Nuclear deformation causes DNA damage by increasing replication stress

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Abstract

Cancer metastasis, i.e., the spreading of tumor cells from the primary tumor to distant organs, is responsible for the vast majority of cancer deaths. In the process, cancer cells migrate through narrow interstitial spaces substantially smaller in cross-section than the cell. During such confined migration, cancer cells experience extensive nuclear deformation, nuclear envelope rupture, and DNA damage. The molecular mechanisms responsible for the confined migration-induced DNA damage remain incompletely understood. While in some cell lines, DNA damage is closely associated with nuclear envelope rupture, we show that in others, mechanical deformation of the nucleus is sufficient to cause DNA damage, even in the absence of nuclear envelope rupture. This deformation-induced DNA damage, unlike nuclear envelope rupture-induced DNA damage, occurs primarily in S/G2 phase of the cell cycle and is associated with stalled replication forks. Nuclear deformation, resulting from either confined migration or external cell compression, increases replication fork stalling and replication stress, providing a molecular mechanism for the deformation-induced DNA damage. Thus, we have uncovered a new mechanism for mechanically induced DNA damage, linking mechanical deformation of the nucleus to DNA replication stress. This mechanically induced DNA damage could not only increase genomic instability in metastasizing cancer cells, but could also cause DNA damage in non-migrating cells and tissues that experience mechanical compression during development, thereby contributing to tumorigenesis and DNA damage response activation.

Introduction

Cell migration is important for various developmental processes and immune surveillance (Munjal and Lecuit, 2014; Weninger et al., 2014). In addition, cell migration is essential for tumor cell invasion and metastasis, which is responsible for more than 80% of all cancer deaths (Chaffer and Weinberg, 2011). During metastasis, cancer cells disseminate from the primary tumor, invade through the surrounding extracellular matrix and into neighboring tissues, ultimately spreading to distant organs via the circulation and lymphatic system (Chaffer and Weinberg, 2011). In this process, cancer cells encounter interstitial spaces of the order of 0.1-20 µm in diameter, i.e., smaller than the size of the cell nucleus (Doerschuk et al., 1993; Stoitzner et al., 2002; Weigelin et al., 2012). Migration through such confined environments puts considerable mechanical stress on the cell nucleus, which constitutes the largest and stiffest organelle (Lammerding, 2011; Swift et al., 2013). As a result, cells frequently experience severe nuclear deformation and nuclear envelope (NE) rupture during confined migration (Bakhoum et al., 2018; Denais et al., 2016; Elacqua et al., 2018; Irianto et al., 2017a; Pfeifer et al., 2018; Raab et al., 2016; Wolf et al., 2013; Xia et al., 2019). Transient loss of NE integrity allows uncontrolled exchange between the nucleoplasm and cytoplasm, exposes the genomic DNA to cytoplasmic components such as nucleases, and leads to DNA damage (Nader et al., 2020; Alvey et al., 2017; Bakhoum et al., 2018; Cho et al., 2017; Cho et al., 2019; Denais et al., 2016; Elacqua et al., 2018; Hatch, 2018; Irianto et al., 2016; Pfeifer et al., 2018; Raab et al., 2016; Shah et al., 2017). Although cells rapidly repair their NE and continue to survive and migrate (Denais et al., 2016; Elacqua et al., 2018; Raab et al., 2016), the acquired DNA damage can increase genomic instability in these cancer cells (Nader et al., 2020; Alvey et al., 2017; Bakhoum et al., 2018; Irianto et al., 2017a; Pfeifer et al., 2018; Zhang et al., 2015) which could further enhance their metastatic potential and resistance to therapies.

While it is now well recognized that confined migration can cause DNA damage, the underlying molecular mechanism remains incompletely understood. Recent reports have implicated loss of DNA repair factors during NE rupture or local exclusion of repair factors due to nuclear deformation as possible mechanisms (Alvey et al., 2017; Cho et al., 2019; Irianto et al., 2016; Pfeifer et al., 2018; Xia et al., 2019). Exposure to cytoplasmic DNases such as TREX1 following NE rupture (Nader et al., 2020; Hatch, 2018; Shah et al., 2017) and mislocalization of organelles like mitochondria post NE rupture (Vargas et al., 2012) have also been suggested as cause of DNA damage. Contributing to the uncertainty about the molecular mechanism responsible for the confined migration-induced DNA damage is that previous studies have provided at times conflicting results on the association between DNA damage and NE rupture. While some studies reported that DNA damage requires NE rupture (De Vos et al., 2011; Irianto et al., 2017a; Pfeifer et al., 2018; Raab et al., 2016; Smith et al., 2019), others found that DNA damage can occur in the absence of NE rupture as cells squeeze their nuclei through tight spaces (Denais et al., 2016; Irianto et al., 2017b).

Using time-lapse microscopy and a broad panel of cell lines co-expressing fluorescent reporters for DNA damage and NE rupture, we found that in some cell lines, mechanical deformation of the nucleus is sufficient to cause DNA damage, while in other cell lines DNA damage is primarily associated with NE rupture. These results provide an explanation for the varied results in previous studies. Furthermore, we show that nuclear deformation led to increased replication stress during confined migration, and that deformation-induced DNA damage frequently occurred at stalled replication forks. Thus, we demonstrate a novel mechanism by which deformation of the nucleus can cause DNA damage in the absence of NE rupture. Intriguingly, deformation-induced DNA damage does not require cell migration, but is also seen in stationary cells subjected to physical compression. Thus, this mechanism could have broad implications during development and in tissues subjected to regular compression, such as solid tumors, skin, or cartilage, where the DNA damage could promote genomic instability and activate apoptosis or senescence pathways.

Results

NE rupture and nuclear deformation lead to DNA damage during confined migration

To address the specific cause of DNA damage during confined migration, we performed a systematic study using a panel of cells consisting of two breast cancer cell lines (MDA-MB-231 and BT-549), a fibrosarcoma cell line (HT1080), and two normal human cell lines (RPE-1 and human skin fibroblasts) that had previously been reported to exhibit DNA damage during confined migration (Denais et al., 2016; Raab et al., 2016). Cells were modified to stably express a fluorescent reporter for DNA damage, 53BP1-mCherry, which localizes to DNA double strand breaks (DSBs) (Denais et al., 2016; Dimitrova et al., 2008; Raab et al., 2016). Treatment with Phleomycin, a DSB inducing agent (Sleigh, 1976), and staining for γ-H2AX, which accumulates at DSBs, was used to validate the 53BP1-mCherry DNA damage reporter (Suppl. Fig. S1). To detect NE rupture, cells were modified to co-express a NE rupture reporter, consisting of a green fluorescent protein with a nuclear localization sequence (NLS-GFP), which spills from the nucleus into the cytoplasm upon NE rupture and is re-imported into the nucleus once NE integrity has been restored (Denais et al., 2016; Elacqua et al., 2018). Using time-lapse microscopy, we monitored cells for DNA damage, NE rupture, and nuclear deformation as they migrated through collagen matrices or custom-built microfluidic devices that mimic the interstitial spaces found in *vivo* (Davidson et al., 2014; Davidson et al., 2015; Denais et al., 2016; Elacqua et al., 2018). The microfluidic devices contain channels with constrictions either $1 \times 5\ \mu m^2$ or $2 \times 5\ \mu m^2$ in cross-section that require extensive nuclear deformation, and larger control channels with $15 \times 5\ \mu m^2$ openings that do not require substantial nuclear deformation while still providing a 3-D cell environment.

For all cell types, migration of cells through the $\leq 2 \times 5\ \mu m^2$ constrictions led to a higher increase in DNA damage than migration through the $15 \times 5\ \mu m^2$ control channels (Fig. 1A-B, 1C-D, Suppl.

Fig. S2A-C). For HT1080 fibrosarcoma cells, RPE-1 retinal epithelial cells, and immortalized human skin fibroblasts, the DNA damage occurred predominantly following NE rupture (Fig. 1A, 1C, 1G; Suppl. Fig. S2D-E; Suppl. Video 1), consistent with a previous report in RPE-1 cells (Raab et al., 2016). In the MDA-MB-231 and BT-549 breast cancer cells, in contrast, DNA damage predominantly occurred in the absence of NE rupture as the cell squeezed the nucleus through the tight constrictions (Fig. 1D-G; Suppl. Fig. S2F; Suppl. Video 2). These data suggest that while DNA damage is associated with NE rupture in some cell lines, nuclear deformation is sufficient to induce DNA damage, even without NE rupture, in other cell lines. Furthermore, the cell line-specific differences may explain the conflicting findings obtained in previous studies. Collectively, our results indicate that DNA damage during confined migration can result from two distinct, albeit overlapping events: nuclear deformation as seen in MDA-MB-231 and BT-549 breast cancer cells; and NE rupture (combined with nuclear deformation) as seen in HT1080, human fibroblasts and RPE-1 cells.

For subsequent studies, we focused on HT1080 and MDA-MB-231 cells to compare and contrast NE rupture and nuclear deformation-associated damage and to identify the underlying mechanism(s). We had previously demonstrated that HT1080 cells migrating through dense collagen matrices exhibit NE rupture and DNA damage (Denais et al., 2016). To investigate deformation-induced DNA damage in MDA-MB-231 cells, we imaged cells co-expressing the NLS-GFP and 53BP1-mCherry reporters as they migrated through dense (1.7 mg/ml) collagen matrices using lattice light-sheet microscopy (LLSM). LLSM allows fast, high resolution, 3-D imaging of cells, while minimizing phototoxicity (Chen et al., 2014). MDA-MB-231 cells predominantly exhibited deformation-induced DNA damage, independent of NE rupture (Fig. 1H-I, Suppl. Video 3). Furthermore, the extent of DNA damage increased with the severity of nuclear deformation in individual cells. These results confirm that MDA-MB-231 cells exhibit deformation-induced DNA damage during migration through confined environments.

Nuclear compression is sufficient to cause DNA damage

Confined migration involves numerous other cellular processes in addition to nuclear deformation. To test whether nuclear deformation is sufficient to cause DNA damage, we applied external compression to cells cultured on flat, 2-D substrates, resulting in substantial nuclear deformation (Fig. 2A-B). Nuclei were deformed to different heights using a custom-built cell compression device (Fig. 2A) that was inspired by previous designs to study cell confinement and compression (Le Berre et al., 2012; Liu et al., 2015). We compressed cells to a height of 2 µm to mimic the nuclear deformation inside the tight constrictions in the microfluidic channels and dense collagen matrices. As baseline control, cells were compressed to a height of 5 µm, corresponding to the height of the microfluidic channels. In this case, the cells deform only moderately (Fig. 2B) compared to unconfined conditions, in which the typical cell height is ≈6-7 µm for MDA-MB-231 cells, but experience similar oxygen and nutrient exchange conditions as in

the more severe 2 µm compression case. For some experiments, we additionally tested the effect of intermediate compression (3 µm height).

Compression of the cells increased DNA damage, as visualized by the appearance of new 53BP1-mCherry foci (Fig. 2C). The DNA damage increased with the extent of cell compression, i.e., decreasing nuclear height (Fig. 2C-D). These findings indicate that nuclear deformation is sufficient to cause DNA damage. New DNA damage occurred within 30 minutes of the start of compression (Fig. 2C), thereby ruling out that DNA damage is caused by limited availability of nutrients and oxygen due to compression, which would be expected to result in more gradual increase and later onset of DNA damage (Quinn et al., 2000). Although compression increased NE rupture (Suppl. Fig. S3A), time-lapse analysis of the NLS-GFP and 53BP1-mCherry reporters revealed that MDA-MB-231 predominantly experienced deformation-induced DNA damage, and not NE rupture-induced DNA damage during compression (Suppl. Fig. S3B), consistent with the results of the confined migration studies (Fig. 1F). Furthermore, while increasing the extent of cell compression from 3 µm height to 2 µm height significantly increased the amount of DNA damage (Fig. 2D), the rate of NE rupture did not increase further with more severe compression (Suppl. Fig. 3A).

To further validate that deformation of the nucleus is sufficient to cause DNA damage, we compressed parts of individual nuclei approximately to a height of 2 µm using an atomic force microscope (AFM) cantilever with a spherical tip (6 µm in diameter) while monitoring nuclear deformation and formation of DNA damage on a light-sheet (LS) microscope (Fig. 2E). The AFM-LS system allows for high-resolution, 3-D imaging of the whole nucleus throughout the compression application (Gavara, 2017; Liu et al., 2019; Nelsen et al., 2020), thereby enabling us to observe the spatio-temporal dynamics of DNA damage (Suppl. Video 4). Experiments in which the spherical tip was brought in contact with the cell without inducing nuclear compression served as controls (Suppl. Video 5). MDA-MB-231 cells showed increased DNA damage upon compression compared to uncompressed controls (Fig. 2F-G). Taken together, these findings support the concept that mechanical deformation of the nucleus or parts of it is sufficient to cause DNA damage.

Deformation induced DNA damage is independent of reactive oxygen species (ROS)

To investigate the mechanism of deformation-induced DNA damage, we examined the role of ROS, which can increase during confined migration (Xia et al., 2019) and lead to oxidative DNA damage (Barzilai and Yamamoto, 2004; Tudek et al., 2010). However, treatment of MDA-MB-231 with N-acetyl cysteine (NAC), a ROS scavenger (Zafarullah et al., 2003), did not reduce DNA damage during confined migration (Suppl. Fig. S4), suggesting that deformation-induced DNA damage in these cells is not caused by increased ROS levels.

Deformation associated DNA damage occurs specifically in S/G2 phase of the cell cycle

A major cause of DNA damage in proliferating cells is DNA replication stress, which occurs in S/G2 phase of the cell cycle (Zeman and Cimprich, 2014). To test whether DNA damage incurred during confined migration or cell compression was associated with a specific cell cycle stage, we modified MDA-MB-231 and HT1080 cells to stably co-express the 53BP1-mCherry DNA damage reporter and a fluorescent cell cycle reporter, FUCCI (fluorescent ubiquitination-based cell cycle indicator). The FUCCI reporter fluorescently labels cells in the G0/G1 stage of cell cycle in red, and cells in S/G2 phase in green (Fig. 3A; Suppl. Video 6) (Sakaue-Sawano et al., 2008). We validated that the cell cycle stages determined by the FUCCI reporter were consistent with those obtained by DNA content assay for both MDA-MB-231 and HT1080 cells (Suppl. Fig. S5A-B).

Strikingly, in MDA-MB-231 cells, which show predominantly deformation-induced DNA damage, most of the DNA damage during confined migration occurred in S/G2 phase of the cell cycle (Fig. 3B-C). Similar results were obtained for external compression of MDA-MB-231 cells (Suppl. Fig. S5C). In contrast, in HT1080 cells, which predominantly experience NE rupture-induced DNA damage, DNA damage occurred equally in both G0/G1 and S/G2 phase of the cell cycle during confined migration (Fig. 3D) and external compression (Suppl. Fig. S5D). Importantly, confined migration did not select for any particular cell cycle stage in either MDA-MB-231 cells (Fig. 3E) or HT1080 cells (Fig. 3F), ruling out that the increased occurrence of DNA damage in S/G2 in the MDA-MB-231 cells was due to enrichment of cells in this cell cycle phase. Taken together, these findings indicate that NE rupture-induced DNA damage occurs independent of cell cycle stage, consistent with it resulting from the influx of cytoplasmic nucleases, which would attack DNA irrespective of cell cycle stage (Nader et al., 2020; Hatch, 2018; Shah et al., 2017). In contrast, deformation-induced DNA damage occurs primarily in the S/G2 phase of the cell cycle, suggesting that it is linked to DNA replication.

Deformation-induced DNA damage occurs at stalled replication forks

Since deformation-induced DNA damage occurred primarily in S/G2 phase of the cell cycle, we hypothesized that this damage could be associated with DNA replication stress. DNA replication requires unwinding and subsequent separation of the double strand DNA (dsDNA) into single strands to allow synthesis of the new complementary DNA strands at the replication fork. Replication forks can stall due to conformational and/or torsional stress of the dsDNA, limited availability of nucleotides for DNA synthesis, and other factors (Labib and Hodgson, 2007; Mirkin and Mirkin, 2007). If not repaired in time, stalled replication forks can collapse and form DSBs (Alexander and Orr-Weaver, 2016), leading to replication stress.

We stained MDA-MB-231 cells migrating through confined spaces for phosphorylated RPA (p-RPA S33), a marker for single stranded DNA (ssDNA) that accumulates at stalled replication forks (Vassin et al., 2009). New 53BP1-mCherry foci were frequently co-localized with p-RPA S33 foci (Fig. 4A), suggesting replication stress contributed to the deformation-induced DNA damage

during confined migration. To further investigate if this DNA damage occurred at stalled replication forks, we modified MDA-MB-231 and HT1080 cells to co-express 53BP1-mCherry and GFP-PCNA, a fluorescent reporter for DNA replication (Brickner et al., 2017). PCNA is a DNA clamp that moves along replicating DNA and accumulates at stalled replication forks (Essers et al., 2005; Franchitto et al., 2008; Shimura et al., 2008). To validate the GFP-PCNA reporter, HT1080 and MDA-MB-231 cells were treated with Hydroxyurea (HU), a DNA replication inhibitor (Koc et al., 2004; Singh and Xu, 2016; Vesela et al., 2017), which caused a substantial increase in GFP-PCNA foci (Suppl. Fig. S6).

Time-lapse microscopy revealed that new 53BP1-mCherry foci co-localized with GFP-PCNA foci significantly more frequently in MDA-MB-231 cells migrating through the tight constrictions (≤ 2 × 5 μm^2) compared to cells migrating through the 15 × 5 μm^2 control channels (Fig. 4B-C; Suppl. Video 7). In contrast, for HT1080 cells, which predominantly experience NE rupture-induced DNA damage, co-localization of 53BP1 and PCNA foci was comparable between cells migrating through constrictions and control channels (Fig. 4D). These findings indicate that in MDA-MB-231 cells, which predominantly exhibit deformation-induced DNA damage, confined migration increases the amount of DNA damage at stalled replication forks compared to baseline levels. MDA-MB-231 cells migrating through collagen matrices showed similar co-localization between new 53BP1-mCherry and GFP-PCNA foci when imaged by LLSM. In these experiments, over 50% of new DNA damage foci occurring at pre-existing PCNA foci, suggesting that the deformation-induced DNA damage occurred at stalled replication forks (Fig 4E, Suppl. Video 8). Collectively, these findings indicate that deformation-induced DNA damage frequently occurs at stalled replication forks and could arise from increased replication stress associated with nuclear deformation.

Nuclear compression leads to increased replication fork stalling

To directly test whether nuclear deformation can cause replication fork stalling and impair DNA replication, we applied external compression to adherent MDA-MB-231 cells. The compression assay allows precise control of the extent and timing of compression, is suitable for large cell numbers, and enables imaging of live or fixed cells before, during, and after compression. MDA-MB-231 cells were compressed to different heights and analyzed for incorporation of a nucleotide analog, EdU, to assess DNA synthesis rates (Salic and Mitchison, 2008). MDA-MB-231 cells subjected to severe compression had significantly reduced EdU incorporation than cells subjected to only mild compression (Fig. 5A-B), suggesting that nuclear deformation reduces DNA replication. To test if the impaired DNA replication during compression was due to replication fork stalling, we stained MDA-MB-231 cells for p-RPA S33 following compression. Cells compressed to a height of 2 µm had significantly more p-RPA S33 foci than cells compressed to the 5 µm control height (Fig. 5C-D), indicating that nuclear deformation increases replication fork stalling and replication stress in these cells.

To further investigate whether nuclear deformation during confined migration leads to increased replication fork stalling, we analyzed MDA-MB-231 and HT1080 cells labeled with GFP-PCNA for formation of new PCNA foci as they migrated through the microfluidic device. MDA-MB-231 cells, but not HT1080 cells, exhibited an increase in GFP-PCNA foci as they migrated through the small constrictions ($\leq 2 \times 5\ \mu m^2$) channels compared to the $15 \times 5\ \mu m^2$ control channels (Fig. 5E), indicating increased replication fork stalling in the MDA-MB-231 cells. Taken together, these findings indicate that mechanical deformation of the cell nucleus, for example, during confined migration or external force application, can lead to replication fork stalling, increased replication stress, and DNA damage.

Discussion

Using a systematic study with a broad panel of cell lines, we showed that DNA damage during confined migration or external cell compression can occur due to at least two separate, but overlapping mechanisms. In some cell lines (MDA-MB-231 and BT-549), nuclear deformation associated with the cell squeezing through tight spaces or external compression is sufficient to cause DNA damage, even without NE rupture, while in other cell lines (HT1080, Human fibroblasts, and RPE-1) DNA damage is predominantly associated with NE rupture. Deformation-induced DNA damage, unlike NE rupture-induced DNA damage, occurs primarily in the S/G2 phase of cell cycle. The deformation-induced DNA damage was located at stalled replication forks, indicating that nuclear deformation is sufficient to cause replication fork stalling and increase replication stress. We have thus identified a novel mechanism for DNA damage linking mechanical stress on the nucleus and the resulting nuclear deformation to increased replication stress in tumor cells.

While we identified cell line specific differences in the cause of DNA damage during confined migration, the molecular mechanisms underlying these differences remain unclear and subject to further investigation. The variability in the susceptibility of different cell lines to deformation-induced DNA damage and NE rupture-induced DNA damage could arise from multiple factors, including cell type or tissue of origin. Intriguingly, all of the cell lines tested that exhibited deformation-induced DNA damage had mutations in the gene encoding p53, while the cells exhibiting NE rupture-induced DNA damage had normal p53 function. Thus, it is tempting to speculate that functional p53 provides resistance to deformation-induced replication stress and DNA damage. Consistent with this idea, p53 can mediate replication stress and restart DNA replication at stalled forks, thereby preventing replication fork collapse and associated DNA damage (Roy et al., 2018). At the same time, differences in the susceptibility of cells to NE rupture-induced DNA damage could be due to differences in the expression of cytoplasmic nucleases (Nader et al., 2020; Hatch, 2018) or other factors.

In contrast to previous reports (Moriarty and Stroka, 2018; Pfeifer et al., 2018; Smith et al., 2019; Xia et al., 2019), we did not find evidence of increased cell cycle arrest during confined migration.

This could be due to differences in the experimental systems used in the various studies: previous experiments used either transwell plates (Pfeifer et al., 2018; Smith et al., 2019; Xia et al., 2019) or long microfluidic channels (Moriarty and Stroka, 2018) and assessed cell cycle progression after 12-24 hours of confined migration. In contrast, in our microfluidic devices, only parts of the cell are within the tight constriction at a given time, similar to the situation of cells migrating in collagen matrices (Wolf et al., 2013), and we evaluated cell cycle stage while the cell was moving during the constriction, which typically occurred within 1-3 hours (Fig. 1A). Thus, our results reflect the cell cycle stage at the time new DNA damage occurs, while the findings from previous reports may represent cell cycle arrest resulting from activation of DNA damage response pathways following migration-induced DNA damage.

Our findings suggest that deformation-induced DNA damage occurs at stalled replication forks due to increased replication fork stalling and replication stress associated with nuclear deformation during confined migration or external compression. However, the cause for this increase in replication stress is still unknown. One possible reason could be increased torsional stress in the DNA due to the physical deformation of the nucleus, which could alter DNA conformation and/or make it more difficult to unwind the DNA ahead of the replication fork. Aberrant activity of Mus81, an endonuclease that cleaves stalled replication forks and allows their resolution to prevent DNA DSBs, could also contribute for deformation-induced DNA damage at stalled forks (Regairaz et al., 2011). Moreover, ATR which plays an important role in mediating replication stress (Zeman and Cimprich, 2014) and has been reported to be mechanosensitive (Kumar et al., 2014), could also play a role in deformation-induced replication stress. Indeed, based on an analysis of the BIOGPS database (www.biogps.org), MDA-MB-231 cells, which experience deformation-induced DNA damage, have lower expression of ATR than HT1080 cells, which primarily exhibit NE rupture-induced DNA damage. This difference may provide one explanation for the increase in replication fork stalling and replication stress during confined migration in MDA-MB-231 cells.

In conclusion, we uncovered a novel link between mechanically induced nuclear deformation and replication stress. Insights from this work are not only relevant to cells migrating through confining environments, but also to other cells and tissues that experience large compressive forces, for example, during development, in mechanically active tissues such as cartilage, or inside solid tumors (Davidson et al., 2009; Heisenberg and Bellaiche, 2013; Mammoto and Ingber, 2010). Mechanically induced DNA damage could cause cell death, senescence, or, if not repaired properly, lead to mutations, genomic deletions, and/or translocations (Irianto et al., 2017a; Varga and Aplan, 2005). Taken together, these findings identify a new mechanism by which mechanical forces can lead to replication stress and increased genomic instability.

Materials and Methods

Cells and cell culture. The breast adenocarcinoma cell line MDA-MB-231 (ATCC HTB-26) and the breast ductal carcinoma cell line BT-549 (ATCC HTB-122) were purchased from American Type Culture Collection (ATCC); the fibrosarcoma cell line HT1080 (ACC 315) was a gift from Peter Friedl and Katarina Wolf and originally purchased from the DSMZ Braunschweig, Germany; the hTERT-immortalized retinal epithelial cell line RPE-1 was a gift from Marcus Smolka; the SV40-immortalized human fibroblasts were purchased from the Coriell Institute. MDA-MB-231, HT1080, RPE-1 and SV40-immortalized human fibroblasts cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS, Seradigm VWR) and 1% (v/v) penicillin and streptomycin (PenStrep, ThermoFisher Scientific). BT-549 cells were grown in RPMI 1640 media supplemented with 10% FBS and 1% PenStrep. All cell lines were cultured under humidified conditions at 37°C and 5% CO_2.

Generation of fluorescently labeled cell lines. All cell lines were stably modified with retroviral vectors to express the DNA damage reporter 53BP1-mCherry (mCherry-BP1-2-pLPC-Puro; obtained from Addgene: Plasmid #19835). Some cell lines were additionally modified to stably co-express one or more of the following lentiviral constructs (vectors listed in parenthesis): cell cycle reporter - FUCCI (pLenti6.2-IRES-G1-Orange-S-G2-M-Green-BlastiS, a gift from Katarina Wolf (Sakaue-Sawano et al., 2008)); replication reporter - GFP-PCNA (pHAGE-CMV-GFP-PCNA-BlastiS, a gift from Nima Mosammaparast (Brickner et al., 2017)) and nuclear rupture reporter - NLS-GFP (pCDH-CMV-NLS-copGFP-EF1-blastiS, (Denais et al., 2016), available through Addgene (#132772)).

Viral modification. Pseudoviral particles were produced as described previously (Hanson et al., 2008). In brief, 293-TN cells (System Biosciences, SBI) were co-transfected with the lentiviral plasmid and lentiviral helper plasmids (psPAX2 and pMD2.G, gifts from Didier Trono) using PureFection (SBI), following manufactures protocol. Retroviral particles were produced using 293-GPG cells, which contain the viral packaging plasmid inside. 293-GPG cells were also transfected with plasmid of interest using PureFection. Lentivirus or retrovirus containing supernatants were collected at 48 hours and 72 hours after transfection, and filtered through a 0.45 µm filter. Cells were seeded into 6-well plates so that they reached 50-60% confluency on the day of infection and transduced at most 3 consecutive days with the viral stock in the presence or absence (for BT-549 cell line) of 8 µg/mL polybrene (Sigma-Aldrich). The viral solution was replaced with fresh culture medium, and cells were cultured for 24 hours before selection with 1 µg/mL of puromycin (InvivoGen) or 6ug/mL of blasticidine S (InvivoGen) for 10 days. Cells were also sorted using fluorescence assisted cell sorting (FACS) to ensure expression of all the fluorescent reporters and maintained in a media with antibiotics to ensure continued plasmid expression.

Construct validation experiments. The 53BP1-mCherry construct used had a truncated version of the 53BP1 protein containing only the DSB binding domain, preventing downstream signaling

(Dimitrova et al., 2008). To validate the 53BP1-mCherry construct, cells expressing 53BP1-mCherry were treated with 20 µg/mL Phleomycin (Cayman Chemical), a radiation mimetic agent derived from Streptomyces, which induces DSBs. Cells were imaged every 10-15 min for accumulation of 53BP1-mCherry foci over a period of 1-2 hours after Phleomycin treatment. For GFP-PCNA construct validation, cells expressing GFP-PCNA plasmid were treated with 5mM HU (a gift from Marcus Smolka), a DNA replication inhibitor for 24 hours to induce replication fork stalling (Singh and Xu, 2016), and increase cellular PCNA expression. Fluorescent images were taken before and after drug treatment, blinded and analyzed for number of PCNA positive cells.

Fabrication and use of microfluidic migration devices. The devices were prepared as described previously (Davidson et al., 2014; Davidson et al., 2015). Microfluidic devices were first assembled by plasma treating the PDMS pieces and coverslips for 5 min, then immediately placing the PDMS pieces on the activated coverslips (pretreated with 0.2M HCl) and gently pressing to form a covalent bond. The finished devices were briefly heated on a hot plate at 95°C to improve adhesion. Devices were filled with 70% ethanol, then rinsed with autoclaved deionized water and coated with extracellular matrix proteins. For all cell lines, except human fibroblasts, devices were coated with 50 µg/mL type-I rat tail collagen (BD Biosciences) in acetic acid (0.02 N) overnight at 4°C. For human fibroblasts, devices were incubated with fibronectin (Millipore) in PBS (2-20 µg/mL) overnight at 4°C. After the incubation, devices were rinsed with PBS and medium before loading the cells (about 50,000-80,000 cells per chamber). Subsequently, devices were placed inside a tissue culture incubator for a minimum of 6-24 hours to allow cell attachment before mounting the devices on a microscope for live-cell imaging. The media reservoirs of the device were covered with glass coverslips to minimize evaporation during live cell imaging. Cells were imaged every 5-10 min for 14-16 hours in phenol-red free medium FluoroBrite DMEM supplemented with either 1% or 10% FBS. For experiments with NAC, cells were treated with 10 µM of NAC (Cayman Chemicals) dissolved in PBS starting one hour prior to the start of migration experiments and the treatment was continued for the length of the experiment.

Single cell invasion assay in collagen. The single cell invasion assays using collagen matrices were performed as described previously (Denais et al., 2016). Briefly, 5 mm round glass coverslips (Warner Instruments) were treated with 1% PEI for 10 min followed by 0.1% Glutaraldehyde for 30 min (to allow consistent bonding between the collagen matrix and the glass coverslip) and washed with PBS before adding the collagen solution. Collagen matrices containing MDA-MB-231 cells were prepared by mixing acidic collagen solution (Becton Dickinson) (supplemented with DMEM, PBS and NaOH to reach a neutral pH of 7.4) with MDA-MB-231 cells suspended in complete DMEM (density of 100,000 cells /mL). Collagen solution was allowed to polymerize at 37°C for 30 min before adding complete medium. Experiments were carried out 48-72 hours after seeding the cells in the collagen matrix.

Cell compression experiments. A custom-built compression device with a PDMS piston (a kind gift from Matthew Paszek) similar to a device published previously (Le Berre et al., 2012; Liu et

al., 2015) and a commercially available version (https://www.4dcell.com/cell-culture-systems/cell-confinement/dynamic-cell-confiner/) was used. Fluorescent beads of either 2, 3 or 5 µm diameter (Invitrogen) were used as spacers to determine the height of compression. Cells were seeded at a density of 10,000/cm^2 on 35-mm dishes with 14-mm glass coverslips (MatTek) and allowed to adhere overnight inside a tissue culture incubator at 37°C. The next day, cells were washed with PBS, supplemented with FluoroBrite media containing the fluorescent beads, and compressed using the device. To induce suction required to lower the PDMS piston for compression, a syringe pump (New Era Syringe Pump Systems, Inc.) with a 60 mL syringe was operated at a flow rate of 0.5mL/min for 2 min. The suction was maintained at 0.01-0.1 mL/min throughout the course of imaging (2 hours) for all experiments. For release of compression, air was pushed inside at the rate of 0.5 mL/min till the PDMS piston was completely released (about 5-8 min).

DNA content analysis. Cell cycle distribution was evaluated using DNA content assay as described previously (Darzynkiewicz et al., 2010). Briefly, cells were trypsinized, collected and fixed with 70% Ethanol, on ice for 30 min. Cells were washed and incubated with PBS containing 5µg/mL RNase A (Omega Bio-Tek) and 10µg Propidium Iodide (PI, Invitrogen) for 15 min at 37°C, followed by cell sorting using Accuri C6 Flow Cytometer (BD Biosciences). A linear gate was placed on the forward and side scatter plot, to eliminate debris and collect only intact cell data. An additional gate on a secondary graph plotting propidium iodide total fluorescence intensity area vs. height was used to exclude doublets and triplets. Samples were run at 35 µL/min, with a core size of 16 µm, until 100,000 gated cells were recorded. DNA content levels were recorded by plotting propidium iodide intensity vs. cell count on a linear scale. Peaks were used to estimate cell cycle phase, and cell count per phase was determined using the Accuri C6 Plus software (BD Biosciences).

Immunofluorescence staining. For validation of the 53BP1-mCherry construct, co-immunofluorescence staining for ɤ-H2AX was performed. Cells cultured on cover slips (pretreated with fibronectin) overnight were fixed with 4% PFA for 20 min at 37°C, permeabilized with PBS containing 0.25% Triton X-100 for 15 min at room temperature, washed, and stained with anti- ɤ-H2AX antibody (Millipore, dilution 1:500). For staining after compression, cells were treated with extraction solution (containing HEPES, NaCl, EDTA, Sucrose, $MgCl_2$ and 0.5% Triton X-100) for 15 min on ice followed by fixation with 4% PFA for 20 min at 37°C, permeabilized with PBS containing 0.25% Triton X-100 for 15 min at room temperature, washed, and stained with anti-p-RPA32 (S33) antibody (Bethyl Laboratories, Inc.; dilution 1:1000). For EdU labeling, cells were pulsed with 10 µM EdU (Jena Bioscience) for 2 hours while they were compressed to different heights. After compression, cells were fixed with 4% PFA for 20 min followed by permeabilization with PBS containing 0.25% Triton X-100 for 15 min at room temperature, washed and labelled for EdU using click-chemistry as described previously (Salic and Mitchison, 2008).

Fluorescence and confocal microscopy. Microfluidic device migration experiments, compression experiments, construct validation experiments, and immunofluorescence staining were imaged on inverted Zeiss Observer Z1 microscopes equipped with temperature-controlled stages (37°C) and CCD camera (Photometrics CoolSNAP KINO) using 20× air (NA = 0.8), 40× water (NA = 1.2) and 63× oil (NA = 1.4) immersion objectives. Airy units for all images were set between 1.5 and 2.5. The image acquisition for migration experiments was automated through ZEN (Zeiss) software with imaging intervals for individual sections between 5-10 min. For live-cell imaging of cell compression, Z-stacks were acquired from fluorescence, reflection and transmission channels in sequential order every 10 min for 2 hours. Images were also taken before compression was started for direct comparison.

AFM-LS. MDA-MB-231 cells were cultured in DMEM/F12 media without phenol red supplemented with 10% FBS (Sigma-Aldrich), 1× antimycotic (Gibco) and 15mM HEPES. A day before the experiment, 50-70% confluent cultures were trypsinized and plated on polyacrylamide gels (stiffness of 55 kPa) coated with collagen as described before (Nelsen et al., 2020). Briefly, 10 µL of activated gel solution was deposited on APTES-treated 40 mm round coverslips, allowed to dry, and attached to a 10 mm diameter glass cloning cylinder (Corning) using vacuum grease (Dow Corning). The gel with cloning rings was treated with EDAC and NHS in PBS, inside the incubator, followed by PBS washes before coating with 50 µg/mL collagen (Rat Tail Type I, Invitrogen) for 30 minutes at 37°C. The collagen solution was washed with PBS and DMEM/F12 medium before adding cells. Cells were plated such that only 1-3 cells were present per field of view at 60× magnification. To compress individual nuclei, we employed the use of atomic force microscopy (AFM) along with light-sheet (LS) microscopy as described previously (Liu et al., 2019; Nelsen et al., 2020). Briefly, a beaded cantilever (beaded in house and calibrated using thermal tuning as described in (Gavara, 2017)) was mounted onto the AFM and lowered over a cell of interest. A small (180 µm) mirror (8531-607-1, Precision Optics Corporation, USA) was placed adjacent to the cell of interest and the objective lens (UplanSAPO 60×/1.2 W, Olympus, Japan) was raised such that the image plane intersected the mirror in order to achieve side-view imaging. A vertical light sheet propagates out of the objective lens and an electrically tunable lens (ETL) was used to lower the waist of the light sheet such that it lied within the cell. Volumetric images were acquired before, during and after compression. Cells were compressed to an approximate height of 2 µm by lowering the cantilever at the rate of 250 nm/s. For control cells, the cantilever was lowered to indent the cell only a few hundred nanometers. Images were acquired for 20 minutes with compression before retracting the cantilever at the rate of 250 nm/s. Cells were imaged for 5 minutes after retraction as well. The AFM-LS system used has an objective lens heater (Hk-100, Thorlabs, Inc, USA) with a PIV controller (Thorlabs, Inc, USA) and a heated scanning stage (Oxford Instruments, UK) to maintain the temperature of the sample at 37°C.

Lattice light-sheet Microscopy (LLSM). Single cell invasion assay in collagen was imaged using the lattice light sheet microscope (LLSM) (Heisenberg and Bellaiche, 2013) housed in the

Advanced Imaged Center at the Howard Hughes Medical Institute Janelia Research Campus. The system is configured and operated as previously described (Chen et al., 2014). Samples were illuminated by a 2D optical lattice generated by a spatial light modulator (SLM, Fourth Dimension Displays). The light-sheet pattern was a square lattice with minimum NA of 0.44 and a maximum NA of 0.54. The sample is excited by 488 nm and 560 nm diode lasers (MPB Communications) at 50% AOTF transmittance and 100 mW initial box power through an excitation objective (Special Optics, 0.65 NA, 3.74-mm WD). Fluorescent emission was collected by detection objective (Nikon, CFI Apo LWD 25XW, 1.1 NA), and detected by a sCMOS camera (Hamamatsu Orca Flash 4.0 v2) at 100 ms exposure time. Acquired data were deskewed as previously described (Chen et al., 2014) and deconvolved using an iterative Richardson-Lucy algorithm. Point-spread functions for deconvolution were experimentally measured using 200nm tetraspeck beads adhered to 5 mm glass coverslips (Invitrogen) for each excitation wavelength.

Image analysis. Image sequences were analyzed using ZEN (Zeiss), ImageJ (Schindelin et al., 2012) or Imaris (BitPlane) using only linear intensity adjustments uniformly applied to the entire image region. For DNA damage analysis, the number of 53BP1 foci were manually counted before, during and after transit through microfluidic channels. Nuclear rupture was detected by an increase of the cytoplasmic NLS-GFP signal. Cell cycle stage was determined by the fluorescence signal of the FUCCI reporter – red was counted as G0/G1 cell cycle stage, and green as S/G2 cell cycle stage. Co-localization of PCNA and 53BP1 was counted manually using image sequences of cells migrating through microfluidic devices. For confocal image stacks of compression experiments, images were three-dimensionally reconstructed and displayed as maximum intensity projections. DNA damage analysis was performed by manually counting 53BP1 foci on these maximum intensity projections of the confocal image stacks. A total of about 200-1000 cells were counted for each condition in the microfluidic migration and compression experiments. Cells were excluded if there was excessive 53BP1 foci at the start of the experiment preventing analysis of new foci for both migration and compression experiments. For AFM-LS experiments, 53BP1 foci were counted manually by examining the full 3D image stacks as well as maximum intensity projections. A total of n = 21 compressed cells and n = 19 control cells were included in the analysis. Three cells (all compression experiments) were excluded from analysis because of excessive 53BP1 foci at the start of the experiment, and two other cells (one control, one compression) were excluded because of ambiguity in whether or not a new 53BP1 focus had formed. To find new 53BP1 foci appearance during migration through collagen matrices, foci were tracked as surfaces over time using Imaris. A total of n = 32 cells were analyzed. For PCNA and 53BP1 foci counting in cells migrating through collagen matrices, surfaces were created using Imaris for both PCNA and 53BP1 foci and shortest surface-surface distance was calculated for each tracked point. A total of n = 21 cells were analyzed. Graphs were generated in Excel (Microsoft), and figures were assembled in Illustrator (Adobe).

Statistical analysis. Unless otherwise noted, all experimental results are from at least three independent experiments. For data with normal distribution, we used either two-sided Student's t-tests (comparing two groups) or one-way analysis of variance (ANOVA) (for

experiments with more than two groups) with post hoc tests. For experiments with two variables and more than two groups, two-way ANOVA was used with post hoc tests. All tests were performed using GraphPad Prism. Welch's correction for unequal variances was used with all *t*-tests, comparing two groups. Post hoc multiple-comparisons testing with Dunnett's correction was performed to determine differences between 5 µm, 3 µm and 2µm compression heights for all compression experiments involving three different heights. For experiments involving individual cells, such as the AFM-LS compression experiments and migration through collagen matrices, a Fisher's test was used. Post hoc multiple-comparisons testing with Tukey's correction was used to determine differences in G0/G1 and S/G2 phases across multiple groups and to determine differences in increase in PCNA foci between HT1080 and MDA-MB-231 cells migrating through confined and control spaces. Unless otherwise indicated, error bars represent s.e.m. Data supporting the findings of this study are available from the corresponding author on reasonable request.

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Figures

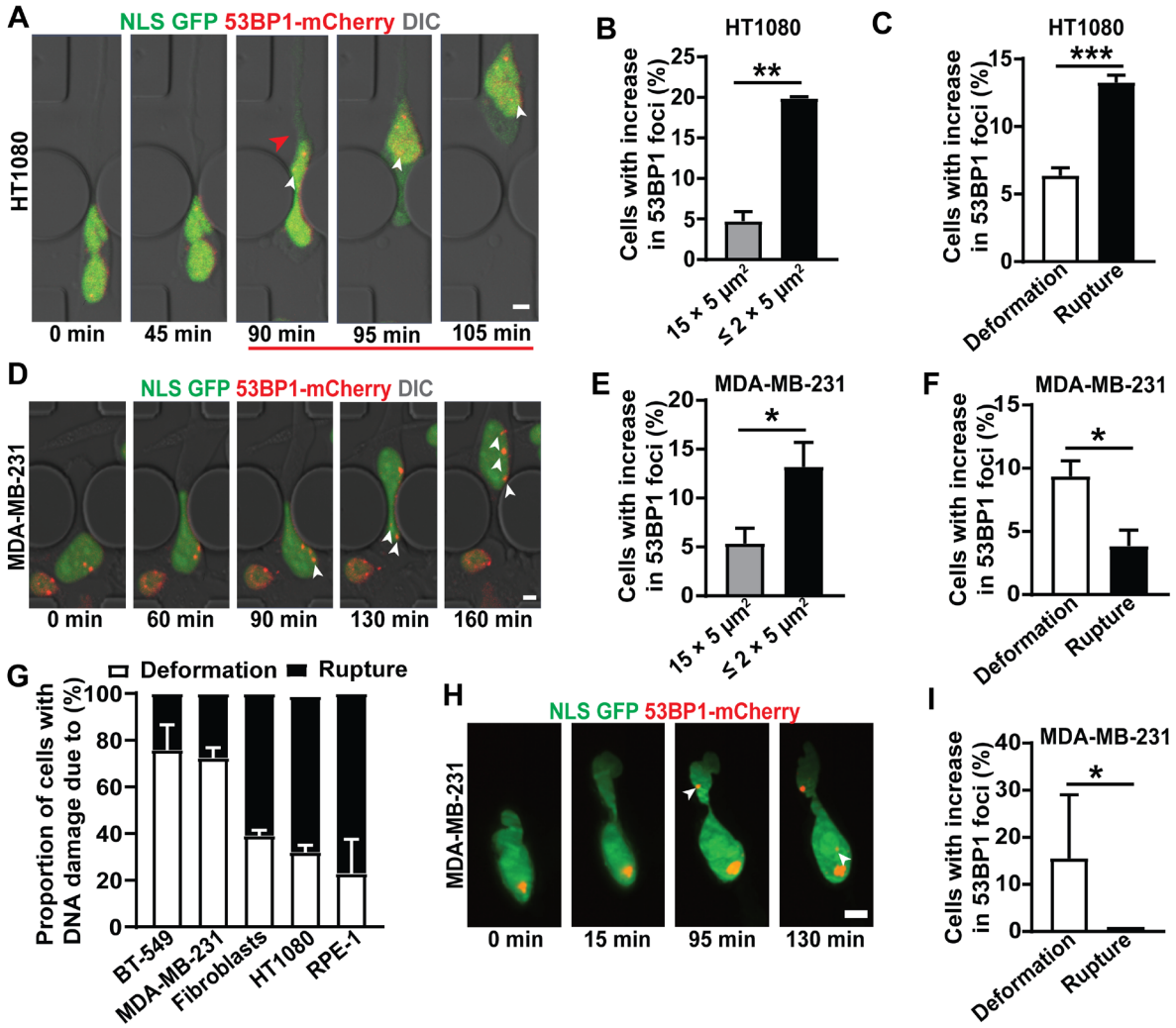

Figure 1: NE rupture and nuclear deformation lead to DNA damage during confined migration. (A) Representative image panel showing a HT1080 fibrosarcoma cell co-expressing NLS-GFP and 53BP1-mCherry exhibiting DNA damage following NE rupture during migration through a 1 × 5 µm^2 constriction in the microfluidic device. Red arrowhead indicates start of NE rupture; the red line indicates the duration of NE rupture; white arrowheads indicated new 53BP1-mCherry foci. Scale bar: 5 µm **(B)** Percentage of HT1080 cells with new DNA damage (53BP1-mCherry foci) during migration through small ≤ 2 × 5 µm^2 constrictions (n = 372 cells) or 15 × 5 µm^2 control channels (n = 268 cells). **, $p < 0.01$ based on unpaired t-test with Welch's correction. **(C)** Percentage of HT1080 cells in which new DNA damage during migration through ≤ 2 × 5 µm^2 constrictions was associated with either NE rupture or with nuclear deformation in the absence of NE rupture. n = 372 cells; ***, $p < 0.001$ based on unpaired t-test with Welch's correction. **(D)** Representative image sequence showing a MDA-MB-231 breast cancer cell co-expressing NLS-GFP and 53BP1-mCherry experiencing new DNA damage during migration through a 2 × 5 µm^2 constriction. White arrowheads indicate new 53BP1-mCherry foci. Scale bar: 5 µm **(E)** Percentage

of MDA-MB-231 cells with new DNA damage (53BP1-mCherry foci) during migration through small ≤ 2 × 5 μm^2 constrictions (n = 381 cells) or 15 × 5 μm^2 control channels (n = 196 cells). *, $p < 0.05$ based on unpaired t-test with Welch's correction. **(F)** Percentage of MDA-MB-231 cells in which new DNA damage during migration through ≤ 2 × 5 μm^2 constrictions was associated with either NE rupture or with nuclear deformation in the absence of NE rupture. *, $p < 0.05$ based on unpaired t-test with Welch's correction. **(G)** Association of new DNA damage incurred during migration through ≤ 2 × 5 μm^2 constrictions with either NE rupture (Rupture) or nuclear deformation without NE rupture (Deformation), for a panel of cell lines. The results correspond to the data presented in Fig. 1C and 1F, and Suppl. Fig. 2D-F. **(H)** Representative image sequence of MDA-MB-231 cell co-expressing NLS-GFP and 53BP1-mCherry incurring DNA damage during migration in a dense (1.7 mg/ml) collagen matrix. White arrowheads indicate new 53BP1-mCherry foci. Scale bar: 5 µm **(I)** Percentage of MDA-MB-231 cells in which new DNA damage during migration through a dense (1.7 mg/ml) collagen matrix was associated with either NE rupture or with nuclear deformation in the absence of NE rupture. n = 32 cells, *, $p < 0.05$ based on Chi-square test.

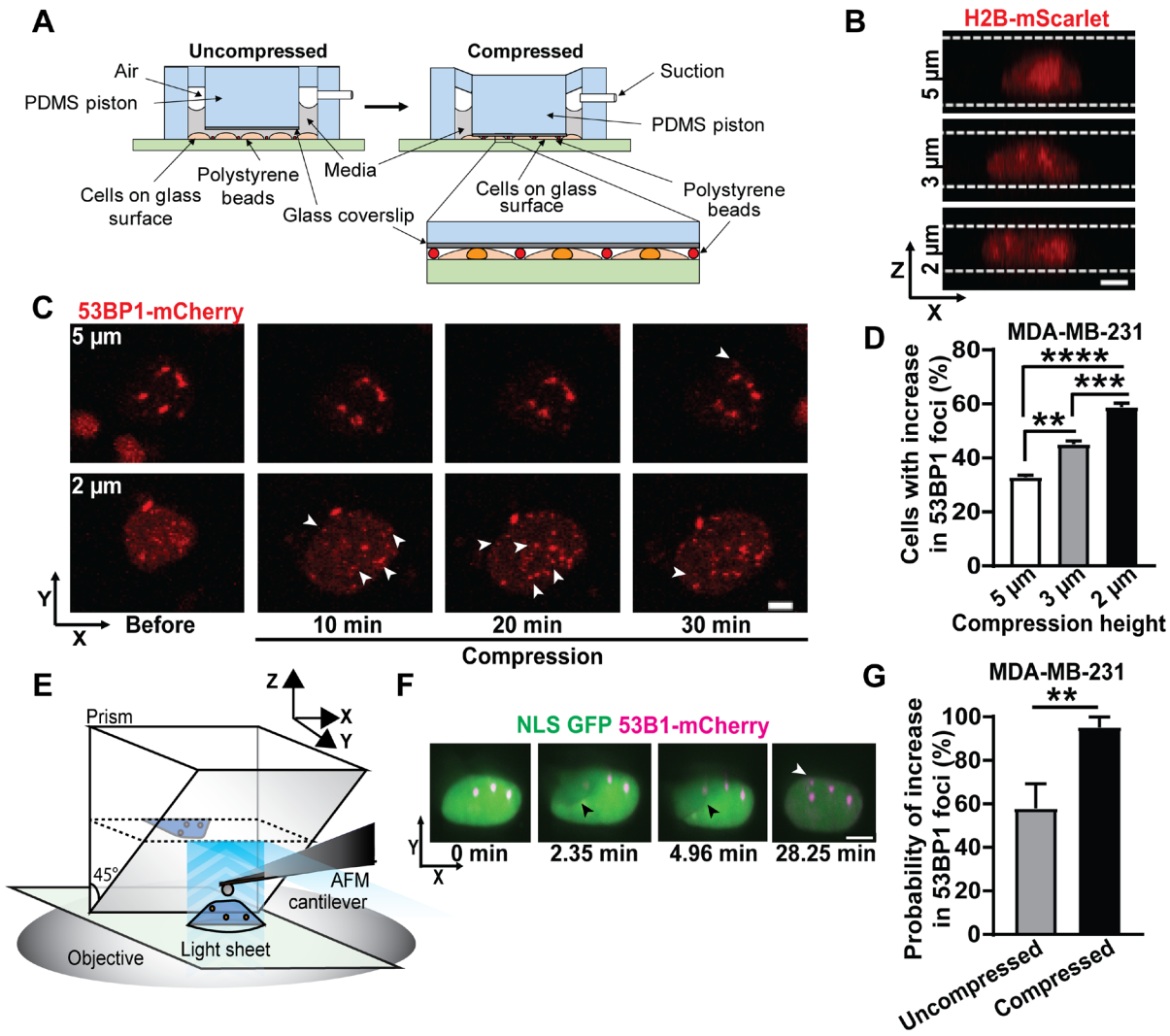

Figure 2: Nuclear compression is sufficient to cause DNA damage. (A) Schematic of the custom-built microfluidic compression device with a PDMS piston. The device is connected to a suction source which causes the PDMS piston, with a small circular coverslip attached, to move down onto the cells. Polystyrene beads serve as spacers to ensure a uniform height between the class coverslip and the glass bottom of the dish. Inset shows cells compressed between the PDMS piston with attached cover slip and the glass surface and the polystyrene beads. **(B)** Representative image sequence showing the nuclear height of a MDA-MB-231 breast cancer cell expressing H2B-mScarlet, compressed to either 5 µm, 3 µm, or 2 µm height using the compression device. White lines indicate the height of the compressed cell. Scale bar: 5 µm **(C)** Representative image sequence showing a MDA-MB-231 breast cancer cell expressing 53BP1-mCherry with new DNA damage formation during compression to either 5 µm (top) or 2 µm (bottom) height. White arrowheads indicate new 53BP1-mCherry foci; black line indicates the duration of compression. Scale bar: 5 µm **(D)** Percentage of MDA-MB-231 cells with new DNA damage (53BP1-mCherry foci) during compression to 5 µm height (n = 500 cells), 3 µm height (n = 378 cells), or 2 µm height (n = 411 cells). **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, based

on ordinary one-way ANOVA with Dunnett's multiple comparison test. **(E)** Schematic overview of the AFM-LS system. A micro-mirror is lowered adjacent to a cell of interest and a vertical light sheet propagates out of the objective illuminating a x-z cross-section of the cell. The image plane is raised to intersect the mirror, capturing the virtual image created by the mirror. The AFM cantilever is positioned between the mirror and cell in order to probe the cell from above while imaging the side-view cross section. **(F)** Representative image sequence showing a MDA-MB-231 breast cancer cell co-expressing NLS-GFP and 53BP1-mCherry experiencing new DNA damage during compression to a height of ~2 µm with an AFM cantilever. Black arrowhead indicates the AFM cantilever; white arrowhead indicates new 53BP1-mCherry foci. Scale bar: 5 µm. **(G)** Percentage of MDA-MB-231 cells with new DNA damage (53BP1-mCherry foci) during compression by an AFM tip (n = 21 cells) or in uncompressed control conditions (n = 19 cells). **, $p < 0.01$ based on Fisher's test.

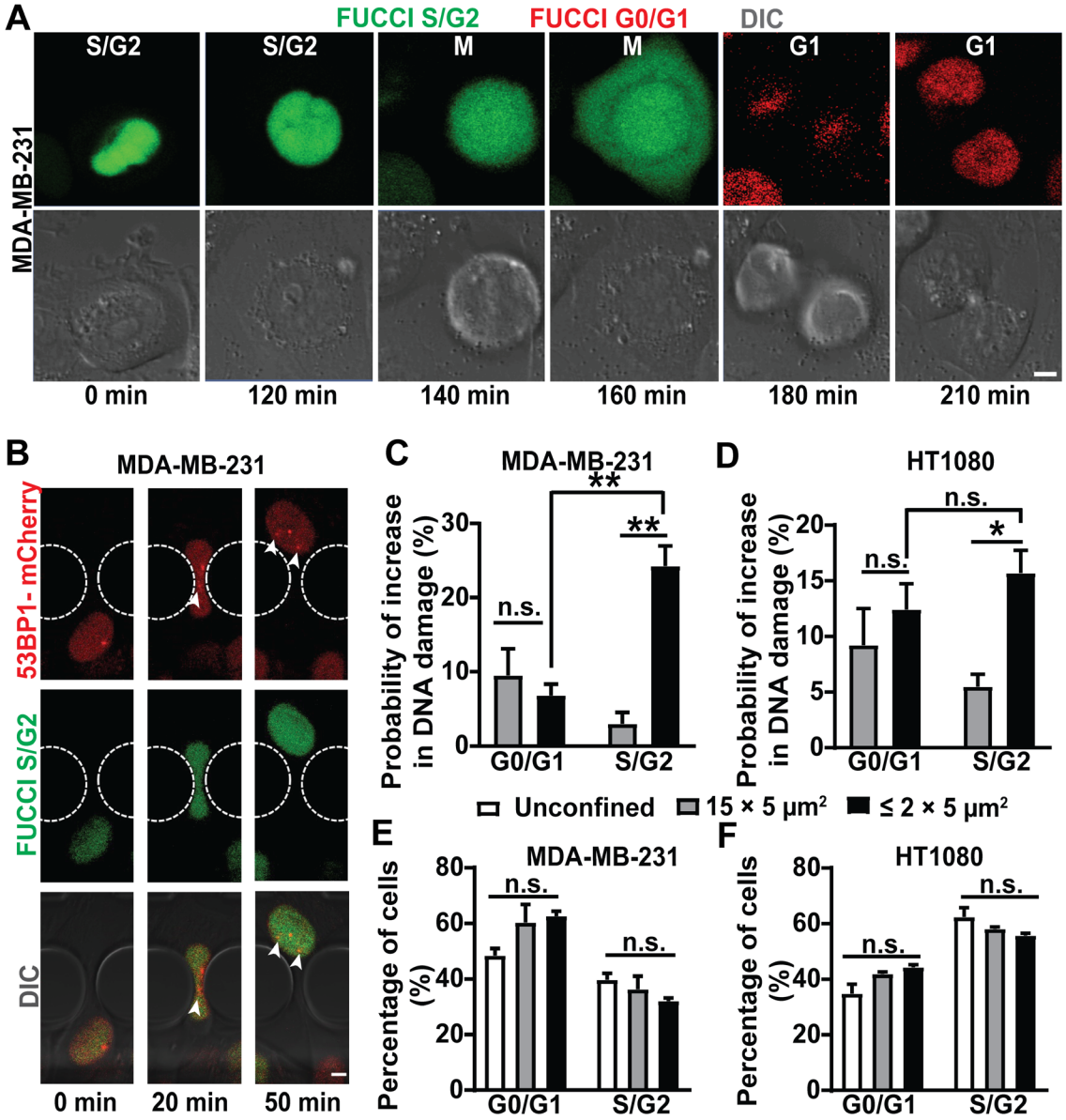

Figure 3: Deformation associated DNA damage occurs specifically in S/G2 phase of the cell cycle. (A) Representative image sequence showing a MDA-MB-231 breast cancer cell expressing FUCCI reporter transitioning from S/G2 to M and to G1 cell cycle phase. Scale bar: 5 µm. **(B)** Representative image sequence of a MDA-MB-231 breast cancer cell co-expressing FUCCI and 53BP1-mCherry experiencing new DNA damage while in S/G2 phase of the cell cycle during migration through a 2 × 5 µm^2 constriction. White arrowheads indicate new 53BP1-mCherry foci. Scale bar: 5 µm. **(C)** Probability of increase in DNA damage (53BP1-mCherry foci) during migration of MDA-MB-231 cells through small ≤ 2 × 5 µm^2 constrictions (n = 327 cells) or 15 × 5 µm^2 control channels (n = 145 cells) as a function of cell cycle phase (G0/G1 or S/G2). **, $p < 0.01$ based on two-way ANOVA with Tukey's multiple comparison test. **(D)** Probability of increase in DNA damage (53BP1-mCherry foci) during migration of HT1080 fibrosarcoma cells through small (≤ 2 × 5 µm^2) constrictions (n = 850 cells) or 15 × 5 µm^2 control channels (n = 371 cells) as a function

of cell cycle phase (G0/G1 or S/G2). *, $p < 0.05$ based on two-way ANOVA with Tukey's multiple comparison test. **(E)** Percentage of MDA-MB-231 cells in G0/G1 or S/G2 phase of the cell cycle in unconfined conditions (n = 3544 cells), or during migration through small (≤ 2 × 5 μm^2) constrictions (n = 327 cells) or 15 × 5 μm^2 control channels (n = 145 cells). Differences were not statistically significant (n.s.) based on two-way ANOVA. **(F)** Percentage of HT1080 cells in G0/G1 or S/G2 phase of the cell cycle in unconfined conditions (n = 6108 cells) or during migration through small (≤ 2 × 5 μm^2) constrictions (n = 850 cells) or 15 × 5 μm^2 control channels (n = 371 cells). Differences were not statistically significant based on two-way ANOVA.

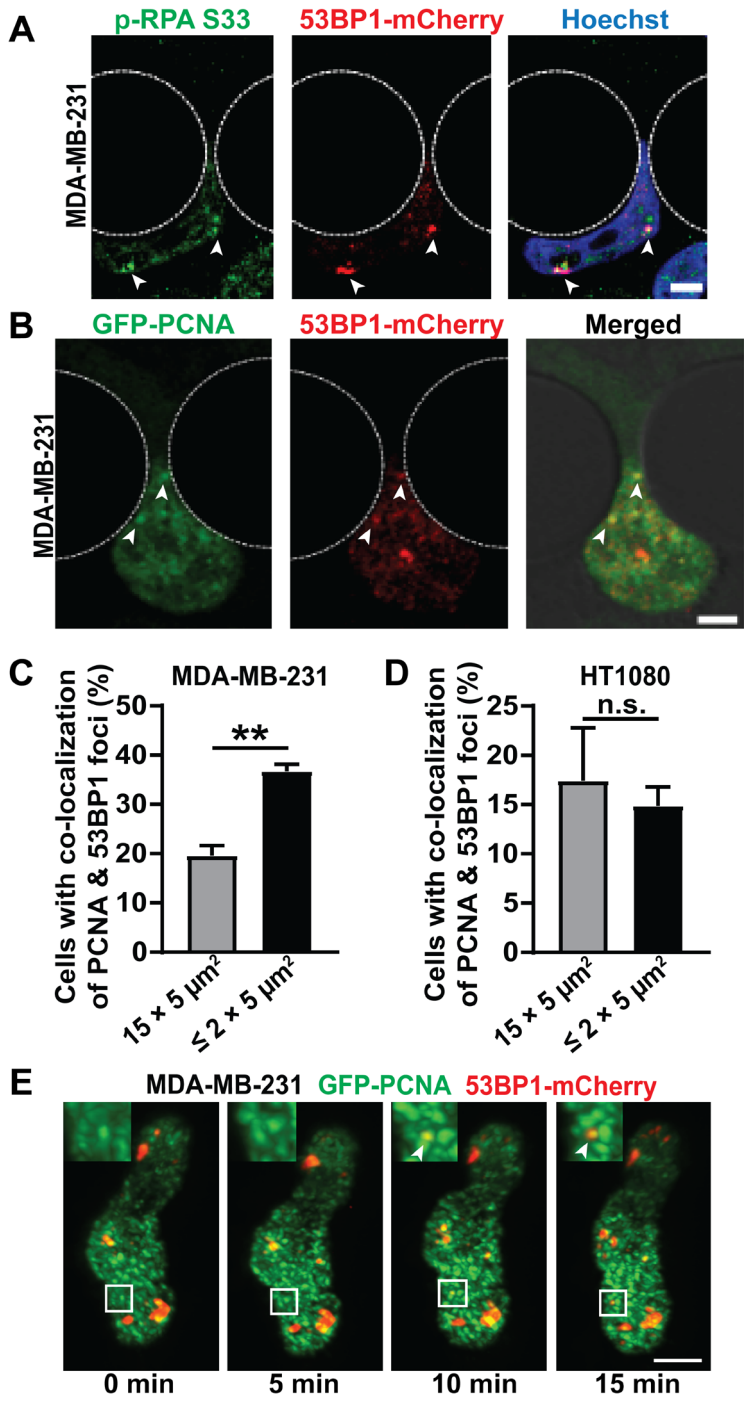

Figure 4: Deformation induced DNA damage occurs at stalled replication forks: (A) Representative image panel of an MDA-MB-231 breast cancer expressing 53BP1-mCherry during migration through a 1 × 5 μm^2 constriction, stained for p-RPA S33 to reveal co-localization between p-RPA S33 foci and 53BP1-mCherry foci. White arrowheads indicate sites of co-localization. Scale bar: 5 µm **(B)** Representative image panel showing a MDA-MB-231 breast cancer cell co-expressing GFP-PCNA and 53BP1-mCherry experiencing new DNA damage at stalled forks during migration through a 2 × 5 μm^2 constriction. White arrowheads indicate sites

of co-localization between new 53BP1-mCherry foci and GFP-PCNA foci. Scale bar: 5 µm. **(C)** Percentage of MDA-MB-231 cells with co-localization between new DNA damage (53BP1-mCherry foci) and stalled replication forks (GFP-PCNA foci) during migration through small (≤ 2 × 5 μm^2) constrictions (n = 584 cells) or 15 × 5 μm^2 control channels (n = 490 cells). **, $p < 0.01$ based on unpaired t-test with Welch's correction. **(D)** Percentage of HT1080 cells with co-localization between new DNA damage (53BP1-mCherry foci) and stalled replication forks (GFP-PCNA foci) during migration through small (≤ 2 × 5 μm^2) constrictions (n = 986 cells) or 15 × 5 μm^2 control channels (n = 641 cells). Differences were not statistically significant based on unpaired t-test. **(E)** Representative image sequence of MDA-MB-231 cell co-expressing GFP-PCNA and 53BP1-mCherry incurring DNA damage at stalled forks during migration in a dense (1.7 mg/ml) collagen matrix. Inset depicts close-up of the region inside the white rectangle to show occurrence of new 53BP1-mCherry foci at stalled replication forks marked by GFP-PCNA foci. White arrowheads indicate site of co-localization between 53BP1-mCherry and GFP-PCNA foci. Scale bar: 5 µm.

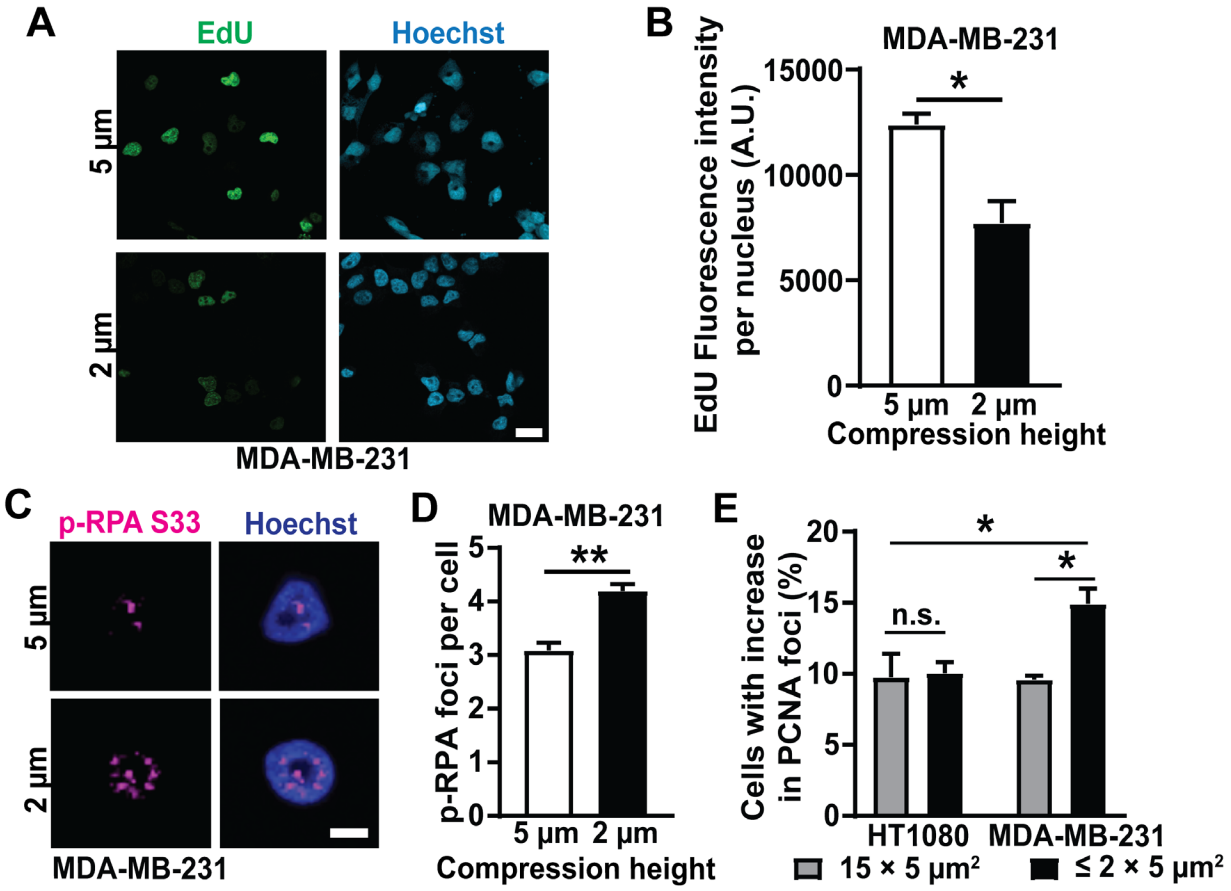

Figure 5: Nuclear deformation leads to increased replication stress. (A) Representative images of MDA-MB-231 breast cancer showing EdU incorporation during either mild (5 µm height, top) or severe (2 µm height, bottom) compression. Scale bar: 20 µm. **(B)** Fluorescence intensity of incorporated EdU per nucleus in MDA-MB-231 cells following compression to either 5 µm height (n = 171 cells) or 2 µm height (n = 184 cells) for 2 hours. *, $p < 0.05$ based on unpaired t-test with Welch's correction. **(C)** Representative images of MDA-MB-231 breast cancer cells showing stalled replication forks (p-RPA S33 foci) during compression to either 5 µm or 2 µm height. Scale bar: 5 µm. **(D)** Average number of p-RPA S33 foci in MDA-MB-231 cells following compression to 5 µm height (n = 247 cells) or 2 µm height (n = 318 cells) for 2 hours. **, $p < 0.01$ based on unpaired t-test with Welch's correction. **(E)** Percentage of MDA-MB-231 and HT1080 cells with increase in stalled replication forks (GFP-PCNA foci) during migration through small (≤ 2 × 5 µm^2) constrictions (n = 584 cells for MDA-MB-231; n = 986 cells for HT1080) or 15 × 5 µm^2 control channels (n = 490 cells for MDA-MB-231; n = 641 cells for HT1080). *, $p < 0.05$ based on two-way ANOVA with Tukey's multiple comparison test.

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