to minimize the use of unfaithful repair mechanisms and/or may assist cell progression to S phase, for a faithful (HR-dependent?) resolution of these breaks.

DSB repair pausing and clustering: The yin and the yang

The fact that in G1, DSBs occurring in transcriptionally active genes exhibit i) delayed repair and ii) clustering, poses a certain number of critical questions about the selective advantage of these mechanisms. First, why would some DSBs be left unrepaired in G1, and second, given that bringing broken DNA ends in close proximity potentiates chromosomal rearrangements, why would cells take such a risk?

Pausing DSB repair at active genes in G1

A hypothesis that could account for delayed repair at active genes in G129 may be an inefficient quick NHEJ-dependent rejoining of DNA ends, due to high...
sterical hindrance imposed by the transcriptional machinery. This would leave a time window for the ends to be processed by nucleases. The absence of a functional HR machinery in G154 would further delay repair at these breaks (discussed in38,55). Once DNA-ends are degraded, a selective advantage may have been given to further pause the repair process in order to avoid mutagenic repair on the coding genome. Notably, DSB repair pausing also occurs at telomeres, at sub-telomeric DSBs, in quiescent cells, in 53BP1 bodies and potentially in the so-called DNA-SCARS during senescence. Thus, it appears that cells may well survive with a small amount of persisting DSBs. We suggest that DSB repair pausing may in some instances be preferable than mutagenic repair, which could trigger abnormal gene expression/product or chromosomal rearrangement. DSB repair pausing may last until a more appropriate cell cycle phase is reached and adequate repair pathways are available.

Function of DSB Clustering

DSB clustering must provide a consequent selective advantage given that it increases the risk of translocations. The first hypothesis is that such centers potentiate repair by concentrating repair factors, as previously suggested in yeast (for review60). However, since we found that clustering in G1 correlates with delayed repair, one should also consider that DSB clusters may serve as “DNA ends shielding centers”. Such nuclear structures could, for instance, be refractory to some repair factors, as proposed for the core of heterochromatin foci from which Rad51 is actively excluded. In such a case, cluster formation may help to inhibit the repair process, alike in 53BP1 bodies, in which DNA repair is likely on hold until cells enter S-phase where these bodies are resolved.47,48

Alternatively, these clusters may play a role in fine tuning the G1/S checkpoint efficacy. Indeed, we did not find evidence of G1 arrest following AsiSI activation61 and resolution of 53BP1 bodies is thought to occur in S phase, indicating that cells harboring unrepaired DSBs in active genes are still likely to progress through the G1/S transition. Notably, the G1 DNA damage checkpoint has been shown to be less robust than the G2/M checkpoint in yeast.62 In higher eukaryotes, while the G1 checkpoint is activated following very low doses of radiation, it can be further alleviated even in presence of a few remaining DSBs.63

Interestingly, G1 checkpoint activation depends on sgs1/exo1 dependent resection in yeast.64 Given that 53BP1 bodies are devoid of single strand DNA binding proteins,47 the absence of long-range resection at these DSBs may diminish checkpoint efficiency allowing cells to go through S-phase. That clustering contributes to inhibit resection hence allowing to bypass the G1/S checkpoint is an exciting possibility that deserves further investigation.

Higher frequency of Translocations as the price to pay

Yet, DSB clustering comes at a cost, as it potentiates illegitimate joining of two distant DNA ends. Former studies clearly established that spatial proximity within the nucleus strongly contributes to translocation biogenesis (see for example39,40,65-68). In addition, genome wide mapping revealed that translocation breakpoints often locate near or within active genes.39–41 Altogether these studies led to the idea that both the higher incidence of DSBs in active genes, combined with their spatial proximity (either due to their belonging to a common TAD or to their juxtaposition within the same transcription factory) accounts for translocation patterns. Our finding unveiled that the propensity of broken DNA to coalesce in G1 may also strongly influence the frequency of translocation between genes. Translocations mainly arise via AltNHEJ in mouse cells,43,69 while in human cells they rely on canonical- XRCC4 and LIG4 dependent-NHEJ.70 Whatever the mechanism, they may arise due to occasional deficient pausing of DSB repair within clusters. Of note our finding that clustering predominantly takes place in G1 raises the possibility that translocations may preferentially occur in G1 arrested cells, which constitute the vast majority of cells in an organism. We believe that deciphering the mechanisms that sustain damaged active gene clustering will now help to understand translocation biogenesis.

Clustering involves the cyto/nucleo-skeleton

The mechanisms underlying DSB mobility and clustering are still only poorly described. ATM-mediated DSB signaling seems to lie at the heart of all DSB mobility events from yeast to human (reviewed in55). In agreement, we found that AsiSI-induced DSB clustering also depends on ATM activity.28 However, it remains to be determined whether this key function of
ATM in DSB motion should be attributed to its role in DNA end processing or in chromatin remodeling. Beyond the involvement of various DNA end processing and DSB repair factors in mobility (reviewed in\textsuperscript{2,60}), we would like to give an emphasis here on the novel evidence that supports a role of cyto- and/or nucleo-skeleton components in DSB mobility and clustering.

The LINC complex

The LInker of Nucleoskeleton and Cytoskeleton (LINC) appears as a key determinant in DSB motion and positioning. This complex embedded in the nuclear membrane transmits forces between the cytoplasm and chromatin by connecting cytoplasmic microtubules (MTs) and the filamentous actin (F-actin) network to the nuclear lamina and chromatin.\textsuperscript{71} Importantly, the LINC complex has been involved in regulating nuclear morphology, stiffness and positioning, as well as modulating chromosome mobility (for review,\textsuperscript{72-74}). In budding and fission yeast as well as in Drosophila the SUN-domain containing proteins (inner membrane LINC complex proteins) Mps3/Sad1/Koi-Spag4 constitute anchoring points for persistent or heterochromatic DSBs.\textsuperscript{21,75,76} Additionally, from yeast to human, SUN-domain proteins influence the repair reaction.\textsuperscript{21,75-79} Moreover, SUN1/2 were found as contributors to unprotected telomere mobility in mice cells.\textsuperscript{80} Importantly, we found that the SUN2 component of the human LINC complex also sustains DSB clustering.\textsuperscript{29}

Cytoplasmic Microtubule network

The LINC complex likely contributes to DSB mobility through a – yet unclear – process that involves microtubules (MTs). Indeed, MT depolymerizing drugs induce a decrease in the mobility of dysfunctional telomeres and IR-induced DSBs in mouse cells.\textsuperscript{80} Similarly in yeast, the mobility of a broken chromosome arm depends on cytoplasmic MT and actin linkage.\textsuperscript{81} Furthermore, disconnection of cytoplasmic MTs from the LINC complex via mutation of the outer LINC component Kms1 leads to reduced gene conversion in S.pombe.\textsuperscript{79} Notably, during meiosis MTs contribute to homologous chromosome motion and pairing through force transmission in the nucleus via the LINC complex in C.elegans.\textsuperscript{82} Hence, a similar mechanism may account for DSB mobility within the nucleus of somatic cells.

In such a case, one should envisage that a signal is transmitted from the nucleus to the cytoplasm following DSB detection that would target the MT cytoskeleton. Interestingly, ATM exerts some cytoplasmic functions (for review\textsuperscript{83}) and bioinformatics analysis of putative ATM/Tel1 targets identified cytoskeleton proteins as overrepresented both in yeast and higher eukaryotes.\textsuperscript{84,85} DDR-mediated cytoskeleton modifications may modify the MTs linkage or forces applied to the LINC hence affecting global chromatin mobility. Enhanced chromatin roaming could occasionally trigger DSB clustering (Fig. 2A left). Alternatively, as mentioned above persistent DSBs are targeted to the nuclear periphery and anchored to the inner LINC components in yeast and Drosophila. Hence, DSB clustering may be achieved via the reorganization of the LINC distribution in the nuclear envelope (NE) (for instance LINC sliding in the NE) rather that movement of the DSBs themselves (Fig. 2A, right). DSB anchorage has yet not been observed in mammalian cells. However, the propensity of the NE to invaginate following damage\textsuperscript{86} calls for further investigations. In both scenarios, sustained signaling due to unrepaired DSBs would trigger changes to the cytoskeleton or/and to its nucleus connection points which would in turn regulate DSBs motion and clustering (Fig. 2).

The actin connection

Members of the actin network have also been identified as contributors to the chromatin mobility and DDR response (see\textsuperscript{2,73} and below). For instance, transcription-induced chromosome mobility,\textsuperscript{87,88} or subtelomeric loci motion in yeast\textsuperscript{89} decrease upon inhibition of actin polymerization and in presence of actin mutants. The function of actin in DSB repair may relate to the ability of cytoplasmic F-actin to regulate chromosomal motion via the above-mentioned LINC complex connection, as during yeast meiosis, where cytoplasmic actin cables regulate mid prophase chromatin movement and telomeric “bouquet” formation.\textsuperscript{90} Yet, recent data suggest that it may also play a more direct role within the nucleus. It is now well admitted that actin can also reside in the nucleus mostly in its monomeric form and as a component of many chromatin remodeling complexes.\textsuperscript{91} However, its
ability to form filaments in the nucleus is hotly debated. Yet, a couple of studies have now described such filaments in different model organisms and conditions.\textsuperscript{92–96} Recently, using a newly developed live actin probe\textsuperscript{94} R.D. Mullins’s laboratory revealed that, upon DNA damage induction by MMS, nuclear actin can form short filaments in a manner that depends on the formin\textsuperscript{2} (FMN2) actin organizer.\textsuperscript{97} In line with these findings, we found that depletion of FMN2 decreases clustering of AsiSI induced DSBs.\textsuperscript{29} Of interest, using another live-actin probe, D. Fisher’s laboratory recently reported that short actin filaments transiently form during G1 in the nucleus of normal cycling cells, in a manner that is regulated by nuclear formins.\textsuperscript{95} Given that clustering occurs in G1 and in a FMN2 dependent manner, it is tempting to hypothesize that these short filaments contribute to DNA end mobility.

In the cytoplasm, molecular motors such as myosin, move along F-actin to sustain active vesicle and macromolecule transport. While in the nucleus chromatin rather exhibits sub-diffusive motion, directional\textsuperscript{22,87,88,98} and ATP-driven\textsuperscript{99} chromatin movements have been reported to occur in few cases upon transcription activation,\textsuperscript{87,88,98} following serum starvation\textsuperscript{99} and also at DNA ends.\textsuperscript{22} Interestingly actin motors have also been found to contribute to DSB response. Indeed chromosome territories relocalization following damage induction\textsuperscript{8} depends on the nuclear Myosin 1 (NM1).\textsuperscript{7} NM1 has previously been implicated in chromatin mobility after starvation or transcription activation\textsuperscript{87,99} and strikingly also promotes interchromosomal TMPRSS2:ETV1 translocation.\textsuperscript{66} In our opinion, taken together these studies support a model whereby DSB clustering is driven, at least in part, by an active-F-actin mechanism (Fig. 2).

\textbf{Figure 2.} Hypotheses regarding cyto/nucleo skeleton driven DSBs clustering. A. The cytoskeleton contributes to DSB mobility and clustering via the LINC complex. (i) ATM dependent modification of the cytoskeleton: Upon DSB detection, a sub-fraction of ATM may translocate to the cytoplasm and modify cytoskeleton components. These changes could generate forces transduced to the nucleus via the LINC complex, embedded in the nuclear membrane. This forces may propagate along the chromatin fiber, known to be anchored to the inner LINC components. This would trigger an enhanced global DNA mobility, eventually leading to DSB clustering (by random ends diffusion). (ii) Alternatively or in addition the ability of DSBs to directly interact with the SUN domain, inner LINC, components (at least in yeast and Drosophila) may also directly impact clustering. In such a scenario, the mobility of the LINC complex within the nuclear envelope (NE) may translate into DSB clustering. B. Hypothesis: Nuclear F-actin drives DSBs mobility and clustering. DSB production on the genome may trigger the nucleation of actin filament at the proximity of DNA ends. DSBs clustering may be achieved via active motion onto F-actin filaments mediated by molecular motors such nuclear myosin 1.
Conclusions
A wealth of data now supports the idea that not only are active genes fragile regions of the genome that undergo recurrent DNA double strand breakage, they are also subjected to tightly regulated and specific repair. Deciphering the mechanisms that contribute to DSB repair pausing and clustering will surely help to understand how translocations arise on the genome and open new avenues to design therapeutic strategies to limit their occurrence following chemotherapy.

Disclosure of interest
The authors report no conflict of interest

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