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Chromosome bridge resolution: Unravelling cell-cycle-dependent mechanisms of biochemical and mechanical nature

Marina Rodríguez Muñoz

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Chromosome bridge resolution: Unravelling cell-cycle-dependent mechanisms of biochemical and mechanical nature

Marina Rodríguez Muñoz

Memòria presentada per Marina Rodriguez Muñoz per optar al Grau de Doctora en Biologia Cel·lular amb Menció internacional per la Universitat Autònoma de Barcelona dins el Programa de Doctorat en Biologia Cel·lular

Directores

Dra. Teresa Anglada Pons

Dra. Anna Genescà Garrigosa

Bellaterra, 2024

La Dra. Teresa Anglada Pons, investigadora post-doctoral del Departament de Biologia Cel·lular, de Fisiologia i d'Immunologia de la Universitat Autònoma de Barcelona,

i la Dra. Anna Genescà Garrigosa, catedràtica del Departament de Biologia Cel·lular, de Fisiologia i d'Immunologia de la Universitat Autònoma de Barcelona,

CERTIFIQUEN:

Que Marina Rodríguez Muñoz ha realitzat sota la seva direcció el treball d'investigació que s'exposa a la memòria titulada "***Chromosome bridge resolution: Unravelling cell-cycle-dependent mechanisms of biochemical and mechanical nature***" per optar al grau de Doctora per la Universitat Autònoma de Barcelona.

Que aquest treball s'ha dut a terme a la Unitat de Biologia Cel·lular del Departament de Biologia Cel·lular, de Fisiologia i d'Immunologia de la Universitat Autònoma de Barcelona i que Marina Rodriguez Muñoz ha fet dues estades al laboratori del Dr. Neil Ganem a la Boston University.

I perquè així consti, firmen el present certificat a Bellaterra, el 14 de febrer de 2024

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ABSTRACT

Chromosome bridge is a common intermediate in mechanisms generating chromosomal instability. Although chromosome bridges are known to be involved in tumorogenesis and tumor progression, the processes governing their resolution remain poorly understood. Diverse models have emerged regarding their resolution; however, these models are conflicting with one another. This dissertation seeks to elucidate the mechanisms involved in chromosome bridge resolution. Our research demonstrates that chromosome bridges can undergo breakage during both cell division and interphase, with different mechanisms governing their breakage at each stage of the cell cycle.

In this thesis, we have used an experimental model based on the CRISPR/Cas9 technology to generate chromosome bridges with defined geometry under exquisite temporal control. Our study revealed that at the exit of mitosis, the resolution of chromosome bridges is intricately linked to the tension forces exerted by the microtubules of the mitotic spindle on the bridge kinetochores. At the exit of mitosis, the length of the bridging chromatin in megabases influences both the timing and the minimum separation between bridge kinetochores needed for bridge breakage. After mitosis, the endonuclease ANKLE1 contributes to the resolution of chromosome bridges as its inhibition led to an increased frequency of chromosome bridges arriving to the interphase of the cell cycle. Notably, we found that ANKLE1 can also resolve bridges during early interphase without necessitating nuclear envelope rupture. Conversely, an additional mechanism that operates during interphase relies on nuclear envelope rupture. Focal adhesions anchoring the bridge to the substrate combined with cell migration results in DNA breakage and nuclear envelope rupture, allowing access to cytoplasmic exonucleases, such as TREX1 for the resolution of chromosome bridges at this stage of the cell cycle.

Altogether, the findings presented in this doctoral dissertation demonstrate that chromosome bridges can undergo breakage at different stages throughout the cell cycle, revealing a diverse and distinctive set of mechanisms governing chromosome bridge breakage.

ABREVIATIONS

53BP1:	p53 binding protein
ANKLE1:	Ankyrin Repeat and LEM Domain Containing 1
APC/C:	Anaphase promoting complex o cyclosome
APOBEC:	C ->U-editing enzyme APOBEC
ATM:	Ataxia-Telangiectasia Mutated
ATR:	Ataxia-Telangiectasia and rad3 related
ATRIP:	ATR-interacting protein
BAF:	Barrier to autointegration factor
BCL9L:	B cell CLL/lymphoma 9-like
BFB:	Breakage-Fusion-Bridge
BLM:	Bloom syndrome protein
BRCA1:	Breast cancer type I Susceptibility
Cas9:	CRISPR associated protein 9
CDK:	Cyclin-dependent kinases
cGAMP:	Cyclic guanosine monophosphate–adenosine monophosphate
cGAS:	Cyclic GMP-AMP synthase
Chk1:	Checkpoint kinase 1
Chk2:	Checkpoint kinase 2
CIN:	Chromosome instability
CK2:	Casein Kinase 2
CRISPR:	Clustered Regularly Interspaced Short Palindromic Repeats
CtIP:	CtBP-interacting protein
DDR:	DNA Damage Response
DNA:	Deoxyribonucleic acid
DNA-PKcs:	DNA-dependent protein kinase catalytic
dNTPs:	Deoxynucleotide triphosphates
DSB:	Double Strand break
EA:	Early Anaphase
ER:	Endoplasmic reticulum
ERK:	Mitogen-activated protein kinase 1
ESCRT-III:	Endosomal sorting complexes required for transport III

ET:	Early Telophase
EXO1:	Exonuclease 1
GFP:	Green Fluorescent Protein
H2B:	Histone H2B
HR:	Homologous recombination
IFNI:	Type I interferon
KIF4A:	Kinesin Family Member 4A
Ku70:	X-ray repair cross-complementing protein 6
Ku80:	X-ray repair cross-complementing protein 5
LA:	Late Anaphase
Lap2β:	Lamina-associated polypeptide 2
LINC:	Linker of nucleoskeleton and cytoskeleton
Lmn A:	Lamin A
Lmn C:	Lamin C
LT:	Late Telophase
Mad2:	Mitotic spindle checkpoint component MAD2
Mcm helicase:	Minichromosome maintenance helicase
MDC1:	Mediator of DNA Damage Checkpoint 1
Mdm2:	Mouse Doble Minute 2
MEK:	Mitogen-activated protein kinase kinase
MIS:	Microsatellite Instability
MN:	Micronuclei
MRN:	MRE11-RAD50-NBS1
MYC:	Myc proto-oncogen protein
NE:	Nuclear envelope
NER:	Nuclear Envelope Rupture
NHEJ:	Non-homologous end-joining
NPC:	Nuclear Pore Complexes
ORC:	Origin Recognition Complex
p16:	cyclin-dependent inhibitor 2A
p21:	Cyclin dependent kinase inhibitor 1A
p53:	Cellular tumor antigen p53
PAM:	Protospacer adjacent motif
PIKKs:	Phosphatidylinositol 3-kinase-like protein kinase
POT1:	Protection of Telomeres 1

RAD51:	DNA repair protein RAD51 homolog 1
RAF:	Rapidly accelerated fibrosarcoma
RAP1:	Repressor-Activator Protein 1
RAS:	H-RAS protein
RFP:	Red Fluorescent Protein
RhoA:	Transforming protein RhoA
RNA:	Ribonucleic acid
RNF168:	ring finger protein 168
RNF8:	Ring Finger Protein 8
RPA32:	Replication protein 2
SAC:	Spindle Assembly Checkpoint
S-CIN:	Segmental Chromosome Instability
sgRNA:	single guide RNA
ssDNA:	single stranded DNA
STING:	Stimulator of interferon genes protein
STRIDE:	Sensitive Recognition of Individual DNA Ends
TERC:	telomerase RNA component
TERT:	telomerase reverse transcriptase
TIN2:	TRF1-Interacting protein
TOPBP1:	DNA topoisomerase 2-binding protein 1
TPP1:	Tripeptidyl peptidase I
TREX1:	Three-prime repair exonuclease 1
TRF1:	Telomere Repeat Binding factor 1
TRF2:	Telomere Repeat Binding factor 2
W-CIN:	Whole Chromosome Instability
XLF:	XRCC4-like factor
XRCC4:	X-ray repair cross-complementing protein
γH2AX:	Phosphorylation of H2AX histone family member X

INTRODUCTION

Cancer accounts for approximately one in six deaths worldwide (WHO, 2020). During the multistep development of human neoplasms, transformed cells acquire the ability to survive, proliferate, and disseminate (Hanahan & Weinberg, 2000; Hanahan, 2022). The acquisition of these abilities by cells as they transition from normalcy to neoplastic growth states depends on eight distinct functional characteristics that are known as the Hallmarks of Cancer as they are shared by cells in most types of cancer. These hallmarks are self-sufficiency in growth signals, insensitivity to antigrowth signals, resistance to cell death, sustained angiogenesis, unlimited replicative potential, activation of tissue invasion and metastasis, dysregulation of cellular energetics, and evasion immune destruction (Figure 1). The acquisition of these hallmarks of cancer occurs through two enabling characteristics: tumor-promoting inflammation and genome instability (Hanahan & Weinberg, 2011). The latter provides fertile ground for additional mutations that lead to the establishment of the distinctive hallmarks of cancer.

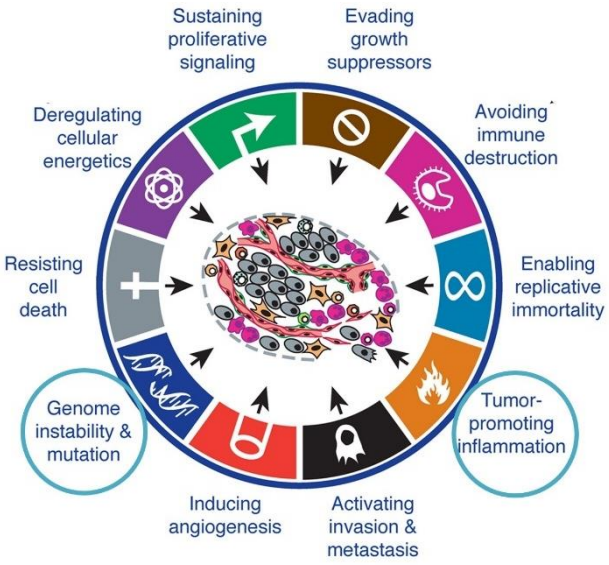


Figure 1. The hallmarks of cancer. Figure adapted from Hannahan and Weinberg (2011). Blue circles highlight the two enabling characteristics of the hallmarks of cancer.

1. Genome instability in human tumor cells

Genome instability refers to the state of the genome in which somatic mutations are generated at a high rate (Shen, 2011). It is not a functional capability *per se* but a characteristic that generates genomic diversity, thus enabling the acquisition of the eight biological capabilities depicted above. Genome instability and the resulting mutability endow cancer cells with genetic alterations needed for the multistep development of human tumors. Conventionally, cancer genomes are believed to undergo a gradual evolution accumulating small-scale changes over many

generations. However, many cancers present a large number of rearrangements that suggest that cancer genomes may sometimes evolve rapidly through drastic episodes of genomic alterations

(Leibowitz et al., 2015; Stephens et al., 2011; Turajlic et al., 2019; Umbreit et al., 2020). Drastic reorganizations are thought to stem from the inherent genome instability within cancer cells.

Mutations generated in cancer cells are diverse and include from single nucleotide mutations to chromosome rearrangements. The type of changes observed in the genome of neoplastic cells and their molecular origin differs from one cancer to another, and even from one individual to another. In some cases, the chromosome set appears normal, but many point mutations can be detected in individual genes. This particular mutation pattern suggests failures in the repair mechanisms that correct replication errors or that ensure the maintenance of genetic information. However, most frequently, cancer cells present highly reorganized karyotypes, with numerous deletions, duplications, and amplifications of some parts of the genome. Such highly rearranged genomes indicate defects in chromosome duplication or segregation during mitosis (Alexandrov et al., 2020; Campbell et al., 2020; Kandoth et al., 2013; Martincorena & Campbell, 2015).

Two major types of genome instability have been described: microsatellite instability (MIS) and chromosomal instability (CIN) (Gizem Sonugür & Akbulut, 2019; Negrini et al., 2010). Whereas MIS is characterized by the change in the number of repetitions of the microsatellite sequences, CIN consists of a high rate of changes in the structure and number of chromosomes. The prevalence of MIS in cancer can vary widely depending on cancer type; for example, MIS is observed in 15% of colorectal cancers, but only in 3% in Lynch syndrome (Boland & Goel, 2010). In contrast, CIN is observed in over 90% of all human solid tumors (Lukow et al., 2021). Regardless of the mechanism underlying these changes, they tend to radically alter the expression and function of numerous genes.

2. Chromosomal instability

CIN is the major form of genome instability in human cancers (Negrini et al., 2010) and can be manifested in two different forms within the genome of cells: whole chromosome instability (W-CIN) and structural CIN (S-CIN). **W-CIN** refers to the condition where there is an increased frequency of gain or loss of chromosomes. It is primarily associated with errors in the segregation of chromosomes during cell division resulting from aberrant mitotic spindles, sister chromatid cohesion defects, improper microtubule-kinetochore attachments, or abnormal cytokinesis, among others. W-CIN leads to alterations in chromosome number, a state also known as aneuploidy. In contrast to W-CIN, **S-CIN** is manifested as abnormalities in the chromosome structure and has been commonly associated with replication stress, telomere dysfunction, and errors in the DNA damage response. Interestingly, while the mechanisms underlying W-CIN and S-CIN are often distinct, they are not mutually exclusive and each one can be the source of the

other one (Böhly et al., 2022; Burrell et al., 2013; Janssen et al., 2011; Siri et al., 2021; Zhang & Kschischo, 2022). Both have an impact on gene dosages.

2.1 Strategies to tolerate chromosomal instability

In the short term, the presence of CIN is associated with a reduced proliferation rate in cells, whereas in the long-term, CIN is often associated with a poor patient prognosis (Zhang & Kschischo, 2022). Nevertheless, it is unknown why CIN is related to more aggressive tumors. To gain more insights, Birkbak and colleagues stratified breast cancer patient samples into low, medium, and high levels of CIN. Whereas low and high CIN groups exhibited better prognoses, those with intermediate levels of CIN correlated with a low survival ratio. These findings strongly suggested that there are mechanisms that allow cells to tolerate intermediate levels of CIN (Birkbak et al., 2011; Dhital & Rodriguez-Bravo, 2023; Roylance et al., 2011). These studies pointed out the necessity to delve into the molecular and cellular processes that allow cells to cope with moderate CIN.

It has been pointed out that loss of *TP53*, which occurs in approximately half of human tumors (Fito-Lopez et al., 2023), could be one of the mechanisms enabling the proliferation of cells with CIN (Sansregret et al., 2018). p53, a classical tumor suppressor protein, is accumulated in the cell nucleus in response to DNA damage, hypoxia, lack of growth factors, or oncogene-induced stress. To maintain cellular homeostasis, p53 levels need to be controlled. Under normal conditions, p53 is regulated by interaction with Mdm2 (Mouse Doble Minute 2), which is an E3 ubiquitin ligase that targets p53 for ubiquitination leading to its degradation through the ubiquitin-proteosome pathway (Figure 2). In response to stress signals, such as the presence of DNA damage, specific proteins belonging to the PIKKs family (Phosphatidylinositol 3-kinase-related kinases) such as ATM (Ataxia-Telangiectasia Mutated) ATR (Ataxia Telangiectasia and rad3 related), or DNA-PKcs (DNA-dependent protein kinase catalytic subunit) are recruited. Subsequently, these kinases phosphorylate the checkpoint kinases Chk1 (Checkpoint Kinase 1) and Chk2 (Checkpoint Kinase 2), which in turn phosphorylate p53 leading to its dissociation from Mdm2. Upon dissociation from Mdm2, p53 translocates to the nucleus and binds to the regulatory region of target genes. One of the targets of p53 is the *p21* gene (also known as *CDKN1A*), which is known to be a universal disruptor of the activity of the cyclin-dependent kinases (CDK) in the CDK-Cyclin complexes (Engeland, 2022; Xiong et al., 1993). By inhibiting the CDK-Cyclin complexes, p21 prevents the phosphorylation of key factors to allow cells to progress through the cell cycle, thereby arresting cells at checkpoints.

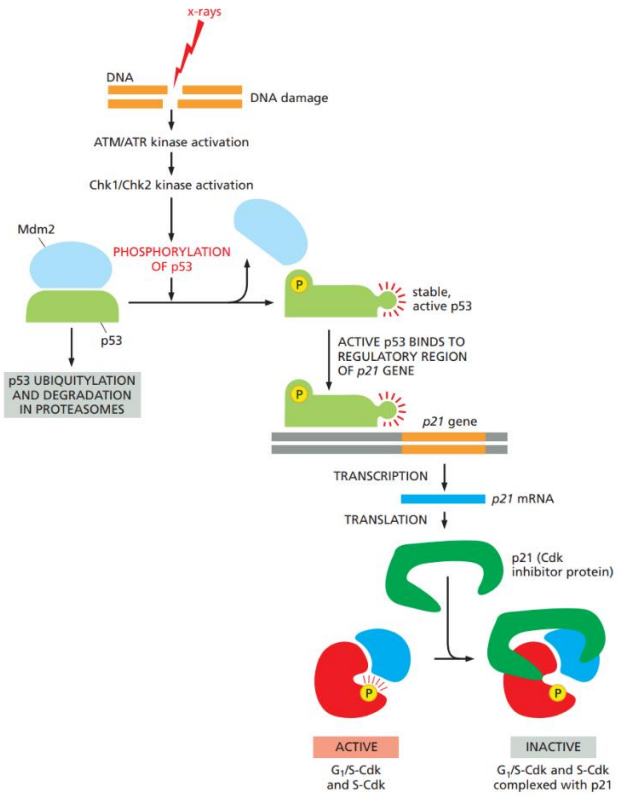


Figure 2. Canonical p53 pathway to halt the cell cycle after DNA damage induction (Alberts et al., 2015).

The specific transcriptional response triggered by p53 depends on the nature and intensity of the stress signal, as well as the cellular context. In the context of aneuploidy, p53 is capable of inducing cell cycle arrest at the G1 stage after chromosome missegregation (Soto et al., 2017). Beyond its pivotal role in halting the cell cycle, p53 serves as a multifaceted guardian of cellular integrity. In the case that the cell presents DNA damage, p53 facilitates DNA repair by activating or repressing a wide range of target genes (Levine & Oren, 2009; Williams & Schumacher, 2016). Moreover, when cellular damage accumulates to a critical point, p53 orchestrates not only cell cycle arrest but also triggers cell death (Childs et al., 2014). p53 is a guardian of the genome and its loss of function permits cells to continue proliferating despite carrying DNA damage. Consequently, disruption of this regulation mechanism is key to enabling tumor cells to tolerate CIN.

Other mechanisms or alterations have been described to allow cells with CIN to proliferate. For example, the loss of BCL9L (B cell CLL/lymphoma 9-like) is known to attenuate cell death after chromosome segregation errors (López-García et al., 2017). Additionally, upregulation of the RAF (Rapidly accelerated fibrosarcoma)/MEK (Mitogen-activated protein kinase kinase)/ERK (Mitogen-activated protein kinase 1) pathway promotes DNA repair in aneuploid cells, leading to adaptation to CIN (Zerbib et al., 2023). Another mechanism is the downregulation of the APC/C (Anaphase-promoting complex) to extend mitosis and prevent extreme chromosome

missegregation (Rodriguez-Bravo et al., 2014). Mutations in the Hippo pathway constitute another mechanism that specifically enables to tolerate tetraploidization (Ganem et al., 2014). Finally, tumor cells present a persistent activation of the inflammatory cGAS-STING-dependent response (Cyclic GMP-AMP synthase-Stimulator of interferon genes protein) that chronically induces immune suppression, impairing the immune system's ability to detect and eliminate abnormal cells, including those with moderate CIN (Bakhoum & Cantley, 2018).

CIN constitutes a double-edged sword for the cell. On one edge, it represents a mechanism for rapid variation of the genome of the tumor cell population that can give rise to a subset of cells with intermediate CIN that possess a higher capacity to adapt, thus increasing their chances to survive and proliferate in a challenging tumor microenvironment. On the other edge, low or high levels of genome damage, can actually hamper the process of cell proliferation due to the activation of cellular mechanisms to repair or eliminate damaged cells. Targeting CIN could offer a promising strategy to eliminate a heterogeneous population of tumor cells. Therefore, it is important to understand the mechanisms that generate CIN during tumorogenesis.

2.2 Mechanisms behind chromosomal instability

The mechanisms responsible for CIN can be categorized into four main groups: defects in the DNA damage response (DDR), telomere dysfunction, replication stress, and defects in cell division.

DNA damage response defects

Cells that present CIN often exhibit structural chromosome abnormalities, including deletions, duplications, inversions, and translocations. Some of these structural chromosome rearrangements arise through inappropriate repair of DNA lesions. Moreover, mutations in genes that control the progression of the cells through the cell cycle may allow cells with impaired genome to divide, thereby facilitating structural chromosome instability.

It is estimated that each human cell can suffer up to 10^5 spontaneous DNA lesions per day (Lindahl & Barnes, 2000) and it is crucial to properly repair them to maintain the genome integrity and prevent chromosomal reorganizations (Jackson & Bartek, 2009). To this end, the cells have the DDR, which consists of a sophisticated and tightly regulated network of signaling pathways for the detection and repair of DNA damage. Depending on the type of DNA damage that occurred, different cellular signaling cascades may be launched. The outcome of these orchestrated cascades may be (1) slowing the cell cycle progression to provide time for DNA repair mechanisms to fix the damage or, (2) in cases where the DNA damage is massive or irreparable, driving the cell to death or to a permanent state of cell cycle arrest.

DNA double-strand breaks (DSBs) are one of the most hazardous types of lesions as they involve both chains of the DNA, thus hindering the re-establishment of the original sequence. Illegitimate

repair of DSBs can cause the formation of unstable chromosome configurations, such as dicentric and ring chromosomes. In this case, mutations in genes of the DDR can contribute to CIN (Martín et al., 2005, 2012). Once a DSB is generated, it is rapidly detected by the MRE11-RAD50-NBS1 (MRN) complex that activates ATM kinase (Blackford & Jackson, 2017). ATM phosphorylate, among others, the histone variant H2AX on serine 139, which is known as γH2AX. This histone modification extends over several megabases flanking the DSB site and serves as a platform for the subsequent recruitment of MDC1 (mediator of DNA damage checkpoint 1) protein. The γH2AX-MDC1 complex interacts with other proteins promoting a positive feedback loop that enhances the phosphorylation of H2AX by ATM, thus amplifying and sustaining the DNA damage signaling (Rogakou et al., 1999; Stucki et al., 2005). MDC1 sustains DSB signaling by recruiting the E3 ubquitin ligase RNF8 (ring finger protein 8), which in turn recruits the E3 ubiquin ligase RNF168 (ring finger protein 168). Together, they initiate a cascade of ubiquitination events essential for the recruitment of repair factors. In eukaryotes, there are two main DSB repair pathways: the homologous recombination (HR) and the non-homologous end-joining (NHEJ) (**Box I**). Several factors influence the DSB repair pathway choice, but at the molecular level this choice is highly regulated by the balance between BRCA1 (breast cancer type 1 susceptibility protein) and 53BP1 proteins (p53 binding protein) (Escribano-Diaz et al 2013; Her and Bunting 2018). Effector proteins from one or the other pathway will ultimately repair the originated DSB. Defects in both pathways have been related to the formation of unstable chromosome rearrangements, which can result in chromosome bridges in proliferating cells (Acilan et al., 2007; Griffin, 2002; Griffin et al., 2000).

Box I. DSB repair pathways

In eucaryotes two major DNA DSB repair pathways exist. The **NHEJ** is the preferential repair pathway in higher eukaryotic cells and consists in the rejoining of the two broken extremes of DNA followed by the DNA end ligation. It is a mechanism with mutagenic potential as it requires minimal homology to ligate the broken ends and can, potentially lead to small insertions and deletions to achieve homology (Her & Bunting, 2018). In response to a DSB, the heterodimer Ku70/80(X-ray repair cross-complementing protein 6/5) is recruited to the break site, and then DNA-PKcs binds to the DNA:Ku complex to maintain the DNA broken ends together (Hammel et al., 2010). The majority of DSBs exhibit sticky ends, necessitating preprocessing by nucleases like Artemis to facilitate their compatibility for ligation. Finally, ligation occurs via DNA ligase IV with the participation of other proteins such as XRCC4 (X-ray repair cross-complementing protein) and XLF (XRCC4-like factor).

HR relies on the availability of a sister chromatid to be used as a template for accurate repair and to generate a high-fidelity copy of homologous DNA. Since sister chromatids are only present from the mid-S phase to the G2 phase, this pathway can only act at this specific moment of the cell cycle. When the HR pathway of repair is launched, the DNA broken ends are resected by the MRN complex (MRE11-Rad50-NBS1) and the CtIP protein (CtBP-Interacting protein). Then, EXO1 (Exonuclease 1) and BLM (Bloom syndrome protein) are essential for an extensive end resection to form a long single-stranded DNA overhang that is rapidly coated by RPA32 (replication protein 2). Finally, various mediator proteins promote the loading of RAD51 (DNA repair protein RAD51 homolog 1) on single-stranded DNA at the DSB site. This leads to the subsequent strand invasion that facilitates the homology-directed repair.

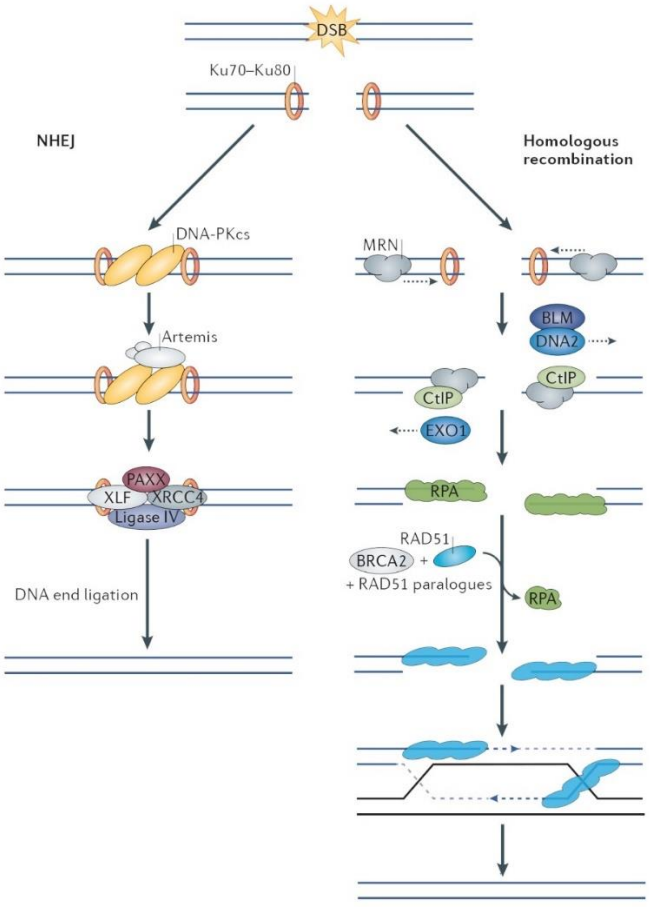


Figure 3. DNA repair pathways: Non-homologous end-joining and homologous recombination. Figure modified from (Schwertman et al., 2016).

During mitosis, DNA repair is a challenging process due to the highly condensed nature of chromatin. In general, cells prioritize faithful chromosome segregation over DNA repair during this stage of the cell cycle (Giunta & Jackson, 2011). However, certain mechanisms can be activated during mitosis to address DNA damage. Interestingly, the signaling of DSBs is truncated during mitosis displaying apical but not downstream components of the DDR (Giunta and Jackson, 2011). The early phosphorylation cascade that includes PIKK activation, H2AX phosphorylation and MDC1 recruitment, still occurs but downstream components of the DDR are not activated. To maintain genome stability in mitosis, CK2 (Casein Kinase 2) phosphorylates MDC1 and TOPBP1 (DNA topoisomerase 2-binding protein 1), which is recruited to DSBs during mitosis forming filamentous structures that can bridge two MDC1 foci (Blackford & Stucki, 2020; Groelly et al., 2023; Leimbacher et al., 2019). Therefore, TOPBP1 links both DNA ends of the DSB together, within the same cell, ensuring their correct segregation.

In conclusion, loss of function of proteins of the DDR leads to increased mutation rates that can have severe consequences by promoting the formation of large rearrangements in the genome that contribute to the generation of CIN, which in turn can facilitate cellular transformation and tumorigenesis.

Telomere dysfunction

The ends of linear chromosomes in eukaryotic cells require a protective nucleoproteic structure termed telomere that, if lost, can lead to CIN. As cells have efficient mechanisms to rejoin two pieces of a chromosome when they are accidentally broken, telomeres have an essential role in ensuring that the natural ends of chromosomes are distinguished from truly broken DNA ends. Telomeres perform this function because they form protective structures that cap the end of linear chromosomes. Telomere functions depend on three elements: the **telomeric DNA**, a telomeric protein component that is mainly composed of the **shelterin complex**, and the **telomerase complex** (Figure 4). Telomeres are characterized by a long stretch of telomeric repeats with a G-rich 3'overhang and its complement C-rich strand. They contain many tandem repeats of a short sequence of DNA that is similar in organisms as diverse as vertebrates (TTAGGG), insects (TTAGG), plants (TTTAGGG), and ciliates (TTGGGG). The telomeric repeat is one of the most diffused DNA sequences in nature. Human telomeric DNA repeats are bound to a six-subunit protein complex termed shelterin (Griffith et al., 1999; Makarov et al., 1997). This complex includes the proteins TRF1 (Telomere Repeat Binding factor 1), TRF2 (Telomere Repeat Binding factor 2), TPP1 (Trypeptidyl peptidase I), TIN2 (TRF1-Interacting protein), RAP1 (Repressor-Activator Protein 1), and POT1 (Protection of Telomeres 1) and participates in repressing the DDR at telomeres thus preventing them from being mistaken as broken ends. This DDR repression by functional telomeres is carried out through two different mechanisms. First,

the telomere-associated proteins of the shelterin complex prevent the activation of the ATM and ATR kinases, which would otherwise induce a cell cycle arrest in response to DNA DSBs. Second, shelterin bound to telomeric DNA repeats ensures the formation of a protective structure that prevents the end of the chromosome from being processed by DNA repair pathways. Proteins of the shelterin complex bound to the repetitive telomeric DNA facilitate the formation of the so-called t-loop structure, which hides the terminal 3'-OH group of the chromosomes. These telomeric loops are formed by invasion of the 3' protruding DNA strand into the double-stranded telomeric DNA located at an earlier position. This single-stranded 3′- overhang is created due to the inability of DNA polymerases to replace the final RNA primer on the lagging strand by DNA and to exonucleolytic degradation of the 5'- end of chromosomes after replication. As a result, telomeric repeats undergo gradual shortening with each cell division. To deal with this problem, telomere DNA sequences recognized by the proteins of the shelterin complex attract the telomerase enzyme that replenishes these sequences each time a cell divides. Telomerase is a template-dependent reverse transcriptase. The enzyme is a ribonucleoprotein composed of a protein moiety with reverse transcriptase activity, TERT (telomerase reverse transcriptase), and a ribonucleic component named TERC (telomerase RNA component), bearing the telomeric template. The shelterin complex regulates telomere length as POT1competes with telomerase to bind the single-stranded 3'-overhang thus affecting telomerase activity (Kelleher et al., 2005; Loayza & De Lange, 2003).

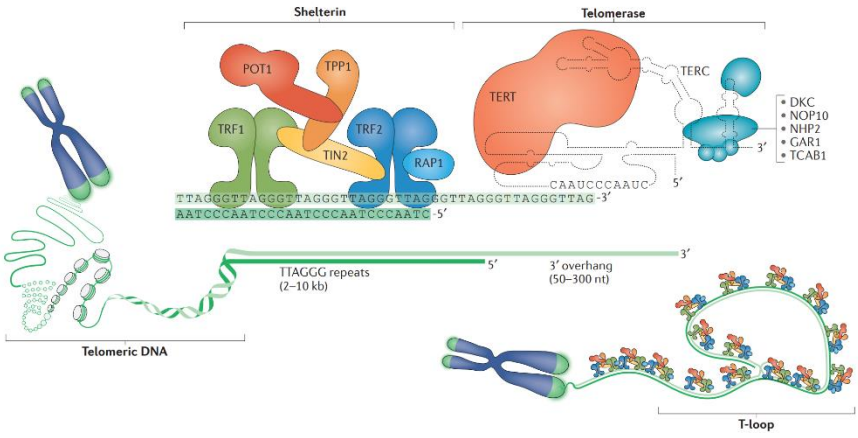


Figure 4. Structure and composition of the telomeres. Human telomeres are composed of three key elements: telomeric DNA, the shelterin complex and the telomerase complex. Figure extracted from (Maciejowski & De Lange, 2017).

In humans, telomerase expression is restricted to specific cell types. In normal conditions, the enzyme is active in germ and stem cells while its expression is not detectable in differentiated

tissues. The telomerase activity is low or absent in most somatic cells. Because of this, telomeric DNA repeats in human somatic cells suffer a progressive erosion. When the telomere length becomes critically short and the tridimensional structure of the telomere can no longer be maintained, cells activate the p16 or p53 pathways to halt cycling (Karlseder et al., 2002; Sharpless et al., 2004; Wang et al., 2013). This phenomenon is known as replicative senescence or Hayflick limit (Hayflick, 1965; Shay & Wright, 2000). However, if the p53 or p16 pathways are compromised, cells can continue proliferating regardless of the nucleolytic erosion of chromosomal ends. Chromosomes with deprotected ends are prone to fuse end-to-end with other unprotected chromosomes and generate dicentric chromosomes. These dicentric chromosomes pose challenges during the exit of mitosis, as the accurate segregation of the rearranged chromosomes becomes a complex process. Consequently, cells harboring such telomere dysfunction-dependent dicentric chromosomes have a higher probability of experiencing CIN.

Excessive erosion of telomeres in human cells with compromised pathways of p16 or p53 is an important cause of CIN in tumors. Nevertheless, reactivation of telomerase and restoration of the telomeric length is important to guarantee the survival of the cells with rearranged genomes and achieve immortality, but also prevent cells from an excessive CIN that would ultimately result in cell death. Reactivation or upregulation of the telomerase to maintain telomere length is a recurrent observation across various cancer types (Shay & Bacchetti, 1997).

In conclusion, telomeres are necessary to safeguard chromosome ends and maintain genome stability. The absence of the proper telomere structure halts the proliferation of normal cells, while a sustained proliferation of cells with dysfunctional telomeres in a permissive gene-environment can trigger CIN.

Replication stress

Accurate replication of the genome is essential to ensure the faithful segregation of the two generated copies between daughter cells. DNA replication is a precise process temporarily and spatially controlled within each cell. It begins at the origins of replication. In eukaryotes, the activation of the origins of replication is controlled by the availability and the subsequent binding of a multisubunit initiator protein called the origin recognition complex (ORC). The initiation of replication is divided into two distinct steps that occur at different times in the cell cycle. The first step occurs only at late mitosis or early G1 when a complex formed by two inactive DNA helicases (Mcm helicases) is loaded onto the DNA at the replication origin. This step is called licensing of the replication origins because initiation of DNA synthesis is permitted only at origins that are preloaded with Mcm helicases. The second step occurs upon transition from the G1 to the S phase of the cell cycle, when Mcm helicases are activated by specialized protein kinases. Upon activation, the two replicative helicases are directed on each of the two complementary

DNA single strands, where they move in opposite directions to begin opening the double-helix. At this point, the additional replication proteins are brought to the DNA, and two complete replication forks move in opposite directions away from the origin of replication, resulting in the initiation of DNA synthesis.

Problems in DNA replication might result in replication stress, one of the main sources of genome instability. Encountering a problem during replication may result in an excess of origin firing, excessive consumption of nucleotides (dNTPs), and ultimately in fork collapse, which produces the accumulation of ssDNA and DSBs at the replication fork. Replication stress can be caused by both exogenous and endogenous sources. For example, overexpression of oncogenes, such as MYC or RAS, increases the number of licensed origins and, consequently, reduces the cellular pool of dNTPs, thus causing replication stress (Saxena & Zou, 2022; Tsegay et al., 2019; Zeman & Cimprich, 2014). Replicative stress can lead to chromosome aberrations during the processes of malignant transformation. Chromosome deletions in regions that are difficult to replicate, such as common fragile sites or early replicating fragile sites are observed in cancer cells.

In response to replication stress, ssDNA at stressed forks is coated by RPA32 which in conjunction with ATR-interacting protein (ATRIP), forms a platform to recruit the checkpoint kinase ATR (Ataxia telangiectasia and Rad3-related) (Zou & Elledge, 2003). Upon activation, ATR phosphorylates many downstream targets, including CHK1 and the tumor suppressor p53. The ATR-CHK1 signaling pathway leads to cell cycle arrest and promotes replication fork stabilization. Additionally, it activates dormant origins in the vicinity of stalled forks to complete the replication of the affected regions (Saldivar et al., 2017).

Cancer cells have elevated levels of replication stress. Moreover, in the specific context of these cells, the ability to bypass checkpoints and enter the M phase adds complexity to the challenge of replication stress, which will have the potential to disrupt mitosis (Gaillard et al., 2015). Intertwines between sister chromatid formed due to replication stress should be resolved before chromosome segregation. Unreplicated regions can fail to segregate correctly by forming common and ultrafine bridges (Finardi et al., 2020), resulting in genome instability, and leading to chromosomal instability.

Defects in cell division

To avoid chromosome missegregation and CIN, mitosis is highly regulated. The successful and accurate progression of mitosis relies on a combination of essential factors. Chromosomes interact with the spindle through the kinetochores, a protein complex that is meticulously assembled on the centromeres (Yamamoto & Capranico, 2021). Establishment of appropriate microtubule attachment to kinetochores is dependent on the tension generated at the kinetochore (Kaitna et al., 2002). To achieve correctly bi-orientated sister chromatids, both kinetochores must be pulled toward opposite poles. Although the kinetochores of sister

chromatids are under tension, the chromatids are held together until anaphase by the protein complex called cohesin. Additionally, the progression of cells through mitosis is regulated by the Spindle Assembly Checkpoint (SAC) that plays a role in inhibiting sister chromatid separation until all the chromosomes are properly attached to the mitotic spindle. The SAC prevents entry into the anaphase by inhibiting the ubiquitylation of cyclin B1 and securin through the ubiquitin ligase APC/C (Nilsson et al., 2008). Collectively, these factors work harmoniously to prevent CIN by ensuring that the two recently generated copies of the genome are faithfully segregated.

When the mechanisms governing mitosis are disrupted, CIN can occur. Mutations in cohesin genes have been observed in different cancer types, although cohesins have multiple roles during the cell cycle and it remains unclear which of these impaired functions contribute to the oncogenic process (Figure 5A) (Cuadrado & Losada, 2020; De Koninck & Losada, 2016; Guacci et al., 1997; Litwin et al., 2018; Michaelis et al., 1997). Another abnormality observed in mitosis is the improper attachment of microtubules to kinetochores. The interaction between microtubules and kinetochores is essential for the proper alignment and segregation of chromosomes during mitosis. As this is a key process, the SAC controls the tension and alignment of the chromosome and prevents the premature separation of sister chromatids. Defects in the SAC increase chromosome missegregation leading to aneuploidy (Li et al., 2009; Michel et al., 2001). Interestingly, the altered expression of SAC proteins, such as Mad2 (Mitotic spindle checkpoint component MAD2), is observed in some cancers (Sjöblom et al., 2006; L. D. Wood et al., 2007). Moreover, abnormal microtubule attachment can occur if there is a reduction in the microtubule turnover rate that would promote more stable attachments, impeding the correction of erroneous microtubules and predisposing cells to CIN (Bakhoum, Genovese, et al., 2009; Bakhoum, Thompson, et al., 2009). However, it can also occur that a kinetochore is attached to both spindle poles, a condition known as merotelic attachment (Figure 5B). This type of attachment is not sensed by SAC because the involved microtubules are under tension, eluding detection, and repair by SAC (Thompson & Compton, 2011). Consequently, merotelic attachments often give rise to anaphase resulting in lagging chromosomes, which in turn can lead to the presence of unsegregated chromatin under the furrow cleavage.

Regarding the polarity mitotic spindle, cells with an extra number of centrosomes can form a multipolar mitotic spindle that a priori might segregate chromosomes in more than two daughter cells. However, multipolar spindles are infrequently observed during anaphase. Indeed, to mitigate the adverse effects of supernumerary centrosomes, cancer cells often cluster centrosomes leading to the formation of bipolar spindles, allowing cancer cells to survive despite the presence of extra centrosomes (Ganem et al., 2009). Despite this clustering, cells with supernumerary centrosomes pass through a transient multipolar spindle stage that leads to a high rate of incorrectly attached chromosomes to the microtubules (Silkworth et al., 2009). Several

studies have reported identified extra centrosomes as an early event in the development of most cancers (Lingle et al., 2002; Neben et al., 2003).

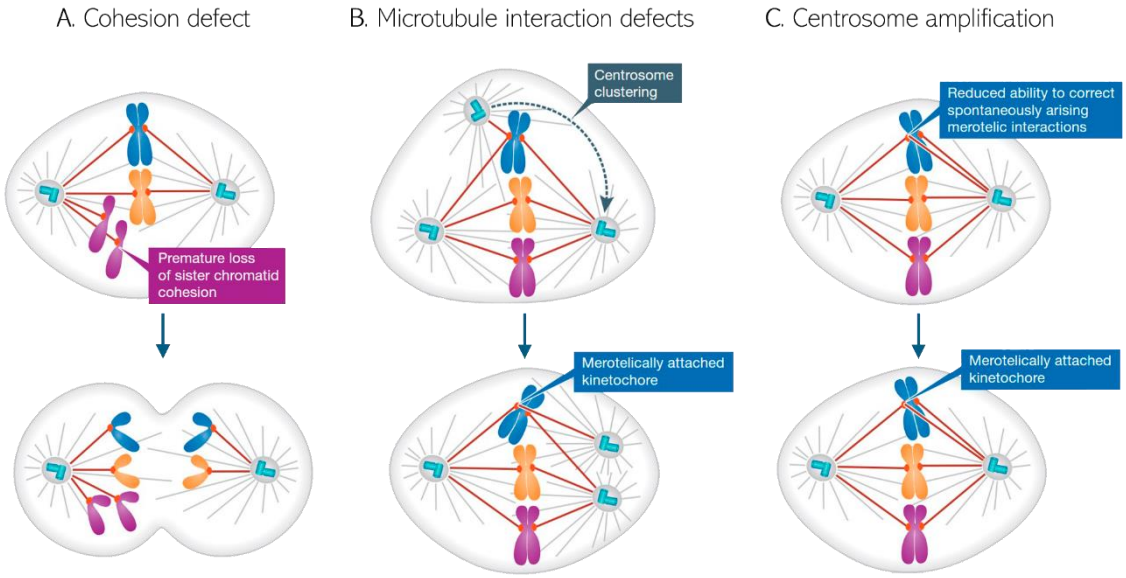


Figure 5. Mitotic defects that lead to CIN. Adapted from (Holland & Cleveland, 2012).

Finally, cytokinesis is the concluding phase of the cell division and involves a concert among constricting forces, membrane remodeling, and endomembrane trafficking. In eukaryotes, the cytoskeletal elements, tubulin, actin and myosin play crucial roles in cytokinesis (Barr & Gruneberg, 2007). Additionally, the process relies on protein kinases such as Aurora B to transmit information about chromosome position. The process of cytokinesis involves distinct stages (Normand & King, 2010). Firstly, microtubules activate RhoA (Transforming protein RhoA), specifying the cleavage site between segregating chromosomes. Secondly, the formation and activation of the actomyosin ring initiates furrow ingression. Thirdly, the constricting furrow compacts central spindle microtubules leading to midbody formation. Finally, abscission occurs as the furrow physically separates the cytoplasm of the daughter cells. Defects in proteins involved in cytokinesis can lead to the failure of the actin-myosin contractile ring or regression of the cleavage furrow. As a result, binucleated daughter cells are formed and, if they progress through the cycle, they become tetraploid cells with an extra centrosome number. Unlike diploid cells with increased CIN, which often die after losing several chromosomes in aberrant mitosis, a tetraploid cell might have a greater chance of survival due to their redundancy in chromosome content (Ganem et al., 2007). Interestingly, polyploidy is found in cancer cells and, in fact, is an event present in over 50% of epithelial cancers (Carter et al., 2012). Nevertheless, there are mechanisms to control the proliferation of polyploid cells. Experimental studies have shown that artificially generated tetraploid cells undergo G1 cell cycle arrest which is regulated by the p53 pathway. Additionally, it has been reported that tetraploid cells with extra centrosomes can trigger

the Hippo tumor suppressor pathway (Ganem et al., 2014). If those control mechanisms are impaired, then chromosomal instability might occur and consequently tumorigenesis.

Altogether, defects in the faithful segregation of chromosomes during mitosis, either due to disturbances in the segregation machinery, in the control mechanisms or in the geometry of the spindle due to the presence of supernumerary centrosomes in polyploid cells, generate CIN.

2.3 Chromosome bridges and micronuclei: Common intermediaries of the different mechanisms that promote CIN

The four major mechanisms that contribute to CIN depend, to a greater or lesser extent, on the formation of two different aberrant nuclear structures (Figure 6): chromosome bridges, structures that connect the two groups of chromosomes in dividing cells, and micronuclei, the small nuclear structures formed by mitotic errors or by chromosome breakage.

Specifically, mutations in the genes involved in the DDR lead to large genome rearrangements such as dicentric chromosomes, which can orient their centromeres towards opposite poles of the cell forming chromosome bridges (Martín et al., 2012), or acentric fragments, which frequently form micronuclei. Furthermore, when the mechanism of CIN is telomere dysfunction, chromosomes fuse end-to-end forming a continuous DNA fiber. As these chromosomes present more than one centromere, the continuous chromatin fiber is frequently stretched simultaneously towards opposite poles of the dividing cell and results in the formation of chromosome bridges (Feijoo et al., 2014). Regarding replicative stress, the success of complete replication is compromised because chromosomes with non-replicated segments interspersed with other replicated segments are generated. These incompletely replicated chromosomes can form chromosome bridges as their centromeres are pulled apart during mitosis (Ganem et al., 2009; Lecona & Fernández-Capetillo, 2014). Finally, when mitotic defects occur, some chromosomes can remain in the equatorial plane bridging between opposite poles or can lag behind the rest of the segregating chromosomes forming micronuclei (Ganem et al., 2009). Thus, the mechanisms of telomere dysfunction, replicative stress, defects in DDR, and defects in cell division lead, to a greater or lesser extent, to the formation of chromosome bridges and micronuclei in dividing cells.

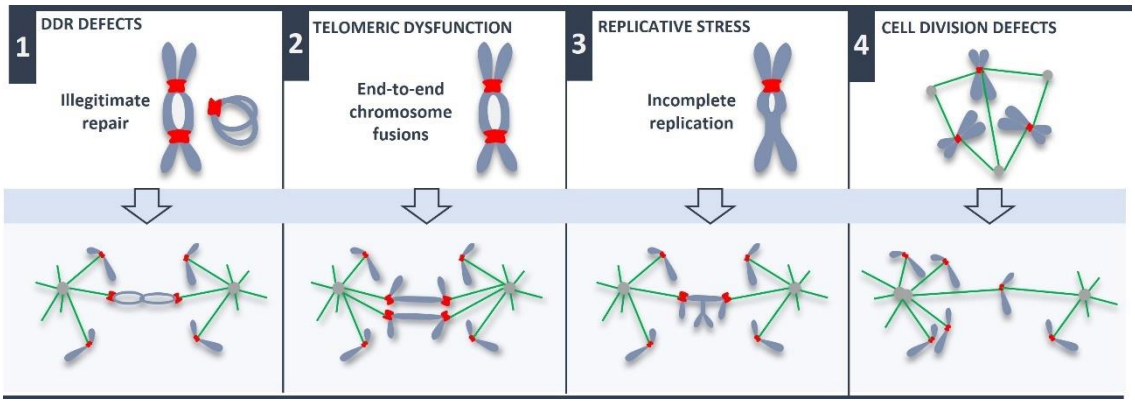


Figure 6. The four basic mechanisms for chromosome bridge formation. Mechanisms that promote CIN generate chromatin bridges during the mitotic anaphase that may persist beyond it, rendering it difficult to segregate the two DNA copies of each replicated chromosome and prolonging cell division. (1) Dicentric or ring chromosomes can be generated by the illegitimate repair of DNA lesions or by (2) the end-to-end fusion of chromosomes with telomeric dysfunction, either by erosion or by defects in telomeric proteins; (3) chromosomes with replicated segments interspersed with unreplicated segments resulting from replicative stress; and (4) chromatids joined to both poles of the mitotic spindle by merolelic junctions lag behind the two masses of segregating chromosomes.

3. Nuclear atypia in cancer

Malignant tumors often present irregular nuclear shape, abnormal chromatin structure, and variations in nuclear size. Irregular nuclear morphologies are termed nuclear atypia and include micronuclei, chromosome bridges, and nuclear buds (Caruso et al., 2008) In certain types of cancer, such as endometrial, ovarian, and mammary adenocarcinomas, the presence of nuclear atypia is associated with an unfavorable prognosis (Erhardt et al., 1989; Gisselsson et al., 2001; Trojani et al., 1984; Vacher-Lavenu et al., 1993).

Nuclear atypia often originates during mitosis. Recently, Tucker and collaborators have analyzed the presence of irregular morphologies during mitosis, such as misaligned chromosomes, anaphase bridges, and lagging chromosomes in primary breast cancer samples including stage I to III from all major subtypes. Misaligned chromosomes were found to be the most common mitotic phenotype observed, followed by lagging chromosomes, chromosome bridges, and multipolar spindles. Specifically, the frequency of chromosome bridges was observed in approximately 10-20% of mitosis of primary breast cancer samples (Tucker et al., 2023).

Detection of defects in cell division in tumor cells can be difficult due to its transient nature. Most of these defects can lead to the formation of anaphase bridges that are transient in time, thus presenting a challenge for their study. Nonetheless, their significance lies in their potential to yield a spectrum of outcomes, significantly exacerbating genome instability. Defects during mitosis can also result in micronuclei, which are easier to detect. Although present in tumors, micronuclei are also observed in healthy conditions. Fenech and Bonassi found that micronuclei are markers of lifestyle factors such as smoking, poor nutrition, and a sedentary lifestyle, among others (Fenech & Bonassi, 2011). Also, Norppa and colleagues showed that healthy individuals with higher

frequencies of micronuclei in peripherical blood cells had more probability of developing cancer later in life (Norppa et al., 2006). Therefore, the presence of micronuclei is not only associated with bad prognosis in cancer patients but also to a higher probability of developing cancer in healthy population. Indeed, the presence of micronuclei has been long used as a marker for diagnosis and prognosis to assess the severity and potential behavior of cancer cells in patients.

Altogether, both chromosome bridges and micronuclei play significant roles in the initiation and the progression of tumorigenic processes. The presence of micronuclei, which can arise from various types of genetic alterations, including chromosome bridges, is a common occurrence in both cancerous and healthy tissues. This suggests that these aberrant structures are involved in the early and late stages of tumor development.

4. Consequences of chromatin bridges and micronuclei

Genetic imbalances

One of the most evident consequences of the presence of micronuclei and chromosome bridges for cells is the imbalanced distribution of chromosomes to daughter cells resulting in alterations of gene doses. Losing a chromosome, or part of it, can lead to deletions and, consequently, to loss of heterozygosity. Moreover, as it will be explored shortly, chromosomes involved in the bridge, or the micronucleus are frequently damaged and if reincorporated to the primary nucleus after mitosis, the broken DNA ends can bind to other DNA ends giving rise to gene imbalances (Maciejowski & De Lange, 2017).

Impaired genetic functions of micronuclei and chromosome bridges

In the context of micronuclei, the integrity of the nuclear envelope (NE) is often compromised, and the number of functional nuclear pores is suboptimal, leading to altered nucleocytoplasmic transport dynamics (Rodriguez-Muñoz et al., 2021). Dysfunctional NE leads to the accumulation of components involved in NE repair, including ESCRT-III (Endosomal sorting complexes required for transport III) along with the BAF (barrier to autointegration factor) (Halfmann et al., 2019). Surprisingly, rather than repairing the NE, the accumulation of these components can exacerbate NE rupture instead (Vietri et al., 2020; Young et al., 2020). Additionally, the impaired nuclear envelope structure can disrupt the distribution of essential factors involved in DNA replication and DNA repair processes. Micronuclei are known to be asynchronized from the cell cycle of primary nuclei giving rise to its incomplete DNA replication. Moreover, although DNA damage in micronuclei is detected, it cannot be repaired. Both defects in DNA replication and repair compromise the integrity of the DNA entrapped in micronuclei (Crasta et al., 2012; Hatch et al., 2013; Krupina et al., 2021; Terradas et al., 2010; Zhang et al., 2015). Moreover, Soto et al, observed that micronuclei exhibit decreased levels of essential kinetochore assembly factors (Soto et al., 2018). This compromised function can result in errors during chromosome

segregation leading to the recurrent missegregation of these micronucleus chromosomes across multiple cell divisions (Soto et al., 2018).

Regarding chromosome bridges, studies have demonstrated a deficient recruitment of nuclear envelope proteins (Liu et al., 2018), a phenomenon like that reported for micronuclei (Liu et al., 2018). Nevertheless, it is noteworthy that chromosome bridges are connected to the primary nuclei, potentially facilitating the recruitment of proteins through diffusion from the primary nuclei (Rodriguez-Muñoz et al., 2021).

Micronuclei and chromosome bridges have defects in their nuclear envelope and also can suffer nuclear envelope rupture, which is an event irreversible that exposes the genetic material of these aberrant structures to the cytoplasmic environment. In the context of nuclear envelope rupture, it has been shown that the endoplasmic reticulum (ER) invades the ruptured micronucleus and chromosome bridge (Liu & Pellman, 2020; Umbreit et al., 2020) and it is proposed to facilitate access to the exonuclease TREX1 (Three-prime repair exonuclease 1) that in the cytoplasmic membrane of the ER. As a consequence, TREX1 might process micronuclear DNA and bridging DNA (Mohr et al., 2021). Nevertheless, it is still not clear the mechanisms of TREX1 as it is an exonuclease that needs an initial break to act. While TREX1's role in DNA processing within bridges and micronuclei has been proposed, it cannot be ruled out the possibility of other nucleases involved in DNA digestion of these aberrant nuclear structures.

Chromothripsis and kataegis of micronuclei and chromosome bridges

The DNA contained in micronuclei and chromosome bridges is prone to suffer damage, but it is also isolated from the DNA contained in the primary nucleus. This observation has raised the possibility that both aberrant structures could lead to chromothripsis. Chromothripsis refers to the phenomenon where tens to hundreds of chromosomal rearrangements affecting one or a few chromosomes or chromosomal segments occur simultaneously rather than accumulating gradually over time. The term chromothripsis derives from Greek referring to chromosome and shattering, reflecting the dramatic and abrupt nature of the rearrangements. Indeed, an analysis conducted by the PCAWG (Pan-Cancer Analysis of Whole Genome Consortium), in which 2658 cancer genomes were examined revealed that 29% of them had chromothriptic events. Notably, certain types of cancer exhibited even higher frequencies (100% of liposarcomas and 77% of osteosarcomas showed chromothriptic events) (Cortés-Ciriano et al., 2020).

Although the mechanisms by which chromothripsis occurs are yet to be understood, the isolation of DNA within micronuclei or chromosome bridges, coupled with the compromised nuclear envelope seems to create a favorable environment for the occurrence of localized chromosomal rearrangements. One possibility is that damage is accumulated in the DNA of these atypical structures due to the disruption of DNA replication and repair processes, leading to the chromothripsis. When micronuclei are reincorporated into the primary nucleus, the NHEJ repair

pathways will be able to rejoin broken DNA ends in an illegitimate manner, giving rise to highly rearranged chromosomes. Nevertheless, it has been observed that even if the DNA is not reincorporated into the primary nucleus, chromothripsis still occurs leading to a second scenario. As the nuclear envelope is ruptured and becomes permeable, cytoplasmic nucleases, including potential yet-to-be-identified DNA processing enzymes, can induce DSBs. One candidate to process the DNA of micronuclei and chromosome bridges is TREX1 (Maciejowski et al., 2015). The fragmentation of DNA in chromosome bridges and micronuclei by TREX1 can explain the localized DNA breaks, as TREX1 only digests the DNA trapped in the chromatin bridge or the micronucleus because these structures have episodes of nuclear envelope rupture that facilitates the access of the cytoplasmic content to the DNA.

The presence of TREX1 on micronuclei and chromosome bridges leads to the formation of ssDNA. This ssDNA can be targeted by APOBEC family proteins (C ->U-editing enzyme APOBEC), which are primarily located in the cytoplasm. APOBEC proteins can induce mutations in the DNA by deaminating cytosine residues, leading to the conversion of cytosine to uracil, which is a characteristic of kataegis (Maciejowski et al., 2015). Therefore, the action of TREX1 generating ssDNA combined with the nuclear envelope rupture on micronuclei and chromosome bridges could be a mechanism that contributes to the generation of kataegis, which is also characteristic of cancer cells.

Innate immune response to micronuclei and chromosome bridges

Upon nuclear envelope rupture, the cell detects the presence of DNA from micronuclei and chromosome bridges, leading to the activation of the innate immune proinflammatory response through the cGAS-STING pathway. This response is a key mediator of inflammation and is mainly activated by foreign DNA from infection, but it has also been reported to be activated also during cellular stress and tissue damage scenarios (Decout et al., 2021). In the context of nuclear envelope rupture, if the cytoplasmic cGAS protein detects DNA, the cGAS-STING pathway is activated, a phenomenon commonly observed in cancer, and can have both suppressive and promoting effects on tumorigenesis (Du et al., 2022; Samson & Ablasser, 2022).

Mackenzie and colleagues demonstrated that a fraction of micronuclei and chromatin bridges show cGAS signal (MacKenzie et al., 2017). Upon binding to DNA, cGAS synthesizes cGAMP (cyclic guanosine monophosphate–adenosine monophosphate), which activates the STING pathway leading to the expression and secretion of IFNI (type I interferon). It is important to note that the cGAS cascade is inhibited by nucleosomes (Flynn et al., 2021; Kujirai et al., 2020a). Therefore, it is possible that the cell's immune response has evolved to distinguish between the well-structured self-DNA from the disorganized and potentially foreign DNA (Flynn et al., 2021; Zierhut et al., 2019). As chromosome bridges are under tension, their chromatin structure can be released, and their nucleosomes lost. Therefore, it is possible that chromosome bridges, but not

micronuclei are able to activate the cGAS-STING pathway. Nevertheless, TREX1 has been identified as a competitor to cGAS, as it can degrade DNA and thus limit cGAS activation at micronuclei (Mohr et al., 2021). The interplay between cGAS and nucleosomes and TREX1 in regulating cGAS activation and function in the context of micronuclei and chromosome bridges is still being investigated.

Self-DNA recognition and the resulting cGAS-STING-mediated inflammation can have dual roles in tumorigenesis. On one hand, the immune response can act as a protective mechanism against cancer by detecting and eliminating abnormal cells. On the other hand, prolonged activation of this pathway can promote an immunosuppressive microenvironment, which can facilitate the invasion of cancer cells (Du et al., 2022). Therefore, the innate immune response triggered by the presence of micronuclei or chromosome bridges and can play a dual role, on one hand a tumor-suppressive function and in the other oncogenic behavior.

5. Chromosome bridge resolution

The resolution of chromosome bridges has been a subject of study and debate in the last years. Various models have proposed mechanisms behind their resolution (Figure 7). Barbara McClintock proposed in 1941 that dicentric chromosomes can bind to opposite spindle poles during metaphase. In this situation, the continuous fiber of each chromatid in a dicentric chromosome is pulled apart to opposite cell poles, culminating in the formation of bridges in anaphase (Figure 7). It was suggested that during the mitotic division, chromosome bridges can be broken giving rise to free DNA ends that can then fuse to other DNA broken ends generating new dicentric chromosomes and therefore new anaphase bridges, initiating a self-perpetuating cycle known as the breakage-fusion-bridge (BFB) cycle (McClintock, 1941).

Recently Jiang and colleagues reported that the endonuclease ANKLE1 (Ankyrin Repeat and LEM Domain Containing 1) is recruited during cytokinesis and suggested that might resolve chromosome bridges to prevent catastrophic damage to the newborn cells (Jiang et al., 2023). Janssen and colleagues, suggested that chromatin under the furrow ingression becomes damaged due to the compression forces (Janssen et al., 2011). Nonetheless, others revealed that the presence of unresolved chromosome bridges can result in cleavage furrow regression resulting in tetraploid cells (Figure 7) (Pampalona et al., 2016). Finally, it has also been reported that chromosome bridges delay abscission and give rise to nucleoplasmic bridges in post-telophases stages (Figure 7) (Steigemann et al., 2009). Nevertheless, it is not clear what determines chromosome bridge resolution.

Alternatively, some authors proposes that breakage during mitosis or cytokinesis does not exist and suggests that all the anaphase bridges arrive to interphase forming nucleoplasmic bridges, resulting in linked cells that behave as separate identities (Figure 7) (Maciejowski et al., 2015;

Umbreit et al., 2020). The main difference between these studies is that one posits that resolution occurs via biochemical processes and the other via mechanical forces. Specifically, Maciejowski and collaborators, showed that chromatin bridges undergo nuclear envelope rupture followed by accumulation of the exonuclease TREX1 which leads to the formation of ssDNA. On the other hand, Umbreit and colleagues propose that the mechanical stretching imposed by daughter cells migrating away from each coupled present focal adhesions on the bridges and is responsible for chromosome breakage.

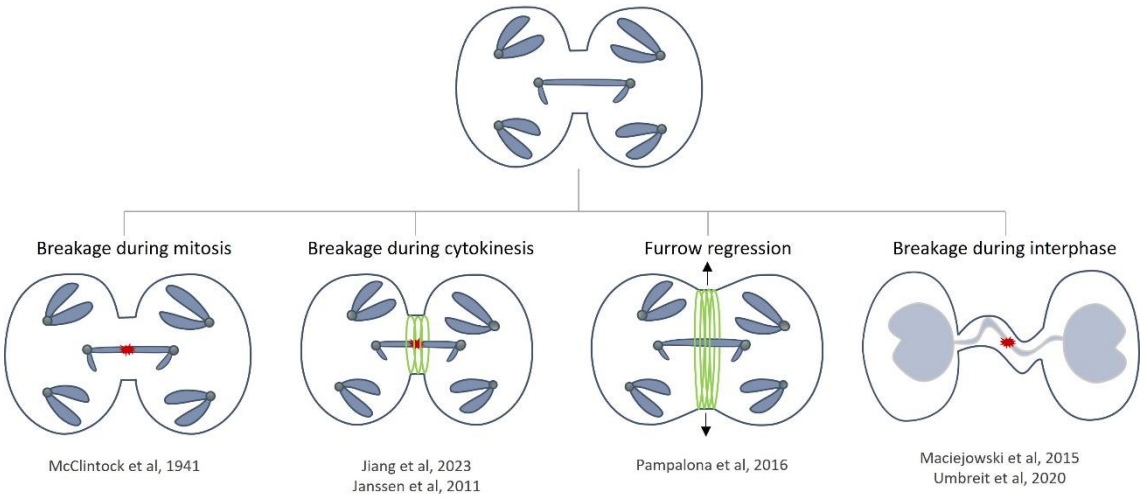


Figure 7: Distinct fates chromosome bridges. From left to right, chromosome bridge breakage during mitosis. Chromosome bridge resolution during cytokinesis by nucleases or compression forces. Regression of the contractile ring due to the presence of an unbroken chromosome bridge. Chromosome bridge resolution during interphase.

Although there are some theories on how chromosome bridges are resolved, controversies still exist within the field. The exact mechanism determining chromosome bridge resolution remains unclear. Recent studies declare that there is no breakage of chromosome bridges during mitosis and the real breakage occurs at interphase by nucleases or mechanical stress. This thesis aims to shed light on this topic and contribute to understanding chromosome bridge resolution.

BACKGROUND, HYPOTHESIS AND OBJECTIVES

BACKGROUND AND HYPOTHESIS

Chromosome bridges, commonly found in environments marked by chromosomal instability, also contribute significantly to the underlying instability. Although they have been described to be involved in tumorigenesis and tumor progression, their features, dynamics, and resolution are still a matter of discussion. In the last decades, efforts have been made to resolve the entanglement of bridge resolution, leading to the emergence of different models that focus on specific cell cycle stages often with conflicting perspectives. As the models proposed have often conflicting perspectives, there is no definite consensus about how chromosome bridges are resolved. We hypothesize that resolution of chromosome bridges might involve multiple mechanisms that are not necessarily mutually exclusive. If confirmed, this would signify a paradigm shift, as the conventional understanding has proposed the resolution of chromosome bridges to be governed by a single mechanism. Our research not only will address the current gaps in the understanding but will also try to comprehend the dynamics governing chromosome bridge resolution from an integrative view.

OBJECTIVES

The main objective of this thesis is to study the mechanisms underlying chromosome bridge resolution across different cell cycle stages and to elaborate an integrative model to provide a comprehensive understanding regarding chromosome bridge resolution. To achieve this general aim, the following specific objectives have been established.

1. To conduct a comprehensive review of studies published related to chromosome bridges and micronuclei to identify the gaps of knowledge.
2. To develop a cellular model that enables the study of chromosome bridges with defined geometric characteristics induced under temporal control in a genomically stable cell line.
3. To investigate the occurrence of chromosome bridges resolution during cell division and interphase.
4. To explore the contribution of mechanisms of biochemical and mechanical nature on chromosome bridge resolution.

RESULTS

CHAPTER 1

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A matter of wrapper: Defects in the nuclear envelope of lagging and bridging chromatin threatens genome integrity

Marina Rodriguez-Muñoz, Teresa Anglada *, Anna Genescà *

Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

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Abstract

The nuclear envelope surrounds the eukaryotic genome and, through the nuclear pore complexes, regulates transport in and out of the nucleus. Correct nucleo-cytoplasm compartmentations are essential for nuclear functions such as DNA replication or repair. During metazoan mitosis, the nuclear envelope disintegrates to allow the segregation of the two copies of DNA between daughter cells. At the end of mitosis, it reforms on each group of chromosomes in the daughter cells. However, nuclear envelope reformation is delayed on lagging chromosomes and DNA bridges. Defects in the coordination between nuclear envelope reformation and chromosome segregation impair the nuclear functions. Mechanical stress to which micronuclei and DNA bridges are subjected to combined with their particular architecture and the altered nuclear functions result in DNA damage. While micronuclei and DNA bridges were considered for more than 100 years as mere indicators of chromosomal instability, rapid technological advances are helping to better understand the biological consequences of these aberrant nuclear morphologies. Recent studies provide interesting evidence that micronuclei and chromatin bridges act as a key platforms for a catastrophic mutational process observed in cancers called chromothripsis and a trigger for the innate immune response. Therefore, they could affect cellular functions by both genetic and non-genetic means.

* Corresponding authors.
E-mail addresses: Marina.Rodriguez.Munoz@uab.cat (M. Rodriguez-Muñoz), Teresa.Anglada@uab.cat (T. Anglada), Anna.Genesca@uab.cat (A. Genescà).

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1. Introduction

Nuclear morphology is not a trivial matter. The nucleus in most normal (non-transformed) cells has a regular and ellipsoid shape, but it is often irregular in cancer cells. Alterations of nuclear structure in tumor cells include changes in nuclear size and shape as well as in the organization of heterochromatin. All these morphological changes are characteristic of certain types of tumors and stages and they have been used in the diagnosis of cancer for many years. For example, the Pap smear developed in the early 20th century by George Papanicolau to simultaneously detect specific nuclear and cytoplasmic features of cancer cells in cervical samples. Changes in the nuclear architecture reveal a characteristic that is transversal to cancer cells: their inability to maintain the integrity of their genome.

Among abnormal nuclear morphologies, micronuclei (MN) have been used for many decades as biomarkers of chromosomal damage, genomic instability and exposure to mutagenic agents [11,21]. Their scoring was considered as the gold standard for detecting exposure to mutagenic agents. However, our perspective on MN has changed dramatically in the last 10 years with recent technological advances for visualizing and recording living cells and massive DNA sequencing. These new approaches have led to relevant progress in our understanding of the biological consequences of MN and have created widespread interest in these nuclear structures. We now know that micronuclei lack structural integrity and they are not fully functional as they cannot properly perform basic nuclear functions such as DNA replication, transcription or DNA damage repair. These new investigations provide evidence that micronucleation can set in motion a deep genome remodelling of cells, thus taking MN to a new dimension: from passive indicators of chromosome instability to sources of DNA damage and karyotypic diversity in cell populations.

Unlike micronuclei, DNA bridges between daughter cell nuclei have long been recognized as a source of DNA damage and intratumoral genetic heterogeneity [14,32]. According to the Breakage-Fusion-Bridge (BFB) model [32], DNA bridges can break and initiate a cycle capable of self-feeding when the broken ends fuse with another broken end. The variety of genome changes caused by the presence of bridges in cells has suddenly spread to unforeseen limits. Classical studies showed that, through BFB cycles, chromosomal bridges could generate mutations, chromosome rupture, amplifications and highly rearranged chromosomes. But it was not until recently that we learned that the repertoire of genomic alterations that can be attributed to DNA bridges is much broader, including phenomena capable of dramatically changing the genome of cells such as duplication of the entire genome and chromothripsis.

The evolution in our knowledge of the consequences of micronuclei and DNA bridges derive, to a great extent, from our understanding that these structures are surrounded by a defective nuclear envelope. Defects in the wrapping of micronuclei and chromatin bridges make not only their nuclear functions to be altered, but also their DNA to be inappropriately exposed to the cytoplasmic environment during the interphase, triggering the innate immune response. Taken together, it suggests that MN and DNA bridges could affect cellular functions by both genetic and non-genetic means.

2. Molecular origin of DNA bridges and micronuclei

There are different molecular mechanisms by which DNA bridges and micronuclei can be formed, but for most of them, mitosis is a common key process. This stage of the cell cycle is inherently stressful for cells. During mitosis, actin and tubulin cytoskeleton suffer dramatic changes to reshape the cell. The nuclear envelope is torn apart, and the replicated chromosomes condense and move inside the cell; first in a congressional movement towards the equatorial cell plane and then, towards the cell poles for segregating the two copies of the replicated DNA between daughter cells. In this stage of the cell cycle, in which changes in the cellular architecture are of such magnitude, the malfunction of the molecular machinery involved has serious consequences for the cell.

Lagging chromosomes during anaphase are prone to be subsequently incorporated into MN. Laggards can originate from different mechanisms. They frequently arise because of erroneous attachments to microtubules, such as those of merotelic chromosomes in which single kinetochores attach to microtubules emanating from different poles [6]. Laggards also result from unattached acentric fragments, which can be derived from unrepaired DNA double-strand breaks caused by DNA replication stress or as a result of misrepair of DNA breaks [4,11]. Lagging chromosomes can also appear due to the untimely loss of sister chromatid cohesion because of the defective removal of cohesins that tether sister chromatids [39] or from abnormal congress of chromosomes before their segregation due to malfunction of microtubule plus-end directed kinetochore motor proteins [12]. Whatever their origin, lagging chromosomes become MN after their nuclear envelope reassemble independently from the primary nucleus, being spatially separated.

As opposed to laggards, bridging chromatin completely spans the segregating masses of chromosomes during anaphase. Bridges are most often the manifestation of dicentric chromatids in which each centromere of a continuous DNA fibre is pulled towards opposite spindle poles. These rearranged chromosomes with two centromeres in a single DNA molecule are most frequently formed because of misjoined DNA breaks or after fusion of chromosomes with

dysfunctional telomeres [13]. Chromatin bridges are also formed during mitosis when the success of complete replication is compromised and it generates chromosomes with non-replicated segments interspersed with other replicated segments [23]. Although to a lesser extent, DNA bridges are also caused by defects in chromatin condensation and sister chromatid cohesion. Chromatin bridges can persist beyond anaphase and form stabilized nucleoplasmic connections between the two daughter cells. At the end of mitosis, the nuclear envelope must reassemble around each group of chromosomes. However, the formation of this envelope on missegregated bridging chromatin, as well as on lagging chromosomes, is usually defective due to the spatial location of these structures in the dividing cell.

3. Defective nuclear envelope on bridging and lagging chromosomes

The nuclear envelope (NE) consists of two lipid bilayer membranes supported by the nuclear lamina and perforated by the nuclear pores. On the nuclear side, the nuclear lamina –a network of intermediate filaments composed of two types of lamin proteins, the B type lamin and the A type (Lmn A/C), and integral membrane proteins– provides mechanical support to the NE and contribute to chromatin organization [16]. Scattered in the envelope, nuclear pores constitute large proteinaceous channels for the selective transport of macromolecules and diffusion of ions and metabolites between the nucleus and the cytoplasm. In eukaryotic cells, the NE has evolved to form a stable physical barrier between the nucleus and the cytoplasm, normally breaking down only during mitosis. NE reassembly is facilitated by the microtubule-mediated accumulation of the DNA cross-bridging barrier-to-autointegration factor (BAF) at the surface of anaphase chromosomes. Relying on its ability to bridge distant DNA sites, BAF shapes a single nucleus from a set of individualized chromosomes at the end of mitosis [38]. Following an initial phase during which BAF localizes on the entire surface of the compact mass of late anaphase chromatin, BAF continues to enrich in central regions of the assembling nuclear rim close to the spindle microtubules. This region of the nuclear envelope near to the pole-to-pole axis of the mitotic spindle is named the “core” domain [17].

To form a functional interphase nucleus, segregating chromosomes have to recruit not only membranes and lamins but also nucleoporins to constitute nuclear pore complexes (NPCs). Therefore, nuclear membrane recruitment and NPC assembly must be coordinated to avoid the formation of a closed NE that lacks NPCs [3]. However, the presence of lagging and bridging chromatin in dividing cells compromises the architecture and the function of the NE on missegregated chromatin. The NE of micronuclei typically possess a lower density of nuclear pores relative to primary nuclei and exhibit inefficient transport of nuclear proteins [19,42,7]. Similar to MN, the NE on chromatin bridges is frequently altered. Although positive for the

chromatin binding BAF, it is typically depleted for many important proteins, including A/C- and B-type Lamins and NPCs [29]. Alterations in NE composition affect the transport of proteins that need to be shuttled through and as a consequence of it, the nuclear functions become defective. For instance, the DNA replication and DNA repair functions in micronuclei are strikingly impaired [7,42]. Therefore, the nucleo-cytoplasmic compartmentation and transport defects of the NE on missegregated bridging and lagging chromosomes are due to an abnormal assembly of NPCs and other NE components.

What causes the NE on lagging and bridging chromosomes to be defective? Why are MN and DNA bridges depleted of NPCs and other NE components? Studies published in the last decade in the fruit fly *Drosophila melanogaster* pointed to a biochemical model to explain a spatial regulation of NE postmitotic reassembly. According to these studies, NE reassembles around segregating chromosomes only once they have left the mitotic midzone where the concentration of active Aurora B enzyme is high [1,2] (Fig. 1). These observations suggested that a surveillance mechanism would be mediated by localized concentrations of active Aurora B kinase: by keeping key substrates phosphorylated, chromosome decondensation and NE reassembly on late-segregating chromosomes that occupy the spindle midzone would be delayed to facilitate their inclusion in the primary nucleus [46]. However, if the missegregated chromosome fails to incorporate in the primary nucleus, delayed NE reformation will finally have impact on the composition and function of the NE that wraps the missegregated chromatin.

Other recent studies point to mechanical causes for the deficient NE assembly on late segregating chromosomes in human cells (Fig. 1). According to these studies, altered NE assembly on lagging chromosomes is not the consequence of a beneficial checkpoint delay, but rather a pathological outcome. NPCs are assembled in two stages, during telophase concomitantly with membrane recruitment on chromatin (hereon referred to as postmitotic assembly) and, later on, during interphase when the nuclear envelope is already sealed [45]. The mechanisms for postmitotic and interphase NPC assembly are fundamentally different and have different kinetics, with the first one being much faster than the second one [10,35]. By using a combination of live-cell imaging and high-resolution 3D electron microscopy, Otsuka and colleagues [36] reported that postmitotic NPC reassembly proceeds by a radial dilation of small membrane openings pre-existing in the endoplasmic reticulum. In contrast to the postmitotic process, the interphase NPC assembly relies on *de novo* membrane fusion as it proceeds by an inside-out evagination of the inner nuclear membrane that grows depth until it fuses with the outer nuclear membrane [35]. Similar to the assembly of other NE components, NPC formation in telophase cells is locally delayed in the so-called “core” NE domains located in the pole-to-

pole axis of the mitotic spindle due to dense spindle microtubules on the DNA surface [5] (Fig. 1). In the subsequent interphase, the core domains of the fully sealed interphasic NE progressively assemble new NPCs, but this new wave of NPC assembly during interphase relies on pre-existing NPCs in adjacent regions of the nucleus. An important consequence inferred from this model is that reduced numbers of functional NPC on the envelope of abnormal nuclear structures that emerge at the end of mitosis, such as MN and chromatin bridges, hinder NPC formation throughout interphase. In agreement with this, Liu and colleagues [25] showed that loosening of spindle microtubule bundling by siRNA-mediated depletion of the kinesin KIF4A partially reversed the defect in NPC and other non-core NE proteins on lagging chromosomes.

Whatever the causes of poor NE assembly on missegregated chromatin are, whether it is due to mechanical or biochemical causes, NE defects in micronuclei and DNA bridges seem to be linked to the position that the chromatin occupies within the dividing cell. Alteration of the NE composition affects not only basic biological processes, such as DNA replication or repair that requires timely transport of proteins but also affects proper nucleo-cytoplasmic compartmentalization.

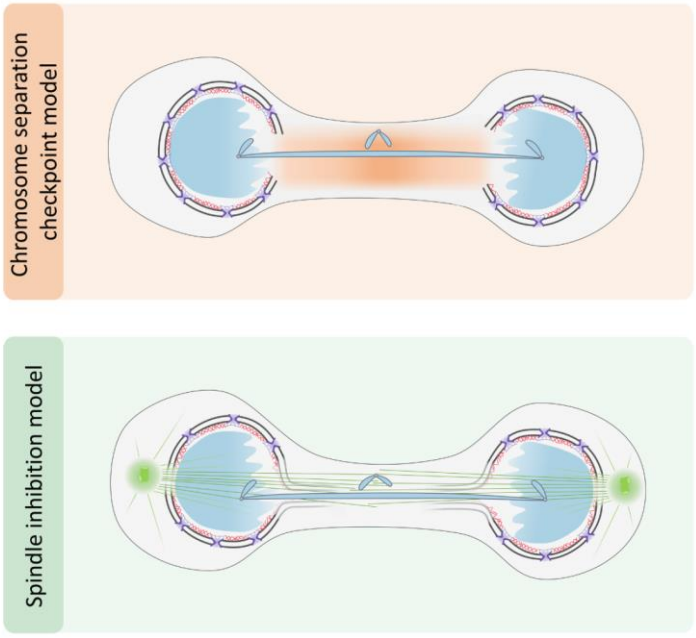


Fig. 1. Two models for nuclear envelope reassembly in telophase. Upper panel: According to the chromosome separation checkpoint model, segregated chromosome masses (light blue) recruit membranes (dark gray) and lamin filaments (red), but NE and NPCs (purple) assembly is locally delayed on chromosome regions close to Aurora B gradient (orange). Under this model, Aurora B activity mediates a surveillance mechanism that prevents chromosome decondensation and NE reassembly until the effective separation of sister chromatids is achieved. Localized delays in nuclear envelope formation have been proposed to facilitate inclusion of late-segregating chromosome fragments. Based on this model, the reassembly of the NE is delayed on lagging chromosomes and DNA bridges. Lower panel: According to the spindle inhibition model, the non-core NE (including NPCs) assembly is inhibited by the mitotic spindle (green) in telophase cells. It is proposed that the chromosome regions in contact with the spindle assemble the core NE (pale gray), whereas the chromosome peripheral regions assemble the non-core NE (dark gray) including NPCs (purple). On the basis of this model,

the NE around lagging chromosomes and DNA bridges resembles the core domain of the NE and fails to recruit NPCs and nuclear lamina (red) despite assembling the core membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Nuclear envelope rupture of micronuclei and chromatin bridges

An intriguing phenomenon was reported in 2012 in human cancer cell lines. Using live-cell imaging, Hetzer's laboratory characterized singular episodes wherein the primary nuclei of proliferating cells from different cancer cell lines become temporarily ruptured during interphase [44]. The interphase loss of the nuclear permeability barrier allowed nucleoplasmic proteins to leak out and cytoplasmic proteins to leak in. Still images in U2OS cells revealed that nuclear ruptures initiated from localized deformations of the NE that expanded forming NE herniations, which are eventually ruptured.

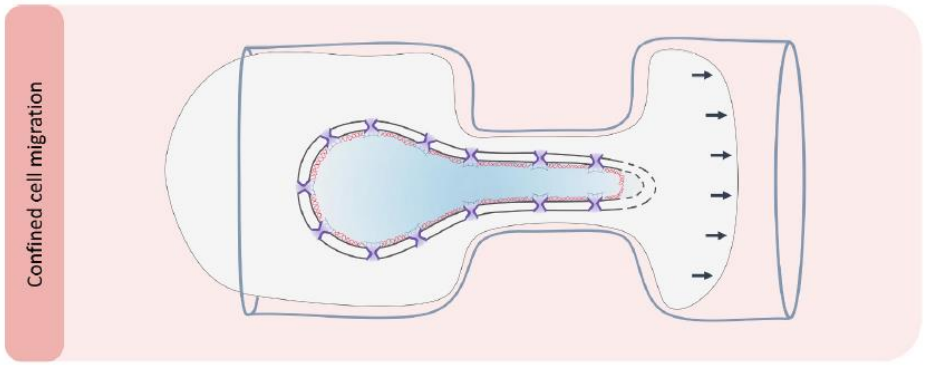
Analysis of NE rupture has identified two significant contributors to NE instability: nuclear lamina organization and mechanical stress (Fig. 2). Experiments in cancer cell lines showing that lamin B1 overexpression is sufficient to prevent NE rupture and that lamin depletion promotes envelope rupture revealed the important role of the nuclear lamina in NE integrity [44]. However, external physical forces can also induce NE rupture. Transient rupture at the leading end of the nucleus was visualized in dendritic cells, fibroblasts, and cancer cells forced to migrate through small pores [8,37]. Significantly, NE rupture in cells subjected to mechanical stress relies on the assembly of contractile actin bundles in the cytoplasm that interact with the nucleus via the linker of nucleoskeleton and cytoskeleton (LINC) complex. Experiments showing that inhibition of cytosolic myosin II activity or loss of the LINC complex reduced the occurrence of NE rupture sustained the notion that NE rupture depends on an increase in intranuclear pressure from actin-based nucleus confinement [20]. According to the emerging model, weak membrane areas caused by defects in lamina organization, rupture when mechanical forces on the nucleus, such as actin-based nucleus compression and stretching, increases the internal pressure on the nuclear membrane.

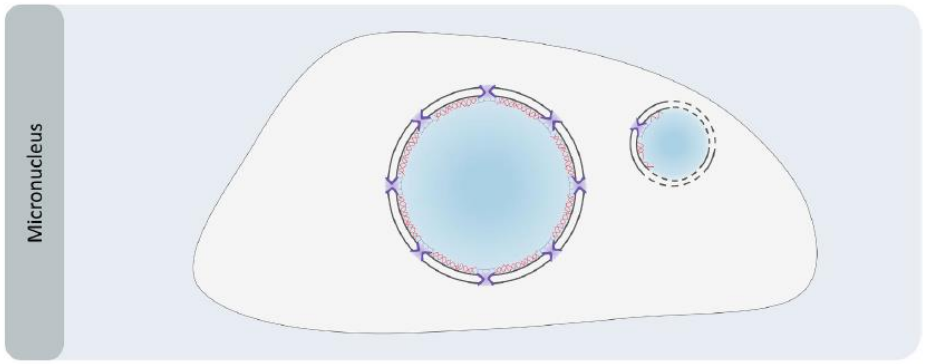
Recent studies have anticipated that nuclear lamina organization defects and mechanical stress may converge in generating NE rupture in relevant pathophysiological processes such as the migration of tumor cells during the process of invasion. Consistent with this concept, a localized detachment of the nuclear membranes from the nuclear lamina in the expanding nuclear blebs of cancer cells migrating through small pores was reported by Denais et al. [8]. Moreover, staining of NE components showed that nuclear pores and lamin B1 were excluded from the ruptured region at the tip of the nucleus [37]. Yet another example of the convergent contribution of mechanical stress and nuclear envelope defects on NE rupture is found in cells born with chromatin bridges. By physically connecting the two nuclei, chromatin bridges exert pulling

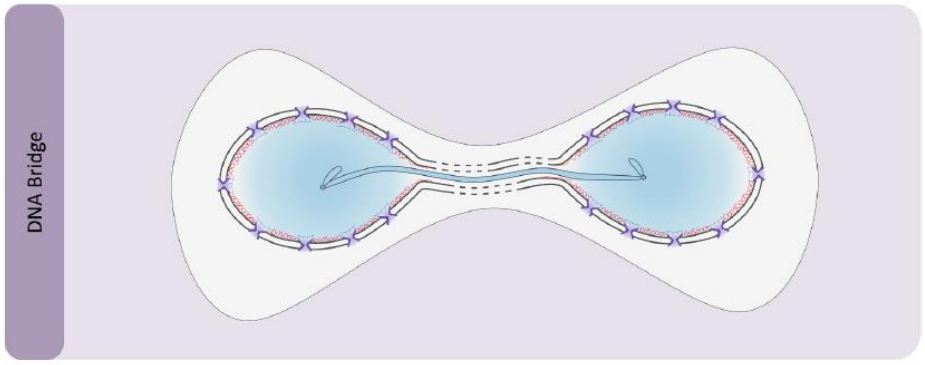
forces on the nucleus and induce abnormal teardrop morphologies in both daughter nuclei. As a consequence of this, nuclear lamins are lost around the chromatin bridges, which are then prone to rupture [29]. Micronuclei also have a high probability of NE rupture. However, in contrast to chromatin bridges, MN are not subjected to heavy mechanical stress. Research suggests strongly that compositional defects, including nuclear lamina defects that manifest with the appearance of gaps in the lamina, are responsible for MN disruption [19,48]. As discussed in the previous section, the defects in the NE composition of MN are probably linked to the position that the missegregated chromatin occupies within the dividing cell.

Transient rupture of the primary nucleus envelope is a source of DNA damage. Intense γH2AX staining, a marker of DNA double-strand breaks (DSBs), was observed at chromatin protrusions of cells that were forced to pass through small pores [8]. The confirmation that DNA damage was caused by migration-induced nuclear deformation and subsequent NE rupture was obtained by live-cell imaging on cells expressing fluorescently labeled 53BP1, another marker of DNA damage [8,22]. Altogether, this suggested that although a transient loss of NE integrity is not fatal for cells, DNA DSBs might arise from it.

Cells are protected against the deleterious consequences of NE rupture by a rapid reestablishment of NE integrity. The envelope of the primary nucleus reseals rapidly during interphase assisted by components of the ESCRT III (endosomal sorting complexes required for transport III) membrane-remodelling machinery [8,37]. However, NE rupture in MN is irreparable as demonstrated with nuclear and cytoplasmic fluorescent reporters [19]. The irreversibility in the loss of compartmentalization of MN potentially arise from its extreme membrane curvature [28]. Recent evidence revealed that high nuclear curvature imposed by a physical external probe on the primary nucleus promotes NE rupture and favors sustained DNA damage [47]. Once launched, loss of NE integrity is probably also permanent in chromatin bridges. In this case, it is not as a result of the curvature of its NE but of the distance between the nuclei to which the bridge is anchored. In a scenario such as that posed in MNs and DNA bridges, in which the cell is unable to repair collapsed NE, damage to the DNA of these structures is exacerbated.







	NE composition defects	Mechanical forces (tension/pressure)	DNA Damage	Reversibility of NE rupture
Confined cell migration	(X)	✓	✓	✓
Micronucleus	✓	(X)	✓	X
DNA Bridge	✓	✓	✓	X

Fig. 2. Mechanisms of nuclear envelope rupture. NE rupture occurs frequently as a result of defects in the organization of the nuclear lamina (red) that give rise to gaps in the lamina meshwork. NE rupture can also occur as a result of increased membrane stress associated with mechanical forces or architectural restrictions. Upper panel: Confined migration of cells leads to the formation of nuclear membrane protrusions at the leading edge of the nucleus as the cell moves through space constrictions. Nuclear blebs form when nuclear membranes (dark gray) detach from the nuclear lamina (red), bulge into the cytoplasm and eventually rupture. DNA double-strand breaks might result from NE openings. Rupture of the primary nucleus during confined cell migration is transient as cells can restore the nuclear membrane integrity during interphase. Middle panel: Micronuclear envelope rupture requires nuclear lamina defects, but the role of mechanical stress is unknown. Disruption of the micronucleus results in DNA damage as a consequence of defects in nuclear functions (deficient DNA replication and DNA repair). Unlike the primary nucleus, micronuclear envelope rupture is irreversible. This is

probably a consequence of the high curvature of the micronuclear envelope. Lower panel: nuclear lamina defects and increased mechanical stress is associated with NE rupture of DNA bridges. DNA damage can result from NE rupture of DNA bridges, although mechanical stress on the DNA bridge due to migration of the connected daughter cells also play an important role. Like micronuclei, NE rupture on bridging DNA is also irreversible. (For interpretation of the references to color in this figure legend, the Reader is referred to the web version of this article.)

5. DNA damage associated with micronuclei

Micronuclei have been associated with DNA damage from the late of 1960s when a pioneering study using cytogenetic approaches reported in human cells the presence of pulverized chromosomes presumably derived from MN [33]. Direct evidence for the presence of massive DNA damage in MN was obtained using γH2AX immunolabeling in irradiated human fibroblasts. Uniform intense γH2AX labeling of MN revealed massive fragmentation of the micronuclear chromatin [41]. Although processes involving a selective degradation of the micronuclear DNA could not be excluded, chromothriptic shattering of micronuclear chromatin would also lead to the reported uniform γH2AX labeling pattern.

Chromothripsis, a mutagenic phenomenon relevant in cancer development, consists of a catastrophic shattering of one or more chromosome regions in a single event, followed by a seemingly random repair of the DNA fragments that lead to the generation of highly rearranged chromosomes [40]. Considering that chromothripsis is most frequently restricted to a single chromosome, the physical isolation of missegregated chromosomes within micronuclei offers a mechanistic explanation for this phenomenon. Indeed, Crasta et al. [7] found that acquisition of DNA damage in MN was often associated with DNA replication. MN undergo defective and asynchronous DNA replication — a defect that was associated with “pulverized” appearance of single chromosomes in micronucleated mitotic cells. By using live-cell imaging, the authors tracked the fate of newly generated MN and reported that chromosome aberrations acquired in MN could be incorporated into the genome as the micronuclear chromatin either reincorporated with the primary nucleus after anaphase or persisted as a micronucleus. Confirmation that genomic rearrangements specifically involved the missegregated chromosomes and occurred within one cell cycle came from the elegant combination of live-cell imaging with single-cell DNA sequencing. DNA sequencing of pairs of daughter cells derived from a micronucleated cell progenitor identified a concentration of rearrangements associated with the gained haplotype in one of the daughters [48]. More recently, an experimental approach to induce the formation of MN from a specific chromosome corroborated the association between micronucleation and chromothriptic genome rearrangements. By selectively inactivating the Y centromere, Ly and colleagues showed both at the cytogenetic and the DNA sequencing level that the missegregated Y chromosome exhibit 120-fold higher probability of developing structural rearrangements

[26,27]. According to these authors, the cascade of events is initiated by centromere inactivation, followed by missegregation and fragmentation of micronucleated chromatin. In the following interphase, canonical non-homologous end joining, but not homology-dependent repair, facilitates the religation of chromosome fragments if the content of the micronucleus was included in the primary nucleus, but not if it was kept separate after the second mitosis.

While the association between MN and chromothripsis is clear, one question remains to be addressed: why might chromosomes sequestered in MN experience massive fragmentation? Micronucleation of a chromosome can lead to DNA cleavage through different mechanisms. A first type of mechanisms depends on the loss of nuclear functions due to NE defects and NE disruption during interphase. The collapse of the micronuclear envelope upon DNA replication initiation slows or stalls DNA synthesis due to leakage of essential nuclear components [19]. DNA damage associated with replication stress might be exacerbated by deficient DNA damage response signalling. Consistent with this, although MN present significant labeling of the DNA damage marker γH2AX, they show poor recruitment of DNA repair factors such as 53BP1 [41,42]. However, the acquisition of massive DNA damage does not always require NE rupture. Defects in the unruptured NE cause micronuclear DNA replication to be slower, and as a consequence of it proliferating cells enter mitosis when replication of micronuclear DNA is not yet complete. The persistence of a large number of unrepaired DNA breaks and unresolved replication intermediates could be catastrophic upon mitotic breakdown of the micronuclear envelope and condensation of chromosomes [7,25]. According to these models, the micronucleated chromosomes undergo an asynchronous DNA synthesis with the primary nucleus thus suffering from a disturbance in its performance of condensation of not yet fully replicated chromosomes, leading eventually to a phenotype of localized shattering in mitosis.

Yet a second mechanism, which is not associated with DNA replication, might potentially link micronucleation with massive DNA damage. Upon disruption of the micronuclear envelope, not only leakage of nuclear proteins, but also an influx of cytosolic proteins into the micronucleus has been observed. NE collapse leads the MN content exposed to the cytoplasmic environment as evidenced by the rapid accumulation of a fluorescent form of the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS) [31]. Although the downstream consequences of cGAS pathway activation on the micronuclear chromosomes are unknown, upon rupture the micronuclear chromosomes become exposed to harmful cytoplasmic components such as endo- and exonucleases. Taken together, several non-mutually exclusive mechanisms associated with defects in nuclear functions arising from defective nuclear envelope could act to promote massive DNA damage in micronuclei.

6. DNA damage associated with chromatin bridges

Several studies have reported an association between the BFB cycles and chromothripsis [24,34]. While the original BFB model places the resolution of chromatin bridges during the last stages of the cell division, more recent studies indicate that they persist through mitosis and cytokinesis to form stabilized nucleoplasmic connections between the two daughter cells. NE rupture of chromatin bridges during interphase is highly correlated with chromothripsis and it was suggested to be a critical initiating event for the localized shattering of DNA in these abnormal nuclear structures [29]. These authors examined the behavior of dicentric chromosomes using spinning-disk confocal imaging and reported that, after persisting for many hours, resolution of chromatin bridges is facilitated by the cytoplasmic 3′ repair exonuclease 1 (TREX1). It was proposed that this enzyme gains access to the bridging chromatin through NE rupture and it contributes to the resolution of the bridge because the amount of ssDNA in the bridge — a likely key step in this process — was shown to be decreased after inhibiting NE rupture. Whole-genome sequencing of clones derived from cells born with DNA bridges showed rearrangements with spatial clustering of breakpoints, a hallmark of chromothripsis [29,30]. However, even though NE rupture frequently associates with DNA breaks, it is unclear whether this is the primary cause of damage on the bridging DNA as it is not known which is the source of the nicks in the double-stranded DNA that allow the exonuclease TREX1 to initiate resection.

In a recent study, Umbreit et al. [43] reported that deformation of bridging chromatin in migrating cells is sufficient to generate simple breaks and local DNA fragmentation. The authors observed that motile cell lines broke DNA bridges during interphase, whereas less motile cell lines rarely underwent bridge breakage. They concluded that mechanical forces generated by the interphase actomyosin cytoskeleton stretch and break chromosome bridges, promoting local chromosome fragmentation. By using a combination of live-cell imaging with single-cell whole-genome sequencing, the authors observed that, in a fraction of the cells born with DNA bridges, the immediate genomic consequences of bridge breakage are relatively simple patterns of copy number alterations localized near the sites of breakage. However, highly complex rearrangements suggestive of errors in the replication of bridging DNA were also observed in some cells suggesting that defective DNA replication generates additional DNA damage and chromothripsis [43]. A possibility is that DNA bridge breakage involves both mechanical and biochemical mechanisms as access to DNA of enzymes, such as cytoplasmic nucleases, might be enhanced by mechanical tension through actomyosin-mediated disruption of the NE.

7. Non-genetic effects of the nuclear envelope rupture of micronuclei and chromatin bridges

Two papers published in 2017 established a connection between aberrantly shaped nuclei and activation of the proinflammatory response [18,31]. The papers described a new way of activating the innate immune system through MN surveillance by cGAS, a cytosolic DNA sensor that induces the production of cytokines through the cGAS-STING pathway, thus extending the effects of nuclear atypia on the non-genetic field. Harding et al. [18] observed that inhibiting the c-NHEJ after high doses of radiation decreased the innate immune response instead of activating it. Because there was a strong G2 cell cycle arrest when c-NHEJ was inhibited in irradiated cells, the authors speculated that the activation of the innate immune response required passing through mitosis and subsequent formation of MN. The authors observed that cGAS is concentrated on disrupted MN and reported that inflammatory cytokine production was inhibited by genetic ablation of cGAS. MacKenzie et al. [31] arrived at the same conclusion using a different experimental system. They reported an accumulation of cGAS in MN formed in mouse embryonic fibroblasts lacking RNase H2, an enzyme that has a role in ribonucleotide excision repair. Consistent with the results reported by Harding et al. [18], they also reported a higher degree of proinflammatory gene expression in cells with radiation-induced MN as compared with non-micronucleated cells. The preceding studies support a model whereby loss of micronuclear envelope integrity can provide access of cytoplasmic factors to micronuclear DNA and by extension, to other nuclear shape defects such as DNA bridges. According to this model, NE rupture leads to cGAS accumulation on the micronuclear and bridging chromatin, which activates cGAS to produce cGAMP and signal to the STING adaptor protein, leading to expression of interferons and pro-inflammatory cytokines [9,15].

While the cGAS-STING pathway evolved in eukaryotic cells as a major mechanism for the detection of bacterial and viral infection, activation of cGAS by self DNA has dual opposite consequences. Whereas it represents a threat to the organism as it triggers autoimmunity, it may also act as an immune surveillance mechanism that detects and potentially suppresses a range of neoplasia-inducing processes. Importantly, these processes have a common denominator: they are based on the formation of aberrant nuclear structures that suffer frequent and irreversible rupture of their wrapping.

8. Conclusions

Nuclear architecture defects are now appreciated as sources of phenomena such as chromothripsis, capable of radically changing the genome of cells. Massive DNA damage has been associated with defects in nuclear functions arising from defective nuclear envelope and ectopic access of cytoplasmic nucleases to missegregated chromatin. Also, mechanical stress

has been identified as an initiating event for chromothripsis. However, not only the repertoire of genetic consequences has expanded but also the type of consequences. We now understand that micronuclei and DNA bridges can also lead to important non-genetic effects through the cGAS-STING pathway, which activates the innate immune system through secretion of cytokines. Therefore, the direct consequences of micronuclei and DNA bridges do not limit to the cells wearing these abnormal structures but they expand to the whole organism.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

[1] O. Afonso, I. Matos, A.J. Pereira, P. Aguiar, M.A. Lampson, H. Maiato, Feedback control of chromosome separation by a midzone Aurora B gradient, Science 345 (6194) (2014) 332–336, https://doi.org/10.1126/science.1251121.

[2] O. Afonso, C.M. Castellani, L.P. Cheeseman, J.G. Ferreira, B. Orr, L.T. Ferreira, J. J. Chambers, E. Morais-de-S´a, T.J. Maresca, H. Maiato, Spatiotemporal control of mitotic exit during anaphase by an aurora B-Cdk1 crosstalk, eLife 8 (2019) 1–31, https://doi.org/10.7554/eLife.47646.

[3] W. Antonin, J. Ellenberg, E. Dultz, Nuclear pore complex assembly through the cell cycle: regulation and membrane organization, FEBS Lett. 582 (14) (2008) 2004–2016, https://doi.org/10.1016/j.febslet.2008.02.067.

[4] R. a Burrell, S.E. McClelland, D. Endesfelder, P. Groth, M.C. Weller, N. Shaikh, E. Domingo, N. Kanu, S.M. Dewhurst, E. Gronroos, S.K. Chew, A.J. Rowan, A. Schenk, M. Sheffer, M. Howell, M. Kschischo, A. Behrens, T. Helleday, J. Bartek, I.P. Tomlinson, C. Swanton, Replication stress links structural and numerical cancer chromosomal instability, Nature 494 (7438) (2013) 492–496, https://doi. org/10.1038/nature11935.

[5] I.J. De Castro, R.S. Gil, L. Ligammari, M.L. Di Giacinto, P. Vagnarelli, CDK1 and PLK1 coordinate the disassembly and reassembly of the nuclear envelope in vertebrate mitosis, Oncotarget 9 (8) (2018) 7763–7773. (www.impactjournals.co m/oncotarget/).

[6] D. Cimini, F. Degrassi, Aneuploidy: a matter of bad connections, Trends Cell Biol. 15 (8) (2005) 442–451, https://doi.org/10.1016/j.tcb.2005.06.008.

[7] K. Crasta, N.J. Ganem, R. Dagher, A.B. Lantermann, E.V. Ivanova, Y. Pan, L. Nezi, A. Protopopov, D. Chowdhury, D. Pellman, DNA breaks and chromosome pulverization from errors in mitosis, Nature 482 (7383) (2012) 53–58, https://doi. org/10.1038/nature10802.

[8] C.M. Denais, R.M. Gilbert, P. Isermann, A.L. McGregor, M. te Lindert, B. Weigelin, P.M. Davidson, P. Friedl, K. Wolf, J. Lammerding, Nuclear envelope rupture and repair during cancer cell migration, Science 352 (6283) (2016) 353–358, https:// doi.org/10.1126/science.aad7297.

[9] Z. Dou, K. Ghosh, M.G. Vizioli, J. Zhu, P. Sen, K.J. Wangensteen, J. Simithy, Y. Lan, Y. Lin, Z. Zhou, B.C. Capell, C. Xu, M. Xu, J.E. Kieckhaefer, T. Jiang, M. Shoshkes- Carmel, K.M.A.A. Tanim, G.N. Barber, J.T. Seykora, S.E. Millar, K.H. Kaestner, B. A. Garcia, P.D. Adams, S.L. Berger, Cytoplasmic chromatin triggers inflammation in senescence and cancer, Nature 550 (7676) (2017) 402–406, https://doi.org/ 10.1038/nature24050.

[10] E. Dultz, J. Ellenberg, Live imaging of single nuclear pores reveals unique assembly kinetics and mechanism in interphase, J. Cell Biol. 191 (1) (2010) 15–22, https:// doi.org/10.1083/jcb.201007076.

[11] M. Fenech, Cytokinesis-block micronucleus cytome assay, Nat. Protoc. 2 (5) (2007) 1084–1104, https://doi.org/10.1038/nprot.2007.77.

[12] C.L. Fonseca, H.L.H. Malaby, L.A. Sepaniac, W. Martin, C. Byers, A. Czechanski, D. Messinger, M. Tang, R. Ohi, L.G. Reinholdt, J. Stumpff, Mitotic chromosome alignment ensures mitotic fidelity by promoting interchromosomal compaction during anaphase, J. Cell Biol. 218 (4) (2019) 1148–1163, https://doi.org/ 10.1083/jcb.201807228.

[13] A. Genescà, J. Pampalona, C. Frías, D. Domínguez, L. Tusell, Role of telomere dysfunction in genetic intratumor diversity, Adv. Cancer Res. (2011) 11–41, https://doi.org/10.1016/B978-0-12-387688-1.00002-8.

[14] D. Gisselsson, L. Pettersson, M. Hoglund, M. Heidenblad, L. Gorunova, J. Wiegant, F. Mertens, P. Dal Cin, F. Mitelman, N. Mandahl, Chromosomal breakage-fusion-bridge events cause genetic intratumor heterogeneity, Proc. Natl. Acad. Sci. USA 97 (10) (2000) 5357–5362, https://doi.org/10.1073/pnas.090013497.

[15] S. Glück, B. Guey, M.F. Gulen, K. Wolter, T.W. Kang, N.A. Schmacke, A. Bridgeman, J. Rehwinkel, L. Zender, A. Ablasser, Designed experiments and analysed the data, Nat. Cell Biol. 19 (9) (2017) 1061–1070, https://doi.org/10.1038/ncb3586.

[16] Y. Gruenbaum, R. Foisner, Lamins: nuclear intermediate filament proteins with fundamental functions in nuclear mechanics and genome regulation, Annu. Rev. Biochem. 84 (2015) 131–164, https://doi.org/10.1146/annurev-biochem-060614- 034115.

[17] T. Haraguchi, T. Kojidani, T. Koujin, T. Shimi, H. Osakada, C. Mori, A. Yamamoto, Y. Hiraoka, Live cell imaging and electron microscopy reveal dynamic processes of BAF-directed nuclear envelope assembly, J. Cell Sci. 121 (15) (2008) 2540–2554, https://doi.org/10.1242/jcs.033597.

[18] S.M. Harding, J.L. Benci, J. Irianto, D.E. Discher, A.J. Minn, R.A. Greenberg, Mitotic progression following DNA damage enables pattern recognition within micronuclei, Nature 548 (7668) (2017) 466–470, https://doi.org/10.1038/nature23470.

[19] E.M. Hatch, A.H. Fischer, T.J. Deerinck, M.W. Hetzer, Catastrophic nuclear envelope collapse in cancer cell micronuclei, Cell 154 (1) (2013) 47–60, https:// doi.org/10.1016/j.cell.2013.06.007.

[20] E.M. Hatch, M.W. Hetzer, Nuclear envelope rupture is induced by actin-based nucleus confinement, J. Cell Biol. 215 (1) (2016) 27–36, https://doi.org/10.1083/ jcb.201603053.

[21] N. Holland, C. Bolognesi, M. Kirschvolders, S. Bonassi, E. Zeiger, S. Knasmueller, M. Fenech, The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current

status and knowledge gaps, Mutat. Res. Rev. Mutat. Res. 659 (1–2) (2008) 93–108, https://doi.org/10.1016/j.mrrev.2008.03.007.

[22] J. Irianto, Y. Xia, C.R. Pfeifer, A. Athirasala, J. Ji, C. Alvey, M. Tewari, R. R. Bennett, S.M. Harding, A.J. Liu, R.A. Greenberg, D.E. Discher, DNA damage follows repair factor depletion and portends genome variation in cancer cells after pore migration, Curr. Biol. 27 (2) (2017) 210–223, https://doi.org/10.1016/j. cub.2016.11.049.

[23] E. Lecona, O. Fernández-Capetillo, Replication stress and cancer: it takes two to tango, Exp. Cell Res. 329 (1) (2014) 26–34, https://doi.org/10.1016/j. yexcr.2014.09.019.

[24] Y. Li, C. Schwab, S.L. Ryan, E. Papaemmanuil, H.M. Robinson, P. Jacobs, A. V. Moorman, S. Dyer, J. Borrow, M. Griffiths, N.A. Heerema, A.J. Carroll, P. Talley, N. Bown, N. Telford, F.M. Ross, L. Gaunt, R.J.Q. McNally, B.D. Young, P. Sinclair, V. Rand, M.R. Teixeira, O. Joseph, B. Robinson, M. Maddison, N. Dastugue, P. Vandenberghe, C. Haferlach, P.J. Stephens, J. Cheng, P. Van Loo, M.R. Stratton, P.J. Campbell, C.J. Harrison, Constitutional and somatic rearrangement of chromosome 21 in acute lymphoblastic leukaemia, Nature 508 (7494) (2014) 98–102, https://doi.org/10.1038/nature13115.

[25] S. Liu, M. Kwon, M. Mannino, N. Yang, F. Renda, A. Khodjakov, D. Pellman, Nuclear envelope assembly defects link mitotic errors to chromothripsis, Nature 561 (7724) (2018) 551–555, https://doi.org/10.1038/s41586-018-0534-z.

[26] P. Ly, L.S. Teitz, D.H. Kim, O. Shoshani, H. Skaletsky, D. Fachinetti, D.C. Page, D. W. Cleveland, Selective Y centromere inactivation triggers chromosome shattering in micronuclei and repair by non-homologous end joining, Nat. Cell Biol. 19 (1) (2017) 68–75, https://doi.org/10.1038/ncb3450.

[27] P. Ly, S.F. Brunner, O. Shoshani, D.H. Kim, W. Lan, T. Pyntikova, A.M. Flanagan, S. Behjati, D.C. Page, P.J. Campbell, D.W. Cleveland, Chromosome segregation errors generate a diverse spectrum of simple and complex genomic rearrangements, Nat. Genet. 51 (4) (2019) 705–715, https://doi.org/10.1038/ s41588-019-0360-8.

[28] P. Ly, D.W. Cleveland, Rebuilding chromosomes after catastrophe: emerging mechanisms of chromothripsis, Trends Cell Biol. 27 (12) (2017) 917–930, https:// doi.org/10.1016/j.tcb.2017.08.005.

[29] J. Maciejowski, Y. Li, N. Bosco, P.J. Campbell, T. de Lange, Chromothripsis and kataegis induced by telomere crisis, Cell 163 (7) (2015) 1641–1654, https://doi. org/10.1016/j.cell.2015.11.054.

[30] J. Maciejowski, A. Chatzipli, A. Dananberg, K. Chu, E. Toufektchan, L.J. Klimczak, D.A. Gordenin, P.J. Campbell, T. de Lange, APOBEC3-dependent kataegis and TREX1-driven chromothripsis during telomere crisis, Nat. Genet. 52 (9) (2020) 884–890, https://doi.org/10.1038/s41588-020-0667-5.

[31] K.J. MacKenzie, P. Carroll, C.A. Martin, O. Murina, A. Fluteau, D.J. Simpson, N. Olova, H. Sutcliffe, J.K. Rainger, A. Leitch, R.T. Osborn, A.P. Wheeler, M. Nowotny, N. Gilbert, T. Chandra, M.A.M. Reijns, A.P. Jackson, cGAS surveillance of micronuclei links genome instability to innate immunity, Nature 548 (7668) (2017) 461–465, https://doi.org/10.1038/nature23449.

[32] B. McClintock, The stability of broken ends of chromosomes in Zea mays, Genetics 26 (2) (1941) 234–282.

[33] H. Kato, A.A. Sandberg, Chromosome pulverization in human binucleate cells following colcemid treatment, J Cell Biol. 34 (1) (1967) 35–45, https://doi.org/ 10.1083/jcb.34.1.35.

[34] K. Nones, N. Waddell, N. Wayte, A.M. Patch, P. Bailey, F. Newell, O. Holmes, J. L. Fink, M.C.J. Quinn, Y.H. Tang, G. Lampe, K. Quek, K.A. Loffler, S. Manning, S. Idrisoglu, D. Miller, Q. Xu, N. Waddell, P.J. Wilson, T.J.C. Bruxner, A.N. Christ, I. Harliwong, C. Nourse, E. Nourbakhsh, M. Anderson, S. Kazakoff, C. Leonard, S. Wood, P.T. Simpson, L.E. Reid, L. Krause, D.J. Hussey, D.I. Watson, R.V. Lord, D. Nancarrow, W.A. Phillips, D. Gotley, B.M. Smithers, D.C. Whiteman, N. K. Hayward, P.J. Campbell, J.V. Pearson, S.M. Grimmond, A.P. Barbour, Genomic catastrophes frequently arise in esophageal adenocarcinoma and drive tumorigenesis, Nat. Commun. 5 (2014) 5224, https://doi.org/10.1038/ ncomms6224.

[35] S. Otsuka, K.H. Bui, M. Schorb, M.J. Hossain, A.Z. Politi, B. Koch, M. Eltsov, M. Beck, J. Ellenberg, Nuclear pore assembly proceeds by an inside-out extrusion of the nuclear envelope, eLife 5 (2016) 1–23, https://doi.org/10.7554/eLife.19071.

[36] S. Otsuka, A.M. Steyer, M. Schorb, J.K. H´erich´e, M.J. Hossain, S. Sethi, M. Kueblbeck, Y. Schwab, M. Beck, J. Ellenberg, Postmitotic nuclear pore assembly proceeds by radial dilation of small membrane openings, Nat. Struct. Mol. Biol. 25 (1) (2018) 21–28, https://doi.org/10.1038/s41594-017-0001-9.

[37] M. Raab, M. Gentili, H. de Belly, H.R. Thiam, P. Vargas, A.J. Jimenez, F. Lautenschlaeger, R. Voituriez, A.M. Lennon-Dum´enil, N. Manel, M. Piel, ESCRT III repairs nuclear envelope ruptures during cell migration to limit DNA damage and cell death, Science 352 (6283) (2016) 359–362, https://doi.org/10.1126/ science.aad7611.

[38] M. Samwer, M.W.G. Schneider, R. Hoefler, P.S. Schmalhorst, J.G. Jude, J. Zuber, D. W. Gerlich, DNA cross-bridging shapes a single nucleus from a set of mitotic chromosomes, Cell 170 (5) (2017) 956–972.e23, https://doi.org/10.1016/j. cell.2017.07.038.

[39] H. Sapkota, E. Wasiak, J.R. Daum, G.J. Gorbsky, Multiple determinants and consequences of cohesion fatigue in mammalian cells, Mol. Biol. Cell 29 (15) (2018) 1811–1824, https://doi.org/10.1091/mbc.E18-05-0315.

[40] P.J. Stephens, C.D. Greenman, B. Fu, F. Yang, G.R. Bignell, L.J. Mudie, E. D. Pleasance, K.W. Lau, D. Beare, L.A. Stebbings, S. McLaren, M.L. Lin, D. J. McBride, I. Varela, S. Nik-Zainal, C. Leroy, M. Jia, A. Menzies, A.P. Butler, J. W. Teague, M.A. Quail, J. Burton, H. Swerdlow, N.P. Carter, L.A. Morsberger, C. Iacobuzio-Donahue, G.A. Follows, A.R. Green, A.M. Flanagan, M.R. Stratton, P. A. Futreal, P.J. Campbell, Massive genomic rearrangement acquired in a single catastrophic event during cancer development, Cell 144 (1) (2011) 27–40, https:// doi.org/10.1016/j.cell.2010.11.055.

[41] M. Terradas, M. Martín, L. Tusell, A. Genesc`a, DNA lesions sequestered in micronuclei induce a local defective-damage response, DNA Repair 8 (10) (2009) 1225–1234, https://doi.org/10.1016/j.dnarep.2009.07.004.

[42] M. Terradas, M. Martín, L. Hern´andez, L. Tusell, A. Genesc`a, Nuclear envelope defects impede a proper response to micronuclear DNA lesions, Mutat. Res. 729 (1–2) (2012) 35–40, https://doi.org/10.1016/j.mrfmmm.2011.09.003.

[43] N.T. Umbreit, C.Z. Zhang, L.D. Lynch, L.J. Blaine, A.M. Cheng, R. Tourdot, L. Sun, H.F. Almubarak, K. Judge, T.J. Mitchell, A. Spektor, D. Pellman, Mechanisms generating cancer genome complexity from a single cell division error, Science 368 (6488) (2020) eaba0712, https://doi.org/10.1126/science.aba0712.

[44] J.D. Vargas, E.M. Hatch, D.J. Anderson, M.W. Hetzer, Transient nuclear envelope rupturing during interphase in human cancer cells, Nucleus 3 (1) (2012) 88–100, https://doi.org/10.4161/nucl.18954.

[45] C. Wandke, U. Kutay, Enclosing chromatin: reassembly of the nucleus after open mitosis, Cell 152 (6) (2013) 1222–1225, https://doi.org/10.1016/j. cell.2013.02.046.

[46] B. Warecki, W. Sullivan, Micronuclei Formation Is Prevented by Aurora, 210 (September) (2018), 171–187. doi: 10.1534/genetics.118.301031.

[47] Y. Xia, I.L. Ivanovska, K. Zhu, L. Smith, J. Irianto, C.R. Pfeifer, C.M. Alvey, J. Ji, D. Liu, S. Cho, R.R. Bennett, A.J. Liu, R.A. Greenberg, D.E. Discher, Nuclear rupture at sites of high curvature compromises retention of DNA repair factors, J. Cell Biol. 217 (11) (2018) 3796–3808, https://doi.org/10.1083/jcb.201711161.

[48] C.-Z. Zhang, A. Spektor, H. Cornils, J.M. Francis, E.K. Jackson, S. Liu, M. Meyerson, D. Pellman, Chromothripsis from DNA damage in micronuclei, Nature 522 (7555) (2015) 179–184, https://doi.org/10.1038/nature14493.

CHAPTER 2

frontiers in Cell and Developmental Biology

ORIGINAL RESEARCH
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Breakage of CRISPR/Cas9-Induced Chromosome Bridges in Mitotic Cells

Marina Rodriguez-Muñoz, Martina Serrat, David Soler, Anna Genescà† and Teresa Anglada*†*

Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain

Chromosomal instability, the most frequent form of plasticity in cancer cells, often proceeds through the formation of chromosome bridges. Despite the importance of these bridges in tumor initiation and progression, debate remains over how and when they are resolved. In this study, we investigated the behavior and properties of chromosome bridges to gain insight into the potential mechanisms underlying bridge-induced genome instability. We report that bridges may break during mitosis or may remain unbroken until the next interphase. During mitosis, we frequently observed discontinuities in the bridging chromatin, and our results strongly suggest that a substantial fraction of chromosome bridges are broken during this stage of the cell cycle. This notion is supported by the observation that the chromatin flanking mitotic bridge discontinuities is often decorated with the phosphorylated form of the histone H2AX, a marker of DNA breaks, and by MDC1, an early mediator of the cell response to DNA breaks. Also, free 3′OH DNA ends were detected in more than half of the bridges during the final stages of cell division. However, even if detected, the DNA ends of broken bridges are not repaired in mitosis. To investigate whether mitotic bridge breakage depends on mechanical stress, we used experimental models in which chromosome bridges with defined geometry are formed. Although there was no association between spindle pole separation or the distance among non-bridge kinetochores and bridge breakage, we found a direct correlation between the distance between bridge kinetochores and bridge breakage. Altogether, we conclude that the discontinuities observed in bridges during mitosis frequently reflect a real breakage of the chromatin and that the mechanisms responsible for chromosome bridge breakage during mitosis may depend on the separation between the bridge kinetochores. Considering that previous studies identified mechanical stress or biochemical digestion as possible causes of bridge breakage in interphase cells, a multifactorial model emerges for the breakage of chromosome bridges that, according to our results, can occur at different stages of the cell cycle and can obey different mechanisms.

Edited by: Yu-Sheng Cong, Hangzhou Normal University, China

Reviewed by:
Marin Barisic, Danish Cancer Society Research Center (DCRC), Denmark
Jin He, Michigan State University, United States

*Correspondence:
Anna Genescà anna.genesca@uab.cat
Teresa Anglada teresa.anglada@uab.cat

†These authors have contributed equally to this work and share last authorship

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INTRODUCTION

An important characteristic of tumors is their ability to adapt. In the face of changes, be they endogenous or exogenous, the neoplastic cell undergoes alterations to generate a variety of phenotypes, some of which give the cell the ability to survive. An important difficulty in combating the adaptive capacity of neoplastic cells is that there is no single mechanism responsible for genome instability, but there are at least four different mechanisms: excessive erosion of telomeric sequences, accelerated DNA replication, impaired DNA repair, and defects in chromosome segregation during mitosis. However, although intrinsically different, the four mechanisms share, to a greater or lesser extent, a common intermediary: the chromosome bridge. This abnormal structure is frequently observed in tumor samples; it occurs 10 times more frequently in neoplasms and premalignant lesions than in healthy tissue (Rudolph et al., 2001; Gisselsson, 2003). Chromosome bridges associate with genomic instability as they can cause the regression of the cleavage furrow and lead to the formation of tetraploid cells in which supernumerary centrosomes hinder the correct segregation of chromosomes (Ganem et al., 2009; Pampalona et al., 2012). Alternatively, bridges can break and lead to DNA damage restricted to the chromosome involved in the bridge, a process known as chromothripsis (Maciejowski et al., 2015, 2020). Despite its close association with genome instability and its importance in tumor initiation and progression, the mechanisms that determine how and when bridges are resolved are unclear.

According to the Breakage-Fusion-Bridge (BFB) model (McClintock, 1939), chromosome bridges can break and initiate a cycle capable of self-feeding when a broken DNA end fuses with another broken end and forms a new unstable chromosome structure. In this way, the BFB cycle can instigate a profund reorganization of the genome. Based on time-lapse observations of living cells, classical studies placed the breakage of chromatin bridges during the last stages of cell division (Hoffelder et al., 2004; Shimizu et al., 2005). However, studies aimed at determining the causes of bridge rupture during mitosis reached controversial conclusions. A rapid shrinking of the chromatin fiber compatible with tangling of the severed chromatin prior to nuclear membrane reformation and completion of cytokinesis was reported by Shimizu et al. (2005). Whereas these observations indicate that mechanical tension would be responsible for severing anaphase bridges, other studies suggest that constricting forces generated by the cleavage furrow break chromosomal DNA (Janssen et al., 2011).

More recent studies argue that the often-visible discontinuities in bridging chromatin during the last stages of mitosis do not correspond to actual breaks (Maciejowski et al., 2015; Umbreit et al., 2020). Unlike the classical models, Maciejowski et al. (2015) and Umbreit et al. (2020) state that chromosome bridges cannot be resolved in anaphase and, therefore, inevitably lead to the

presence of chromatin in the cleavage plane. According to these authors, stabilized nucleoplasmic bridges persist in daughter cells for a considerable period of time as the presence of chromatin in the cleavage plane inhibits abscission, the final stage of cytokinesis (Steigemann et al., 2009). From there, destabilization of the bridge due to mechanical stress (Umbreit et al., 2020) or enzymatic digestion after loss of nuclear envelope function (Maciejowski et al., 2015, 2020) will eventually lead to bridge resolution in the interphase. In summary, different models have been proposed to explain how and when chromosome bridges are resolved, and it is unclear at present whether they can break during mitosis or, alternatively, remain invariably unbroken until the end of mitosis to break in the next interphase. It is important to distinguish between these two possibilities, as the mechanisms that determine the breakage of bridges may be different in the different stages of the cell cycle.

By using different methods for the detection of DNA breakage, we found that the discontinuities frequently observed in bridging chromatin at the end of mitosis mostly correspond to real breaks. Whereas some bridges remain unbroken and form nucleoplasmatic bridges, by employing experimental models to produce bridges with defined characteristics, we demonstrate that others resolve during mitosis, and their breakage is associated with the distance between the kinetochores involved in the bridge.

MATERIALS AND METHODS

Cell Culture

HEK 293T and 293T Phoenix cells were cultured in MEM, and U2OS cells were propagated in 1:1 DMEM:Ham's F10 medium. All media were supplemented with 10% Fetal Bovine Serum (FBS). MCF10A were grown in DMEM:F12 with 5% horse serum, 100 ng/mL cholera toxin, 10 μg/mL insulin, 20 ng/ml epidermal growth factor, and 0.5 μg/mL hydrocortisone. RPE1 Cas9 Tet-ON cells were obtained from Iain Cheeseman's Laboratory and cultured in DMEM:F12 with 10% tetracycline free FBS (FBS-TET-12A, Labclinics, Barcelona, Spain). U2OS LacO/LacI-GFP-CENPT were a kind gift from Iain Cheeseman's Laboratory (Gascoigne et al., 2011) and were cultured in 10 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside, I6758-1G, Merck, Kenilworth, NJ, United States) and 200 μg/mL Hygromycin B (H3274, Merck). U2OS GFP-MDC1 were obtained from the Laboratory of Manuel Stucki and were grown in 250 μg/mL of geneticin (11811023, Thermo Fisher Scientific, Waltham, MA, United States). Penicillin/streptomycin (100 U/mL) was added to the media, and cell cultures were maintained in a humidified incubator at 37 °C and 5% CO_2.

Plasmid Construction

A retroviral plasmid was obtained by cloning the GFP-Lap2β sequence (a gift from Ulrike Kutay's Laboratory) into the pLNCX2-mCherry-CHMP4B plasmid (a gift from Sanford Simon; Addgene plasmid #116923) between the AgeI and NotI restriction sites, thus removing the mCherry-CHMP4B gene. Guide RNA for the subtelomeric region of chromosome 2 (50-ATATTAAGGGCTCCCCGTCG-30) was designed using the CCTop - CRISPR/Cas9 target online predictor platform (Stemmer et al., 2015) and was cloned into lenti-sgRNA blast (gift from Brett Stringer, Addgene #104993) between the BsmBI restriction sites. The cloning protocol for lentisgRNA blast is described in addgene website[1]. All plasmids were verified by Sanger sequencing. After sequencing, plasmids were amplified by transformation into Stbl3 (C737303, Invitrogen, Waltham, MA, United States) or DH5α competent cells (18258012, Thermo Fisher Scientific) and purified using the NucleoBond PC kit (740573.100, Macherey-Nagel, Bethlehem, PA, United States) following the manufacturer's instructions. The DNA concentration was measured with a NanoDrop 2000 spectrometer (Thermo Fisher Scientific).

Viral Particle Production and Transduction

Lentivirus or retrovirus particles were produced in HEK 293T or 293T Phoenix, respectively. Cells were transfected at 70% confluency with 1 μg/mL of total DNA plasmid following the protocol from Trono's Laboratory website[2]. For lentivirus particle production, the DNA concentration of all plasmids was adjusted to a ratio 4:3:1 (transfer plasmid:psPAX2:pMD2.G; Supplementary Table 1). The supernatant was harvested 48 h later, concentrated through centrifugal filters (UFC910008, Merck), and stored at -80°C.

Cells were transduced with viral particles (Supplementary Table 1) overnight using medium with 4 μg/mL polybrene. For retroviral transduction, the procedure was repeated three times. Cells with the antibiotic-resistance cassette were selected, with 2.5 μg/mL blasticidin (15205, Merck) or 250 μg/mL geneticin (11811023, Thermo Fisher Scientific) for 2 weeks or 2 μg/mL puromycin (P8833, Merck) for 1 week. Transduced fluorescent cells were enriched by fluorescent activated cell sorting (FACS) by using a BD FACSJazz (BD Biosciences, Franklin Lakes, NJ, United States) equipped with a 488 nm laser and two detectors (530/40 and 692/40 nm).

Methods to Generate Chromosome Bridges

Chromosome bridges were generated using different methodologies: (1) To generate bridges with irradiation, cells were exposed to 2.5 Gy using an IBL-437C R-137 Cs irradiator (dose rate of 5.10 Gy/min) and incubated at 37 °C and 5% CO_2 for 24 h prior to fixation. (2) To generate bridges using an inducible ectopic kinetochore function, IPTG was remove from the media to

[1] https://media.addgene.org/data/plasmids/104/104993/104993-attachment_fbZFcCQmsWQB.docx
[2] https://www.epfl.ch/labs/tronolab/wp-content/uploads/2019/06/LV_production.pdf

allow the interaction between LacO and LacIGFP- CENPT (after seven washes with PBS), and the cells were fixed 24 h later. (3) To generate bridges with the CRISPR/Cas9 based model in cells expressing sgRNA targeting subtelomere regions, Cas9 was expressed by adding 1 μg/ml doxycycline (631311, Clontech, Mountain View, CA, United States) to the medium for 15 h, and a washout of 24 h was performed before the cells were fixed. When indicated, cells were synchronized in G2 phase with 9 μM RO3306 (SML0569, Sigma-Aldrich, Saint Louis, MO, United States) for 18 h and were released for 43–120 min.

BrdU Incorporation

For the BrdU pulse, cells were seeded in 35-mm-diameter petri dishes and after 24 h, 10 μM 5-bromo-20-deoxyuridine (BrdU) was added to the culture for 8 h and then substituted with 10 μM BrdU for an additional 16 h.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde (PFA) for 15 min, permeabilized with 1x PBS/0.5% Triton-X-100 solution for 20 min, and blocked with 1x PBS/0.5% BSA/0.15% glycine for 15 min. For BrdU labeling, after permeabilization, cells were treated with 2 M HCl for 20 min and incubated in a borate buffer (pH = 9) for 5 min at room temperature. For the CtIP labeling, after PFA, cells were additionally incubated in ice-cold methanol and acetone for 30 min and 1 min, respectively. Then, a blocking step was performed in 1x PBS/1% FBS/5% BSA. After the blocking step, primary antibodies were incubated overnight at 4 °C. Alternatively, BrdU antibody was incubated for 3 h at room temperature. All antibodies are listed in Supplementary Table 2. After three washes with 1x PBS/0.1% Tween 20 or after a 15 min wash in PBS/1% Triton-X-100 (for the BrdU immunofluorescence), the secondary antibodies listed in Supplementary Table 2 were incubated for 1 h at room temperature. Finally, all samples were washed, briefly rinsed with distilled water, progressively dehydrated in alcohol, and mounted on glass microscope slides with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, United States) containing 0.25 μg/mL 40,6-diamidino-2-phenylindole (DAPI).

Sample visualization and image acquisition were performed using an Olympus BX61 epifluorescent microscope (Olympus, Hamburg, Germany) equipped with a CV-M4 + CL camera (JAI, Großwallstadt, Germany) and CytoVision software (Applied Imaging, Newcastle, United Kingdom). Colocalization was determined visually as the coincidence in space of the protein of interest with each γH2AX focus. When indicated, imatge analysis and measurements were performed using ImageJ (Schindelin et al., 2012).

Fluorescence in situ Hybridization

Cells were fixed in methanol:acetic acid (3:1) for 10 min, rinsed twice with 2x SSC, and dehydrated in ethanol. Additionally, for oligo-FISH, samples were incubated with formaldehyde-

MgCl2 buffer for 10 min. Samples were denatured for 3 min at 73 °C in formamide 70%/2x SSC buffer and underwent progressive dehydration. The Chr2 whole-chromosome probe (Vysis probes, Des Plaines, IL, United States) was also denatured at 73 °C for 5 min prior to hybridization for 12 h at 37 °C. Instead, the LacO-TxRed probe (5'-CATGTGGAATTGTGAGCGGATAACAATTTGTGG-3') was hybridized for 1 h at room temperature. Following hybridization, slides were rinsed with 0.4x SSC/0.3% NP-40 for 2 min at 55 °C and 2x SSC/0.1% NP-40 for 2 min at room temperature. Finally, samples were dehydrated and counterstained with DAPI (0.25 μg/mL). Samples were visualized using an Olympus BX61 microscope, as described in the immunofluorescence section.

SensiTive Recognition of Individual DNA Ends

For the STRIDE assay, cells were seeded into 24 x 24-mm coverslips, and chromosome bridges were induced with the CRISPR/Cas9 system. Samples were fixed in PFA 4%, and coverslips were sent embedded in 1x PBS to intoDNA (Krakow, Poland). The dSTRIDE methodology was applied according to the protocol previously described by Kordon et al. (2019).

Live-Cell Imaging

For time-lapse experiments, cells were seeded onto MatTek dishes (P35G-1.5-14-C, MatTek, Ashland, Massachusetts, MA, United States) and allowed to attach for 48 h. The day of the experiment, metaphase or early anaphase (EA) cells were identified using the RFP-H2B signal. Images from five z-stacks (with a 2-μm separation between planes) were acquired every 3 min for 30–60 min with a Zeiss Axio Observer Z1 inverted fluorescent microscope (Zeiss, Oberkochen, Germany) equipped with Zen blue software (Zeiss) and an AxioCam MRm càmera (Zeiss). Cells were maintained under controlled conditions (5% CO_2 and 37 °C) during the experimental time course.

Statistics

Fisher's exact test was used to determine differences between categorical variables. For continuous variables, data normality was analyzed using the D'Agostino-Pearson omnibus K2 normality test. If the data were normally distributed, a t-test was used for unpaired statistical analysis; the Mann–Whitney test was applied for unpaired non-parametric analysis. Statistical analysis and graph plotting were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, United States) and p-values < 0.05 were considered statistically significant.

RESULTS

We used three different methods to generate chromatin bridges: moderate-dose radiation exposure, inducible function of an ectopic kinetochore by expression of the LacI-GFP-CENPT chimeric protein in cellular clones stably transfected with LacO (Gascoigne et al., 2011;

Supplementary Figure 1A), and CRISPR/Cas9-mediated DNA double-strand breaks on chromosome 2 (Supplementary Figure 1B) or chromosome 4 (Umbreit et al., 2020; Supplementary Figure 1C). In addition to chromosome bridges generated by the three methods described above, we examined those spontaneously formed in genomically unstable U2OS cells. While spontaneously formed bridges and those induced by radiation were randomly generated throughout the cell genome, those obtained by the CRISPR/Cas9 system in RPE1 cells were formed under exquisite spatio-temporal control and mostly involved the chromosomes against which guide RNAs were designed (Supplementary Figures 1B,C; Umbreit et al., 2020). As for the inducible kinetochore experimental model, although they theoretically should also be specific, we frequently observed the formation of non-specific bridges. In this model, the inhibition of the interaction between the LacI-GFP-CENPT chimeric protein and the ectopic LacO sequences is not complete and consequently, DNA bridges accumulate with cell passage.

The Frequency of Chromosome Bridges Decreases as Cells Exit Mitosis and Progress to Interphase

It has been proposed that mammalian dicentric chromosomes can withstand mitotic spindle and cytokinetic ring forces to invariably persist through cell division and form long chromatin bridges between daughter cells at the interphase (Maciejowski et al., 2015; Umbreit et al., 2020). Using the CRISPR/Cas9 experimental system, we detected a progressive reduction in the frequency of chromosome bridges as cells advanced from cell division to interphase (Figure 1), a reduction that was consistent with a progressive resolution of chromosome bridges during the last stages of cell division. While we observed bridges in 83.33% of cells in EA and 81.13% in late anaphase (LA), the frequency of cells with bridges decreased to 40.91 and 37.09% in early and late telophase (ET and LT), respectively, and we only detected bridges with GFP-BAF (a sensitive reporter of chromosome bridges in interphase as its signal is not compromised by bridge stretching) in 9.79% of interphase cells at the G1 stage of the cell cycle (positive for cyclin D1). The differences in the frequencies of bridges between mitotic and interphase cells cannot be attributed to a low proliferation index as most of the interphase cells were cycling (92.12% of interphase cells incorporated BrdU after a 24-h pulse) (Supplementary Figure 2). A similar stage-associated reduction in the frequencies of bridges was also observed using other experimental systems (83.33, 58.49, 32.89, and 24.39% of irradiated MCF10A cells at EA, LA, ET, and LT, respectively, and 6.90% in interphase visualized by GFP-Lap2β; Supplementary Figure 3). These findings suggested that fundamental aspects of bridge-breakage mechanisms remained to be clarified, as the observed decrease in the frequency of bridges is not compatible with the claim that all or most bridges persist through mitosis and cytokinesis.

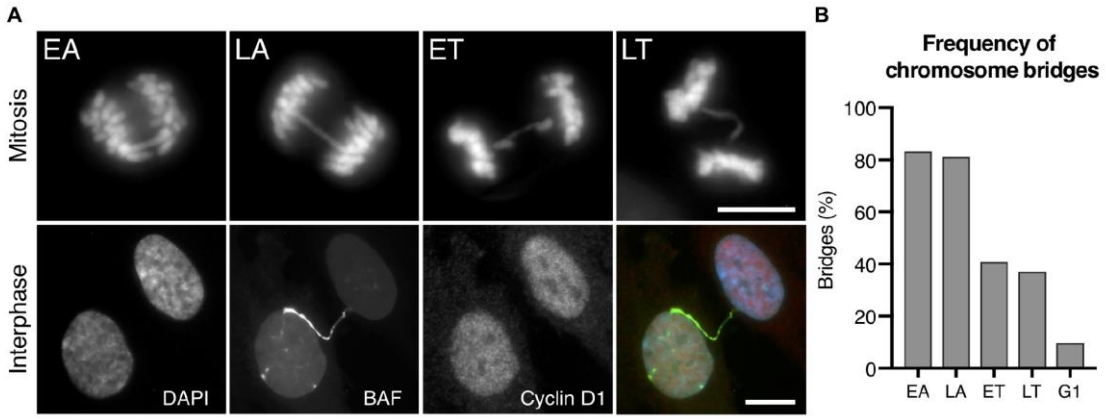


FIGURE 1 | Frequencies of chromosome bridges in RPE1 Cas9 sgRNA Chr4 cells. (A) Representative images of bridges during the last stages of mitosis (EA, early anaphase; LA, late anaphase; ET, early telophase; LT, late telophase) and interphase. Chromosome bridges are visualized with DAPI (blue) during mitosis and with GFP-BAF (green) during interphase. To restrict the analysis to G1 cells, immunofluorescence of cyclin D1 (red) was performed, and its labeling is shown in red. Scale bar = 10 μm. (B) Percentage of bridges during the last stages of mitosis and in interphase cells at the G1 stage. Chromosome bridges were induced with the CRISPR/Cas9 Chr4 methodology, and cells were synchronized with RO3306 and released for 43–120 min to enrich the mitotic or interphase cell populations, respectively (n = 227 cells in mitosis and 1032 cells in G1).

Breakage of Chromosome Bridges During Mitosis

Indirect evidence for mitotic bridge breakage came from the observation that bridges in mitotic cells were frequently labeled with markers of DNA rupture. To determine the extent to which mitotic bridges were labeled in a manner compatible with their severing, we immunodetected the phosphorylated form of the histone variant H2AX (then termed γH2AX), which is a chromatin marker that flags regions in the genome that contain DNA breaks (Rogakou et al., 1998, 1999). In the CRISPR/Cas9 experimental cell model, a visible discontinuity in the chromatin fiber was observed in 43.84% of mitotic bridges (considering all stages of mitosis together), and 73.03% of them presented γH2AX signaling (Figures 2A,B). Conversely, 91.52% of the continuous bridges did not show γH2AX signaling (Fisher's exact test, $p < 0.0001$; Figure 2B). Regarding the location of the γH2AX signal on the DNA bridge (Figure 2A), three main categories were defined: (a) a single γH2AX focus usually in the middle of the bridge; (b) two γH2AX foci flanking the discontinuity of the intervening chromatin fiber; or (c) multiple γH2AX foci spanning the chromatin bridge. Interestingly, most of the continuous bridges positive for γH2AX staining displayed a single or two very close γH2AX foci in the middle of the bridge (82.14%), probably indicating a recent breakage. In contrast, the predominant labeling pattern in the discontinuous subset of chromatin bridges was a γH2AX focus located at each end of the discontinuity (76.92%). Only a small fraction (9.74%) of the discontinuous bridges positive for γH2AX displayed multiple γH2AX foci over the chromatin.

The observed association between the morphology of the bridges and their γH2AX labeling pattern was especially informative when we classified the bridges according to the stage of

mitosis (Figure 2C). Whereas the fraction of continuous bridges decreased from EA (63.04%) to LT (42.25%), and most of them were devoid of γH2AX foci, bridges with visible discontinuity increased complementarily, and most of them showed a γH2AX signal. Therefore, the γH2AX-based method for the indirect detection of DNA breaks indicates that a substantial fraction of chromosome bridges is broken during the final stages of cell division.

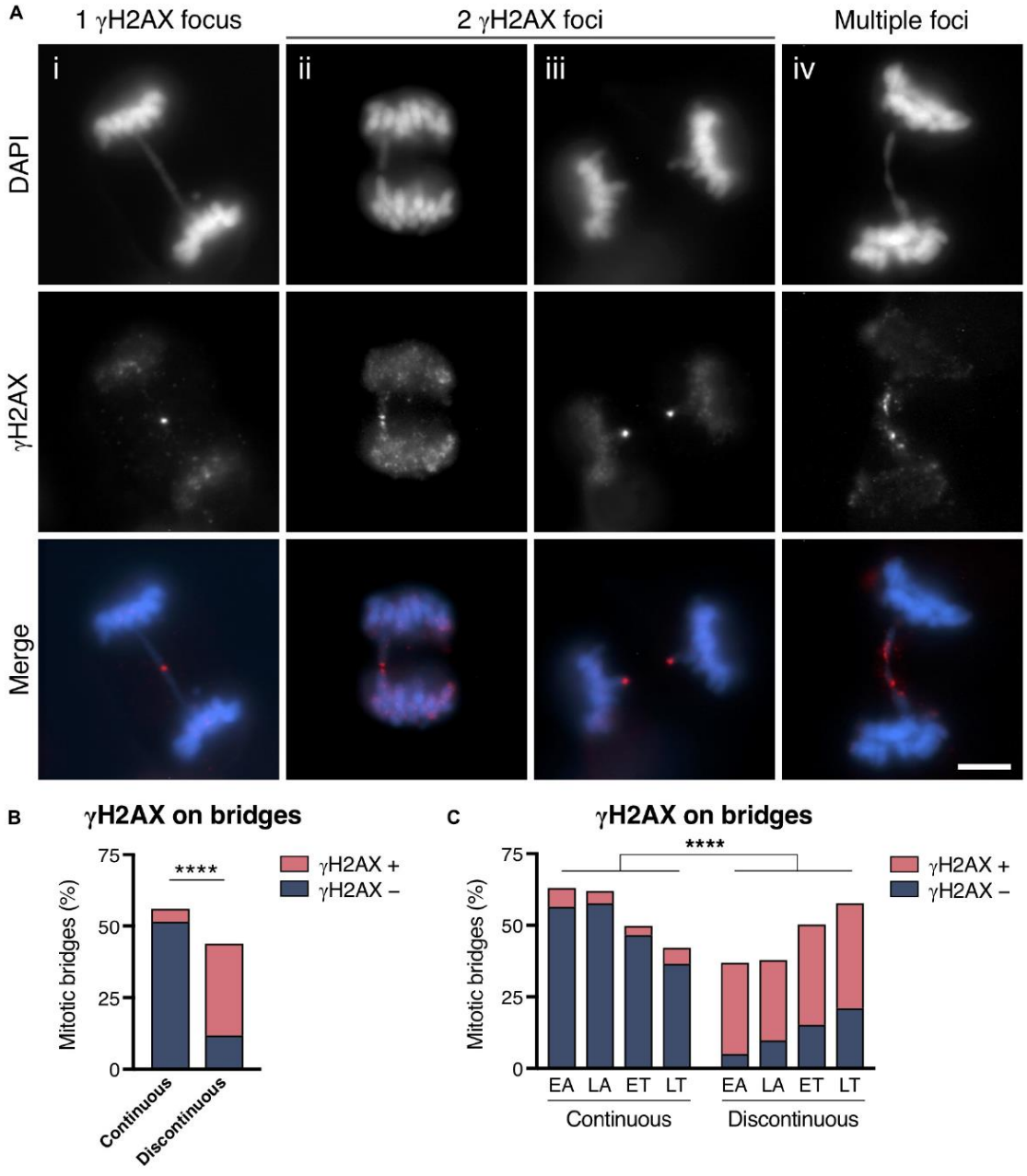


FIGURE 2 | Chromosome bridge breakage is marked with γH2AX. (A) Representative images of chromosome bridges (DAPI, blue) that exhibit γH2AX labeling (red). Representative bridges with (i) a single γH2AX focus in the middle of the bridge, (ii, iii) two γH2AX foci flanking the discontinuity of a chromosome bridge, and (iv) γH2AX labeling spanning the chromosome bridge. Scale bar = 5 μm. (B,C) Frequency of chromosome bridges γH2AX-positive and -negative classified according the continuity or discontinuity of the DAPI staining for (B) all mitotic stages together and for (C) cells segregated by phase (EA, early anaphase; LA, late anaphase; ET, early telophase; LT, late telophase). Asterisks indicate statistical differences between continuous and discontinuous bridges regarding the γH2AX labeling (Fisher's exact test, ****$p < 0.0001$; $n = 609$ from 5 replicates). Chromosome bridges were induced with the CRISPR/Cas9 Chr4 methodology, and cells were

synchronized with RO3306 and released for 43 or 50 min to enrich the number of cells in the anaphase and telophase stages of mitosis, respectively.

In addition to methods based on monitoring of histone modification at the damage sites, we used a method for the direct detection of DNA DSBs (double-strand breaks) in chromosome bridges during mitosis. The method we used is abbreviated STRIDE (section "Sensitive Recognition of Individual DNA Ends), and it enables the direct in situ detection of 3'OH DNA ends even when they are individual DNA double-strand cuts (Kordon et al., 2019). It consists of the conjugation of deoxynucleotide analogues to free DNA ends and their detection by hybridization with fluorescent nucleotides after rolling circle amplification. The STRIDE assay was applied to the detection of free DNA ends in compacted mitotic chromatin in RPE1 cells. In the RPE1 CRISPR/Cas9 experimental model, a total of 82 chromosome bridges were identified in mitotic cells, and 65.15% presented STRIDE labeling at the chromatin flanking the discontinuity (Figure 3). In addition, 12.5% of the continuous bridges also presented STRIDE labeling. Taken together, these results indicate that most of the discontinuities observed in chromosome bridges reflect an actual breakdown of the chromatin fiber.

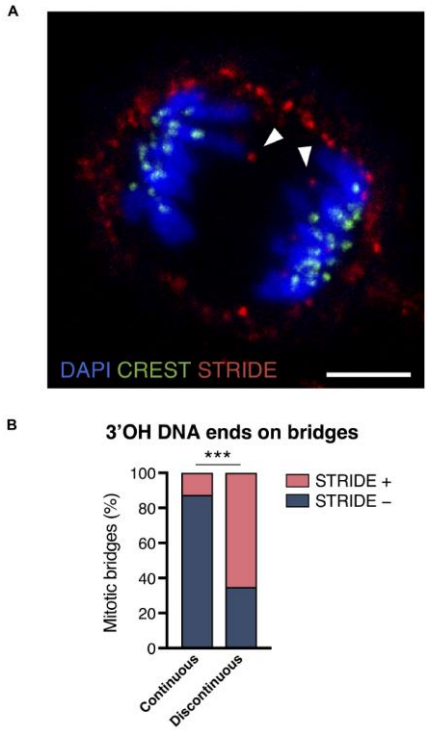


FIGURE 3 | Broken chromosome bridges exhibit 3'OH DNA ends. (A) Representative image of STRIDE (red) detecting the DNA ends flanking the discontinuity of the chromosome bridges (DAPI, blue). Kinetochores are labeled with CREST antibody (green). Arrowheads indicate the 3'OH DNA ends of a broken bridge. Scale bar = 5 μm. (B) Frequency of STRIDE-positive and -negative chromosome bridges classified according to the continuity or discontinuity of DAPI staining. Chromosome bridges were induced with the CRISPR/Cas9 methodology, and cells were synchronized with RO3306 and released for 43 min to enrich the anaphase and telophase populations (Fisher's exact test, $***p < 0.001$; $n = 82$ chromosome bridges).

DNA Ends in Mitotic Chromosome Bridges Are Sensed as Broken Ends

Upon appearance of a DSB, cells activate the DNA damage response (DDR), which comprises two main stages: the initial detection of DNA breaks followed by downstream events leading to cell cycle arrest and DNA repair (Jackson and Bartek, 2009). Numerous factors involved in DNA break signaling, processing, and repair accumulate at damaged sites and form focal structures. However, although the recruitment of DDR factors to DSBs in interphase cells is efficient, the condensed structure of chromatin in mitosis is known to make it difficult to recruit or retain DDR factors to the chromatin flanking the broken ends (Giunta et al., 2010; Heijink et al., 2013; Alcaraz Silva et al., 2014). Given the important role of MDC1 (mediator of DNA damage checkpoint 1) in the early steps of the DDR (Coster and Goldberg, 2010; Jungmichel and Stucki, 2010), we investigated its recruitment to broken bridges in the CRISPR-cell model by immunodetecting MDC1 in fixed cells. We found that, similar to γH2AX, MDC1 labeled the chromatin flanking chromosome bridge discontinuities (Fisher's exact test for MDC1 labeling continuous and discontinuous bridges, $p < 0.0001$; Figure 4A). Furthermore, given that γH2AX provides a docking site for the DDR-mediator protein MDC1, we wanted to assess whether γH2AX and MDC1 coincided at the broken ends of chromosome bridges. We found that MDC1 colocalized with γH2AX in the bridges of mitotic cells as it does in interphase cells (94.52 and 91.08% colocalization in mitotic bridges and interphase cells, respectively; Fisher's exact test, $p = 0.31$; Figures 4B,C). In a complementary manner, the MDC1 signal was absent from those bridges without γH2AX labeling.

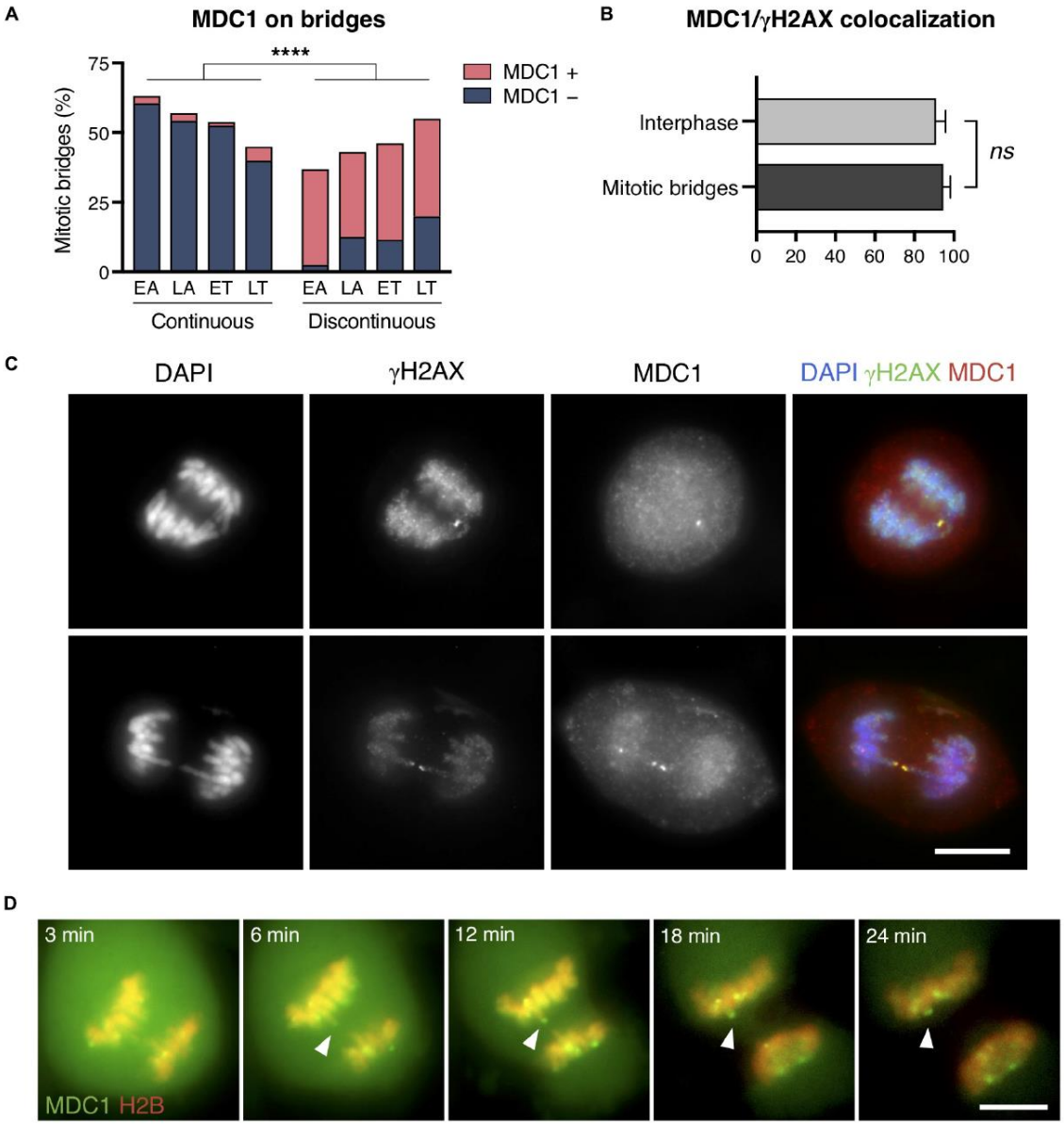


FIGURE 4 | MDC1 recruitment to chromatin bridges in mitotic cells. (A) Frequency of MDC1-positive and -negative staining chromosome bridges during the last stages of mitosis (EA, early anaphase; LA, late anaphase; ET, early telophase; LT, late telophase). Frequencies of MDC1 recruitment to continuous and discontinuous chromosome bridges (continuity was assessed with DAPI; Fisher's exact test, $****p < 0.0001$; $n = 208$ chromosome bridges from two replicates). Chromosome bridges were induced with the CRISPR/Cas9 Chr4 methodology, and cells were synchronized with RO3306 and released for 43 or 50 min. (B) Percentages of MDC1/γH2AX colocalization in interphase cells vs. in chromosome bridges during mitosis. Error bars indicate SD (Fisher's exact test, *ns* $p > 0.05$; $n = 213$ γH2AX foci in interphase cells, n D 146 γH2AX foci in mitotic bridges; two replicates). (C) Representative images for the colocalization of MDC1 (red) and γH2AX (green) at chromosome bridges (DNA in blue). Scale bar = 10 μm. (D) Representative images of a time-lapse of MDC1 (green) recruitment to chromosome bridges (red) in U2OS GFP-MDC1/RFP-H2B cells. Images from anaphase entrance to 24 min later are shown. Bridges were spontaneously induced. Arrowheads highlight an MDC1 focus at the end of a broken bridge. Scale bar = 10 μm.

Next, we evaluated in living cells the dynamics of MDC1 recruitment to chromatin bridges in mitosis. To this end, we used a human osteosarcoma cell line U2OS stably expressing GFP-tagged MDC1 and RFP-H2B in which the endogenous MDC1 was knocked out using CRISPR/Cas9 (Leimbacher et al., 2019). Time-lapse recording of living cells showed recruitment

of the fluorescent protein on bridges in mitotic cells followed by retraction of the chromatin fiber (Figure 4D and Supplementary Video 1). Therefore, we conclude that DNA ends in broken bridges of mitotic cells are detected and signaled by the DDR cellular machinery.

When instead of DSBs the lesion produces single-stranded DNA fragments, replication protein A (RPA) is known to detect them and act as a platform to recruit other factors involved in DNA repair. Using an anti-RPA32 antibody in the CRISPR/Cas9 Chr4 experimental model, we found that although most mitotic bridges showed no RPA32 signal, in 22.05% of the bridges in mitotic cells, we observed a long RPA32 signal extending like a filament physically connecting the two groups of recently segregated chromosomes (Figure 5A). As shown in Figure 5B, RPA32 signaling was mostly detected at telophase, indicating that it might require some time for RPA32 to be visible after DNA breakage. When present, the RPA32 signal coincided with the chromatin discontinuity, and it was frequently flanked by γH2AX signals (41.30%; n = 46 chromosome bridges). Therefore, our results indicate that bridge rupture in mitosis does not always proceed through DSB formation. Sometimes, nicks can occur that affect only one strand of DNA with the subsequent formation of long RPA32-labeled single-stranded DNA fragments. Altogether, we conclude that chromosome bridges severed during mitosis exhibit apical aspects of the DDR, as they are marked with γH2AX and MDC1 and sometimes with RPA.

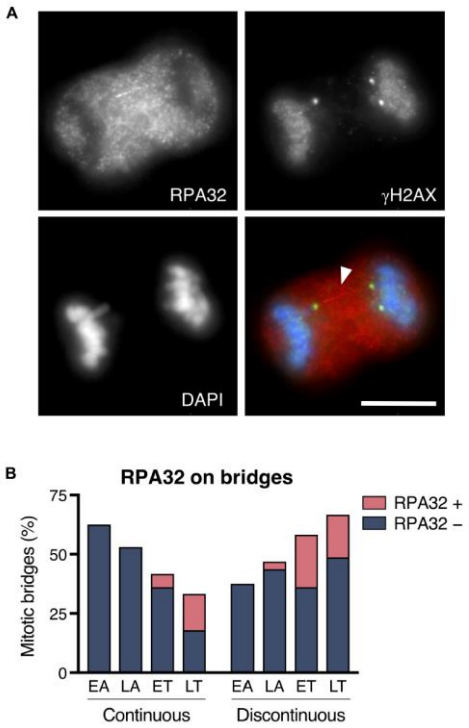


FIGURE 5 | Replication protein A recruitment to chromosome bridges in mitotic cells. (A) Representative image for the RPA32 labeling on chromosome bridges. RPA32 (red), flanked by γH2AX (green), forms a filament-like structure that connects the two groups of segregated chromosomes (blue). Arrowhead highlights the RPA filament. Scale bar = 10 μm. (B) Distribution of RPA-positive and -negative staining in continuous and discontinuous chromosome bridges during the last stages of mitosis (EA, early anaphase; LA, late anaphase; ET,

early telophase; LT, late telophase). Chromosome bridges were induced with the CRISPR/Cas9 Chr4 methodology, and cells were synchronized with RO3306 and released for 43 or 50 min (n = 127 chromosome bridges).

DNA Ends in Mitotic Chromatin Bridges Are Not Processed for Repair

Having established that mitotic cells detect and signal the broken ends of DNA bridges, we next examined the behavior of 53BP1 (p53-binding protein 1), BRCA1 (breast cancer type 1 susceptibility protein), and CtIP (CtBP-interacting protein), three proteins involved in the second stage of the DDR. At a molecular level, the balance between 53BP1 and BRCA1 plays an important role in the choice of a DSB repair pathway, and CtIP is responsible for DNA end-resection initiation (Sartori et al., 2007; Escribano-Díaz et al., 2013; Ceccaldi et al., 2016). By using an anti-53BP1 antibody in the CRISPR/Cas9 Chr4 experimental system and in irradiated MCF10A cells, we found that, in marked contrast to interphase cells, 53BP1 was excluded from mitotic chromatin (average number of 53BP1 foci per cell was 2.43 and 2.56 in RPE1 and MCF10A interphase cells, respectively, and 0 in mitotic cells). It is important to note that, whereas in interphase cells most 53BP1 foci colocalized with γH2AX foci (RPE1: 90.33%, 243/269 foci; MCF10A: 94.55%, 191/202 foci; Figure 6A upper panel), in broken mitotic bridges γH2AX did not recruit 53BP1 (RPE1: 0/71 foci; MCF10A: 0/47 foci; Figure 6A lower panel). A similar pattern was found when we detected BRCA1 (Figure 6B). Whereas focal structures of BRCA1 on γH2AX-decorated chromatin were observed in interphase cells (RPE1: 69.40% colocalization in BRCA1 positive cells in interphase, 195/281 foci; MCF10A: 56.50%, 113/200 foci), colocalization of this factor with γH2AX was absent in chromatin bridges of mitotic cells (RPE1: 0/71 foci; MCF10A: 0/53 foci). CtIP was also missing in those broken chromosome bridges marked with γH2AX (RPE1: 0/66 foci; MCF10A: 0/41 foci; Figure 6C), thus indicating that DNA end-resection was not triggered at the mitotic DNA ends. Collectively, these findings suggest that the DNA repair machinery cannot be fully launched in those bridges broken during mitosis. Thus, bridges may remain unrepaired while the ends of the broken chromosome are pulled apart.

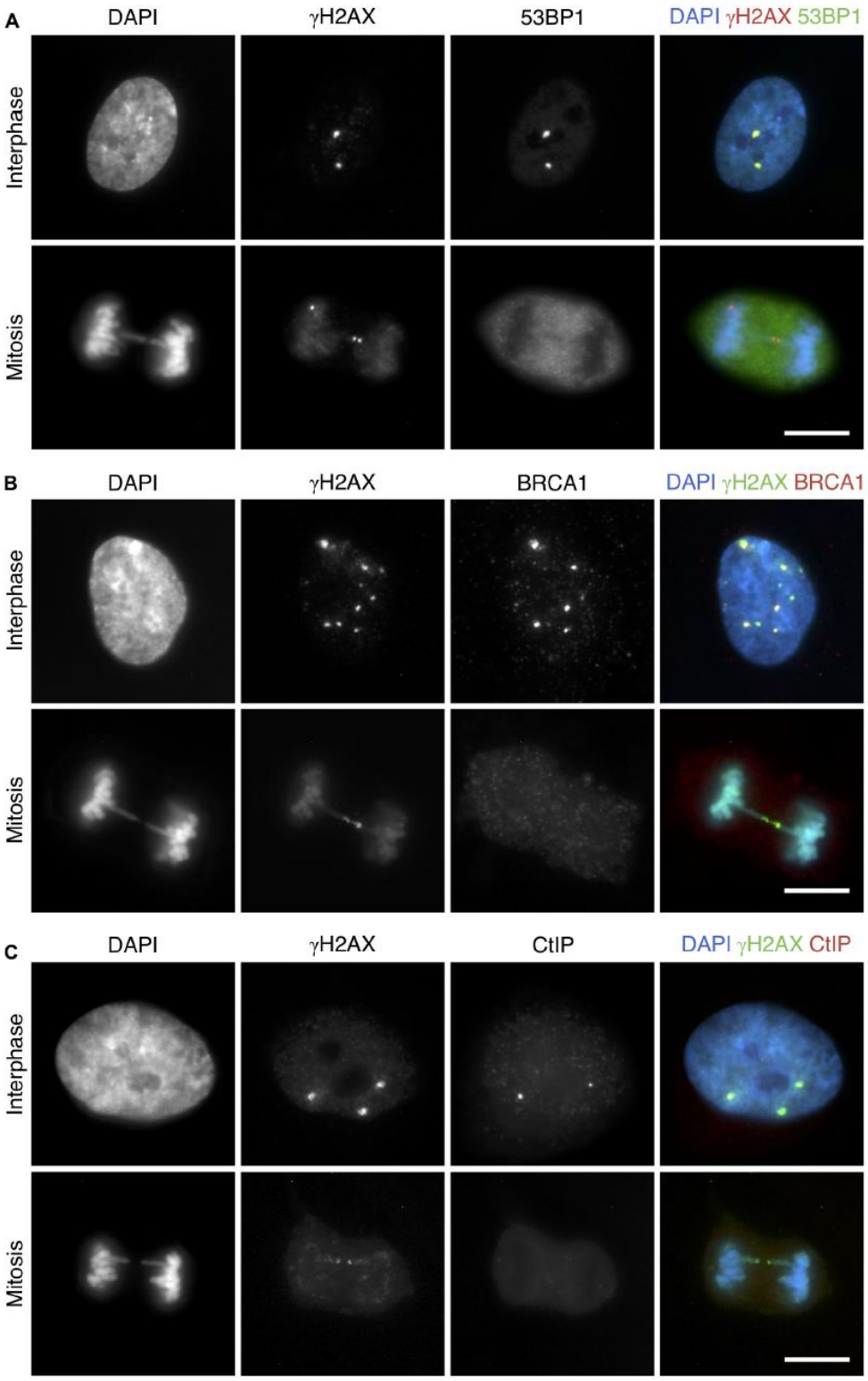


FIGURE 6 | 53BP1, BRCA1, and CtIP are not recruited to broken chromosome bridges in mitotic cells. Representative images for the colocalization of (A) 53BP1 (green) with γH2AX (red), (B) BRCA1 (red) with γH2AX (green), and (C) CtIP (red) with γH2AX (green). Upper panels correspond to interphase cells, and lower panels show mitotic cells with chromosome bridges. DNA is counterstained with DAPI (blue). All images correspond to RPE1 cells with CRISPR/Cas9-induced chromosome bridges using sgRNA Chr4. Cells were synchronized with RO3306 and fixed after a 45-min release. Scale bar = 10 μm.

Breakage of Bridges in Mitotic Cells Is Associated With their Stretching

During the last stages of mitosis, microtubules of the mitotic spindle stretch the bridging chromatin toward the opposite poles of the cell. To determine whether the increasing distance between the poles of the mitotic cell is associated with bridge rupture, we detected pericentrin, a component of centrosomes, in the RPE1 CRISPR/Cas9 Chr4 experimental model. We found no association between the distance between cell poles and the breakage of bridges in mitotic cells (signaled with γH2AX) (t-test; $p = 0.46$; Figure 7). In concordance with this result, we found no differences between cells with broken and unbroken bridges when measuring the average distance between non-bridge kinetochores by using a CREST antibody (Mann–Whitney test; $p = 0.62$; Figures 8A–C). Therefore, the separation between cell poles does not determine chromosome bridge breakage.

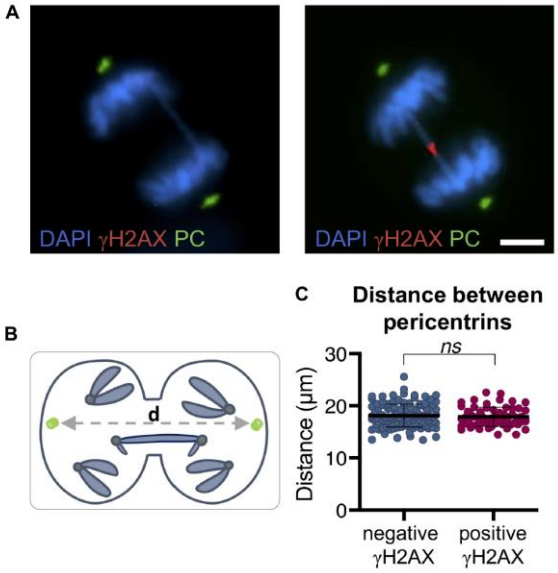


FIGURE 7 | Distance between pericentrins is not associated with bridge breakage in RPE1 Cas9 sgRNA Chr4 cells. (A) Representative images of mitotic chromosome bridges (DAPI, blue) immunolabeled with γH2AX (red) and pericentrin (green). Scale bar = 5 μm. (B) Schematic illustration of the measurement of the distance between pericentrins. Pericentrins are depicted in green, d = distance between pericentrins. (C) Distance between pericentrins classified by γH2AX labeling of the bridge. The mean and SD are indicated (t-test, *ns* $p > 0.05$; n = 183). After CRISPR/Cas9 chromosome bridge induction, cells were synchronized with RO3306 and fixed after a 43-min release.

Although there was no association between bridge breakage and cell pole distance, we frequently observed that the bridge kinetochores were displaced from the cluster of nonbridge kinetochores (Figure 8A, panel i). When we measured the distance of this displacement (Figure 8C) we found that it was longer for unbroken chromosome bridges than for the broken ones (Mann–Whitney test, $p < 0.0001$; Figure 8D), thus indicating that unbroken bridges were under tension, which was released by breakage. In fact, the displacement was only significant on unbroken bridges of cells at the EA and LA stages of mitosis (Mann– Whitney test; $p < 0.01$; Figure 8E), which is precisely when mitotic spindle microtubules generate maximum tension on segregating chromosomes.

To confirm the role of mechanical stress in bridge resolution, we then measured the distance between the bridge kinetochores and found that there were significant differences between broken and unbroken bridges: the longer the distance between the bridge kinetochores, the higher the probability of bridge breakage (Mann–Whitney test, $p < 0.0001$; Figure 8F). It is important to note that this association was only observed in EA and LA, but not in telophase (Mann–Whitney test; $p < 0.01$; Figure 8G). Furthermore, when cells were classified according to the distance between bridge kinetochores, we found that in this experimental system (RPE1 CRISPR/Cas9 Chr4) a minimum distance of 5.96 μm between centromeres was needed for bridges to break (Figures 8F,G). Once the bridge kinetochores had exceeded this distance, we found broken bridges, but also unbroken bridges, which could indicate that in addition to tensile forces there may be other factors that influence the breakage of bridges during mitosis (Figure 8H). Similar results were observed for bridges induced in U2OS cells with the LacO system (Supplementary Figure 4) giving support to our conclusion.

In order to further corroborate this finding, we used the system RPE1 CRISPR/Cas9 with a sgRNA targeting the q-arm subtelomeric region of Chr2 as it allowed us to generate bridges with a different distance in base pairs between bridge centromeres (Supplementary Figure 1). While subtelomeric target sequences in Chr4 are at 139.4 and 50.7 Mb from the centromere, for Chr2 there is only one target sequence, and it is at 147.6 Mb from the centromere; thus, the intercentromeric distance is longer for Chr2 than for Chr4 bridges. Interestingly, we found that for bridges induced with the Chr2 CRISPR/Cas9 system, the minimum distance between bridge kinetochores from which bridges begin to break is 8.2 μm (Supplementary Figure 5), longer than the 5.96 μm obtained for bridges induced with the Chr4 RNA guides. This result reveals a relationship between the centromere distance in base pairs of the dicentric chromosome and the minimum separation in micrometers between bridge kinetochores from which bridge breakage occurs.

Finally, as the guide RNA for RPE1 Cas9 Chr4 targets a sequence located at the end of both, the p- and the q-arm (Supplementary Figure 1C), in some mitotic cells we identified bridging rings. This particular kind of bridges are formed when a break in the p-arm plus a break in the q-arm of chromosome 4 are induced and fusion between sister chromatids leads to one short and one long bridge (Figure 8A, panel iii). In these double bridges, we frequently found one broken and one unbroken bridge sharing only a pair of kinetochores, confirming that with the same kinetochore separation two bridges of different intercentromeric base pair length may respond differently to the tension exerted by the microtubules of the mitotic spindle. Although we cannot rule out the contribution of other causes to the resolution of chromatin bridges, our results

suggest that the mechanical stress to which bridges are subjected may contribute to their resolution.

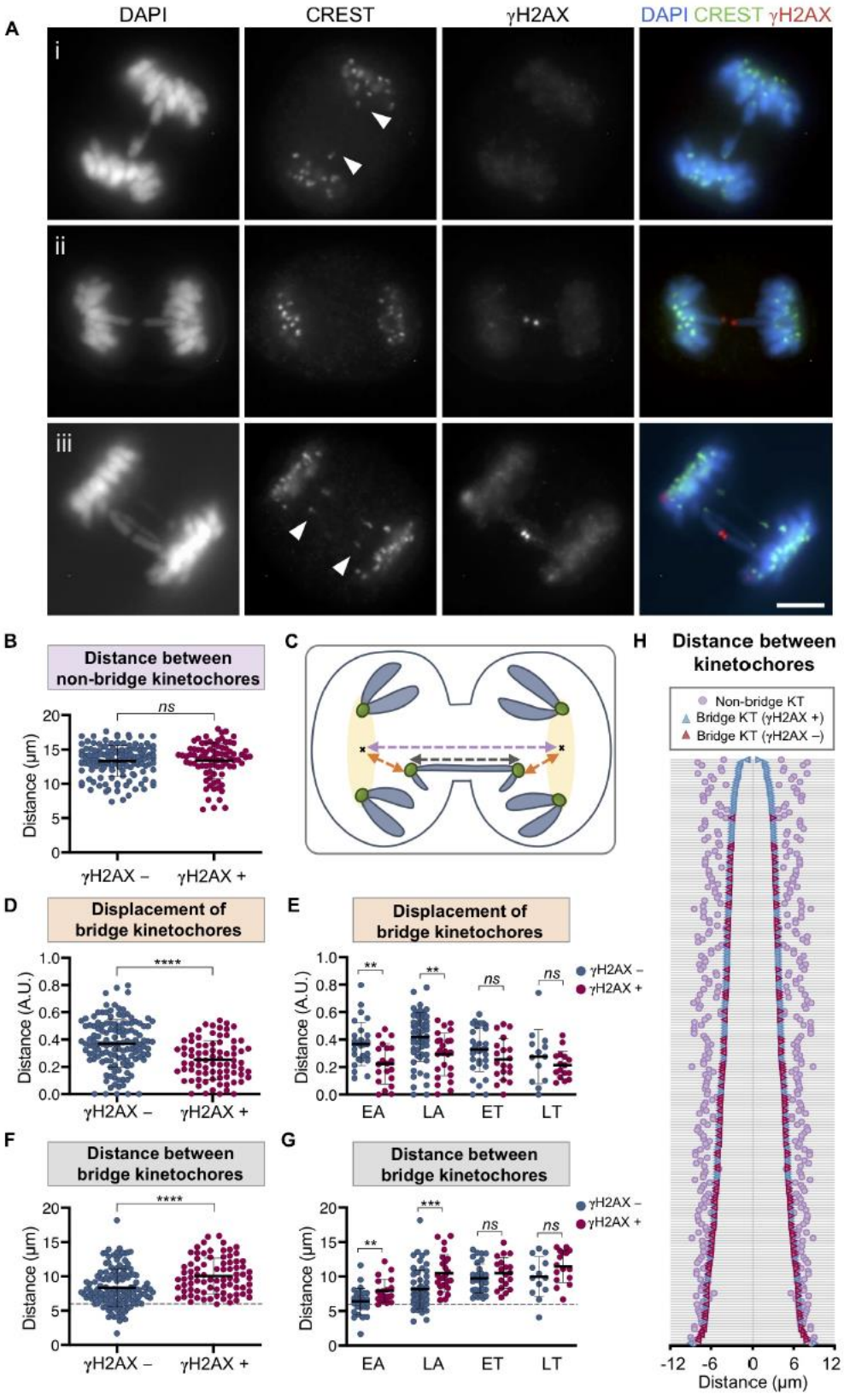


FIGURE 8 | Chromosome bridge breakage is associated with the separation between bridge kinetochores in RPE1 Cas9 sgRNA Chr4 cells. (A) Representative images for the immunofluorescent labeling of kinetochores (CREST, green) and γH2AX (red) on chromosome bridges (DAPI, blue). (i) Arrowheads indicate the CREST

signals of a continuous bridge (γH2AX-negative) that are displaced away from the non-bridge kinetochores of the cell. (ii) Representative image of a broken bridge (γH2AX-positive) with no displacement of the bridge kinetochores. (iii) Ring chromosome with one broken and one unbroken bridge sharing kinetochores (arrowheads). Scale bar = 5 μm. (B) Distance between the non-bridge kinetochores of cells with bridges classified according to their γH2AX labeling in unbroken (γH2AX-negative) and broken (γH2AX-positive) bridges. The mean and SD are indicated (Mann–Whitney test, *ns* $p > 0.05$; $n = 216$ cells from two replicates). (C) Schematic illustration for measurements taken of the distances between kinetochores (green): distance between bridge kinetochores (dark gray line), regions including the non-bridge kinetochores (yellow highlighted), centroid of the region (black cross), distance between centroids (pale purple line), distance between the bridge kinetochores and the centroid referred to as displacement (orange line). (D,E) Displacement of bridge kinetochores from the cluster of non-bridge kinetochores classified according to the γH2AX labeling of the bridge. For (D) all mitotic stages together and for (E) cells segregated by phase (EA, early anaphase; LA, late anaphase; ET, early telophase; LT, late telophase). Displacement is calculated as the distance between the bridge kinetochores and the centroid of non-bridge kinetochores (in orange in the scheme) and normalized to the distance between centroids of the cluster of non-bridge kinetochores (in pale purple in the scheme). The mean and SD are indicated. Asterisks indicate statistical differences between unbroken and broken bridges (Mann–Whitney test, ****$p < 0.0001$, **$p < 0.01$, *ns* $p > 0.05$; $n = 212$ from two replicates). (F,G) Distance between bridge kinetochores (dark gray line in the diagram) classified according to the γH2AX labeling of the bridge in broken (γH2AX-positive) and unbroken (γH2AX-negative) bridges for (F) all mitotic stages together and for (G) segregated phases. The mean and SD are indicated. Asterisks indicate statistical differences between γH2AX-negative and γH2AX-positive bridges (Mann–Whitney test, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *ns* $p > 0.05$; $n = 212$ from two replicates). Dashed line at 5.96 μm indicates the minimum distance from which bridges begin to break. (H) Graph displaying the distances between the kinetochores (KT) involved in the bridge (distances between kinetochores of γH2AX-negative bridges are represented in blue and those of γH2AX-positive bridges in red). Each horizontal line represents a cell. Cells are ordered according to the distance between bridge kinetochores. Pale purple dots represent the distance between the non-bridge kinetochores of each cell. All images and analyses correspond to chromosome bridges induced by the CRISPR/Cas9 Chr4 system and cells synchronized with RO3306 and fixed after 43 and 50 min of release ($n = 212$ from two different experiments).

DISCUSSION

Our results show that there is no single cell cycle stage at which chromosome bridges are broken. Although we found that some bridges persisted beyond telophase and formed nucleoplasmic connections between the two daughter cell nuclei, most of the chromosome bridges broke during the anaphase and telophase stages. Thus, we conclude that chromosome bridges may break during the last stages of cell division. Our conclusion is based on several observations: firstly, on immunofluorescent analysis of mitotic cells, in which the discontinuous chromatin fiber of bridges is frequently labeled with intense γH2AX staining on both sides of the discontinuity. Secondly, on the time-lapse recording of mitotic cells expressing a fluorescent form of the DDR factor MDC1, as we observed chromosome bridge end retraction after MDC1 recruitment to DNA. Finally, confirmation of bridge breakage during mitosis was obtained by the STRIDE assay, as 30-OH free ends were detected in the bridges of mitotic cells. However, bridge rupture in mitosis does not always proceed through DSB formation. Sometimes, nicks can occur in mitosis that affect only one strand of DNA with the subsequent formation of long RPA32-labeled single stranded DNA fragments.

The Ends of Broken Chromosome Bridges in Mitotic Cells Are Detected but Not Repaired

Although genome stability needs to be maintained during cycles for cells to pass on their hereditary material to the next generation, cells suppress DNA repair during mitosis as changes in chromatin structure necessary for repair would interfere with the correct segregation of chromosomes during mitosis (Blackford and Stucki, 2020). Thus, cells prioritize completion of mitosis over activation of a full DDR and repair of DNA damage. According to this notion, our data show that the recruitment to broken bridges of key downstream factors that regulate DSB repair pathway choice such as 53BP1, BRCA1 and CtIP is blocked during mitosis. However, the cellular response to DNA breaks is only partially disrupted in broken bridges during mitosis as upstream events such as H2AX phosphorylation and MDC1 recruitment still occur. A similar specialized response to DNA breaks in mitosis was previously described for DNA breaks induced by exposure to ionizing radiation (Giunta et al., 2010) or laser microirradiation (Alcaraz Silva et al., 2014). Furthermore, 53BP1 was found to be actively removed from mitotic chromatin as it dissociates from endogenously arising DNA DSBs at the G2/M boundary (FitzGerald et al., 2009; Nelson et al., 2009). Thus, the truncated nature of the DDR in mitotic cells does not depend on how DNA injury was induced or whether injury was induced before or after entering mitosis.

It has been suggested that the activation of early DDR events in mitotic cells may facilitate recognition of DNA damage and its repair during the following cell cycle (Giunta et al., 2010). Although this is plausible for breaks induced in most regions of the genome, it cannot be conceived of when the DNA ends are pulled apart, such as in the case of a broken chromosome bridge. It is unlikely that the two DNA ends derived from a broken bridge will be rejoined in the daughter cells, as repair proteins cannot overcome the tension of the mitotic spindle that pulls the two ends toward opposite poles (Doherty and Jackson, 2001; Blackford and Stucki, 2020). Therefore, once produced, the breakage of the chromosome bridge is likely permanent.

Intense Stretching and Constriction of the Condensed Chromosome of Bridges in Dividing Cells

We found an association between the distance between bridge kinetochores and the probability of breakage of the chromosome bridge. These results indicate that the mechanical tension to which the chromosome bridge is subjected during the last stages of mitosis would contribute to its breakage. However, we cannot exclude the possibility that additional mechanisms, such as those based on biochemical digestion or mechanical compression, could contribute to the breakage of chromosome bridges.

Apart from the particular characteristics of each bridge, at least two additional components need to be considered to understand the behavior of chromosome bridges during mitosis: the complexity of mitotic chromosomal decondensation and the magnitude of the forces applied to

the bridging chromosome. It could be speculated that, because the mitotic chromosome is highly condensed, it would decondense to prevent breakage when it is stretched to opposite poles. However, the mitotic chromosome must be able to withstand mechanical stress and viscous resistance as premature decondensation of chromatin would cause the sister chromatids to become entangled and missegregated. According to this notion, chromosome segregation and decondensation are tightly orchestrated with cytokinesis at the exit of mitosis (Kitagawa and Lee, 2015). As for the forces applied on the chromosome bridge, spindle microtubules can generate pushing or pulling forces by adding or losing subunits from their ends. At the single-molecule level, cytoskeletal proteins generate small forces. However, during cell division these proteins function cooperatively to generate forces in the range of nanonewtons and serve to accurately move separated chromatids over distances of micrometers (Scholey et al., 2003). Our results suggest that the force generated by hundreds to thousands of cytoskeletal force generators acting cooperatively must be able to break chromosome bridges during anaphase.

Considering that previous studies identified mechanical stress or biochemical digestion as possible causes of bridge breakage in interphase cells, a multifactorial model emerges for the breakage of chromosome bridges that, according to our results, can occur at different stages of the cell cycle and can obey different mechanisms. Therefore, we offer support for the validity of the BFB model as a mechanism capable of self-feeding to generate new changes in the genome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AG and TA: conceptualization and supervision. MR-M: design of guide RNAs. MR-M and MS: methodology and results. DS: specialized technical support. TA, MR-M, and MS: formal analysis. AG: writing—original draft preparation, project administration and funding acquisition. TA, AG, and MR-M: writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at:

https://www.frontiersin.org/articles/10.3389/fcell.2021. 745195/full#supplementary-material

REFERENCES

Alcaraz Silva, B., Stambaugh, J. R., Yokomori, K., Shah, J. V., and Berns, M. W. (2014). DNA damage to a single chromosome end delays anaphase onset. J. Biol. Chem. 289, 22771–22784. doi: 10.1074/jbc.M113.535955

Blackford, A. N., and Stucki, M. (2020). How cells respond to DNA breaks in mitosis. Trends Biochem. Sci. 45, 321–331. doi: 10.1016/j.tibs.2019.12.010

Ceccaldi, R., Rondinelli, B., and D'Andrea, A. D. (2016). Repair pathway choices and consequences at the double-strand break. Trends Cell Biol. 26, 52–64. doi: 10.1016/j.tcb.2015.07.009

Coster, G., and Goldberg, M. (2010). The cellular response to DNA damage: a focus on MDC1 and its interacting proteins. Nucleus 1, 166–178. doi: 10.4161/nucl. 11176

Doherty, A. J., and Jackson, S. P. (2001). DNA repair: how Ku makes ends meet. Curr. Biol. 11, 920–924. doi: 10.1016/S0960-9822(01)00555-3

Escribano-Díaz, C., Orthwein, A., Fradet-Turcotte, A., Xing, M., Young, J. T. F., Tká˘c, J., et al. (2013). A cell cycle-dependent regulatory circuit composed of 53BP1-RIF1 and BRCA1-CtIP controls DNA repair pathway choice. Mol. Cell 49, 872–883. doi: 10.1016/j.molcel.2013.01.001

FitzGerald, J. E., Grenon, M., and Lowndes, N. F. (2009). 53BP1: function and mechanisms of focal recruitment. Biochem. Soc. Trans. 37, 897–904. doi: 10. 1042/BST0370897

Ganem, N. J., Godinho, S. A., and Pellman, D. (2009). A mechanism linking extra centrosomes to chromosomal instability. Nature 460, 278–282. doi: 10.1038/ nature08136

Gascoigne, K. E., Takeuchi, K., Suzuki, A., Hori, T., Fukagawa, T., and Cheeseman, I. M. (2011). Induced ectopic kinetochore assembly bypasses the requirement for CENP-A nucleosomes. Cell 145, 410–422. doi: 10.1016/j.cell.2011.0 3.031

Gisselsson, D. (2003). Chromosome instability in cancer: how, when, and why? Adv. Cancer Res. 87, 1–29. doi: 10.1016/S0065- 30X(03)87164-6

Giunta, S., Belotserkovskaya, R., and Jackson, S. P. (2010). DNA damage signaling in response to double-strand breaks during mitosis. J. Cell Biol. 190, 197–207. doi: 10.1083/jcb.200911156

Heijink, A. M., Krajewska, M., and Van Vugt, M. A. T. M. (2013). TheDNA damage response during mitosis. Mutat. Res. 750, 45–55. doi: 10.1016/j.mrfmmm.2013. 07.003

Hoffelder, D. R., Luo, L., Burke, N. A., Watkins, S. C., Gollin, S. M., and Saunders, W. S. (2004). Resolution of anaphase bridges in cancer cells. Chromosoma 112, 389–397. doi: 10.1007/s00412-004-0284-6

Jackson, S. P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature 461, 1071–1078. doi: 10.1038/nature08467

Janssen, A., Van Der Burg, M., Szuhai, K., Kops, G. J. P. L., and Medema, R. H. (2011). Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. Science 333, 1895–1898. doi: 10.1126/ science.1210214

Jungmichel, S., and Stucki, M. (2010). MDC1: the art of keeping things in focus. Chromosoma 119, 337–349. doi: 10.1007/s00412-010-0266-9

Kitagawa, M., and Lee, S. H. (2015). The chromosomal passenger complex (CPC) as a key orchestrator of orderly mitotic exit and cytokinesis. Front. Cell Dev. Biol. 3:14. doi: 10.3389/fcell.2015.00014

Kordon, M. M., Zar¸ebski, M., Solarczyk, K., Ma, H., Pederson, T., and Dobrucki, J. W. (2019). STRIDE—a fluorescence method for direct, specific in situ detection of individual single- or double-strand DNA breaks in fixed cells. Nucleic Acids Res. 48:e14. doi: 10.1093/nar/gkz1118

Leimbacher, P. A., Jones, S. E., Shorrocks, A. M. K., de Marco Zompit, M., Day, M., Blaauwendraad, J., et al. (2019). MDC1 Interacts with TOPBP1 toMaintain Chromosomal Stability during Mitosis. Mol. Cell 74, 571.e8–583.e8. doi: 10. 1016/j.molcel.2019.02.014

Maciejowski, J., Chatzipli, A., Dananberg, A., Chu, K., Toufektchan, E., Klimczak, L. J., et al. (2020). APOBEC3-dependent kataegis and TREX1-driven chromothripsis during telomere crisis. Nat. Genet. 52, 884–890. doi: 10.1038/s41588-020-0667-5

Maciejowski, J., Li, Y., Bosco, N., Campbell, P. J., and De Lange, T. (2015). Chromothripsis and kataegis induced by telomere crisis. Cell 163, 1641–1654. doi: 10.1016/j.cell.2015.11.054

McClintock, B. (1939). The behavior in successive nuclear divisions of a chromosome broken at meiosis. Proc. Natl. Acad. Sci. U.S.A. 25, 405–416.

Nelson, G., Buhmann, M., and Von Zglinicki, T. (2009). DNA damage foci in mitosis are devoid of 53BP1. Cell Cycle 8, 3379–3383. doi: 10.4161/cc.8.20.9857

Pampalona, J., Frías, C., Genescà, A., and Tusell, L. (2012). Progressive telomere dysfunction causes cytokinesis failure and leads to the accumulation of polyploid cells. PLoS Genet. 8:e1002679. doi: 10.1371/journal.pgen.1002679

Rogakou, E. P., Boon, C., Redon, C., and Bonner, W. M. (1999). Megabase chromatin domains involved in DNA double-strand breaks in vivo. J. Cell Biol. 146, 905–916. doi: 10.1083/jcb.146.5.905

Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J. Biol. Chem. 273, 5858–5868. doi: 10.1074/jbc.273.10.5858

Rudolph, K. L., Millard, M., Bosenberg, M. W., and DePinho, R. A. (2001). Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. Nat. Genet. 28, 155–159. doi: 10.1038/88871

Sartori, A. A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., et al. (2007). Human CtIP promotes DNA end resection. Nature 450, 509–514. doi: 10.1038/ nature06337

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. doi: 10.1038/nmeth.2019

Scholey, J. M., Brust-mascher, I., and Mogilner, A. (2003). Cell division. Nature 422, 746–752. doi: 10.1038/nature01599

Shimizu, N., Shingaki, K., Kaneko-Sasaguri, Y., Hashizume, T., and Kanda, T. (2005). When, where and how the bridge breaks: anaphase bridge breakage plays a crucial role in gene amplification and HSR generation. Exp. Cell Res. 302, 233–243. doi: 10.1016/j.yexcr.2004.09.001

Steigemann, P., Wurzenberger, C., Schmitz, M. H. A., Held, M., Guizetti, J., Maar, S., et al. (2009). Aurora B-mediated abscission checkpoint protects against tetraploidization. Cell 136, 473–484. doi: 10.1016/j.cell.2008. 12.020

Stemmer, M., Thumberger, T., Del Sol Keyer, M., Wittbrodt, J., and Mateo, J. L. (2015). CCTop: an intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. PLoS One 10:e0124633. doi: 10.1371/journal.pone.0124633

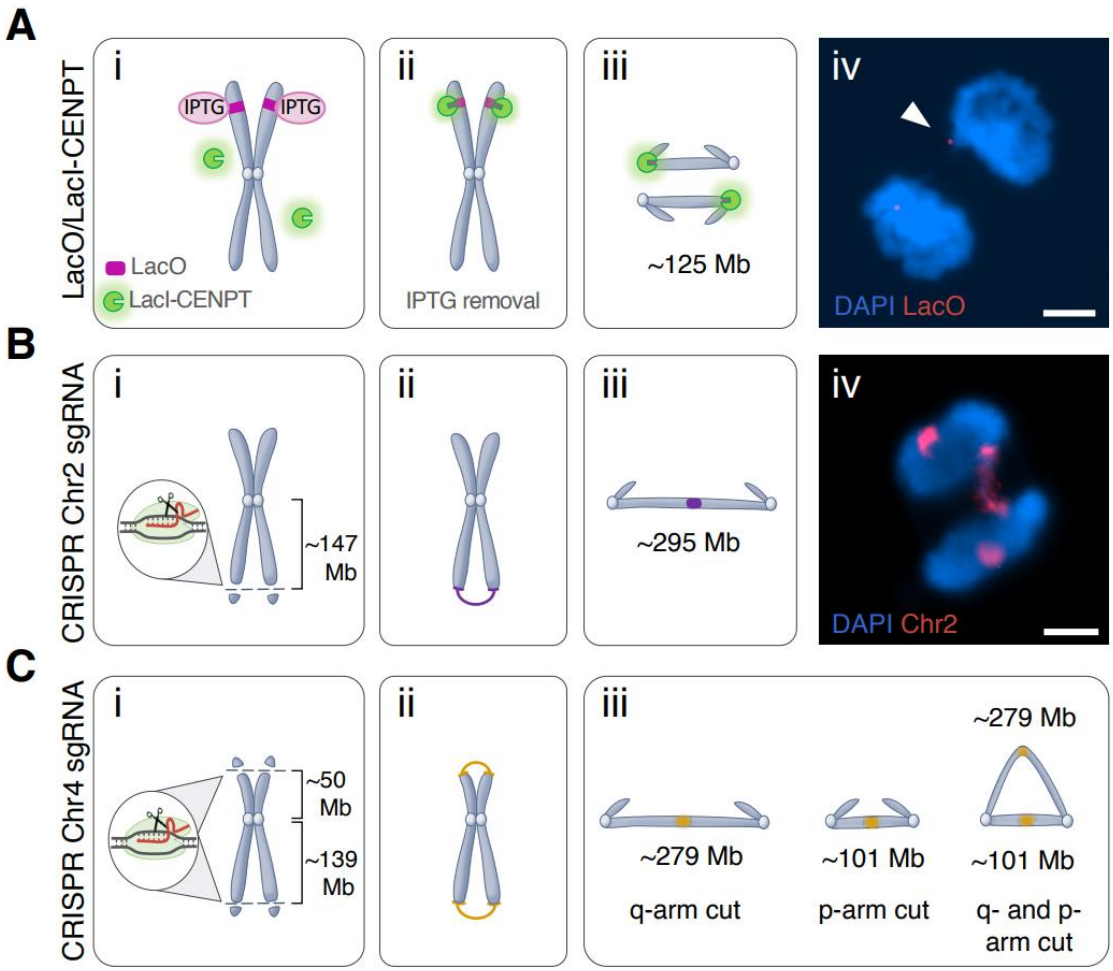
Umbreit, N. T., Zhang, C.-Z., Lynch, L. D., Blaine, L. J., Cheng, A. M., Tourdot, R., et al. (2020). Mechanisms generating cancer genome complexity from a single cell division error. Science 368:eaba0712. doi: 10.1126/science.aba0712

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

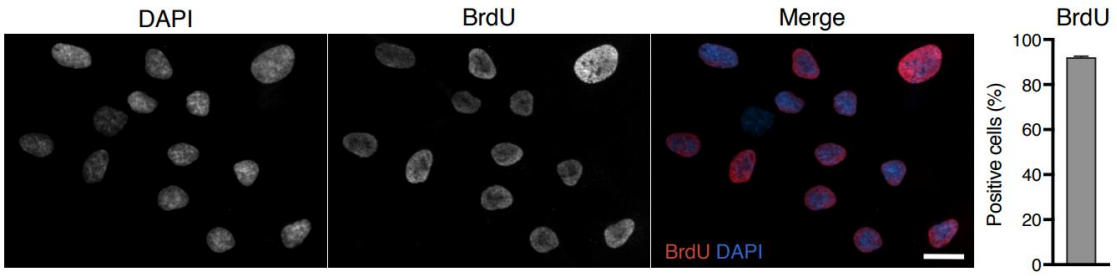
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SUPPLEMENTARY MATERIAL

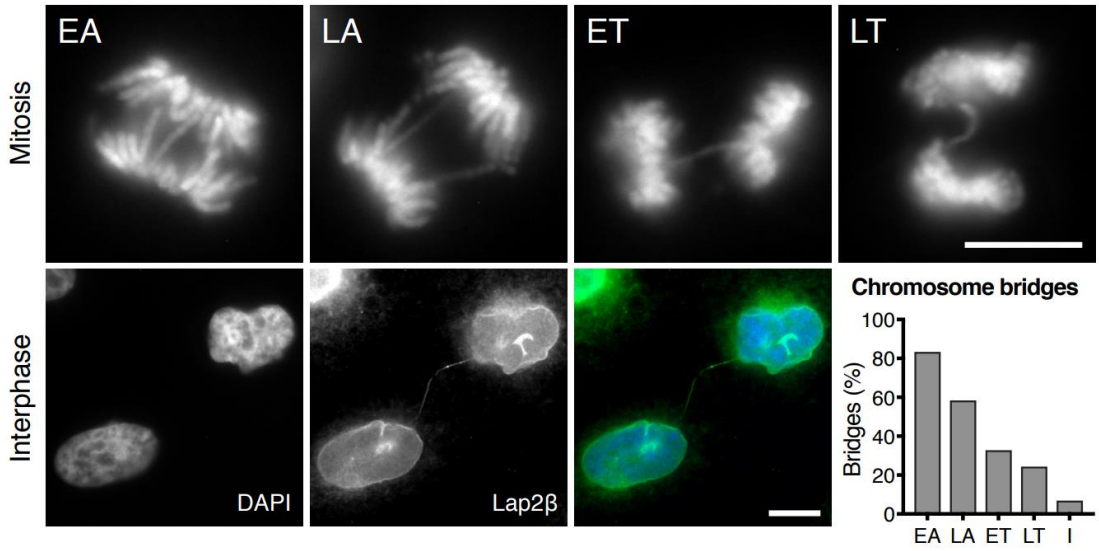
1.1 SUPPLEMENTARY FIGURES



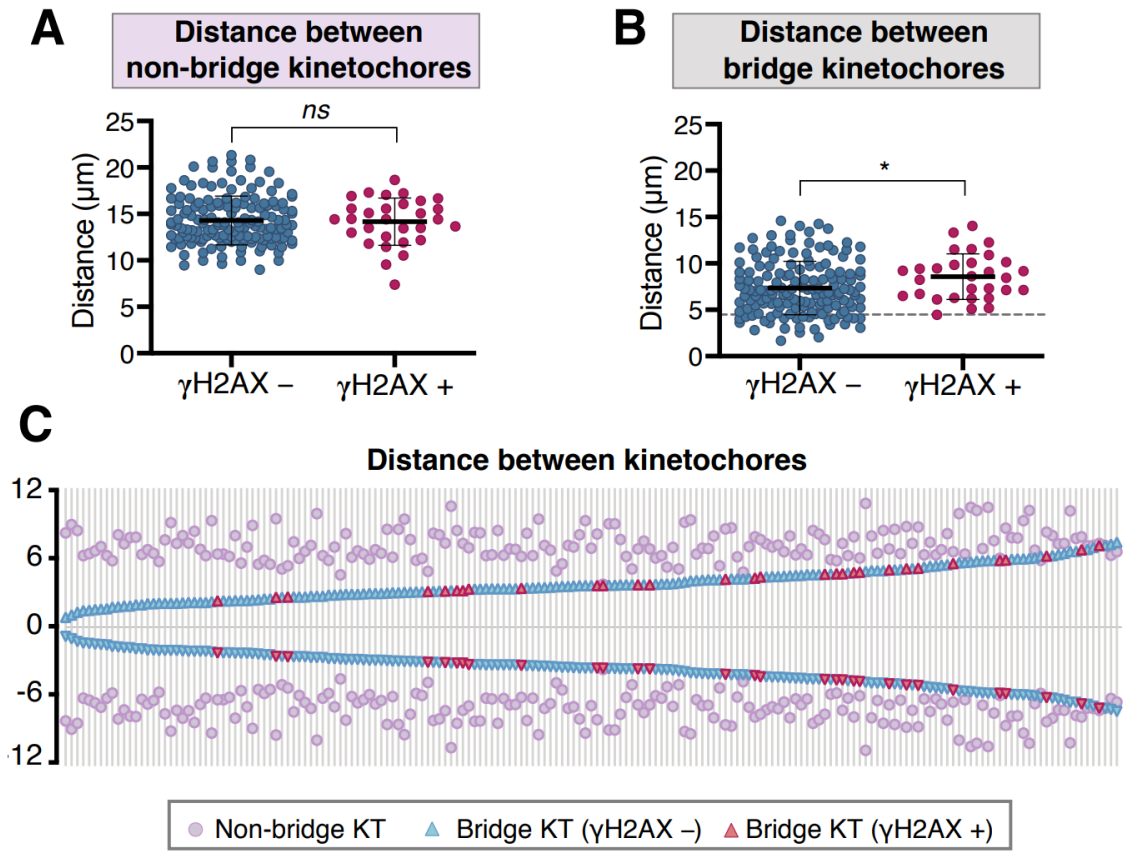
Supplementary Figure 1. Methods used for the induction of chromosome bridges of defined characteristics. (A) Schematic illustration of the LacO/LacI-GFP-CENPT experimental model to induce chromosome bridges (Gascoigne et al, 2011). (i) A LacO array (magenta) was introduced in the p-arm of Chr1. IPTG (Isopropyl β-D-1-thiogalactopyranoside) prevents the interaction between LacO and the chimeric protein LacI-GFP-CENPT (green). (ii) After IPTG removal from the media, the LacIGFP-CENPT can interact with the LacO array and form an ectopic kinetochore. (iii) Two chromosome bridges with a distance of approximately 125 Mb between the endogenous and the ectopic kinetochores are formed during the next mitosis. (iv) Representative image of a chromosome bridge (DAPI, blue) labelled with the LacO probe (red) in U2OS cells. Arrowhead indicates the LacO signal. Scale bar = 5 μm. (B) Schematic illustration of CRISPR/Cas9 sgRNA Chr2 system to generate bridges. After Cas9 expression, (i) a cut in the subtelomeric region of Chr2 q-arm is produced, (ii) leading to an uncapped chromosome end, which can be repaired by fusion with the sister chromatid after replication, (iii) generating a dicentric chromosome with a distance of 295 Mb between the centromeres. (iv) Representative image of a bridge induced with CRISPR/Cas9 sgRNA Chr2 in RPE1 cells. Whole chromosome 2 painting is shown in red, and DNA is counterstained with DAPI (blue). Scale bar = 5 μm. (C) Schematic illustration of CRISPR/Cas9 sgRNA Chr4 system to generate bridges (Umbreit et al, 2020). (i) sgRNA Chr4 can cut both ends of Chr4 and (ii) they can be repaired with their sister chromatid. Thus, different bridges can arise from this system. (iii) The fusion between the q-arms of chromosome 4 generates bridges of 279 Mb intercentromeric distance, and the fusion of p-arms generates a bridge of 101 Mb. When both chromosome ends are cut and fused, a ring chromosome with a long and a short arm sharing a pair of centromeres can be generated.



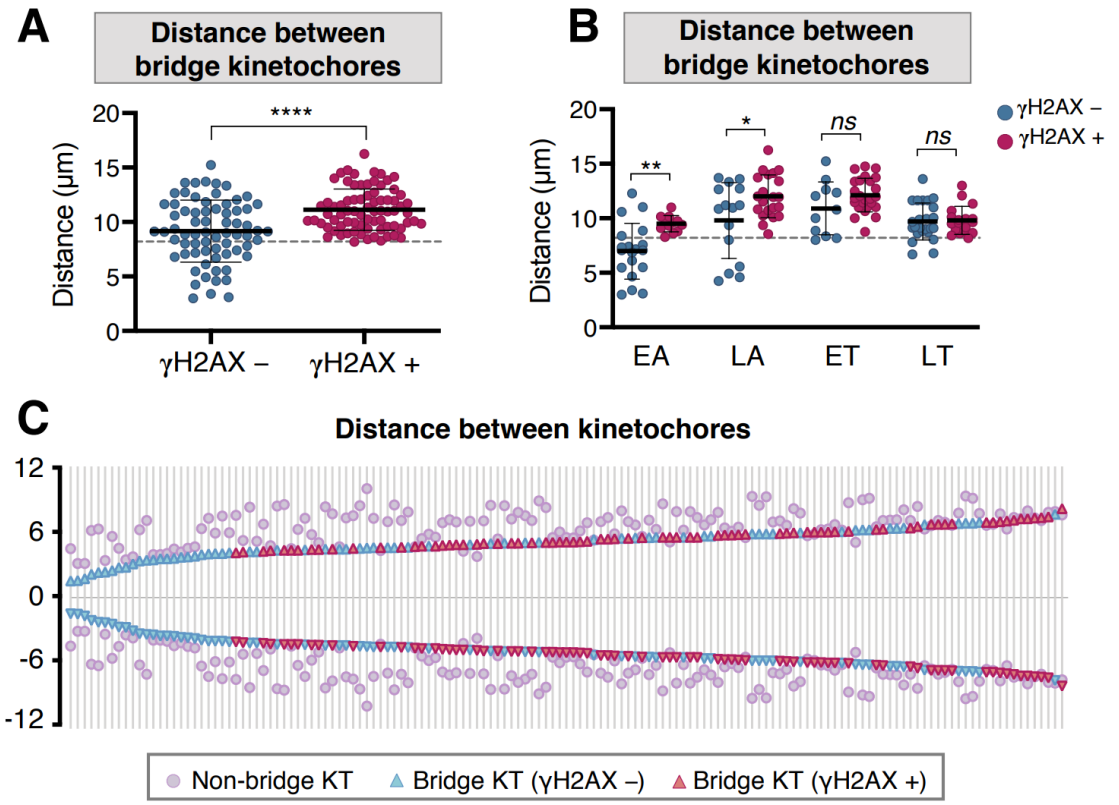
Supplementary Figure 2. Proliferation index in RPE1 Cas9 sgRNA Chr4 cells. (A) Representative image of the BrdU immunofluorescent labelling (red) after a 24-h BrdU-incubation. Cell nuclei are counterstained with DAPI (blue). Scale bar = 20 µm. (B) Percentage of BrdU-positive cells. Error bars indicate SD, *n* = 878 interphase cells from three independent replicates.



Supplementary Figure 3. Frequencies of chromosome bridges in irradiated MCF10A cells. Representative images of chromosome bridges during the last stages of mitosis and in interphase. Chromosome bridges are visualized with DAPI (blue) during mitosis and with GFP-Lap2β (green) during interphase. Scale bar = 10 µm. Graph showing the percentage of bridges during the last stages of mitosis and in interphase (*n* = 247 for mitosis and 1493 for interphase cells). EA = early anaphase, LA = late anaphase, ET = early telophase, LT = late telophase, I = interphase.



Supplementary Figure 4. Chromosome bridge breakage is associated with the separation between bridge kinetochores in U2OS LacO/LacI-GFP-CENPT cells. (A) Distance between nonbridge kinetochores in cells with broken (γH2AX-positive) and unbroken (γH2AX-negative) bridges. The mean and SD are indicated (Mann–Whitney test, *ns* $p > 0.05$; $n = 181$). (B) Distance between bridge kinetochores classified according to the γH2AX labelling of the bridge considering all mitotic stages together. The mean and SD are indicated. Asterisks indicate statistical differences between γH2AX-negative and γH2AX-positive bridges (Mann–Whitney test, * $p < 0.05$). Dashed line at 4.48 µm indicates the minimum distance from which bridges begin to break. (C) Graph displaying the distances between the bridge kinetochores (KT) of γH2AX-negative (blue) or γH2AX-positive (red) bridges. Each line represents a cell. Cells are ordered according to the distance between bridge kinetochores. Pale purple dots represent the distance between the non-bridge kinetochores of each cell. All cells were synchronized with RO3306 and fixed after a 120-minute release.



Supplementary Figure 5. Chromosome bridge breakage associated with bridge kinetochore distances in RPE1 Cas9 sgRNA Chr2 cells. (A, B) Distance between bridge kinetochores classified according to the γH2AX labelling of the bridge for (A) all mitotic stages together or (B) segregated by phase (EA = early anaphase, LA = late anaphase, ET = early telophase and LT = late telophase). The mean and SD are indicated. Asterisks indicate statistical differences between γH2AX-negative and γH2AX-positive bridges (t-test, **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, *ns* $p > 0.05$; $n = 145$). Dashed line at 8.2 µm indicates the minimum distance from which bridges begin to break. (C) Graph displaying the distances between the bridge kinetochores of γH2AX-negative (blue) or γH2AX-positive (red) bridges. Each line represents a cell. Cells are ordered according to the distance between bridge kinetochores. Pale purple dots represent the distance between the non-bridge kinetochores of each cell. All cells were synchronized with RO3306 and fixed after a 43- or 65-minute release.

1.2 SUPPLEMENTARY VIDEO

Supplementary Video 1. Representative video showing MDC1 recruitment to spontaneously induced chromosome bridges in U2OS GFP-MDC1/RFP-H2B cells. The arrowhead indicates the formation of an MDC1 focus at the end of a broken bridge. The time interval between frames is 3 minutes, and the total video duration is 42 minutes. Imaging was performed with a 60x oil objective. Scale bar = 5 µm.

1.3 SUPPLEMENTARY TABLES

Supplementary Table 1. List of plasmids used.

Plasmid	Reference	Selection	Vector Type
BAF-GFP	Umbreit *et al.*, 2020	Neomycin	Lentiviral
H2B-RFP	Addgene #26001	Cell sorter	Lentiviral
LAP2β-GFP	This study	Neomycin	Retroviral
Lenti sgRNA Chr4	Umbreit *et al.*, 2020	Puromycin	Lentiviral
Lenti sgRNA Chr2	This study	Blasticidin	Lentiviral
psPAX2	Addgene #12260	-	Packaging
pMD2.G	Addgene #12259	-	Packaging

Supplementary Table 2. List of antibodies used.

Antibody	Host	Reference	Working dilution
Primary antibodies			
Anti-53BP1	Rabbit	Abcam, ab21083	1:2000
Anti-BRCA1	Mouse	Abcam, ab16781, clone MS13	1:500
Anti-BrdU	Mouse	Abcam, ab8152	1:50
Anti-Cyclin D1	Rabbit	Abcam, ab16663, clone SP4	1:100
Anti-CREST	Human	Antibodies Incorporated, 15-234	1:50
Anti-MDC1	Mouse	Sigma-Aldrich, M2444, clone MDC1-50	1:250
Anti-Pericentrin	Rabbit	Abcam, ab4448	1:1500
Anti-RPA32/RPA2	Mouse	Abcam, ab2175, clone 9H8	1:500
Anti-γH2AX (Ser 139)	Mouse	Millipore, 05-636, clone JBW301	1:1000
Anti-γH2AX (Ser 139)	Rabbit	Abcam, ab81299, clone EP854(2)Y	1:500
Secondary antibodies			
Anti-human FITC	Goat	Antibodies Incorporated, 52-241-0100	1:100
Anti-mouse Cyanine Cy™3	Goat	Jackson ImmunoResearch Inc., 115-165-146	1:500
Anti-rabbit Alexa Fluor ®488	Goat	Thermo Fisher Scientific, A-11034	1:500
Anti-rabbit Alexa Fluor ®568	Donkey	Thermo Fisher Scientific, A-10042	1:500

CHAPTER 3

Engineering Chromosome Bridges through CRISPR/Cas9: Deciphering the Impact of Intercentromeric Distance on the Resolution Dynamics

INTRODUCTION

Understanding the resolution dynamics of chromosome bridges is crucial to unravel the intricacies of genomic stability, a pivotal aspect of cancer research. Over the last years, several studies have explored the mechanisms underlying the resolution of chromosome bridges. However, a definitive consensus on the subject remains elusive. It is plausible that various resolution mechanisms exist and are redundant, act in distinct scenarios and/or cell cycle moments or are influenced by the geometric and intrinsic characteristics of the bridges. Considering the existing uncertainties, one of the main objectives of this study is to address the need for controlled experimentation models by creating chromosome bridges with precisely defined characteristics.

While some researchers have argued that the breakage of chromosome bridges during mitosis may be merely apparent (Maciejowski et al., 2015; Steigemann et al., 2009; Umbreit et al., 2020), our findings confirm the real occurrence of such events(Rodriguez-Muñoz et al., 2021). Furthermore, most bridges are resolved during the exit of mitosis, with only a small fraction progressing intact to the interphase of the cell cycle. Thus, it is crucial to understand the mechanisms that operate in the unique context of cell division. Mitosis is a process inherently stressful for the cell, with chromatin achieving the maximum level of compaction and organization to ensure the proper distribution of the genetic information between the two daughter cells. In eukaryotic cells, chromosome segregation is facilitated by the assembly of the microtubule-based mitotic spindle. Within minutes, paired sister chromatids balanced at the center of the mitotic spindle are pulled apart by the pulling forces of mitotic spindle microtubules. Therefore, investigating chromosome bridge resolution mechanisms in mitosis requires bridges with well-defined geometric characteristics and precise temporal control of induction due to the brief window of time available for their study.

Numerous approaches can be used to generate chromosome bridges, including massive induction of DNA breaks by ionizing radiation (Giunta et al., 2010) or chemical agents (Mardin et al., 2015), and widespread telomere deprotection (Van Steensel et al., 1998). However, these methods, while useful for answering questions related to genomic instability, are not ideal for studying the mechanisms of chromosome bridge resolution at mitosis due to substantial variability between the originated bridges in a context where the characteristics of the chromosome involved in the bridge might influence the probability of breakage (Rodriguez-Muñoz et al., 2021). In the last years, CRISPR/Cas9 has emerged as a revolutionary technique for genome editing with multiple applications in the biomedical research field (Pickar-Oliver & Gersbach, 2019; Ran et al., 2013). Cas9 enzyme is an RNA-guided endonuclease that can

recognize and cleave target DNA, thus generating a blunt DNA double-strand break (DSB) on a specific genome site. Recently described consequences of on-target Cas9-mediated DNA breakage are the formation of micronuclei and chromosome bridges in dividing cells (Leibowitz et al., 2021; Umbreit et al., 2020). Thus, this technology can be a valuable tool for inducing chromosome bridges under controlled conditions.

Leveraging the CRISPR/Cas9 technology, we employed single guide RNA (sgRNA) species to target unique DNA sequences, engineering chromosome bridges with a specific number of base pairs between the two centromeres and ensuring the formation of dicentric chromosomes with defined intercentromeric distances. The Cas9 system's activation is regulated by the addition of doxycycline and cell synchronization at the G2-M transition is achieved by inhibition of CDK1 (Vassilev, 2006). This strategy allows precise temporal control over the generation of chromosome bridges with defined characteristics and significantly reduced variability. Such precision provides a robust engineered model allowing us to pursue an additional goal which is to examine the mechanistic basis of chromosome bridge resolution during mitosis. By using CRISPR/Cas9 to engineer chromosome bridges, we contribute valuable insights into the resolution mechanisms operating during mitosis, shedding light on the importance of bridge intercentromeric distance in this intricate process.

METHODS

Cell Culture

Telomerase-immortalized retinal pigment epithelial 1 (RPE-1) cells expressing Cas9 under a doxycycline-inducible promoter (a gift from Iain Cheesman's Laboratory) were grown in DMEM:F12 (Biowest, Riverside, UK) supplemented with 10% tetracycline-free fetal bovine serum (FBS) (FBS-TET-12A, Labclinics, Barcelona, Spain). HEK293T were cultured in minimal essential medium supplemented with 10% FBS. The media for all cell lines was supplemented with Penicillin/streptomycin (100U/mL) and cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

Single guide RNA design, plasmids construction and amplification

Single guide RNAs targeting intergenic regions of chromosomes of interest were selected by searching in the reference human genome 38 for protospacer-adjacent motif (PAM) on the subtelomeric regions. Then, the preselected candidates were tested using the CCTOP CRISPR/Cas9 target online predictor (Stemmer et al., 2015) based on high on-target predictions (>0.7) to choose our definite candidates. The guide RNAs (Table I) were cloned into a lentiviral blasticidin-resistance backbone that contains the scaffold guide RNA (gift from Brett Stringer,

Addgene #104993) using the BsmBI restriction enzyme following the cloning protocol described in the Addgene website[1].

Table I. Design of single guide RNAs

Chromosome arm involved	Targeting sequence + PAM	Cut site (hg38)
Chr2q	ATATTAAGGGCTCCCCGTCGGGG	240,709,919
Chr1p	TGACGTTGGACAGCCGCTGGAGG	2,860,114
Chr3q	AGGGCTGGTCACCATTCAAGAGG	195,507,432
Chr6p	CCCCCCGCATCTGTGTTTACTGT	835,245
Chr20q	GCTGCGCATCCAGCTCGCCGGGG	63,855,717

Plasmid amplification was performed using Stbl3 cells kit following manufacturer's instructions (C737303, Invitrogen, Waltham, MA, United States). Purification of plasmids was carried out using NucleoBond PC kit (740573.100, Macherey-Nagel, Bethlehem, PA, United States). Sanger sequencing was performed to validate plasmids in the UAB genomic facility and DNA concentration was determined using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, USA).

Viral particle production and transduction

Lentiviral particles were generated using HEK293T cells. At a confluency of 70% cells were transfected with a total DNA plasmid concentration of 1 µg/mL (Table II), following Trono's Lab protocol[2]. DNA concentration was adjusted to a 4:3:1 ratio (transfer plasmid:psPAX2:pMD2.G). After 48 hours, the supernatant was collected, concentrated using centrifugal filters (UFC910008, Merck), and stored at -80 °C. Cells were transduced overnight with viral particles in a medium containing 4 µg/mL polybrene. Cells were selected with 2.5 µg/mL of blasticidin (15205, Merck) for two weeks.

Table II. List of plasmids used.

Plasmid name	Antibiotic resistance	Vector type	Reference
sgChr2	Blasticidin	Lentiviral	This study
sgChr1	Blasticidin	Lentiviral	This study
sgChr3	Blasticidin	Lentiviral	This study
sgChr6	Blasticidin	Lentiviral	This study
sgChr20	Blasticidin	Lentiviral	This study
psPAX2	-	Packaging	Addgene #12260
pMD2.G	-	Envelope	Addgene #12259

[1]https://media.addgene.org/data/plasmids/104/104993/104993-attachment_fbZFcCQmsWQB.docx
[2] https://www.epfl.ch/labs/tronolab/wp-content/uploads/2019/06/LV_production.pdf

Generation of chromosome bridges: CRISPR/Cas9 DSB induction and cell cycle synchronization

Cells were seeded on glass coverslips or in 60 mm Petri dishes depending on the purpose of the experiment. The day after seeding, Cas9 expression was induced by adding 1µg/mL doxycycline to the medium (631311, Clonetech, Mountain View, CA, United States) for 15 hours. Then, cells were rinsed seven times with PBS and were returned to the incubator for five hours. Cells were synchronized in G2/M transition by incubating them with 9 µM RO3306 (SML0569, Sigma-Aldrich, Sant Louis, MO, United States) for 18 hours. Cells were fixed with 4 % paraformaldehyde at 43-120 minutes after RO3306 release to analyze chromosome bridges in cells from anaphase to telophase stage. For analyses at the metaphase stage of mitosis, immediately after RO3306 release cells were treated for 1 h and 30 minutes with 0.05 µg/mL Colcemid. Afterwards, hypotonic shock with KCl 0.075M for 13 minutes at 37 °C and methanol:acetic (3:1) fixation was performed. Cell suspensions were dropped onto slides to obtain metaphase spreads.

Fluorescence *In situ* Hybridization

Slides with metaphase spreads were treated with pepsin/HCL solution at 37 °C for 90 seconds post-fixated with formaldehyde-MgCl2 for 10 minutes and dehydrated in ethanol. The hybridization mix included a pancetromeric PNA probe conjugated with FITC (AAACACTCTTTTTGT-AGA) (Panagene, Daejeon, Korea) and a pantelomeric PNA probe conjugated with Cy3 (CCCTAA) (PE Byosystems, Foster City, CA, USA). DNA on the slides underwent denaturation at 80 °C for 90 seconds. Hybridization took place at room temperature for 2 hours, followed by post-hybridization washes using 70 % formamide and Tris-NaCl-Tween20 buffer. Finally, samples were dehydrated and counterstained with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, United States) containing 0.25 µg/mL 4′,6-diamidino-2-phenylindole (DAPI).

For locus-specific probes or chromosome painting, after carnoy fixation cells were rinsed with 2x SSC, denatured for 3 minutes at 73 °C in formamide 70 % / 2x SSC buffer and dehydrated in ethanol. The Chr2 whole chromosome probe (Vysis probes, Des Plaines, IL, United States) or CEP Chr6 (Vysis probes) were also denatured at 73 °C for 5 minutes before their hybridization with samples for 12 hours at 37 °C. Following hybridization, slides were rinsed with 0.4x SSC / 0.3 % NP40 for 2 minutes at 73 °C and 2x SSC / 0.1 % NP-40 for 2 minutes at room temperature. Samples were dehydrated and counterstained with DAPI. Imaging and visualization were carried out utilizing Olympus BX61 microscope (Olympus, Hamburg, Germany) equipped with a CV-M4+ CL camera (JAI, Großwallstadt, Germany) and CytoVision software (Applied Imaging, Newcastle, United Kingdom). Image analysis was performed using ImageJ (Schindelin et al., 2015).

Immunofluorescence and imaging

After fixation, cells were permeabilized with 1x PBS / 0.5 % Triton-X-100 solution for 15 minutes, and blocked with 1x PBS/0.5 % BSA / 0.15 % glycine for 15 minutes. Afterwards, primary antibodies were incubated overnight at 4 °C (CREST-Human from Antibodies Incorporated at 1:50 concentration, γH2AX-Mouse from Millipore using a concentration of 1:500). Then, cells were washed 3 times with 1x PBS/0.1 % Tween 20 and incubated with secondary antibodies (Human-FITC from Antibodies Incorporated used at 1:100; Mouse-Cy3 from Jackson ImmunoResearch Inc. using a concentration of 1:500) for 1 hour at room temperature. Finally, cells were washed 3 times with PBS / 0.1 % Tween20 for 7 minutes each and rinsed in distilled water. Coverslips were mounted on glass microscope slides using Vectashield mounting medium containing DAPI (0.25 μg/mL). Image acquisition was performed using a Zeiss Axio Observer Z1 microscope equipped with AxioCam MRm camera (Zeiss, Oberkochen, Germany) and motorized stage at the bioimaging platform of the Institut d'Investigació Biomèdica de Bellvitge (IDIBELL). To obtain images of cells in the last stages of mitosis two rounds of imaging were performed. First, a general view of each sample was obtained using 10x/0.3 objective and DAPI filter set. Then, cells of interest were manually identified and selected. In a second round of imaging, selected cells were relocalized and imaged with DAPI, FITC and Cy3 filter sets using a 100x/1.4 objective. Nine z-stacks were acquired for each cell with a step size of 0.7 um between planes. Image analysis and measurements were performed using ZEN software v3.7 (Zeiss).

Statistics

The Chi-square test was used to determine differences between categorical variables. Correlations were tested for simple linear regression. Statistical analysis and graph plotting were performed using GraphPad Prism 8 (GraphPad Software, San Diego, USA). P-values < 0.05 were considered statistically significant.

RESULTS

1. Design and validation of a cell-based experimental system for the generation of chromosome bridges with defined intercentromeric distances using CRISPR/Cas9

CRISPR/Cas9 technology is currently mostly applied to gene editing and regulation (Ran et al., 2013), but we and others have used it for other purposes (Ma et al., 2016; Rodriguez-Muñoz et al., 2021; Umbreit et al., 2020). In this study, we report the design of five sgRNAs that target specific genomic sites to generate chromosome bridges with precisely defined intercentromeric distances. Specifically, these sgRNA were tailored to recognize subtelomeric regions of Chr2q, Chr1p, Chr3q, Chr6p, and Chr20q, thereby determining the distance from the cut site to the

centromere of each chromosome according to the individual length in megabases of each chromosome arm (Figure 1A). As a result, the selected sgRNA covered spanning distances between 36 and 147 Mb (Figure 1B). Once directed to its target site, the Cas9 enzyme induces a DSB that cleaves the chromosome in two pieces: one centric and one acentric fragment. If the broken ends are not faithfully repaired by the cell's DNA repair machinery to reconstitute the original chromosome, the acentric fragment generated will probably missegregate and result in a micronucleus in the next cell division (Leibowitz et al., 2021). Regarding the centric fragment, it can lead to the formation of a dicentric chromosome bridge in the next anaphase after DNA end ligation of their sister chromatids.

Single guide RNAs were introduced individually in genetically stable immortalized human RPE-1 cells containing a *Cas9* gene with a doxycycline-inducible promoter to trigger a sgRNA-directed cut on DNA under precise spatiotemporal control (Gascoigne et al., 2011). To estimate the frequency of mitotic cells with chromosome bridges, cells were synchronized by inhibiting CDK1 using RO3306, thus halting them at the G2/M checkpoint. After Cas9 induction and cell synchronization, chromosome bridges were obtained for the five sgRNA cell lines generated, and the specificity of the assay was validated by either locus-specific probes or painting against the targeted chromosomes (Figure 1D). Moreover, chromosome bridges in mitotic cells exhibited morphological differences depending on the sgRNA used. Specifically, sgRNAs targeting regions more distant from centromeres, such as for Chr2q and Chr1p, led to dicentric chromosomes with longer bridging chromatin connecting the two chromatid groups and their kinetochores localizing with the kinetochores of the rest of chromosomes. In contrast, sgRNAs targeting regions closer to centromeres, such as Chr6p and Chr20q, led to dicentric chromosomes with shorter bridging chromatin and their kinetochores dislocated from those of the rest of chromatids (Figure 1E). Therefore, morphological features of chromosome bridges during mitosis are inherently influenced by the length of the bridging chromatin, which is determined by the number of base pairs between the cut site and the centromere of the targeted chromosome.

After confirming that the five designed sgRNAs allow the formation of bridges and noting that these bridges exhibit diverse morphologies according to the distance that separates the cut site from the centromere of each chromosome, our objective was to assess how often bridges were observed in the five cell lines. During the exit of mitosis, the frequency of cells with chromosome bridges differed among the different cell lines generated (Chr2q: 22.94 % of bridges; Chr1p: 19.32 %; Chr3q: 16.02 %; Chr6p: 12.68 %; Chr 20q: 9.57 %; Figure 1C). Although we could observe formation of chromosome bridges in the five designed RPE-1 Cas9 cell lines, the percentage of chromosome bridges in cells at the last stages of mitosis was lower as the intercentromeric

distance of the generated dicentric decreased (p = 0.0013; Figure S1). Taken together, using the cell-based experimental system designed, we observed differences in the frequency of bridges at the exit of mitosis that could be associated with the length of the bridging chromatin.

Upon having established the experimental system's capacity to induce specific chromosome bridges we aimed to elucidate whether the observed differences among sgRNA cell lines in the frequency of chromosome bridges are derived from variations in chromosome bridge formation or from distinct dynamics of bridge resolution. Initially, we evaluated the efficacy of Cas9 guided by the different sgRNAs in producing a break on the targeted chromosome end. To estimate the efficiency of breakage in each of the five cell lines, we examined chromosome spreads of cells at the metaphase stage of mitosis for the presence of telomere signal-free ends indicative of cleavage on the targeted chromosome. As the stage of mitosis at which break induction is scored is before the formation of the chromosome bridges, the results obtained will not be affected by differences in resolution dynamics among the different sgRNA cell lines. To ensure a sizable sample for analysis, each cell line was treated with doxycycline followed by a double synchronization process – first at the G2/M boundary using RO3306 and then at the metaphase stage of mitosis with Colcemid (Figure 1F). After the double synchronization, metaphase spreads were obtained for each cell line and the targeted chromosomes were identified by reverse G bands. Telomere sequences were detected using FISH with pan-telomeric DNA probes and the centromeres of all chromosomes with pan-centromeric DNA probes (Figure 1G). No differences in the efficiency of Cas9 induced DSB were observed among the five cell lines engineered as the frequency of chromosomes with telomere-free signals were similar for all of them (Chr2q: 30.61 %; Chr1p: 31.37 %; Chr3q: 30.00%; Chr6p: 26.09 %; Chr20q: 29.63 %; Figure 1H). Thus, we can conclude that the engineered model generates deprotected ends with a similar frequency among the five sgRNA cell lines obtained. Hence, differences observed in the number of bridges are not related to differences in the efficacy of the Cas9 directed by sgRNA to cut chromosomes, but to differences in the dynamics of bridge resolution that need to be investigated.

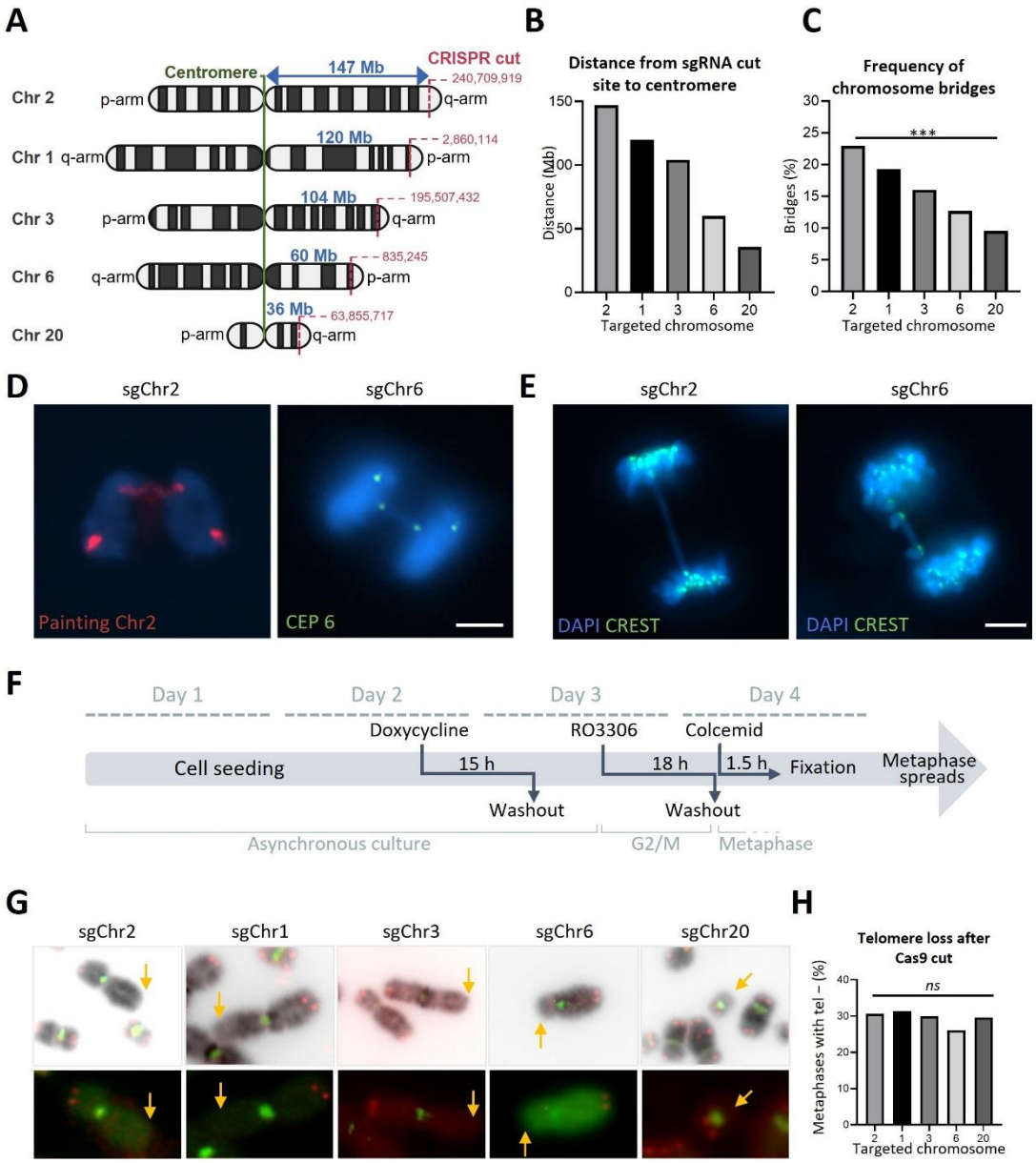


Figure 1. Design and validation of sgRNAs for generation of chromosome bridges with different intercentromeric distances (A) Schematic illustration of sgRNAs directed cut sites on human chromosome arms 2q, 1p, 3q, 6p and 20q. The distance between the cut site and the centromere is indicated in megabases. **(B)** Graph of the distance in megabases from the CRISPR/Cas9 sgRNA cut site to the centromere of each chromosome. **(C)** Graph showing the frequency of chromosome bridges observed for each sgRNA cell line. Chi-square test; p = 0.0009. A minimum of 200 cells were analyzed for each sgRNA cell line. **(D)** Representative images of chromosome bridges and detection of specific chromosomes by FISH probes. **(E)** Representative images of chromosome bridges with long intercentromeric distances (Chr2) or short intercentromeric distances (Chr6) at the exit of mitosis. Kinetochores were immunodetected with CREST antibody and DNA was counterstained with DAPI. **(F)** Experimental design for the generation of chromosome bridges by CRISPR/Cas9 and cell synchronization to obtain an enriched population of cells in metaphase. RO3306 was used for cell synchronization at the G2/M checkpoint and Colcemid to arrest cells at the metaphase. **(G)** Representative images of telomeres (red) and centromere (green) in metaphase spreads from the five sgRNA cell lines after CRISPR/Cas9 directed cut. Telomere signal absence on chromosome ends of targeted chromosomes is highlighted with arrows. **(H)** Graph of the percentage of telomeric signal-free ends on the targeted chromosome for each of the five sgRNA cell line (Chi-square test, *ns* $p > 0.05$; number of metaphases analyzed: 49 for Chr2q, 51 for Chr1p, 30 for Chr3q, 23 for Chr6p and 27 for Chr20q sgRNA cell lines).

2. Impact of the bridging chromatin length on chromosome bridge resolution timing in mitosis

After verifying the capability of the CRISPR/Cas9 system to induce chromosome bridges, our focus turned to investigating the behavior of these bridges during the final stages of mitosis. In an initial approach, we explored time-lapse microscopy to provide real-time insights into the evolution of chromosome bridges throughout the exit of mitosis. Nonetheless, the high spatial and temporal resolution required to obtain valuable information about chromosome bridge dynamics during mitosis makes it difficult to achieve a sufficient sample size. Therefore, we adopted an alternative strategy using fixed samples. As detailed in Supplementary Figure S2, cells in the last stages of mitosis were morphologically classified into four categories considering the degree of chromatin condensation and separation of chromatid groups towards cell poles: early anaphase (EA), late anaphase (LA), early telophase (ET) and late telophase (LT). Moreover, to ensure sufficient sample size for each category, following CRISPR/Cas9 induction RPE-1 cells were synchronized at G2/M transition with the CDK1 inhibitor RO3306 and fixed at precise time points after release (Figure 2A). This comprehensive approach facilitated the capture of a sufficient number of cells in each of the last stages of cell division, thus enabling a profound analysis of chromosome bridge dynamics for each targeted chromosome using the designed sgRNA.

The frequency of chromosome bridges significantly decreased as cells progressed from EA to LT in each of the five CRISPR/Cas9 sgRNA cell lines (Figure 2B), thus indicating that a substantial fraction of chromosome bridges is indeed resolved by mechanisms operating during mitosis. However, at EA the frequency of chromosome bridges differed among the sgRNA cell lines and was directly related to the distance between the cut site and the centromere of the targeted chromosome. In fact, for the sgRNA cell lines in which the targeted site is far from the centromere, the percentage of bridges at EA precisely matched the percentage of metaphases with telomeric signal-free ends for each targeted chromosome (30.00 % EA with bridge and 30.61 % metaphases with telomere signal-free end for sgRNA Chr2q; 31.48 and 31.37 % for sgRNA Chr1p; Figures 2B and 1H). In contrast, for the sgRNA cell lines in which the targeted site is closer to the centromere, the percentage of EA cells with chromosome bridges was significantly lower than the values obtained for telomeric signal-free ends. In the specific case of Chr20q sgRNA, which induces the closest cut to the centromere among the five cell lines generated, only 18.53 % of cells in EA exhibited chromosome bridges, a percentage that is far from the 29.63 % of metaphases showing telomere signal-free end on chromosome 20. This observation reinforces the idea that a subset of chromosome bridges formed in the cell lines in which the sgRNA-directed cut site is close to the centromere undergoes resolution at the onset of anaphase before becoming identifiable as

chromosome bridges. This implies that the mechanisms responsible for bridge resolution during mitosis would act from the beginning of anaphase and have a strong impact on chromosome bridges with shorter bridging chromatin.

To obtain a broader picture of chromosome bridge behavior during mitosis and investigate if their dynamics depend on the length of the bridging chromatin, chromosome bridges were segregated into continuous and discontinuous structures (Figure 2C). Discontinuous chromosome bridges refer to those bridging structures that have lost the continuity of the DNA fiber indicating that have already undergone resolution. Among the discontinuous chromosome bridges, we distinguished between those recently broken bridges, in which the two broken ends were still very close to each other and exhibited one single γH2AX foci flanking the discontinuity, and bridge remnants, in which the broken ends were more distant but still facing each other. This categorization allowed us to obtain information at the population level on the progression of chromosome bridges in their process for resolution, transitioning from continuous bridges to recently broken bridges and, ultimately, to bridge remnants (Figure 2C). Results obtained in cells at EA in the five sgRNA cell lines revealed that between 26-52 % of the identified chromosome bridges were continuous structures and the rest have already initiated resolution at this stage of mitosis (Figure 2B). The proportion between continuous and discontinuous chromosome bridges decreased for all targeted chromosomes as the mitosis advanced from EA to LT. These results indicate that most chromosome bridges undergo resolution during anaphase with only a small fraction remaining intact until the end of mitosis. Specifically, in sgRNA cell lines in which the cut site is close to the centromere (Chr6p and Chr20q sgRNA cell lines), all chromosome bridges underwent resolution during mitosis and in LT cells only bridge remnants were observed, appearing in a small fraction of cells (3.92% and 1.79% for Chr6p and Chr20q sgRNA cell lines, respectively). In contrast, in sgRNA cell lines in which the cut site is distant from the centromere, continuous chromosome bridges were still detected in LT cells (2.86 %, 3.45 %, and 2.00 % for Chr2q, Chr1p, and Chr3q sgRNA cell lines, respectively). These results suggest that the longer the distance in megabases between centromeres, the greater the likelihood of chromosome bridges to persist connecting the two masses of chromosomes giving rise to daughter cells born with chromosome bridges after mitosis.

In conclusion, our study reveals that most chromosome bridges undergo resolution during mitosis and highlights a correlation between the length of the bridging chromatin and the timing of resolution, with shorter bridging chromosomes experiencing an earlier resolution than the longer ones. The frequency of chromosome bridges decreases as mitosis advances, coinciding with mitotic spindle elongation, suggesting a potential involvement of pulling forces by kinetochore

microtubules attaching the dicentric chromosomes. These insights emphasize an interplay between the chromatin structure and the mechanical forces influencing the resolution of chromosome bridges during mitosis.

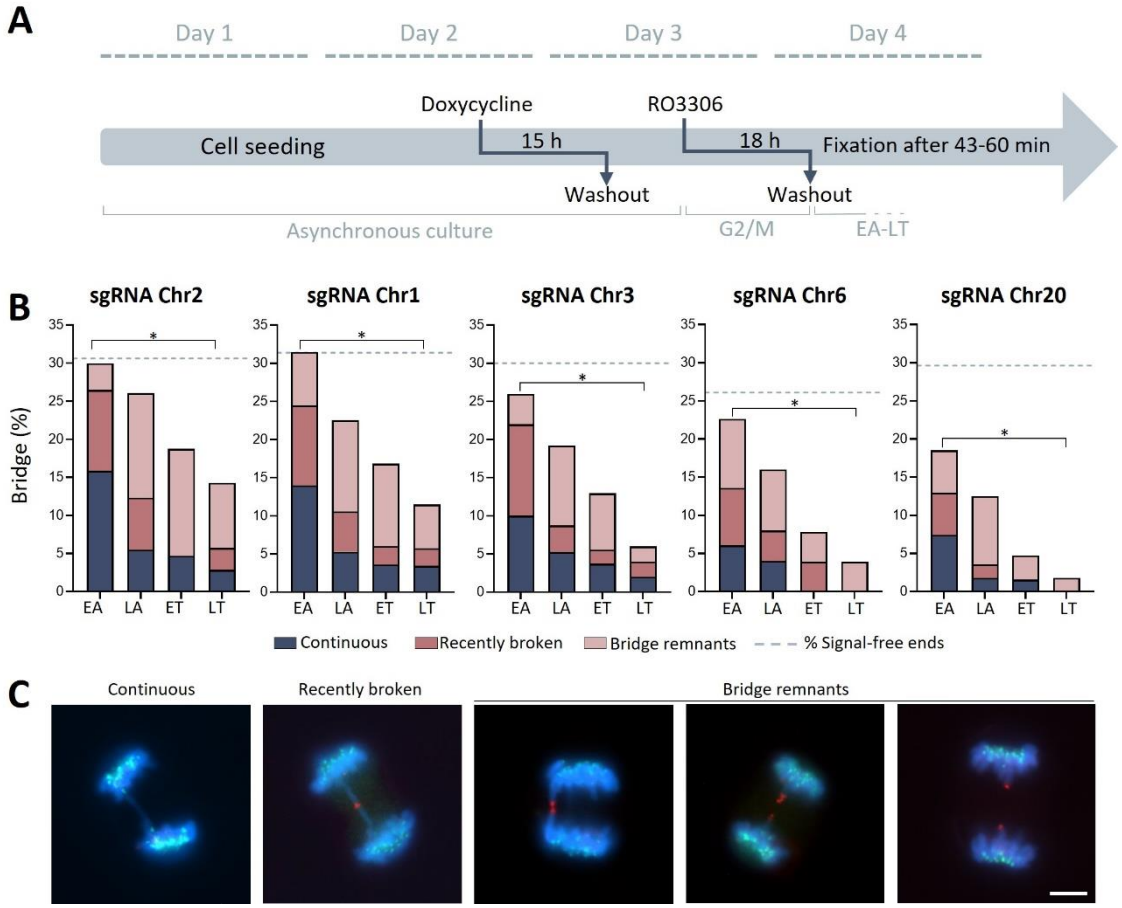


Figure 2. Dynamics of CRISPR/Cas9-induced chromosome bridges at the exit of mitosis. (A) Timeline scheme illustrating the experiment carried out from the activation of the CRISPR/Cas9 nuclease using doxycycline to the analysis of enriched populations of cells at the final stages of mitosis. RO3306 was used for cell synchronization at G2/M and cells were fixed at 43, 50, and 60 minutes after RO3306 washout. **(B)** Frequencies of chromosome bridges for each sgRNA cell line. Categories of continuous, recently broken, and discontinuous chromosome bridges are included. Note that graphs are ordered from left to right by decreasing distance from the cut site to the centromere of the targeted chromosome. A minimum of 50 cells were analyzed for each sgRNA cell line (Chr2q, Chr1p, Chr3q, Chr6p, and Chr20q) and stage (EA = early anaphase, LA = late anaphase, ET = early telophase, LT = late telophase). The dotted horizontal line represents the percentage of metaphases with telomeric signal-free ends on the targeted chromosome arm for each sgRNA cell line. Asterisk refers to $p < 0.05$ for Chi-square test **(C)** Representative images of cells with continuous chromosome bridges, recently broken bridges or bridge remnants. γH2AX (red) and CREST (green) were immunodetected and DNA was stained with DAPI (blue). Scale bar = 5 μm.

3. Influence of the mitotic spindle pulling forces on chromosome bridge resolution

To explore the potential influence of pulling forces exerted by the mitotic spindle on chromosome bridge resolution, we next focused on analyzing the separation of bridging kinetochores in cells at the mitotic exit. If tension forces impact the breakage of chromosome bridges, we anticipate

observing a correlation between the length in megabases of the bridging chromatin and the separation in micrometers between the bridge kinetochores when bridge breakage occurs. To test this hypothesis, we measured the interkinetochoric distance in both continuous and recently broken chromosome bridges (Figures 3Ai and 3Aii). Notably, bridge remnants were excluded from this analysis due to the loss of connection between bridge kinetochores upon resolution, leading to two centric fragments independently pulled towards opposite cell poles.

Analysis of continuous chromosome bridges revealed variability in the distance between bridging kinetochores among cells of the same sgRNA cell line, probably because of the gradual separation between the spindle poles during the mitotic exit (Figure 3B**).** Interestingly, the median distance between the kinetochores of continuous chromosome bridges decreases as the number of megabases from the cut site to the centromere is reduced (Table III). This suggests that the greater amount of chromatin between the centromeres of the dicentric chromosome facilitates the maintenance of bridge integrity. Furthermore, the maximum distance at which the bridging kinetochores can separate while maintaining their integrity differs significantly among the different sgRNA cell lines and correlates with the number megabases from the cut site to the centromere (Figures 3B and 3C; Table III; $R^2 = 0.9278$). Therefore, the amount of chromatin between the two centromeres of the bridge dictates the maximum distance at which bridge kinetochores can separate at mitosis.

After examining the distance between the two kinetochores of continuous chromosome bridges, we aimed to determine to what extent bridge breakage is influenced by the amount of chromatin between the bridge centromeres. Hence, we measured the bridge kinetochore separation in those recently broken bridges across the five sgRNA cell lines. We selected this type of chromosome bridges because it provides a picture of the precise moment immediately after bridge breakage. Remarkably, the bridging chromatin tethered between kinetochores in the recently broken bridges was observed fully extended in the five cell lines (Figure 3Aii), indicating that chromosome bridges are under tension upon DNA breakage. Regarding the separation between bridging kinetochores, there was not a specific separation at which chromosome bridges of each sgRNA cell line break. Instead, bridge resolution occurred within a range of bridge interkinetochore distances (Figure 3D). Notably, although the range varies among the different sgRNA cell lines, the median separation between bridge kinetochores correlates with the distance between the sgRNA cut site and the centromere of the targeted chromosome (Table III). Interestingly, the minimum separation between kinetochores needed for bridges to become broken significantly correlates with the distance in megabases from the cut site to the centromere of each targeted

chromosome (Figure 3D and 3E; $R^2 = 0.9770$), with longer chromosome bridges requiring more separation between the bridge kinetochores for their resolution.

In summary, the results reported here indicate that chromosome bridge resolution at the mitotic exit is influenced by tension forces to which the bridges are subjected while they are stretched towards opposite poles. This tension, within the context of mitosis, is provided by the kinetochoric microtubules of the mitotic spindle, which stretch sister chromatids toward opposite poles. In the specific case of chromosome bridges, microtubules from opposite cell poles exert opposing forces on each kinetochore of the dicentric chromosome, hereby placing them under tension and allowing the chromosome bridge to break once a certain separation between its two kinetochores is reached.

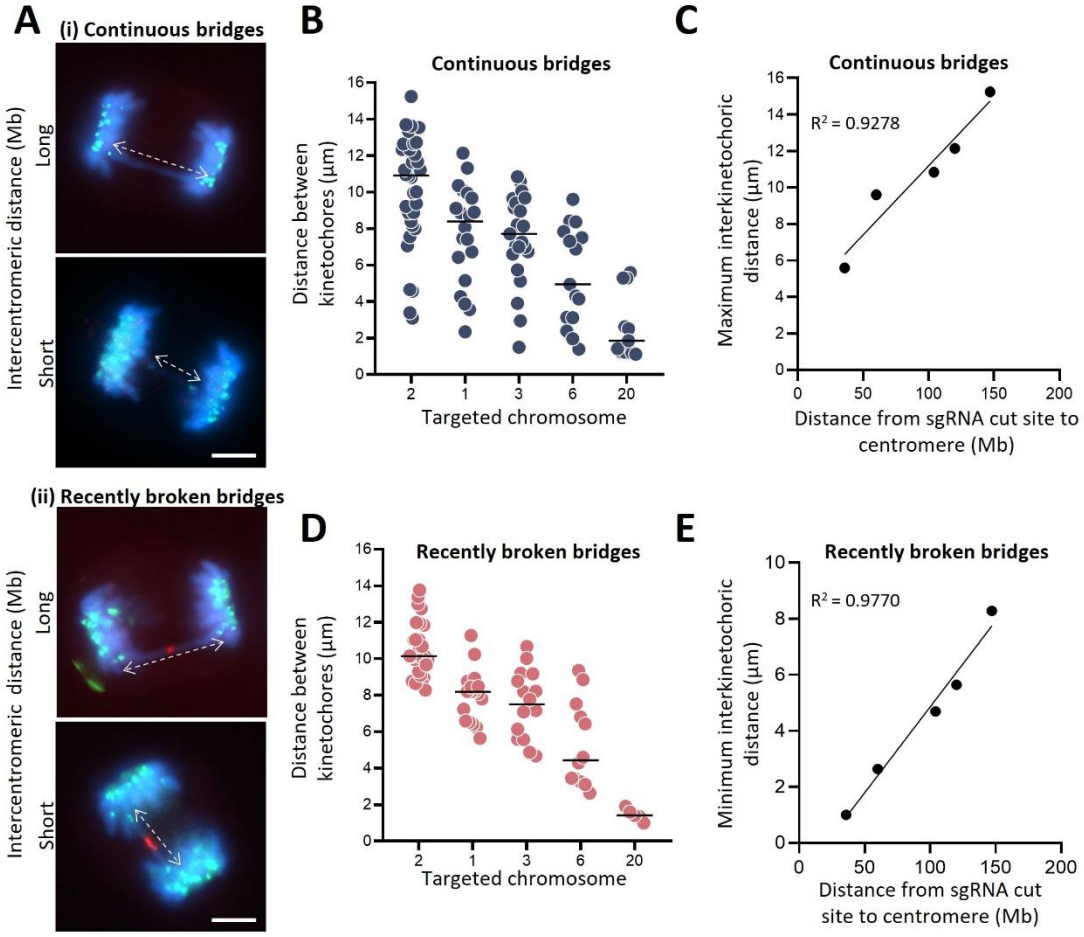


Figure 3. Chromosome bridge resolution dynamics. (A) Representative images of chromosome bridges with short or long intercentromeric distances that are (i) continuous or (ii) recently broken. Kinetochores are shown in green, γH2AX in red and DAPI in blue. Note pronounced kinetochore delocalization for bridges with short intercentromeric distances. Scale bar = 5 µm. **(B)** Distance between bridge kinetochores of continuous chromosome bridges. X axis numbers refer to the targeted chromosome arm in each sgRNA cell line **(C)** Correlation between the maximum interkinetochoric distance in micrometers and the distance between the cut site and the centromere in megabases for continuous bridges of the five sgRNA cell lines (Linear regression; $p = 0.0084$). **(D)** Bridge kinetochore distance of recently broken chromosome bridges categorized by sgRNA cell lines. **(E)** Correlation between the minimum interkinetochoric distance in micrometers and the distance between the cut site and the centromere in megabases for recently broken bridges (Linear regression; $p = 0.0015$).

Table III. Distance between bridging kinetochores of continuous and recently broken bridges for each sgRNA cell line.

Chromosome arm involved (sgRNA cell line)	Distance from cut site to centromere (Mb)	Median distance continuous bridges (um)	Maximum distance continuous bridges (um)	Median distance at breakage (um)	Minimum distance to break (um)
Chr2q	147	10.92	15.24	10.14	8.28
Chr1p	120	8.40	12.14	8.18	5.64
Chr3q	104	7.71	10.84	7.50	4.67
Chr6p	60	4.95	9.61	4.44	2.64
Chr20q	36	1.86	5.59	1.41	1.00

DISCUSSION

Despite chromosome bridges were discovered over a century ago, the mechanisms underlying their resolution remain only partially understood and are still a subject of discussion. Here, we provide crucial insights into the mechanism governing chromosome bridge resolution during the exit of mitosis, a stage in which uncertainties have arisen regarding the resolution of this aberrant structure. In a seminal study on the involvement of chromosome bridges in self-perpetuating cycles of bridge-breakage-fusion, Barbara McClintock suggested that chromosome bridges might be resolved during mitosis (McClintock, 1941). In studies published more recently, the investigations started from an unproven premise that the chromosome bridges were not resolved during mitosis but later, once the daughter cells had already entered interphase (Maciejowski et al., 2015; Umbreit et al., 2020). However, in a previous study carried out in our laboratory we obtained evidence indicating that the bridges could be broken during mitosis (Rodriguez-Muñoz et al., 2021). This evidence included the observation of markers of DSBs, such as γH2AX foci on the mitotic bridging chromatin, and detection of 3'OH free DNA ends using the STRIDE assay (Kordon et al., 2020). In the present study, we demonstrate that chromosome bridges can be resolved during mitosis and show the influence of the pulling forces exerted by mitotic spindle microtubules in their resolution.

To study the behavior of chromosome bridges during mitosis, it is essential to (1) temporally control chromosome bridge induction to ensure the examination of first-generation bridges, (2) obtain bridges of defined geometry so that differences in the length of the bridging chromatin does not impact on bridge resolution during mitosis, and (3) use a genomically stable cell line to prevent interference from spontaneously formed chromosome bridges. In our study, using CRISPR/Cas9 technology, we have successfully engineered an experimental system capable of generating chromosome bridges with defined intercentromeric distances in the RPE-1 genome-

stable cell line under temporal control by regulating the expression of an inducible Cas9. Our experimental approach has been demonstrated to be highly specific as the individual chromosomes involved in the bridges were checked to be the specific ones targeted by the Cas9 sgRNAs. Other researchers have also tried to create models that generate unique bridges with specific features under temporal control. For instance, Gascoigne and colleagues created a system to ectopically recruit kinetochore proteins (Gascoigne et al., 2011), an elegant design that albeit two inconvenience: (1) the model was constructed in an unstable genome cell line and (2) it uses the less effective Lac Operator system in eukaryotic cells (Kallunki et al., 2019). Similarly, Umbreit et al. (2020) utilized the CRISPR/Cas9 technology combined with the Tet-ON system to induce chromosome bridges with temporal and chromosomal specificity control, although having the potential to generate two types of chromosome bridges as the designed sgRNAs target the two ends of the same chromosome. The generation of bridges with varying sizes hinders the study of the mechanisms of chromosome bridge resolution based on the size of the bridging chromatin. Our model provides an ideal platform to unravel the intricacies associated with chromosome bridges during mitosis as it distinguishes itself by meticulously controlling the characteristics of the bridges, allowing to explore their resolution and dynamics systematically.

The rapid nature of mitosis presents an important additional challenge to its investigation; thus, to achieve a large population of mitotic cells, synchronization was essential. There exist drugs to synchronize cells in mitosis, such as nocodazole or monastrol, but they interfere with microtubule dynamics and are strong pro-apoptotic agents (K. W. Wood et al., 2001). As the initiation of mitosis relies on the accumulation of the cyclin B/CDK1 complex (Gong & Ferrell, 2010; Hochegger et al., 2008) and RO-3306, function as a selective inhibitor of this complex (Vassilev, 2006), the drug offers an alternative method to isolating mitotic cells. RO3306 arrested cells enter mitosis rapidly after release from the G2/M block thus allowing controlled examination of the different mitotic stages. Therefore, the engineered CRISPR/Cas9 design combined with G2/M synchronization permits the exhaustive study of chromosome bridges in mitotic cells, serving as a valuable tool for studying their resolution in this stage of the cell cycle.

To examine whether the size of the bridge influences its resolution, we have established five unique RPE-1 Cas9 sgRNA cell lines capable of generating chromosome bridges with a defined intercentromeric number of base pairs each. We mimicked the geometric features of bridges that originate in human cells by creating CRISPR/Cas9 cuts guided to span the entire diversity of chromosomal arm lengths present in the human genome, which in turn will generate bridges with different megabase lengths spanning between the two groups of segregating masses of chromosomes. Our study provides evidence supporting the occurrence of chromosome bridge

breakage during mitosis. The noticeable reduction in bridge frequency observed as mitosis advances from early anaphase to late telophase across all the sgRNA cell lines indicates the resolution of a significant fraction of chromosome bridges during the exit of mitosis. Notably, our observations reveal a progression from continuous bridges to recently broken structures and, ultimately, to bridge remnants, thus demonstrating that the observed discontinuity of the chromatin fiber is not merely apparent. While the bridge resolution mechanism investigated in the present study primarily acts during anaphase, our findings highlight differences in resolution dynamics based on the size of bridging chromatin. Chromosome bridges with long intercentromeric distances can resolve during both anaphase and telophase and some of them reach the end of mitosis as continuous chromatin filaments. In contrast, most chromosome bridges with short intercentromeric distances resolve during early anaphase stages. Thus, our results demonstrate the influence of bridging chromatin size on the mitotic bridge resolution timing.

The decrease in the frequency of chromosome bridges occurs in a concerted manner with spindle elongation during anaphase. Consequently, it is tempting to speculate about the involvement of pulling forces exerted by the microtubules that are attached to the kinetochores of dicentric chromosomes on bridge resolution in mitosis. Although it was estimated that the pulling force generated by the spindle might be insufficient to break mitotic chromosomes (Houchmandzadeh et al., 2002), single-molecule experiments provided evidence of the influence of multiple factors in the magnitude of stretching forces under various buffer conditions (Bustamante et al., 2000; Cui & Bustamante, 2000). These authors challenge the notion of a definite tensile strength, as it is influenced by parameters such as the rate of stretching, the length of the molecule, and other solvent factors. Additionally, considering the flexibility, contractility of the DNA molecule, and chromatin compaction is essential for evaluating a possible role of microtubule-generated forces on chromosome bridge resolution. Therefore, theoretical and experimental efforts are imperative to further understand the spindle mechanobiology in the context of chromosome bridge resolution.

During mitosis, the pulling forces on kinetochores are opposed by the elastic forces of the stretched chromatin (Pickett-Heaps et al., 1986). This explains why chromosome bridges of the same length in base pairs do not break at the same separation between their kinetochores, instead, we observe a range of distances at which resolution occurs. While pulling forces might not be solely responsible for chromosome bridge resolution, the high correlation observed between the minimum separation required for the breakage and the distance in megabases of the bridging chromatin of our five cell lines points to a mechanism of chromosome bridge resolution

during the exit of mitosis that relies on spindle-dependent tensing of the bridging chromatin. We understand that those chromosome bridges with less DNA between the two centromeres have reduced tolerance to tension forces by the mitotic spindle that is evidenced by a marked dislocalization of their bridge kinetochores. Thus, we infer that a complex equilibrium exists between tensile spindle forces and chromatin elasticity.

Altogether, the results presented in this study indicate that the mechanism of chromosome bridge resolution in mitosis is triggered soon after metaphase: initially impacting on chromosome bridges with shorter intercentromeric distances and rapidly also influencing those with longer intercentromeric distances. Nevertheless, the mechanism for chromosome bridge resolution during mitosis might not be exclusive. Other mechanisms, such as the compressive forces exerted by the contractile ring in animal cells (Janssen et al), or biochemical mechanisms such as nucleases (Jiang et al., 2023), need to be explored and considered. Experimental models, like the one developed here, can be highly valuable in undertaking chromosome bridge investigations.

REFERENCES

Bustamante, C., Smith, S. B., Liphardt, J., & Smith, D. (2000). Single-molecule studies of DNA mechanics. Current Opinion in Structural Biology, 10(3), 279–285. https://doi.org/10.1016/S0959-440X(00)00085-3

Cui, Y., & Bustamante, C. (2000). Pulling a single chromatin fiber reveals the forces that maintain its higher-order structure. Proceedings of the National Academy of Sciences of the United States of America, 97(1), 127–132. https://doi.org/10.1073/PNAS.97.1.127

Gascoigne, K. E., Takeuchi, K., Suzuki, A., Hori, T., Fukagawa, T., & Cheeseman, I. M. (2011). Induced ectopic kinetochore assembly bypasses the requirement for CENP-A nucleosomes. Cell, 145(3), 410. https://doi.org/10.1016/J.CELL.2011.03.031

Giunta, S., Belotserkovskaya, R., & Jackson, S. P. (2010). DNA damage signaling in response to double-strand breaks during mitosis. The Journal of Cell Biology, 190(2), 197. https://doi.org/10.1083/JCB.200911156

Gong, D., & Ferrell, J. E. (2010). The Roles of Cyclin A2, B1, and B2 in Early and Late Mitotic Events. Molecular Biology of the Cell, 21(18), 3149. https://doi.org/10.1091/MBC.E10-05-0393

Hochegger, H., Takeda, S., & Hunt, T. (2008). Cyclin-dependent kinases and cell-cycle transitions: does one fit all? Nature Reviews. Molecular Cell Biology, 9(11), 910–916. https://doi.org/10.1038/NRM2510

Houchmandzadeh, B., Wieschaus, E., & Leibler, S. (2002). Establishment of developmental precision and proportions in the early Drosophila embryo. Nature, 415(6873), 798–802. https://doi.org/10.1038/415798A

Jiang, H., Kong, N., Liu, Z., West, S. C., & Chan, Y. W. (2023). Human Endonuclease ANKLE1 Localizes at the Midbody and Processes Chromatin Bridges to Prevent DNA Damage and cGAS-STING Activation. Advanced Science (Weinheim, Baden-Wurttemberg, Germany), 10(12). https://doi.org/10.1002/ADVS.202204388

Kallunki, T., Barisic, M., Jäättelä, M., & Liu, B. (2019). How to Choose the Right Inducible Gene Expression System for Mammalian Studies? Cells, 8(8). https://doi.org/10.3390/CELLS8080796

Kordon, M. M., Zarebski, M., Solarczyk, K., Ma, H., Pederson, T., & Dobrucki, J. W. (2020). STRIDE-a fluorescence method for direct, specific in situ detection of individual single- or double-strand DNA breaks in fixed cells. Nucleic Acids Research, 48(3). https://doi.org/10.1093/NAR/GKZ1118

Leibowitz, M. L., Papathanasiou, S., Doerfler, P. A., Blaine, L. J., Sun, L., Yao, Y., Zhang, C. Z., Weiss, M. J., & Pellman, D. (2021). Chromothripsis as an on-target consequence of CRISPR-Cas9 genome editing. Nature Genetics, 53(6), 895–905. https://doi.org/10.1038/S41588-021-00838-7

Ma, H., Tu, L. C., Naseri, A., Huisman, M., Zhang, S., Grunwald, D., & Pederson, T. (2016). Multiplexed labeling of genomic loci with dCas9 and engineered sgRNAs using CRISPRainbow. Nature Biotechnology, 34(5), 528–530. https://doi.org/10.1038/NBT.3526

Maciejowski, J., Li, Y., Bosco, N., Campbell, P. J., & De Lange, T. (2015). Chromothripsis and kataegis induced by telomere crisis. Cell, 163(7), 1641. https://doi.org/10.1016/J.CELL.2015.11.054

Mardin, B. R., Drainas, A. P., Waszak, S. M., Weischenfeldt, J., Isokane, M., Stütz, A. M., Raeder, B., Efthymiopoulos, T., Buccitelli, C., Segura-Wang, M., Northcott, P., Pfister, S. M., Lichter, P., Ellenberg, J., & Korbel, J. O. (2015). A cell-based model system links chromothripsis with hyperploidy. Molecular Systems Biology, 11(9). https://doi.org/10.15252/MSB.20156505

McClintock, B. (1941). The Stability of Broken Ends of Chromosomes in Zea Mays. Genetics, 26(2), 234. https://doi.org/10.1093/GENETICS/26.2.234

Pickar-Oliver, A., & Gersbach, C. A. (2019). The next generation of CRISPR–Cas technologies and applications. Nature Reviews Molecular Cell Biology 2019 20:8, 20(8), 490–507. https://doi.org/10.1038/s41580-019-0131-5

Pickett-Heaps, J. D., Tippit, D. H., Cohn, S. A., & Spurck, T. P. (1986). Microtubule dynamics in the spindle. Theoretical aspects of assembly/disassembly reactions in vivo. Journal of Theoretical Biology, 118(2), 153–169. https://doi.org/10.1016/S0022-5193(86)80131-X

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nature Protocols, 8(11), 2281–2308. https://doi.org/10.1038/NPROT.2013.143

Rodriguez-Muñoz, M., Serrat, M., Soler, D., Genescà, A., & Anglada, T. (2021). Breakage of CRISPR/Cas9-Induced Chromosome Bridges in Mitotic Cells. Frontiers in Cell and Developmental Biology, 9. https://doi.org/10.3389/FCELL.2021.745195

Schindelin, J., Rueden, C. T., Hiner, M. C., & Eliceiri, K. W. (2015). The ImageJ ecosystem: An open platform for biomedical image analysis. Molecular Reproduction and Development, 82(7–8), 518–529. https://doi.org/10.1002/MRD.22489

Steigemann, P., Wurzenberger, C., Schmitz, M. H. A., Held, M., Guizetti, J., Maar, S., & Gerlich, D. W. (2009). Aurora B-mediated abscission checkpoint protects against tetraploidization. Cell, 136(3), 473–484. https://doi.org/10.1016/J.CELL.2008.12.020

Stemmer, M., Thumberger, T., Del Sol Keyer, M., Wittbrodt, J., & Mateo, J. L. (2015). CCTop: An Intuitive, Flexible and Reliable CRISPR/Cas9 Target Prediction Tool. PLOS ONE, 10(4), e0124633. https://doi.org/10.1371/JOURNAL.PONE.0124633

Umbreit, N. T., Zhang, C. Z., Lynch, L. D., Blaine, L. J., Cheng, A. M., Tourdot, R., Sun, L., Almubarak, H. F., Judge, K., Mitchell, T. J., Spektor, A., & Pellman, D. (2020). Mechanisms generating cancer genome complexity from a single cell division error. Science (New York, N.Y.), 368(6488). https://doi.org/10.1126/SCIENCE.ABA0712

Van Steensel, B., Smogorzewska, A., & De Lange, T. (1998). TRF2 Protects Human Telomeres from End-to-End Fusions. Cell, 92(3), 401–413. https://doi.org/10.1016/S0092-8674(00)80932-0

Vassilev, L. T. (2006). Cell cycle synchronization at the G2/M phase border by reversible inhibition of CDK1. Cell Cycle (Georgetown, Tex.), 5(22), 2555–2556. https://doi.org/10.4161/CC.5.22.3463

Wood, K. W., Cornwell, W. D., & Jackson, J. R. (2001). Past and future of the mitotic spindle as an oncology target. Current Opinion in Pharmacology, 1(4), 370–377. https://doi.org/10.1016/S1471-4892(01)00064-9

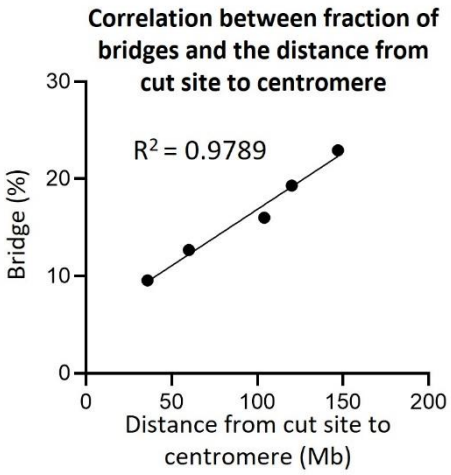


Figure S1. Plot of the correlation between the frequency of chromosome bridges observed and the distance from cut site to centromere in megabases for each sgRNA cell line (Linear regression; $p = 0.0013$).

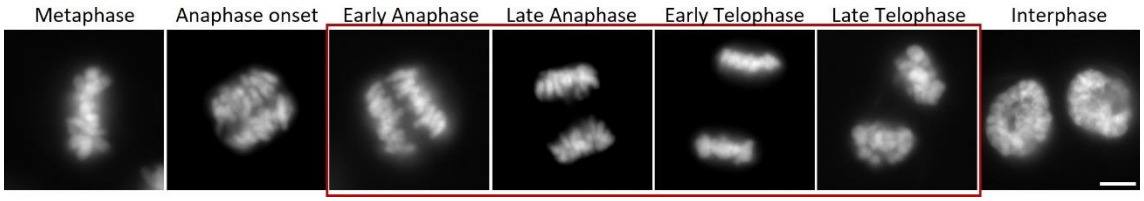


Figure S2. Morphological classification of RPE-1 cells in the last stages of mitosis. Representative images of cells from metaphase to the next interphase of the cell cycle. Scale bar = 5 μm. Chromosome bridge analysis was restricted to cells in the stages included in the box. As dicentric chromosomes connecting the two segregating masses of chromatids cannot be identified until a separation between the two chromatid groups is visible, cells in the anaphase onset were excluded from the analysis. For the classification of cells in anaphase and telophase stages of mitosis the degree of chromatin condensation and the separation of chromatid groups towards cell poles were considered by using DAPI staining. Early and late anaphase cells were segregated considering the distance between the chromatid groups and the alignment of the chromosome ends, defining EA as having closely spaced, irregularly contoured chromatid groups, and LA as featuring widely spaced, smooth-edged chromatid groups. Regarding early and late telophase distinction, the degree of chromatin decondensation and the size of the region occupied by each chromatid group were considered. ET cells exhibited distant chromatid groups, with slight chromatin decondensation still allowing to trace the boundaries of some chromosomes. In contrast, LT cells displayed a greater extent of chromatin decondensation, with no discernible edges for individual chromosomes, forming two rounded masses of chromatin.

CHAPTER 4

Unveiling Chromosome Bridge Resolution beyond Mitosis: Contribution of Physical- and Biochemical-based mechanisms

INTRODUCTION

Nuclear architecture defects, termed "nuclear atypia", are major features of cancer and have been used to establish tumor grade and prognosis for more than a century. Among them, chromosome bridges and micronuclei have scaled their importance in the processes of carcinogenesis as recent studies have shown that they are not only strategic biomarkers of chromosome instability but also drivers of it. Micronuclei and chromosome bridges undergo diverse types of mutational processes that range from simple DNA breaks to extensive fragmentation of selected chromosome or chromosome arms by a mutational process termed chromothripsis (Leibowitz et al., 2021; Maciejowski et al., 2015; Umbreit et al., 2020; Zhang et al., 2015). These previously unappreciated findings have reawakened widespread interest in nuclear abnormal structures such as micronuclei and chromosome bridges. However, the mechanisms that explain how these structures act as key platforms for DNA rearrangements are still controversial.

Micronuclei and chromosome bridges usually derive from errors in mitosis. At the exit of mitosis, the cleavage plane occupied by unsegregated chromatin needs to be cleared to ensure a faithful cell division. At this stage of the cell cycle, the presence of unsegregated chromatin under the segmentation furrow can cause the spontaneous regression of the furrow (Pampalona et al., 2016), which can proceed at this stage without damaging the DNA. However, it has also been described that experimentally induced lagging or bridging chromosomes acquire substantial DNA damage during cytokinesis both in budding yeast (Cuylen et al., 2013) and in human cells (Gascoigne & Cheeseman, 2013; Janssen et al., 2011). Restricted localization of damage markers (γH2AX and 53BP1) on DNA close to cleavage furrow and decreased γH2AX foci in cells treated with cytokinesis inhibitors suggested that the missegregating chromosomes become mechanically damaged during cytokinesis by cleavage furrow-generated forces. In addition to compressive cytokinetic forces, mechanical tensile forces during mitosis were identified as a cause of breakage in the particular case of chromosome bridges (Rodriguez-Muñoz et al., 2021) (Unpublished results-Chapter 3). Using a CRISPR/Cas9 experimental system to induce chromosome bridges with a defined intercentromeric distance, we showed an association between the separation between bridge kinetochores and the probability of breakage of the chromosome bridge during mitosis. In these studies, breakage of the bridging chromatin during mitosis was confirmed by the detection of the phosphorylated histone H2AX and confirmed by the detection of 3'-OH free ends through the STRIDE assay.

At the interphase of the cell cycle, some of the causes of DNA damage of micronuclei and chromosome bridges have been associated with defects in their nuclear envelope (NE) (Rodriguez-Muñoz et al., 2022). Densely bundled spindle microtubules in the midzone at the end

of cell division might limit the access of non-core NE components (Haraguchi et al., 2008) and compromise the reassembly of nuclear pore complexes on laggards and bridges (Liu et al., 2018). Previous work on micronuclei in cultured cells identified this irreversible NE assembly defect as a cause of DNA damage as it leads to a defective recruitment of DNA repair factors (Terradas et al., 2010, 2012) and to a delayed DNA replication (Crasta et al., 2012), which can cause massive DNA double-strand breaks (DSBs) if the cell enters mitosis while the micronucleus is undergoing replication. In summary, these studies identified the observed abnormal nuclear repair and replication protein recruitment in micronuclei as a potential source of chromosome pulverization. Similar to micronuclei, chromosome bridges also have difficulty forming their NE (Liu et al., 2018). Because of these wrapping defects, cells born with chromosome bridges may have difficulty carrying out basic processes such as damage repair and DNA replication, which can affect the integrity of their DNA.

Once believed to be highly stable, the defective NE of micronuclei and chromosome bridges has recently been demonstrated to be fragile and prone to release its content into the cytoplasm after an irreversible process of nuclear envelope rupture (NER), an event that involves an interphase loss of the nuclear permeability and merging of normally separated nuclear and cytoplasmic components (Hatch et al., 2013; Zhang et al., 2015). Chromosomes from micronuclei and bridges exposed to the cytoplasmic environment activate the cGAS-STING surveillance pathway. When this occurs, cells exhibit a proinflammatory transcriptional response (Harding et al., 2017; MacKenzie et al., 2017). Besides being detected by cytoplasmic sensors responsible for inducing an innate immune response, irreversible membrane breakdown of micronuclei and chromosome bridges also exposes their chromosomal DNA to cytoplasmic enzymes such as nucleases, which have been described to be involved in their processing.

Two apparently irreconcilable models have been proposed to explain the resolution of chromosome bridges during the interphase of the cell cycle. One model proposes that the resolution of chromosomal bridges occurs by a mechanism of biochemical nature that depends on the collapse of the NE (Maciejowski et al., 2015; Maciejowski & De Lange, 2017). According to this first model, the nucleoplasmic bridges connecting daughter cells would be broken at interphase by a mechanism that depends on the cytoplasmic exonuclease TREX1 (three-prime repair exonuclease 1). This nuclease would digest the DNA of the bridge now exposed to the cytoplasmic environment due to the rupture of the NE. The second model proposes that the resolution of chromosomal bridges occurs by mechanical causes and does not depend on the breakdown of the NE (Umbreit et al., 2020). According to this second model, the forces generated by the actomyosin cytoskeleton due to the independent migration of the two cells connected by a chromosome bridge would generate both simple breaks and local

fragmentation of the chromosome bridge. Each of the proposed models faces challenges. The TREX1-based model relies on exonuclease activity but necessitates an unexplained initial break. On the other hand, complex rearrangements and multiple breakpoints observed in DNA just after bridge resolution might be difficult to explain if only attributed to actomyosin skeleton forces. Therefore, considering the limitations in the existing proposed models, it becomes evident that additional research is essential to understand the mechanisms underlying the resolution of chromosome bridges after mitosis.

METHODS

Cell Culture

Immortalized non-transformed human retinal pigment epithelial (RPE-1) cells expressing Cas9 under a Tet-ON promoter were obtained from Iain Cheesman's Laboratory and were cultured in DMEM:F12 supplemented with 10% tetracycline-free Fetal Bovine Serum (FBS; FBS-TET-12A, Labclinics, Barcelona, Spain). All experiments were performed in cells depleted of p53 by siRNA or in cells constitutively expressing shRNAs against p21 and retinoblastoma (shp21/Rb). HEK293T cells were grown in MEM medium supplemented with 10% FBS. The media for all cell lines was supplemented with Penicillin/streptomycin (100U/mL) and cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

Plasmids

For plasmid amplification Stbl3 (C737303, Invitrogen, Waltham, MA, United States) or DH5α competent cells (18258012, Thermo Fisher Scientific) were used. Plasmid purification was performed using the NucleoBond PC kit (740573.100, Macherey-Nagel, Bethlehem, PA, United States) according to the manufacturer's guidelines. All plasmids underwent validation through Sanger sequencing and the concentration of DNA was determined using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific). All the plasmids used are listed in Table SI. GFP-BAF and sgRNA for Chr4 were kind gifts from David Pellman's Laboratory, shp21/Rb was obtained from Titia de Lange Laboratory and pTRIP-CMV-tagRFP-cGAS was a gift from Nicolas Manel 's laboratory.

Viral particle production and transduction

Lentiviral particles were generated in HEK293T. At a confluency of 70%, cells were transfected with a total DNA plasmid concentration of 1 μg/mL, following the Trono's Lab protocol[1]. DNA concentration was adjusted to a 4:3:1 ratio (transfer plasmid:psPAX2:pMD2.G). After 48 hours,

[1] https://www.epfl.ch/labs/tronolab/wp-content/uploads/2019/06/LV_production.pdf

the supernatant was collected, concentrated using centrifugal filters (UFC910008, Merck), and stored at – 80 °C.

Cells were transduced overnight with viral particles in medium containing 4 µg/mL polybrene. Cells carrying the antibiotic-resistance cassette were treated for one-week with 2 µg/mL puromycin (P8833, Merck), two weeks with 250 µg/mL geneticin (11811023, Thermo Fisher Scientific) or 2.5 µg/mL of blasticidin (15205, Merck) for selection. Transduced fluorescent cell population were enriched using fluorescent activated cell sorting (FACS) with BD FACSJazz (BD Biosciences, Franklin Lakes, NJ, United States) equipped with 488 nm laser and two detectors (530/40 and 692/40 nm).

Chromosome bridge formation and cell treatments

In order to generate chromosome bridges using the CRISPR/Cas9 model within cells expressing sgRNA directed at subtelomeric regions of Chr4, Cas9 expression was triggered by adding 1 µg/mL doxycycline (631311, Clonetech, Mountain View, CA, United States) to the medium for 15 hours. Afterwards, doxycycline was removed from the media by seven washes with PBS and cells were allowed to generate chromosome bridges for at least 8 hours. When indicated cells were treated with ML7 (#ab120848, Abcam, Cambridge, UK) at a final concentration of 20 µM. ML7 was added to the cells 24 h after doxycycline washout.

siRNA transfections

siRNA transfections were performed using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions using a final siRNA concentration of 10 nM. p53 siRNA or siRNA for the nuclease screening were on-target plus SMARTpool (Dharmacon, Lafayette, CO, United States).

Live-cell imaging

For live-cell imaging experiments, RPE-1 cells stably expressing GFP-BAF were cultured in glass-bottomed 12-well cell culture dishes (Cellvis, Mountain View, USA) and treated with doxycycline for 15h. After washout, imaging was performed using a Nikon TE2000-E2 inverted microscope equipped with the Nikon Perfect Focus system (Nikon, Tokyo, Japan). The microscope was enclosed within a controlled environment at 37 °C and 5% humidified CO_2. Bright-field and fluorescent images were obtained every 10 minutes using a x20/0.5 Plan Fluor objective. Multiple points were imaged, and for each designated point of interest, a 2x2 grid of images with 15% tile overlap was captured to have a broader field of view. Cells were followed for 72 h and the acquired images were analyzed using ImageJ. Cells in mitosis were identified using the brightfield and the GFP-BAF signal was tracked.

Immunofluorescence

Cells were fixed using 4 % paraformaldehyde for 15 minutes. Subsequent permeabilization was performed with 1x PBS/0.5 % Triton-X-100 for 15 minutes. After a blocking with 1x PBS/0.5 % BSA/0.15 % glycine for 15 minutes, primary antibodies (Table SII) were incubated overnight at 4 °C. Then, cells were washed 3 times with 1x PBS/0.1 % Tween 20 and were subsequently incubated with secondary antibodies (Table SII) for 1 hour at room temperature. Finally, cells were washed 3 times with PBS/0.1 % Tween20 for 5 minutes each and rinsed in distilled water. Coverslips were mounted on glass microscope slides using Vectachield mounting medium (Vector Laboratories, Inc., Burlingame, CA, United States) containing 0.25 µg/mL 4',6-diamidino-2-phenylindole (DAPI).

Sample visualization and imaging were carried out utilizing an Olympus BX61 microscope (Olympus, Hamburg, Germany), equipped with a CV-M4+ CL camera (JAI, Großwallstadt, Germany) and CytoVision software (Applied Imaging, Newcastle, United Kingdom). Image analysis was performed using ImageJ (Schindelin et al., 2012).

Tissue immunostaining and confocal imaging

Breast cancer progression tissue microarray slides were provided by the Cooperative Human Tissue Network (CHTN). Mounted tissue samples were deparaffinized using Histoclear II (National diagnostics, Charlotte, USA) and rehydrated via an ethanol:water gradient. For immunofluorescence, antigen unmasking was performed on rehydrated tissue sections using a citric acid retrieval buffer (VectorLabs; Newark, CA, USA). Heat-mediated antigen retrieval was performed using Decloaking Chamber NxGen (Biocare Medical; Concord, CA, USA) at 110 °C for 12 minutes, followed by cooling to room temperature (~30 – 60 min). Tissue sections were rinsed twice with PBS and were blocked for 1 hour in 10 % goat serum in TBS. Primary emerin antibody (10351-1-AP, Proteintech, Rosemont, IL, USA) was diluted in 10 % goat serum in TBS and incubated overnight at 4°C in a humidified chamber. Following the primary antibody, slides were rinsed twice with 1x TBS-0.1 % Tween20 and incubated with species-specific fluorescent secondary antibody (Molecular Probes) and 2.5 µg/mL Hoechst for 1 hour at room temperature in a dark humidified chamber. Tissue samples were rinsed twice with 1x TBS-0.1 % Tween20 and were mounted using Prolong Gold Antifade (Invitrogen). Confocal images were obtained using a Nikon Ti-E inverted microscope equipped with a C2+ laser scanning confocal head with 405 nm, 488 nm, 561 nm, and 640 nm laser lines. Z-stacks were acquired with a series of 0.5–1 µm optical slices.

Statistics

To determine differences between categorical variables, Chi square test was performed. For continuous variables, data normality was assessed using the D'Agostino-Pearson omnibus K2 normality test. Non-normally distributed data was analyzed by the Mann-Whitney test. Correlation between variables was performed using the Pearson correlation. Statistical analysis and graph plotting were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, United States). Statistically significant differences were considered when the p-value was lower than 0.05.

RESULTS

Chromosome bridges are often observed in immortal cell lines because of the instability acquired in cell genomes over passages *in vitro*. However, this aberrant nuclear structure is not exclusive to cells that are propagated *in vitro*, since chromosome bridges are also observed in samples obtained from human tumors, and not only in mitotic cells but also interphase cells (Figure 1). This observation is relevant because chromosome bridges are related to the inability to maintain genome integrity, a characteristic of cells in the process of malignant transformation.

To explore the evolution of chromosome bridges sustained to post-telophase stage and investigate the mechanisms governing their resolution, we generated chromosome bridges by inducing DNA DSBs on chromosome 4 using CRISPR/Cas9 technology in the immortalized non-transformed human retinal pigment epithelial (RPE-1) cell line (Rodriguez-Muñoz et al., 2021; Umbreit et al., 2020). After Cas9 DSB induction, loss of chromosome end protection allowed the ligation of sister chromatid ends, thus generating chromosome bridges in the subsequent mitosis. Chromosome bridges obtained by the CRISPR/Cas9 system were formed under exquisite spatiotemporal control minimizing potential variations that might mask the results.

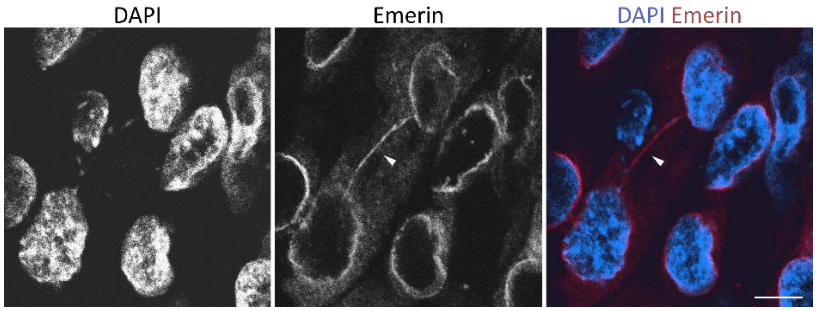


Figure 1. Chromosome bridges in interphase cells of a human primary tumor breast sample. Immunofluorescent detection of emerin (red) in cells counterstained with DAPI (blue) in a sample of invasive ductal breast carcinoma grade 3. Arrowheads indicate a chromosome bridge. Scale bar = 10 μm.

1. Chromosome bridge resolution with breakage of the DNA molecule can be identified as a visible discontinuity in the bridging chromatin

When investigating the mechanisms involved in the resolution of chromosome bridges during the interphase of cell cycle, it may be useful to know how long the bridges take to resolve since the machinery responsible for their resolution might be available only in defined windows of time. Thus, to know the specific moment in which each bridge is resolved, RPE-1 cells stably expressing fluorescent barrier-to-autointegration factor (GFP-BAF) were live-imaged for 72h after CRISPR/Cas9 bridge induction (Figure 2A). The accumulation of BAF on chromosome surfaces at the end of mitosis assists daughter cells in the formation of a single nuclear envelope on each group of segregated chromosomes, endowing BAF with a heightened sensitivity for detecting chromosome bridges that is not compromised even by stretching of the bridge. Chromosome bridges were considered intact if a continuous GFP-BAF signal was observed connecting the two primary nuclei. Among cells at the interphase, bridge lifetime was very variable. While some bridges broke just after the cell ends mitosis, other bridges were maintained as a continuous fiber connecting the two daughter cells for a long time before they eventually broke (range of lifetime between 20 and 740 minutes; Figure S1A). Bridges remained unresolved for a median of 120 minutes after the cell completed mitosis. When discontinuity was finally observed in the GFP-BAF signal, it was usually accompanied by a sudden change in the morphology of the bridge that consisted in a rapid retraction of the two bridge remnants towards its primary nucleus and a rapid movement of the daughter cells away from each other (Figure 2A), indicating that the bridge was indeed resolved at this time point.

To confirm a direct correspondence between BAF discontinuity and breakage of the bridging DNA, we assessed on fixed samples at the single-cell level whether markers of breakage or disruption of the double-stranded DNA molecule could be detected on the chromosome ends flanking the BAF signal discontinuity. Whereas γH2AX serves as an excellent marker for DNA breaks on chromosome bridges during mitosis (Rodriguez-Muñoz et al., 2021), its effectiveness is compromised in interphase. γH2AX was observed in the base of the chromosome bridge, where the chromatin fiber is not as stretched as in the middle (Figure 2B, Figure S1A). This might occur because the stretching of the chromosome bridge during interphase causes the loss of nucleosomes from the chromatin in the bridge, thus preventing the observation of the γH2AX signal. Additionally, we also detected long stretches of DNA coated by the replication protein A (RPA32) on 48.8% of the chromosome bridges with discontinuous GFP-BAF signal (Figure 2C; Figure S1B), indicating that the generation of single-stranded DNA (ssDNA) fragments might depend on the processing of broken ends of the DNA of chromosome bridges. These results

indicate that the discontinuity of the BAF signal and the observed retraction of the two ends of chromosome bridges are associated with a breakage of the DNA molecule.

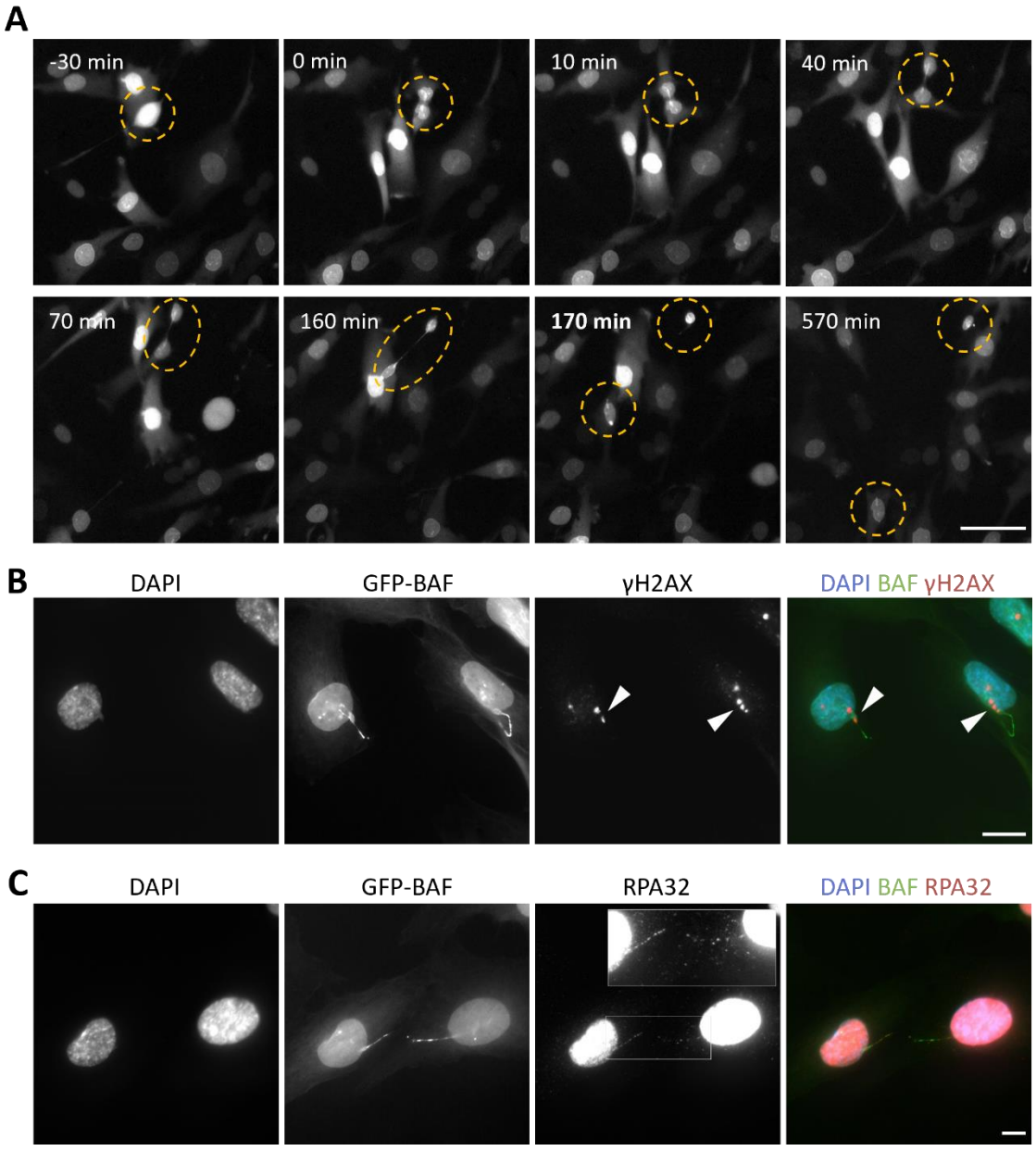


Figure 2. Discontinuity on BAF signal is associated with chromosome bridge resolution and DNA breakage. Representative images of chromosome bridges induced in RPE-1 cells using sgRNA Chr4. **(A)** General view of live imaging of GFP-BAF taken every 10 minutes after doxycycline release. Images from interphase onset to chromosome bridge resolution. Dashed yellow line highlights a chromosome bridge and minutes in bold letters indicate the time of chromosome bridge resolution. Scale bar = 50 μm. **(B)** Representative image of a chromosome bridge in interphase cells. DAPI (blue) labels DNA, nuclear envelope is detected by GFP-BAF (green), and γH2AX (red) is immunodetected. Arrowheads indicate γH2AX of the bridge. Scale bar = 20 μm. **(C)** Representative image of chromosome bridges in interphase cells. DAPI (blue) labels DNA, nuclear envelope is detected by GFP-BAF (green), and the repair factor RPA32 (red) is immunodetected. Inset shows RPA32 along bridging DNA. Scale bar = 20 μm.

2. Diverse mechanisms for bridge resolution after mitosis: Insights into bridge lifetime and nuclear envelope rupture

Once we verified the breakage of the DNA fiber in discontinuous bridges, we aimed to analyze if chromosome bridges suffer nuclear envelope rupture (NER). The observation that some bridges exhibit a heightened signal of GFP-BAF indicated a potential anomaly in the nuclear envelope. We classified GFP-BAF signal into basal-BAF, patched-BAF and bright-BAF. Regions with bright- and patched-BAF could potentially indicate locations where an increased recruitment of BAF occurred to uphold the integrity of the nuclear envelope. Consequently, these areas might indicate disruption of the nuclear envelope's structure. To assess NER, we tested if the content of the bridging structures was accessible to the cytoplasmic environment. To investigate this question, we used a fluorescent reporter for cGAS (cGAMP synthase), a cytoplasmic protein with a DNA-binding domain that belongs to the nucleotidyl transferase family and can activate the innate immune response because of the presence of DNA in the cytoplasm of mammalian cells. We observed the RFP-cGAS signal in all the bridges with bright-BAF and 86.36% of bridges with patched-BAF but in none of the bridges with basal-BAF signal (Figure 3Ai and 3B). These results indicate that, in contrast to chromosome bridges with basal-BAF signal, the nuclear envelope of those chromosome bridges that have recruited higher amounts of GFP-BAF is abnormally permeable and is not functionally isolating the nuclear content from the cytoplasm. In other words, the often-irreversible breakdown of the bridge envelope offers a mechanism through which DNA inside the nucleus becomes exposed to the cytoplasmic sensor cGAS.

NER has been associated with a lack of the so-called non-core NE proteins in micronuclei (Liu et al., 2018; Rodriguez-Muñoz et al., 2022; Samwer & Gerlich, 2018). To explore whether the absence of these proteins explains the observed abnormal permeability of the chromosome bridge envelope, we investigated the presence of core and non-core nuclear envelope proteins in the bridge envelope. Emerin, a core protein of the nuclear envelope, was present in all the basal-BAF bridges examined. Also, emerin signal was maintained, either partially or completely, in 97.83 % of patched-BAF bridges and in all the bright-BAF bridges (Figure 3Aii and 3C). In the case of non-core proteins, lamina B1 colocalization with BAF signal was observed in more than 66 % of the bridges, and it was absent in bright-BAF bridges (Figure 3Aiii and 3D). In conclusion, the bright-BAF pattern on chromosome bridges coincides with the loss of non-core proteins and reveals a loss of nucleo-cytoplasmatic compartmentalization. Thus, bright-BAF and patched-BAF patterns are markers of NER of chromosome bridges.

After confirming NER on chromosome bridges, we proceeded to examine the process of bridge resolution in live cells. Firstly, we aimed to analyze changes in the GFP-BAF signal over time as the cells with bridges progressed through the cell cycle to establish whether there is a direct

temporal relationship between the rupture of the envelope and the resolution of the bridge. Two different patterns could be clearly distinguished in the dynamics of chromosome bridges as detected by its GFP-BAF pattern (Figure 3E and 3F). Whereas 37.5 % of bridges proceed with a BAF fluorescent intensity similar to that found on the primary nucleus (Figure 3E), 62.5 % of the bridges recruit high levels of GFP-BAF leading to increased brightness of the envelope on discrete regions of the bridge or heightened BAF signal over all the bridge before becoming discontinuous (Figure 3F). By comparing the timepoint of NER onset for each chromosome bridge, as ascertained by BAF signal pattern, with the timepoint of bridge discontinuity, it becomes evident that the two timepoints do not present a correlation ($R^2 = 0.0013$, Figure S2). Therefore, although NER occurs prior to chromosome bridge visible discontinuity, bridge lifetime does not correlate with the timepoint NER occurs. However, the lifetime of bridges suffering NER is significantly longer than that of bridges resolving without perturbing their nuclear envelope (median time of 160 minutes versus 90 minutes; $p = 0.0098$; Figures 3G and 3H). This finding suggests the existence of at least two distinct mechanisms for resolving chromosome bridges: a NER independent and a NER associated bridge resolution mechanism.

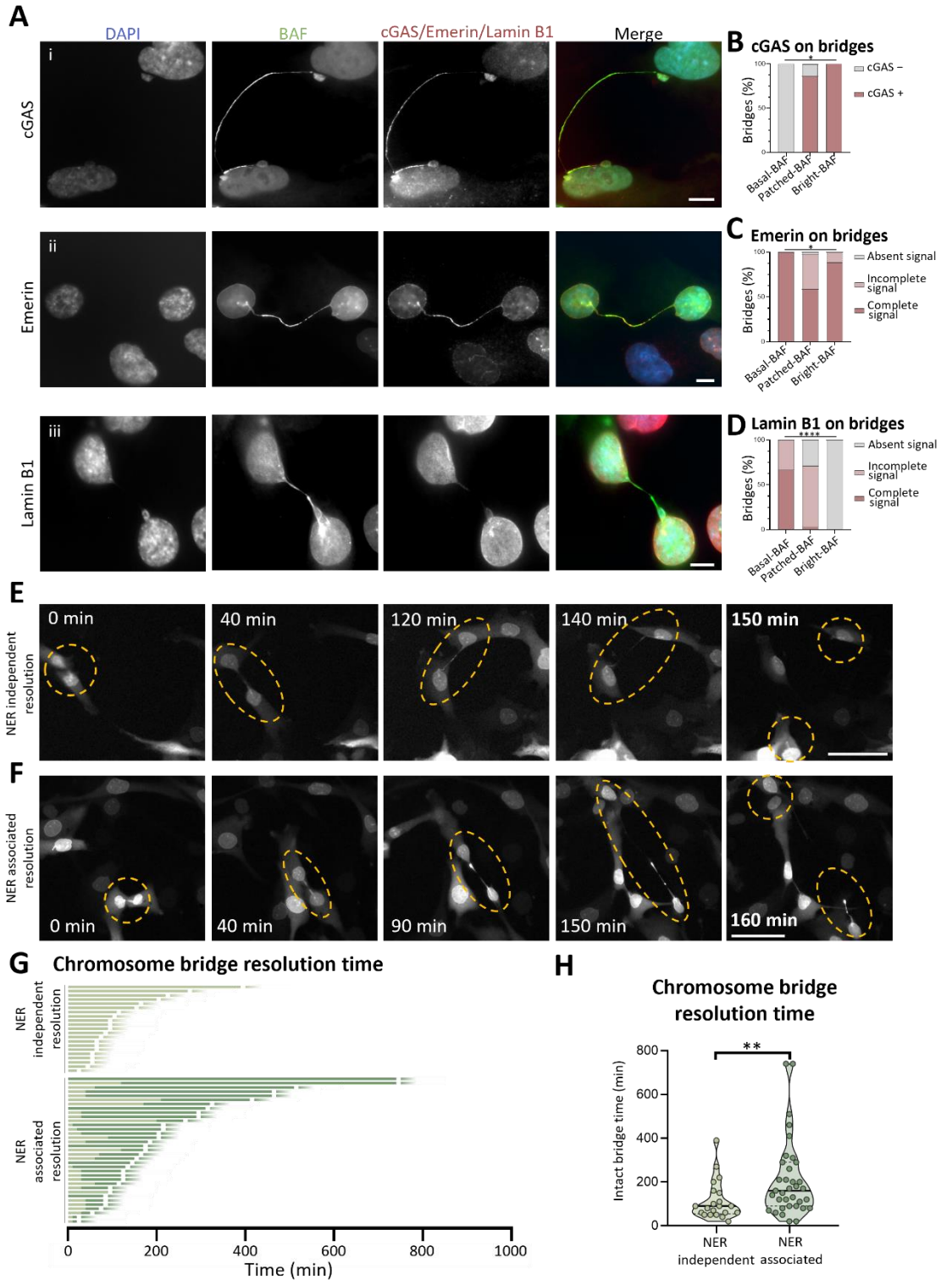


Figure 3. Two distinct mechanisms of chromosome bridge resolution. **(A)** Representative images of chromosome bridges induced in RPE-1 cells using sgRNA Chr4 observed by GFP-BAF (green) and **(i)** RFP-cGAS (red), **(ii)** immunodetected with emerin (red) or **(iii)** lamin B1 (red). In all images DNA is counterstained with DAPI (blue). Scale bar = 10 µm. **(B)** Distribution of cGAS-negative and -positive staining in basal-BAF, patched-BAF or bright-BAF chromosome bridges during interphase. $n = 45$; Chi square test, $p = 0.0134$. **(C, D)** Frequency of chromosome bridges classified according to GFP-BAF signal pattern displaying absent, incomplete or complete signal of **(C)** emerin, $n = 73$; Chi square test, $p = 0.0345$ or **(D)** lamin B1, $n = 43$; Chi square test, $p < 0.0001$. **(E, F)** Representative sequence of images of live imaging of RPE-1 cells expressing GFP-BAF taken every 10 minutes. Scale bar = 50 µm. Bold letters indicate the resolution timepoint. The yellow dashed line

highlights a chromosome bridge that **(E)** displays basal-BAF signal, indicating NER independent resolution or **(F)** heightened BAF signal, indicating that it has undergone NER before resolution. **(G)** Graph classifying bridges coursing independently of NER or undergoing NER before resolution. Each line represents a cell that enters interphase with a chromosome bridge. Light green indicates bridges with basal-BAF and dark green indicates patched-BAF or bright-BAF signal on chromosome bridges. White space represents the moment the chromosome bridge becomes discontinuous. Time is indicated in minutes being timepoint zero set up at the interphase onset for each cell. **(H)** Bridge lifetime in minutes categorized based on whether they undergo NER (dark green) or not (light green) before discontinuity. Median and 95% CI are represented. Asterisks indicate statistically differences between NER independent resolution of bridges and NER associated resolution (Mann-Whitney test; $^{**}p < 0.01$); $n = 56$ from three replicates.

3. Resolution of chromosome bridges with unperturbed envelope: ANKLE1 contribution

We observed by live-cell imaging that those chromosome bridges that do not resolve during mitosis and are sustained to post-telophase stages, will eventually break in the interphase of the cell cycle. To explore the contribution of endogenous nucleases in the resolution of chromosome bridges we conducted a live-cell imaging-based siRNA screen targeting 36 human proteins known to exhibit exo- or endonuclease activity. Specifically, pooled siRNA screen targeting to individually knock down the selected nucleases was used in the CRISPR/Cas9 RPE-1 cell model and the GFP-BAF signal was monitored for 72 hours after bridge induction with doxycycline. We hypothesize that specific exo- and endonucleases might be important for promoting bridge resolution. If true, loss of these specific nucleases would be expected to significantly increase the frequency of cells born with chromosome bridges at the interphase of the cell cycle.

Among all the nucleases tested, the endonuclease ANKLE1 was identified as potentially involved in the resolution of chromosome bridges since ANKLE1 inhibited cells showed an increased frequency of chromosome bridges in interphase after CRISPR/Cas9 induction as compared to the non-inhibited cells. Cells born with bridges represented 43 % of the population in siANKLE1 conditions versus 21 % ANKLE1 proficient cells (Figure 4A). The frequency of chromosome bridges was also increased in ANKLE1-inhibited samples even when the CRISPR/Cas9 system of bridge induction was not activated (3.55 % of spontaneously generated bridges with proficient ANKLE1 versus 9.14% of spontaneous bridges with ANKLE1 inhibition). (Figure 4A). These findings strongly suggest that the endonuclease ANKLE1 has a role in facilitating the resolution of chromosome bridges before interphase.

To explore whether the ANKLE1-mediated mechanism of bridge resolution is linked to NER, live-cell imaging of GFP-BAF was performed from 8 hours to 48 hours after bridge induction. Whereas in cells proficient for ANKLE1, 36 % of bridges coursed without NER before their resolution, after inhibiting ANKLE1 the fraction of NER-independent bridge resolution decreased to 28 % (Figure 4B and 4D). Moreover, our findings show that bridges that resolved without suffering NER tended to have a longer lifetime than in control conditions despite endonuclease

inhibition (median of 90 minutes versus 150 minutes; Figure 4E). Altogether, our results indicate that ANKLE1 would be involved in a NER-independent mechanism of bridge resolution. As it has been recently described that ANKLE1 recruitment occurs during cytokinesis (Jiang et al., 2023), we next explored if the inhibition of myosin II, a key protein to from the contractile ring during cytokinesis, would impact the NER independent mechanism of chromosome bridge resolution. Strikingly, our experiments revealed that the fraction of bridges that resolved via a NER-independent mechanism decreased from 36 to 17.24 % when cells were exposed to the myosin kinase inhibitor ML7 (Figure 4F). Moreover, ML7 addition prolonged the lifetime of bridges that resolve with an unperturbed NE in a similar extent to what was observed after ANKLE1 inhibition (median time of 125 minutes for control cells versus 150 minutes in ML7 treated cells) (Figure 4G). Altogether, these findings indicate that ANKLE1 recruited during cytokinesis under the furrow cleavage play a significant role in facilitating bridge resolution after the end of mitosis or at early interphase through a mechanism that proceeds independently of NER.

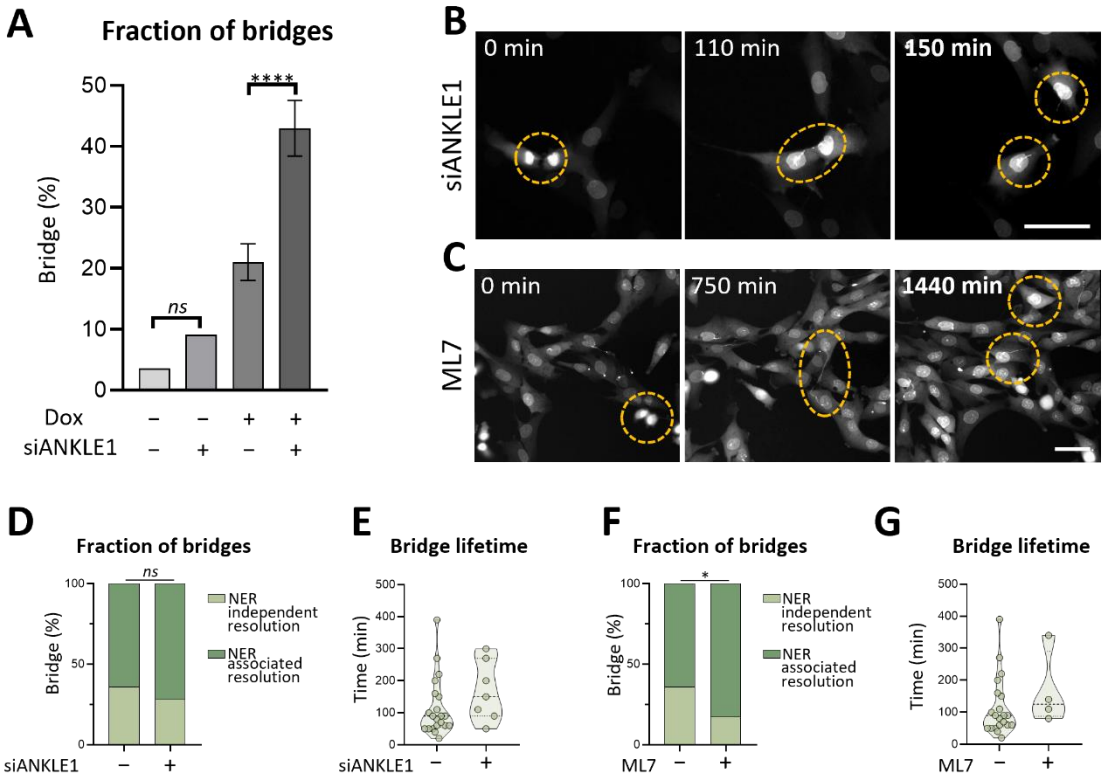


Figure 4. Resolution of chromosome bridges mediated by ANKLE1. (A) Fraction of bridges observed in cells treated with or without doxycycline in ANKLE1 proficient or siANKLE1 samples. Whiskers depict standard deviation and asterisks represent statistically significant differences (Chi square test; **** *p*-value < 0.0001; *ns* = no significant). A minimum of 150 cells were analyzed for each sample. Three independent replicates were performed for each condition in the RPE-1 cells treated with doxycycline. **(B, C)** Representative images of live-cell imaging of GFP-BAF RPE-1 cells treated with doxycycline **(B)** siANKLE1 or **(C)** ML7 treated samples. The yellow dashed line highlights a chromosome bridge and bold letters indicate the timepoint of bridge breakage. Scale bar = 50 μm. **(D, F)** Fraction of chromosome bridges classified according to their BAF-GFP signal pattern

into NER-independent or NER-associated resolution in cells treated with doxycycline and **(D)** siANKLE1, n = 56 for control condition and n = 26 for siANKLE1 or **(F)** ML7, n = 56 for control condition or n = 24 for ML7 treated condition. Chi square test; * $p < 0.05$; *ns* = no significative. **(E, G)** Chromosome bridge lifetime in minutes classified according to their BAF-GFP signal pattern into NER-independent or NER-associated resolution in cells treated with doxycycline and **(E)** siANKLE1 or **(G)** ML7 treated samples.

5. Chromosome bridges with ruptured nuclear envelope: A two-step mechanism for bridge resolution

The ANKLE1-dependent mechanism does not seem to be the only mechanism contributing to the resolution of bridges after mitosis as 64 % of bridges have an extended lifetime and proceed with nuclear envelope rupture. To investigate the mechanism underlying the NER-associated bridge resolution, we immunodetected TREX1 on chromosome bridges that suffered NER (Figure 5A). TREX1 exonuclease resides in the cytosolic side of the endoplasmic reticulum membranes and has been proposed to contribute to bridge resolution at the interphase of the cell cycle(Maciejowski et al., 2015; Maciejowski & De Lange, 2017). Interestingly, whereas TREX1 signal was not detected on chromosome bridges with basal-BAF signal, 96.6 % of bridges with patched-BAF and 81 % of bridges with bright-BAF exhibited TREX1 signal along the bridge (Figure 5C). Given TREX1's capacity to produce single-stranded DNA. RPA32, a marker for ssDNA was immunodetected. We observed RPA32 signal on bridges with ruptured NE (38.1 % patched-BAF, 53.3 % bright-BAF), but not on bridges with unruptured nuclear envelope (Figure 5D). Therefore, our results suggest that access of TREX1 to chromosome bridges is reliant upon envelope rupture to process the bridging DNA.

After obtaining evidence of recruitment of cytoplasmic TREX1 exonuclease and resection of DNA end of the bridge, our focus was directed towards identifying the causes of NER. Notably, paxillin was observed at discrete regions along the bridge (Figure 5B). Since paxillin localizes at focal adhesions to permit the cell to attach to the substrate, it is possible that forces exerted by the actomyosin cytoskeleton during cell migration could induce the rupture of the nuclear envelope of those bridges attached to the substrate by focal adhesions. To investigate this possibility, we deliberately inhibited activation of myosin by the addition of ML7 drug, thereby restricting cell mobility and weakening cell adhesion to the substrate. Cells were tracked by live imaging and our analysis revealed a delayed onset of NER in cells treated with ML7, indicating an association between focal adhesion and the initiation of NER (median time of NER onset 30 minutes in the untreated sample versus 100 minutes in the ML7 treated sample, Figure 5F). Thus, NER of chromosome bridges might be triggered by the tensile forces generated by the independent migration of the two daughter cells walking away from each other while keeping the adhesion of the chromosome bridge on the substrate.

Once the origin of NER has been demonstrated, it becomes necessary to understand how the initial DNA nick is generated to facilitate the action of TREX1. Thus, we examined the occurrence of DNA breaks during the bridge lifetime. γH2AX labelling increased gradually on bridges as the rupture of the nuclear envelope progressed from unruptured to completely ruptured stages (Figure 5E) (28.57 % of basal-BAF, 45.9 % of patched-BAF and 56.52 % of bright-BAF bridges presented γH2AX labeling). As aforementioned, BAF pattern of the bridge is related to cellular mobility and the presence of focal adhesion, therefore focal adhesion might impact not only the NE but also the DNA integrity of the bridge. When cell mobility was restricted with ML7 drug, bridges had an extended lifetime (median time of 160 minutes versus 605 minutes; Figure 5G), suggesting that the break of DNA molecule is delayed. Based on these findings, the initial breakage of the bridging DNA and the disruption of its NE – essential for the subsequent processing by TREX1 – rely on the migration of cells away from each other while the bridge remains anchored to the substrate.

In summary, the NER-associated mechanism for resolving bridges involves a multi-step process. Initially, tension on the bridging DNA caused by cell migration and anchorage of the bridge through focal adhesion causes NER and DNA breakage. The broken ends of the bridging DNA inside the nuclear envelope provide the substrates and conditions necessary for the digestion of the DNA by the exonuclease TREX1. Finaly, the resulting single-stranded DNA is then protected by RPA32 to prevent extensive degradation.

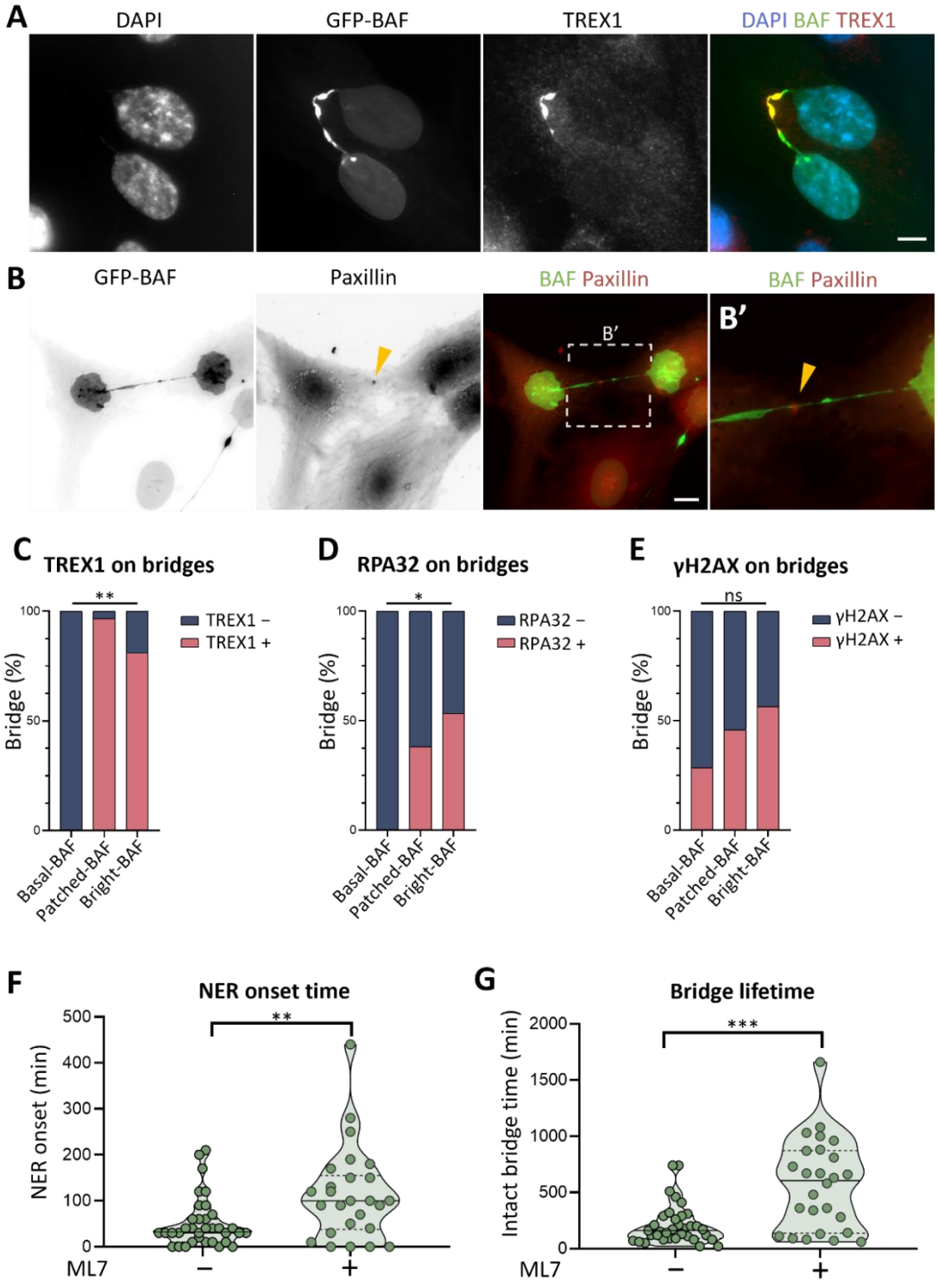


Figure 5. NER-associated resolution of chromosome bridges. **(A, B)** Representative images of chromosome bridges seen by GFP-BAF (green) and **(A)** TREX1 (red) or **(B)** paxillin (red). Scale bar = 10 μm. **(B')** Inset of the bridge area including paxillin signal highlighted with a yellow arrowhead. **(C, D, E)** Graphs of the percentage of chromosome bridges in RPE-1 cells sgChr4 after doxycycline induction categorized by basal-, patched- or bright-BAF signal and with or without labelling of **(C)** TREX1, n = 52, Chi square test, p = 0.0045 **(D)** RPA32, n = 62, Chi square test, p = 0.035 or **(E)** γH2AX, n = 129, Chi square test, *ns= no significant* . **(F, G)** Comparison of **(F)** NER onset and **(G)** bridge lifetime, in control cells (n = 35) and in cells treated with ML7 drug (n = 26); Mann-Whitney test; ** p = 0.0056; *** p = 0.0004. Dashed line indicates the median and dotted line the CI for 25-75 %.

DISCUSSION

In the last years, diverse theories have emerged regarding chromosome bridge resolution mechanisms, including resolution during mitosis, or interphase, and even keeping unresolved leading to cell tetraploidization (Janssen et al., 2011; Maciejowski et al., 2015; McClintock, 1941; Pampalona et al., 2016; Shimizu et al., 2005; Steigemann et al., 2009; Umbreit et al., 2020). Each mechanism of resolution will have a different impact on the cell, shaping the level of chromosome instability that cells may experience, and the spectrum of abnormalities formed. Here, we demonstrate that there is not a single mechanism for chromosome bridge resolution, but there are multiple mechanisms that have the potential to resolve bridges, and their action depends on the cell cycle stage.

In this study, we identified two main mechanisms for chromosome resolution that act beyond telophase (Figure 6). The first mechanism, orchestrated by ANKLE1, operates independently of NER and its functionality appears crucial in the cellular transition from cell division to interphase, resulting in bridges that last for short in the interphase. The second mechanism requires two hits: an initial DNA break and the rupture of the nuclear envelope, followed by the degradation of the broken ends of the bridging DNA by the cytoplasmic exonuclease TREX1. When chromosome bridges are resolved through this NER-dependent mechanism, their lifetime is on average longer than when chromosome bridges are resolved by the ANKLE1 NER-independent mechanism.

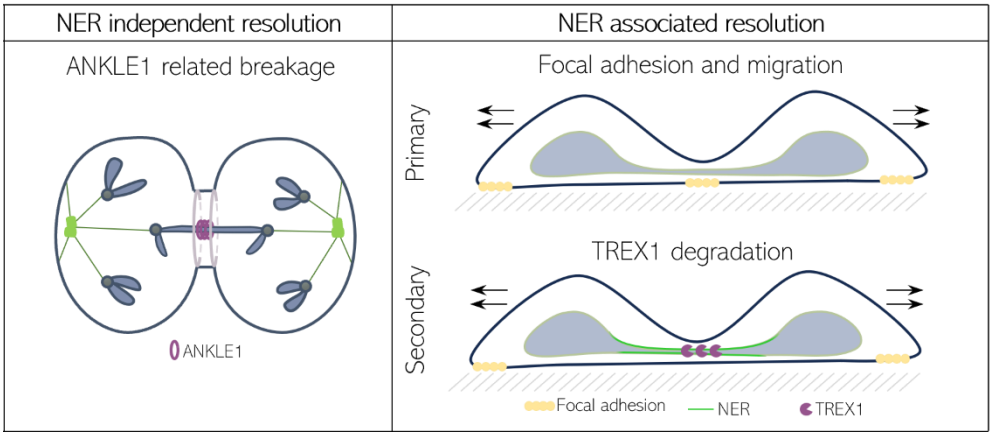


Figure 6. Illustration of the integrative model proposed.

Nuclease screening revealed ANKLE1 as a participant in the resolution of chromosome bridge at the transition between cell division and the early interphase cell cycle. Inhibition of ANKLE1 via siRNA or impeding its recruitment with ML7 resulted in a significant increase in the percentage of cells with chromosome bridges transitioning to interphase. These findings align with those reported by Hong and colleagues who illustrated the resolution of chromosome bridges in *Caenorhabditis elegans* embryos through LEM3, the analog for human ANKLE1 (Hong et al., 2018). Furthermore, recent work by Jiang and colleagues, (2023) showed that ANKLE1

endonuclease accumulates at the bulge-like structure of the midbody via its ankyrin repeats providing a path for a possible role of ANKLE1 in resolving chromosome bridges during the late stages of cell division. Our results confirm the role of ANKLE1 in resolving chromosome bridges at the end of cell division but also in a NER independent manner at early interphase. Additionally, the consequences of bridges being resolved by ANKLE1 were addressed by Jiang and colleagues that reported a prolonged activation of DNA damage response and elevated levels of 53BP1, a protein recruited at DSB sites in ANKLE1 knockout cells, highlighting ANKLE1's role in preventing excessive DNA damage (Jiang et al., 2023). Whether operative during cytokinesis or early interphase, the ANKLE1-dependent mechanism of bridge resolution might create a quite clean breakage event with reduced likelihood of extensive breakage of the bridging DNA and chromothripsis.

While a fraction of bridges can be resolved through ANKLE1, a significant percentage of cells with unresolved bridges reach interphase. Live-cell imaging showed that most of them proceed with NE rupture, corroborating studies from other researchers (Ganem & Pellman, 2012; Gordon et al., 2012; Lim et al., 2016; Liu et al., 2018; Maciejowski et al., 2015). Over the last decade, conflicting perspectives have characterized the ongoing debate regarding the resolution of chromosome bridges during interphase and the implication of the NER on their resolution. Some authors propose a mechanism for chromosome bridge resolution at the interphase based on the action of TREX1 exonuclease that depends on the occurrence of NER (Maciejowski et al., 2015), while others attribute it to focal adhesions (Umbreit et al., 2020) and affirm that the resolution is independent of NER. Each model presents weaknesses that challenge its standalone viability. As TREX1 has exonuclease activity, the model of bridge resolution based on TREX1 depends on the requirement for an unexplained initial break. Conversely, complex genome rearrangements and multiple breakpoints identified in the DNA of cells immediately after bridge resolution are challenging to solely attribute to focal adhesions. Our study introduces a potential reconciliation: both mechanisms may operate synergistically. The cooperative operation of TREX1 and mechanical forces projected by focal adhesions in resolving chromosome bridges could address the weaknesses inherent in individual theories. Focal adhesion could facilitate the required initial break for TREX1 as well as the irreversible breakdown of the nuclear envelope, while TREX1 might contribute to the generation of multiple breaks concentrated in discrete chromosomes.

We propose a model for bridge resolution in the interphase based on the sequential interplay of mechanisms of physical and biochemical nature. In accordance with our model, others described that the independent migration of the daughter cells connected by a bridge adhered to the substrate instigate mechanical tension to trigger an initial break on the bridging DNA and the

rupture of the nuclear envelope (Diz-Muñoz et al., 2013; Hatch & Hetzer, 2016). These two events will permit the recruitment of the cytoplasmic TREX1 and its action on the chromosome bridge (Fouquerel et al., 2019; Maciejowski et al., 2015; Maciejowski & De Lange, 2017; MacIejowski & Hatch, 2020). Our investigation suggests a potential link between the actomyosin cytoskeleton and the initiation of NE rupture, which probably might be transmitted by the LINC complex which binds cytoskeleton and NE (Hatch & Hetzer, 2016; Starr & Fridolfsson, 2010; Umbreit et al., 2020). According to this notion, live imaging experiments inhibiting the phosphorylation needed of the light chain of the activation of myosin II with ML7. These assays revealed a delay in NE rupture and an extended bridge lifetime upon focal adhesion destabilization, demonstrating a correlation between cell adhesion and NE rupture initiation. This reinforces the hypothesis that the actomyosin cytoskeleton acts as the initiator for bridge resolution during interphase in an associated manner with NE rupture. Subsequent digestion of the DNA inside the ruptured nuclear envelope by TREX1 might generate complex genome reorganizations as those observed in the phenomena of chromothripsis and kataegis.

Altogether, our results indicate that bridges can be resolved after mitosis by at least two independent mechanisms (Figure 6). A first mechanism operating after mitosis or early interphase based on the action of the endonuclease ANKLE1. A second mechanism for bridge resolution requires NER and would obey a multi-step mechanism. An initial break in the bridging DNA, which is locally strained as it is anchored by focal adhesions as daughter cells freely migrate away from each other. Tension on the bridge triggers breakage of the DNA and loss of compartmentation by rupture of the nuclear bridge envelope. Then, the broken ends of the bridging DNA inside a ruptured nuclear envelope will provide the substrates and conditions needed for the cytoplasmic TREX1 to digest the bridging DNA.

According to the data outlined in this article, the majority of bridges that reach the interphase will undergo breakage whether through the ANKLE1 mechanism or via focal adhesions followed by TREX1 recruitment. Nevertheless, there exists the potential for a subset of bridges to endure unresolved.

Recent studies have unveiled a repertoire of genomic alterations that can be ascribed to the presence of chromosome bridges in cells that include phenomenon capable to radically change the cell genome such as whole genome doubling, chromothripsis and kataegis. Notably, our findings, alongside others demonstrating post-mitotic breakage do not refute the classical model of BFB. The crux of matter is that breakage, whether occurring in mitosis or post-mitosis, triggers a feedback loop enabling the generation of novel genomic changes. Therefore, the core aspect of

BFB cycle remains intact: broken ends can fuse, initiating a mechanism that perpetuates new genomic alterations within cells.

REFERENCES

Crasta, K., Ganem, N. J., Dagher, R., Lantermann, A. B., Ivanova, E. V., Pan, Y., Nezi, L., Protopopov, A., Chowdhury, D., & Pellman, D. (2012). DNA breaks and chromosome pulverization from errors in mitosis. Nature, 482(7383), 53–58. https://doi.org/10.1038/NATURE10802

Cuylen, S., Metz, J., Hruby, A., & Haering, C. H. (2013). Entrapment of chromosomes by condensin rings prevents their breakage during cytokinesis. Developmental Cell, 27(4), 469–478. https://doi.org/10.1016/J.DEVCEL.2013.10.018

Diz-Muñoz, A., Fletcher, D. A., & Weiner, O. D. (2013). Use the force: membrane tension as an organizer of cell shape and motility. Trends in Cell Biology, 23(2), 47–53. https://doi.org/10.1016/J.TCB.2012.09.006

Fouquerel, E., Barnes, R. P., Uttam, S., Watkins, S. C., Bruchez, M. P., & Opresko, P. L. (2019). Targeted and Persistent 8-Oxoguanine Base Damage at Telomeres Promotes Telomere Loss and Crisis. Molecular Cell, 75(1), 117-130.e6. https://doi.org/10.1016/J.MOLCEL.2019.04.024

Ganem, N. J., & Pellman, D. (2012). Linking abnormal mitosis to the acquisition of DNA damage. The Journal of Cell Biology, 199(6), 871–881. https://doi.org/10.1083/JCB.201210040

Gascoigne, K. E., & Cheeseman, I. M. (2013). Induced dicentric chromosome formation promotes genomic rearrangements and tumorigenesis. Chromosome Research, 21(4), 407. https://doi.org/10.1007/S10577-013-9368-6

Gordon, D. J., Resio, B., & Pellman, D. (2012). Causes and consequences of aneuploidy in cancer. Nature Reviews. Genetics, 13(3), 189–203. https://doi.org/10.1038/NRG3123

Haraguchi, T., Kojidani, T., Koujin, T., Shimi, T., Osakada, H., Mori, C., Yamamoto, A., & Hiraoka, Y. (2008). Live cell imaging and electron microscopy reveal dynamic processes of BAF-directed nuclear envelope assembly. Journal of Cell Science, 121(Pt 15), 2540–2554. https://doi.org/10.1242/JCS.033597

Harding, S. M., Benci, J. L., Irianto, J., Discher, D. E., Minn, A. J., & Greenberg, R. A. (2017). Mitotic progression following DNA damage enables pattern recognition within micronuclei. Nature 2017 548:7668, 548(7668), 466–470. https://doi.org/10.1038/nature23470

Hatch, E. M., Fischer, A. H., Deerinck, T. J., & Hetzer, M. W. (2013). Catastrophic Nuclear Envelope Collapse in Cancer Cell Micronuclei. Cell, 154(1), 47. https://doi.org/10.1016/j.cell.2013.06.007

Hatch, E. M., & Hetzer, M. W. (2016). Nuclear envelope rupture is induced by actin-based nucleus confinement. Journal of Cell Biology, 215(1), 27–36. https://doi.org/10.1083/JCB.201603053/VIDEO-7

Hong, Y., Sonneville, R., Wang, B., Scheidt, V., Meier, B., Woglar, A., Demetriou, S., Labib, K., Jantsch, V., & Gartner, A. (2018). LEM-3 is a midbody-tethered DNA nuclease that resolves chromatin bridges during late mitosis. Nature Communications 2018 9:1, 9(1), 1–11. https://doi.org/10.1038/s41467-018-03135-w

Janssen, A., Van Der Burg, M., Szuhai, K., Kops, G. J. P. L., & Medema, R. H. (2011). Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. Science (New York, N.Y.), 333(6051), 1895–1898. https://doi.org/10.1126/SCIENCE.1210214

Jiang, H., Kong, N., Liu, Z., West, S. C., & Chan, Y. W. (2023). Human Endonuclease ANKLE1 Localizes at the Midbody and Processes Chromatin Bridges to Prevent DNA Damage and cGAS-STING Activation. Advanced Science (Weinheim, Baden-Wurttemberg, Germany), 10(12). https://doi.org/10.1002/ADVS.202204388

Leibowitz, M. L., Papathanasiou, S., Doerfler, P. A., Blaine, L. J., Sun, L., Yao, Y., Zhang, C. Z., Weiss, M. J., & Pellman, D. (2021). Chromothripsis as an on-target consequence of CRISPR-Cas9 genome editing. Nature Genetics, 53(6), 895–905. https://doi.org/10.1038/S41588-021-00838-7

Lim, S., Quinton, R. J., & Ganem, N. J. (2016). Nuclear envelope rupture drives genome instability in cancer. Molecular Biology of the Cell, 27(21), 3210. https://doi.org/10.1091/MBC.E16-02-0098

Liu, S., Kwon, M., Mannino, M., Yang, N., Renda, F., Khodjakov, A., & Pellman, D. (2018). Nuclear envelope assembly defects link mitotic errors to chromothripsis. Nature, 561(7724), 551–555. https://doi.org/10.1038/S41586-018-0534-Z

Maciejowski, J., & De Lange, T. (2017). Telomeres in cancer: tumour suppression and genome instability. Nature Reviews. Molecular Cell Biology, 18(3), 175–186. https://doi.org/10.1038/NRM.2016.171

MacIejowski, J., & Hatch, E. M. (2020). Nuclear Membrane Rupture and Its Consequences. Annual Review of Cell and Developmental Biology, 36, 85–114. https://doi.org/10.1146/ANNUREV-CELLBIO-020520-120627

Maciejowski, J., Li, Y., Bosco, N., Campbell, P. J., & De Lange, T. (2015). Chromothripsis and kataegis induced by telomere crisis. Cell, 163(7), 1641. https://doi.org/10.1016/J.CELL.2015.11.054

MacKenzie, K. J., Carroll, P., Martin, C. A., Murina, O., Fluteau, A., Simpson, D. J., Olova, N., Sutcliffe, H., Rainger, J. K., Leitch, A., Osborn, R. T., Wheeler, A. P., Nowotny, M., Gilbert, N., Chandra, T., Reijns, M. A. M., & Jackson, A. P. (2017). cGAS surveillance of micronuclei links genome instability to innate immunity. Nature, 548(7668), 461. https://doi.org/10.1038/NATURE23449

McClintock, B. (1941). The Stability of Broken Ends of Chromosomes in Zea Mays. Genetics, 26(2), 234. https://doi.org/10.1093/GENETICS/26.2.234

Pampalona, J., Roscioli, E., Silkworth, W. T., Bowden, B., Genescà, A., Tusell, L., & Cimini, D. (2016). Chromosome Bridges Maintain Kinetochore-Microtubule Attachment throughout Mitosis and Rarely Break during Anaphase. PloS One, 11(1). https://doi.org/10.1371/JOURNAL.PONE.0147420

Rodriguez-Muñoz, M., Anglada, T., & Genescà, A. (2022). A matter of wrapper: Defects in the nuclear envelope of lagging and bridging chromatin threatens genome integrity. Seminars in Cell & Developmental Biology, 123, 124–130. https://doi.org/10.1016/J.SEMCDB.2021.03.004

Rodriguez-Muñoz, M., Serrat, M., Soler, D., Genescà, A., & Anglada, T. (2021). Breakage of CRISPR/Cas9-Induced Chromosome Bridges in Mitotic Cells. Frontiers in Cell and Developmental Biology, 9. https://doi.org/10.3389/FCELL.2021.745195

Samwer, M., & Gerlich, D. W. (2018). A core problem in nuclear assembly. Nature, 561(7724), 467–468. https://doi.org/10.1038/D41586-018-06668-8

Shimizu, N., Shingaki, K., Kaneko-Sasaguri, Y., Hashizume, T., & Kanda, T. (2005). When, where and how the bridge breaks: anaphase bridge breakage plays a crucial role in gene amplification and HSR generation. Experimental Cell Research, 302(2), 233–243. https://doi.org/10.1016/J.YEXCR.2004.09.001

Starr, D. A., & Fridolfsson, H. N. (2010). Interactions between nuclei and the cytoskeleton are mediated by SUN-KASH nuclear-envelope bridges. Annual Review of Cell and Developmental Biology, 26, 421–444. https://doi.org/10.1146/ANNUREV-CELLBIO-100109-104037

Steigemann, P., Wurzenberger, C., Schmitz, M. H. A., Held, M., Guizetti, J., Maar, S., & Gerlich, D. W. (2009). Aurora B-mediated abscission checkpoint protects against tetraploidization. Cell, 136(3), 473–484. https://doi.org/10.1016/J.CELL.2008.12.020

Terradas, M., Martín, M., Hernández, L., Tusell, L., & Genescà, A. (2012). Nuclear envelope defects impede a proper response to micronuclear DNA lesions. Mutation Research, 729(1–2), 35–40. https://doi.org/10.1016/J.MRFMMM.2011.09.003

Terradas, M., Martín, M., Tusell, L., & Genescà, A. (2010). Genetic activities in micronuclei: is the DNA entrapped in micronuclei lost for the cell? Mutation Research, 705(1), 60–67. https://doi.org/10.1016/J.MRREV.2010.03.004

Umbreit, N. T., Zhang, C. Z., Lynch, L. D., Blaine, L. J., Cheng, A. M., Tourdot, R., Sun, L., Almubarak, H. F., Judge, K., Mitchell, T. J., Spektor, A., & Pellman, D. (2020). Mechanisms generating cancer genome complexity from a single cell division error. Science (New York, N.Y.), 368(6488). https://doi.org/10.1126/SCIENCE.ABA0712

Zhang, C. Z., Spektor, A., Cornils, H., Francis, J. M., Jackson, E. K., Liu, S., Meyerson, M., & Pellman, D. (2015). Chromothripsis from DNA damage in micronuclei. Nature, 522(7555), 179–184. https://doi.org/10.1038/NATURE14493

SUPPLEMENTARY DATA.

Table SI. List of plasmids used

Plasmid name	Antibiotic resistance	Vector type	Reference
sgChr4	Puromycin	Lentiviral	Umbreit et al, 2020
GFP-BAF	Geneticin	Lentiviral	Umbreit et al, 2020
sh p21/Rb	Blasticidin	Lentiviral	Umbreit et al, 2020
pTRIP-CMV-tagRFP-cGAS	-	Lentiviral	Addgene #86676
psPAX2	-	Packaging	Addgene #12260
pMD2.G	-	Envelope	Addgene #12259

Table SII. List of antibodies used

Antibody	Host	Reference	Working dilution
Primary antibodies			
Emerin	Rabbit	10351-1-AP	1:500
γH2AX	Mouse	Millipore, 05-636, clone JBW301	1:1000
Lamin B1	Rabbit	Abcam, ab16048	1:500
RPA32/RPA2	Mouse	Abcam, ab2175, clone 9H8	1:500
TREX1	Rabbit	Abcam, ab185228	1:200
Paxillin	Mouse	UAB Microscopy Facility	1:500
Secondary antibodies			
Anti-mouse Cyanine Cy™3	Goat	Jackson ImmunoResearch Inc., 115-165-146	1:500
Anti-rabbit Alexa Fluor 568	Donkey	Thermo Fisher Scientific, A-10042	1:500

SUPPLEMENTARY FIGURES.

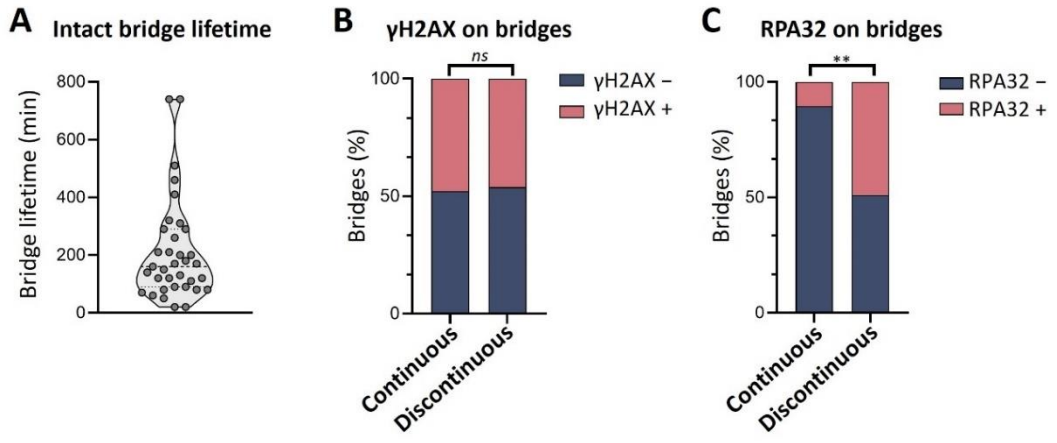


Figure S1: Chromosome bridge lifetime and markers of resolution. (A) Bridge lifetime from interphase entry to their resolution. Chromosome bridges are considered resolved when the pattern of BAF becomes discontinuous. Dashed line indicates the median and dotted line the CI for 25-75 %. n = 56 **(B)** Frequency of γH2AX-positive and -negative chromosome bridges classified according to the continuity or discontinuity of the GFP-BAF signal. Chi square test; *ns* = no significative; n = 129 chromosome bridges from three independent replicates. **(C)** Frequency of RPA32-positive and -negative chromosome bridges classified according to the continuity or discontinuity of the GFP-BAF signal. Chi-square test; ** p-value = 0.0038; n = 62 chromosome bridges from three independent replicates.

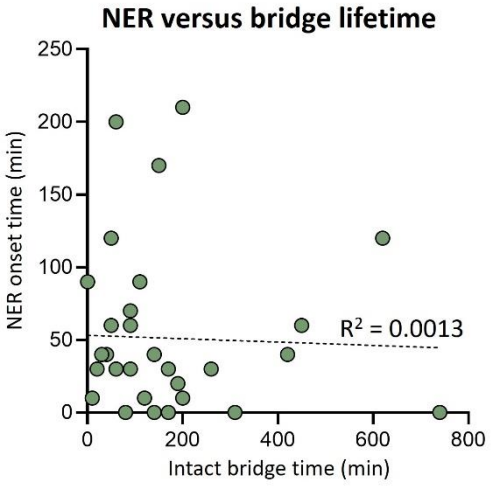


Figure S2: Nuclear envelope rupture does not correlate with bridge lifetime. Plot showing no significant correlation between bridge lifetime and NER onset (linear regression; $p = 0.83$; $R^2 = 0.0013$). $n = 35$. Bridges were induced in RPE-1 cells using sgRNA Chr4 and visualized with GFP-BAF. NER was considered positive when chromosome bridges expressed a more brightness signal than the main nucleus.

DISCUSSION

A common characteristic of cells derived from solid tumors is the presence of interphasic nuclei with abnormal morphologies and atypical mitotic shapes (Beale, 1860; Gisselsson et al., 2002; Jin et al., 2007; Papanicolaou et al., 1941; Singh & Lele, 2022). Cancerous cells often manifest high levels of genome reorganizations due to mechanisms that typically promote CIN, such as telomere dysfunction, DDR defects, replication stress, and mitosis defects (reviewed in the Introduction Section). Common intermediaries to the different processes contributing to CIN are chromosome bridges, which completely span the segregating masses of chromosomes during anaphase and can persist as nucleoplasmic connections at the interphase. These pathological bridges that can be visualized using DNA-crosslinking dyes frequently manifest defects in their nuclear envelope (Results Section - Chapter 1). Despite chromosome bridges being discovered over a century ago, the mechanisms underlying their resolution are still under discussion and the consequences for cells harboring chromosome bridges can differ depending on how and when bridges are resolved.

The research presented in this disseration has been developed using a cell-based model for generation of chromosome bridge of unique characteristics under temporal control. This model has been consistently employed throughout all the studies presented here to mitigate variability derived from the bridge's origin. Results obtained demonstrate that bridge resolution can proceed through different mechanisms of biochemical or mechanical nature that act leveraging the scenarios occurring in cells at different moments of the cell cycle.

1. Chromosome bridge resolution during mitosis

The results presented in Chapters 2 and 3 reveal that most chromosome bridges undergo breakage at the exit of mitosis, primarily due to mechanical forces. Our findings conflict with statements by other researchers who suggest that chromosome bridges formed at the anaphase invariably persist through mitosis and develop into stable nucleoplasmic connections between daughter cells (Maciejowski et al., 2015; Maciejowski & De Lange, 2017; Steigemann et al., 2009; Umbreit et al., 2020). These discrepancies might be attributed to the fact that the above-referenced studies focused on examining chromosome bridges during interphase, potentially neglecting an exhaustive investigation of chromosome bridge dynamics during mitosis. Their studies in mitotic cells did not include the evaluation of markers of DNA breakage on chromosome bridges and consisted only of live-cell imaging experiments involving time intervals of 10 minutes between image acquisition, possibly resulting in missing anaphase bridge resolution due to the rapid nature of the mitotic process.

We obtained four independent observations that support the notion that breakage of the DNA molecule in chromosome bridges during mitosis is real. Firstly, we observed that the frequency

of chromosome bridges diminished as cells progress through the last stages of mitosis, indicating that most bridges are resolved during mitosis. Secondly, the mitotic cells examined frequently exhibited the DNA DSB marker γH2AX flanking disrupted chromatin bridges demonstrating that discontinuities visible microscopically in chromosome bridges indeed represent real breakage of the DNA molecule. Thirdly, using the STRIDE assay, a direct method to detect 3'OH free-ends at the DNA molecule, we confirmed that the DNA flanking discontinuities in chromosome bridges at the end of mitosis in fact present 3'OH ends thus corresponding to real breakage of the DNA molecule. Fourthly, in live imaging recordings of mitotic cells expressing the DDR mediator protein MDC1 conjugated to GFP, we witnessed the retraction of chromosome bridge ends after MDC1 recruitment to the broken bridging DNA. All these observations demonstrate chromosome bridge breakage during mitosis. Hence, challenging previous premises established by others, our observations firmly demonstrate the resolution of chromosome bridges during mitosis.

After confirming the breakage of a fraction of chromosome bridges in mitosis, we delved into the fate of the resulting fragments. Repair of DNA ends from broken chromosome bridges does not occur during mitosis for two reasons, the extreme condensation of the mitotic chromatin (Giunta et al., 2010) and the rapid and progressive separation of the broken DNA ends toward opposite cell poles. As expected, we did not detect recruitment of downstream effectors of DNA repair to the bridge during mitosis. Recently, TOPBP1 has been reported to ensure genome integrity during mitosis by maintaining the two ends of broken DNA pulled to the same pole (Bagge et al., 2021; Blackford & Stucki, 2020; Gallina et al., 2016; Leimbacher et al., 2019). However, in the context of chromosome bridges, the two ends that would need to be maintained together are actively pulled apart by the spindle microtubules. It is challenging to conceive how molecules like TOPBP1 could retain these two ends close to each other. Therefore, considering that downstream effectors of DNA repair are not recruited on chromosome bridges during mitosis and that the two ends of broken chromosome bridges are pulled apart, it is possible to conclude that the breakage detected by γH2AX and STRIDE on chromosome bridges will actually result in an DNA rupture. Consequently, the resulting fragments will be separately incorporated in the daughter cells where they may fuse illegitimately to other free DNA ends, exacerbating CIN.

Our initial study (Results Section -Chapter 2) shed light on a potential correlation between the likelihood of bridge breakage and the bridge kinetochore separation in micrometers based on two observations. Firstly, our results showed that bridge kinetochores were frequently displaced from the cluster of non-bridge kinetochores, thus indicating that they were opposing resistance to the mitotic spindle generated tension. This result is in accordance with previous observations from

our research group when investigatingd tetraploid cell formation by non-resolved chromosome bridges (Pampalona et al., 2016). Secondly, we found that the longer the distance between the bridge kinetochores in micrometers, the higher the probability of bridge breakage during anaphase. These findings suggest that the mechanical stress experienced by chromosome bridge during the last stages of mitosis contributes to its breakage.

To further investigate the causes of chromosome bridge breakage during mitosis we employed CRISPR/Cas9 technology to induce DNA breaks at selected sites of the genome and thus generate chromosomal bridges with defined intercentromeric lenght in megabases (Results Section-Chapter 3). Using this approach five different sgRNA cell lines were generated, and we observed that for each cell line, the minimum separation between kinetochores needed for bridge breakage was different and correlated with their bridging chromatin length. Consequently, a larger base pair distance between bridge centromeres increases the likelihood of the bridge to persist intact, resulting in daughter cells born with a bridge. Taken together, our results indicate that the breakage at mitotic exit could be explained by the action of inherent mechanical forces. Specifically, the continuous DNA fiber experiences tension as its two kinetochores are pulled towards opposite poles by the microtubules of the mitotic spindle, potentially causing the breakage. Moreover, the tension depends on the distance in base pairs between centromeres. This correlation is unsurprising from a physical standpoint since longer chromosome bridges will require additional separation to accumulate enough tension from the microtubules attached to bridge kinetochores to break the bridge.

Regarding the forces acting on the chromosome bridge, mitotic spindle microtubules exert push-and-pull forces by adding or shedding subunits from their ends. At the level of individual molecules, cytoskeletal proteins generate forces at the piconewton scale and movements at the nanometer scale. However, during cell division, these proteins act collectively, forming ensembles capable of generating forces in the nanonewton range and moving chromatids over micrometer distances (Scholey et al., 2003). During mitosis, DNA undergoes significant condensation, presenting a challenge for the DNA within the bridges to decondense instead of breaking. The tension applied to kinetochores is countered by the elasticity of the stretched chromatin (Pickett-Heaps et al., 1986). This dynamic explains why chromosome bridges of equivalent base pair lengths do not uniformly break at identical separations between their kinetochores. Instead, chromosome bridges resolve at a range of distances.

Mitotic bridge breakage is an additional proof supporting the BFB cycle model, showcasing how chromosome bridges exacerbate genome instability. However, it is important to note that not all

bridges experience breakage during the exit of mitosis, some of them persist and could potentially be resolved through alternative mechanisms.

2. Chromosome bridge resolution during cytokinesis and early interphase

The distinct scenarios during mitosis and cytokinesis suggest that the causes behind chromosome bridge breakage might be different. At the exit of mitosis, pulling forces by the mitotic spindle responsible for segregating the two copies of each chromosome, now devoid of nuclear envelope, can contribute to chromosome bridge breakage (Results-Chapter 2 and 3). However, some bridges remain intact and cells harboring them arrive at cytokinesis undergoing a scenario shift involving disassembly of the mitotic spindle, reassembly of the nuclear envelope, and cytoplasm abscission. Although the NE reassembles around segregating chromosomes, the presence of lagging and bridging chromatin during cell division compromises both the structure and function of the nuclear envelope surrounding unsegregated chromatin (Crasta et al., 2012; Hatch et al., 2013; Maciejowski et al., 2015; Terradas et al., 2012). DNA trapped in the mitotic midzone shows deficient NE assembly, which is attributed to either sustained activity of the Aurora B kinase (Afonso et al., 2014, 2019) or to dense cytoskeleton fibers hindering proper NE reassembly on unsegregated chromatin (Otsuka et al., 2016).

ANKLE1 endonuclease might be responsible for chromosome bridge resolution during **cytokinesis**. Our conclusion is based on the observation that inhibition of ANKLE1 led to a higher fraction of cells with chromosome bridges reaching the interphase, indicating a role of ANKLE1 in chromosome bridge resolution prior to interphase. Our results align with other studies that suggested that ANKLE1 is recruited at the midbody region to resolve trapped chromatin in *C. elegans* and human cells (Jiang et al., 2023). ANKLE1 contains a GIY-YIG domain (Brachner et al., 2012), which is capable of cleaving branched DNA (Freeman et al., 2023; Przanowski et al., 2023; Song et al., 2020). In the context of cytokinesis, the unsegregated DNA that is under the segmentation furrow might be mildly decondensed, thus ANKLE1 can recognize this branched-like DNA and proceed to its degradation. Taken together, this finding strongly suggests that ANKLE1 is responsible for chromosome bridge breakage during cytokinesis.

Although ANKLE1 is recruited at the midbody region during cytokinesis, it might be responsible for chromosome bridge breakage in **early interphase**. According to our results, during the interphase, two types of bridges coexist, short-lifetime bridges, which resolve independently of NER, and long-lifetime bridges, which experience NER prior to their resolution (Results-Chapter 4). ANKLE1 role during interphase stems from the observation that either when ANKLE1 is inhibited or its recruitment impeded, the fraction of bridges that resolve in a short time and through a NER-independent mechanism, diminishes. ANKLE1 is expressed in the cytoplasm

during interphase due to its nuclear export signal (Zlopasa et al., 2016) and is recruited to the spindle midzone during cytokinesis. Therefore, cells with chromosome bridges resolved by ANKLE1 during the interphase might have already recruited this enzyme before transitioning into interphase. Collectively, we propose that ANKLE1 might resolve chromosome bridges during cytokinesis but also in early interphase.

Resolution of chromosome bridges during cytokinesis or early interphase has been very controversial. Recent studies (Maciejowski et al., 2015; Umbreit et al., 2020) start from the unproven premise that bridges do not break during cytokinesis. It is important to note that these studies rely on widely spaced timelapse monitoring of live cells, which might not be ideal for analyzing brief processes like mitosis or cytokinesis. In contrast, classical studies supported resolution of bridges during cytokinesis based on the observation of markers of DNA damage on unsegregated chromatin under ingressing furrow region that were rescued when cytokinesis was inhibited (Janssen et al., 2011). These authors suggested that the cleavage furrow ingression forces were responsible for DNA breakage, yet an exhaustive study was required for its corroboration. Although we cannot exclude the contribution of mechanical ingression forces, our work suggests that ANKLE1 serves as a biochemical mechanism of resolution during cytokinesis and early interphase stages to resolve chromosome bridges trapped under the furrow. It is conceivable that ANKLE1 could act as a priming nuclease for TREX1 or other exonucleases, leading to the degradation of DNA as suggested by Jiang et al (2023). However, a major challenge for this hypothesis is that TREX1's does not have access to DNA, and according to our results ANKLE1 can proceed without NER. Altogether, during the cytokinesis, ANKLE1 would be recruited to clean the area beneath the furrow ingression and to prevent regression of the contractile ring thereby averting tetraploidization. Nevertheless, due to the rapid process of cytokinesis, ANKLE1 might face time constraints to resolve chromosome bridges within cytokinesis and, once recruited to the bridging DNA, could potentially address chromosome resolution during the early interphase. The determination of why some bridges are resolved through ANKLE1 during either cytokinesis or early interphase, while others are not, remains unresolved.

3. Chromosome bridge resolution during interphase

Understanding the resolution of bridges during interphase requires considering two unique circumstances that did not play a role in bridge resolution during cell division: (1) normal interphase eukaryotic cells store their chromatin in a single nucleus with assembled nuclear envelope (Samwer et al., 2017), and (2) the cell migration during interphase is fundamental for its homeostasis and relies on cell's ability to adhere to the substrate through focal adhesions (Gkretsi

& Stylianopoulos, 2018; Martino et al., 2018; Merino-Casallo et al., 2022). Cells harboring chromosome bridges function as independent entities that migrate apart from each other (Umbreit et al., 2020) yet remaining connected by chromosome bridges with typically defective NE. These features suggest that bridge resolution during interphase might rely on the combined effects of mechanical and biochemical processes.

Cells harboring chromosome bridges can progress through the interphase of the cell cycle and frequently exhibit a collapse of the bridge nuclear envelope. Analysis of live-cell images has enabled us to outline a sequence of observations. In general, cells with chromosome bridges are born with an unruptured nuclear envelope surrounding the daughter cells primary nuclei and the bridge. Meanwhile, the two recently born daughter cells migrate away from each other. Abruptly, these bridges undergo NER, yet the cells continue migrating. Suddenly the bridge becomes discontinuous. Although NER precedes bridge breakage, the timepoint NER occurs does not correlate with the moment of bridge breakage, thus suggesting a potential facilitative role of NER in the breakage event.

At the interphase of the cell cycle chromosome bridge resolution depends on their anchorage to the substrate through focal adhesions that in turn causes NER and permits recruitment of TREX1 (Results Section-Chapter 4). This conclusion is based on four observations. Firstly, chromosome bridges often exhibit paxillin, a key component of focal adhesions. Therefore, chromosome bridges are anchored to the substrate through focal adhesions. Secondly, by attenuating focal adhesion strength through inhibiting phosphorylation of myosin light chain, both bridge resolution and NER are delayed, demonstrating that chromosome bridge resolution and NER depend on the adhesion of the daughter cells to the substrate. Thirdly, TREX1 is recruited only in bridges that exhibit NER, thus supporting the idea that upon NE collapse, most bridges recruit the ER-associated exonuclease TREX1. Fourthly, the exonuclease TREX1 requires an initial break to start to degrade DNA. Bridges that exhibit bright BAF are more likely to have DSBs than basal-BAF bridges. Considering the association between bright-BAF and focal adhesion, it can be inferred that focal adhesion might play a role in facilitating the first nick on the DNA needed for the action of TREX1. Altogether, our observations permit us to establish that migration apart of cells with chromosome bridges combined with the presence of focal adhesion under bridging chromatin might facilitate NER and breakage of the DNA molecule inside the bridge, both of them are events needed to enable the exonuclease TREX1 to be recruited and to digest the DNA of the chromosome bridges.

Contribution of focal adhesions on chromosome bridge resolution was previously described by Umbreit and colleagues but their study contemplates bridge resolution as a single mechanism

that is restricted in a window time (Umbreit et al., 2020). They studied bridges in cells cultured on top of different stiffness substrates, corroborating the role of focal adhesions in orchestrating the breakage of chromosome bridges. Moreover, the idea that focal adhesions are responsible for NER is in agreement with Hatch and colleagues that associated NER with the actomyosin cytoskeleton through LINC, specifically in regions distinguished by compromised membranes and disorganized lamina (Hatch & Hetzer, 2016), as is the case of chromosome bridges.

The observation that nuclear envelope ruptured regions exhibit TREX1 aligns with studies conducted on micronuclei, primary nuclei, and on chromosome bridges generated by other methods (Maciejowski et al., 2015; Mohr et al., 2021). Maciejowski and colleagues suggested that the TREX1 intervention could explain the resolution chromosome bridges after a spontaneous event of NER (Maciejowski et al., 2015). Unlike the model described by these authors, we understand the resolution of interphase bridges as a more complex process in which actions of a mechanical nature are combined, to induce NER and a first nick in the DNA of the bridge, that permit the action of biochemical factors such as TREX1 exonuclease. Moreover, another limitation of this study is that TREX1 is an exonuclease and thus relies on an initial nick in the DNA. Recent studies have demonstrated that forces generated by focal adhesion can induce DNA breakage (Hatch & Hetzer, 2016), thus potentially serving as the facilitator for the initial break required by TREX1 to initiate DNA degradation. While focal adhesion might be notably contributing to nick the DNA, we cannot exclude that other mechanisms acting during the previous mitosis, such as microtubule-generated forces or ANKLE1, can cause the initial break.

Altogether, the resolution of chromosome bridges during interphase is a result of the synergy between mechanical and biochemical processes. Cells born in interphase with chromosome bridges behave as two separate entities that migrate apart from each other yet connected by the chromosome bridge that is often anchored to the substrate through focal adhesion. The combination of this phenomenon initiates bridge resolution during interphase and triggers NER. Subsequently, TREX1 is recruited to the bridge and digest the DNA within the ruptured nuclear envelope. Notably, the exonuclease requires an initial break that could potentially result from either focal adhesions or alternative mechanisms.

4. Dynamics of chromosome bridge resolution: Towards an integrative model for chromosome bridge resolution

While a fraction of the bridges is resolved during mitosis, some of the bridges persist until telophase or the next interphase. Taking into account all the bridges analyzed, we conclude that the number of chromosome bridges observed, gradually decreases as cells progress through the different stages of the cell cycle, each one with its specific environment. Firstly, during mitosis

resolution occurs in a scenario devoid of nuclear envelope and in a context where the mitotic spindle has a pivotal role driving mitosis. Secondly, during cytokinesis the bridge is under the influence of the contractile ring that can recruit proteins that have the capacity to degrade the DNA. Thirdly, during interphase, the cells harboring bridges have reassembled the nuclear envelope and they freely move apart. Thus, chromosomes contained in bridges at the interphase are separated from the cytoplasmic environment unless the nuclear envelope is collapsed. Finally, it is conceivable that not all bridges are broken, as there may be a subset of them remaining unbroken.

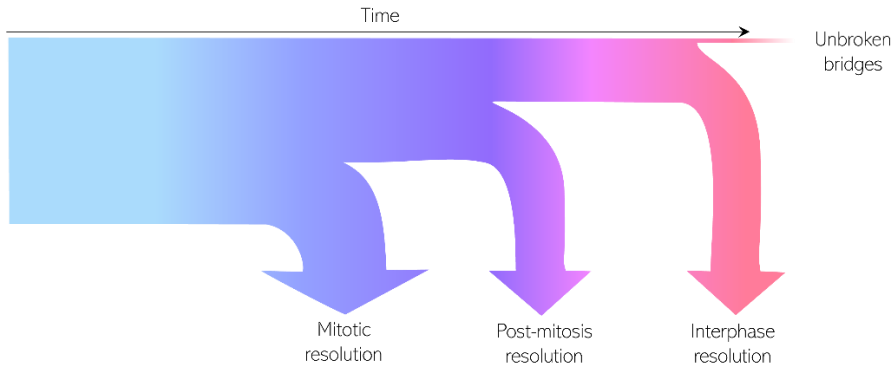


Figure 8. Dynamics of bridge resolution.

The current prevailing understanding held that bridge resolution relied on a single mechanism directed by TREX1 (Maciejowski et al., 2015) or stretching forces by the cytoskeleton (Umbreit et al., 2020). However, the results presented in this dissertation demonstrate that chromosome bridges are resolved by diverse mechanisms, each tailored to the specific context of the different moments of the cell cycle (Figure 8). As a result, we perceive resolution of chromosome bridges as a multifactorial process.

Considering that bridge resolution occurs throughout the cell cycle tailored by unique environmental conditions in each phase, it is not surprising that we find that the resolution of the bridge obeys several mechanisms of different natures (Figure 8). During mitosis, breakage occurs primarily due to mechanical factors due to the tensile forces exerted by the mitotic spindle. Moreover, some bridges persist beyond telophase, and the ANKLE1 endonuclease recruited during cytokinesis degrades chromosome bridges resulting in chromosome bridge breakage. Finally, bridges that have not resolved neither during mitosis due to mechanical causes nor afterwards due to biochemical mechanisms, will have a new opportunity to do so at the interphase. Cells born with unresolved chromosome bridges, despite being connected by the nucleoplasmic bridge are independent and migrate away from each other. In these circumstances,

we comprehend that the resolution of chromosome bridges in cells during interphase relies on a series of mechanical and biochemical processes. Adhesion forces of bridges to the substrate contribute to NER and DNA breakage. These events, in turn, facilitates TREX enzyme entry to complete the bridge resolution.

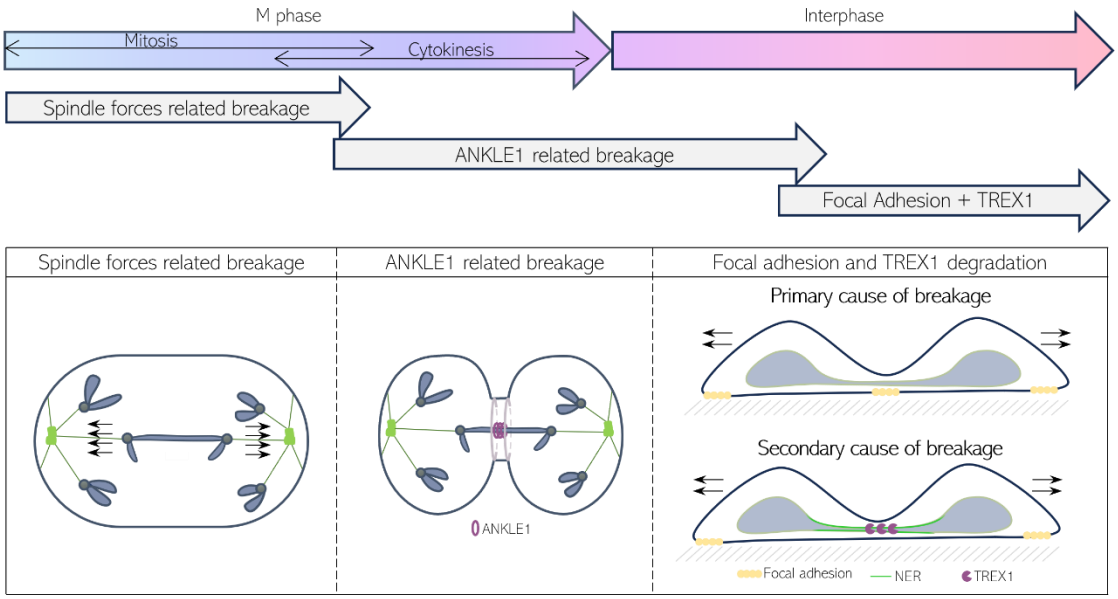


Figure 9. Integrative model of chromosome bridge resolution.

Comprehending the different mechanisms by which bridges resolve is crucial as the potential outcomes resulting from these mechanisms of resolution can vary significantly. We speculate that at the **cellular level**, the extent of the DNA damage might depend on the mechanism. Processes that act in a localized manner, such as microtubule pulling forces or ANKLE1, will probably generate less damage than if the mechanism involves an enzyme like TREX1 that degrades DNA along the area of the chromosome bridge undergoing NER. At the **organism level,** NER leads to the exposure of the nuclear contents to the cytoplasm, triggering potential consequences such as the activation of the cytosolic DNA-binding protein cGAS, which can initiate the innate immune response. This activation is particularly potent when cGAS interacts with DNA that lacks nucleosome organization (Kujirai et al., 2020), as it may occur in chromosome bridges. However, the accumulation of BAF (Guey et al., 2020) or TREX1 (Mohr et al., 2021; Zhou et al., 2021) can hinder the cGAS-STING pathway. Thus, chromosome bridges with heightened BAF or processed by TREX1 might also difficult cGAS signaling. Given the uncertainties, further research is needed to determine whether chromosome bridges with NER activate the immune system.

The research outlined in the dissertation contributes toward resolving long-standing debates surrounding the resolution of chromosome bridges. This dissertation unveils a model for chromosome bridge resolution, highlighting both mechanical and biochemical processes that

operate in a cell cycle-dependent manner. This research has critical implications for understanding genomic instability arising in cancer cells, where the presence of chromosome bridges may contribute to cycles of DNA alterations leading to CIN.

CONCLUSIONS

CONCLUSIONS

1. The revision conducted to identify gaps of knowledge revealed that the impact of nuclear envelope integrity on chromosome bridge resolution is not well-defined.
2. An experimental model using CRISPR/Cas9 has been successfully established enabling the generation of chromosome bridges with defined geometry under temporal control.
3. Most chromosome bridges are resolved in mitosis as evidenced by the observable markers of DNA double-strand breaks and by the presence of 3'OH DNA ends.
4. The length of the bridging chromatin determines the moment of resolution and the required separation between kinetochores for bridge breakage. Therefore, tension forces exerted by the mitotic spindle on bridge kinetochores contribute to chromosome bridge resolution.
5. Chromosome bridges that withstand tension forces can be resolved after mitosis through a biochemical mechanism reliant on the endonuclease ANKLE1.
6. A subset of chromosome bridges is resolved during the interphase by a two-step mechanism. Initially, the anchorage of the bridge to the substrate through focal adhesions in migrating cells causes both the first DNA nick and nuclear envelope rupture. Then, loss of the permeability barrier allows TREX1 action on the nicked DNA of the bridge.

Altogether, the findings presented in this dissertation reveal that chromosome bridges undergo breakage during the cell division or interphase. Bridge resolution is orchestrated by distinct mechanisms, either of a biochemical or mechanical nature, tailored to the specific context of the different moments of the cell cycle.

REFERENCES

A

Acilan, C., Potter, D. M., & Saunders, W. S. (2007). DNA repair pathways involved in anaphase bridge formation. Genes, Chromosomes & Cancer, 46(6), 522–531. https://doi.org/10.1002/GCC.20425

Afonso, O., Castellani, C. M., Cheeseman, L. P., Ferreira, J. G., Orr, B., Ferreira, L. T., Chambers, J. J., Morais-De-Sá, E., Maresca, T. J., & Maiato, H. (2019). Spatiotemporal control of mitotic exit during anaphase by an aurora B-Cdk1 crosstalk. ELife, 8. https://doi.org/10.7554/ELIFE.47646

Afonso, O., Matos, I., Pereira, A. J., Aguiar, P., Lampson, M. A., & Maiato, H. (2014). Feedback control of chromosome separation by a midzone Aurora B gradient. Science, 345(6194), 332–336. https://doi.org/10.1126/SCIENCE.1251121/SUPPL_FILE/AFONSO.SM.PDF

Alberts, B., Heald, R., Johnson, A. (Alexander D.), Morgan, D. O., Raff, M. C., Roberts, K. (Keith), & Walter, P. (Professor). (2015). Molecular biology of the cell. 70.

Alexandrov, L. B., Kim, J., Haradhvala, N. J., Huang, M. N., Tian Ng, A. W., Wu, Y., Boot, A., Covington, K. R., Gordenin, D. A., Bergstrom, E. N., Islam, S. M. A., Lopez-Bigas, N., Klimczak, L. J., McPherson, J. R., Morganella, S., Sabarinathan, R., Wheeler, D. A., Mustonen, V., Boutros, P., … Yu, W. (2020). The repertoire of mutational signatures in human cancer. Nature, 578(7793), 94–101. https://doi.org/10.1038/S41586-020-1943-3

B

Bagge, J., Oestergaard, V. H., & Lisby, M. (2021). Functions of TopBP1 in preserving genome integrity during mitosis. Seminars in Cell & Developmental Biology, 113, 57–64. https://doi.org/10.1016/J.SEMCDB.2020.08.009

Bakhoum, S. F., & Cantley, L. C. (2018). The Multifaceted Role of Chromosomal Instability in Cancer and Its Microenvironment. Cell, 174(6), 1347–1360. https://doi.org/10.1016/J.CELL.2018.08.027

Bakhoum, S. F., Genovese, G., & Compton, D. A. (2009). Deviant kinetochore microtubule dynamics underlie chromosomal instability. Current Biology : CB, 19(22), 1937–1942. https://doi.org/10.1016/J.CUB.2009.09.055

Bakhoum, S. F., Thompson, S. L., Manning, A. L., & Compton, D. A. (2009). Genome stability is ensured by temporal control of kinetochore-microtubule dynamics. Nature Cell Biology, 11(1), 27–35. https://doi.org/10.1038/NCB1809

Barr, F. A., & Gruneberg, U. (2007). Cytokinesis: Placing and Making the Final Cut. Cell, 131(5), 847–860. https://doi.org/10.1016/J.CELL.2007.11.011

Beale. (1860). Examination of sputum from a case of cancer of the pharynx and the adjacent parts.

Birkbak, N. J., Eklund, A. C., Li, Q., McClelland, S. E., Endesfelder, D., Tan, P., Tan, I. B., Richardson, A. L., Szallasi, Z., & Swanton, C. (2011). Paradoxical relationship between chromosomal instability and survival outcome in cancer. Cancer Research, 71(10), 3447–3452. https://doi.org/10.1158/0008-5472.CAN-10-3667

Blackford, A. N., & Jackson, S. P. (2017). ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. Molecular Cell, 66(6), 801–817. https://doi.org/10.1016/J.MOLCEL.2017.05.015

Blackford, A. N., & Stucki, M. (2020). How Cells Respond to DNA Breaks in Mitosis. Trends in Biochemical Sciences, 45(4), 321–331. https://doi.org/10.1016/J.TIBS.2019.12.010

Böhly, N., Schmidt, A. K., Zhang, X., Slusarenko, B. O., Hennecke, M., Kschischo, M., & Bastians, H. (2022). Increased replication origin firing links replication stress to whole chromosomal instability in human cancer. Cell Reports, 41(11). https://doi.org/10.1016/J.CELREP.2022.111836

Boland, C. R., & Goel, A. (2010). Microsatellite instability in colorectal cancer. Gastroenterology, 138(6). https://doi.org/10.1053/J.GASTRO.2009.12.064

Brachner, A., Braun, J., Ghodgaonkar, M., Castor, D., Zlopasa, L., Ehrlich, V., Jiricny, J., Gotzmann, J., Knasmüller, S., & Foisner, R. (2012). The endonuclease Ankle1 requires its LEM and GIY-YIG motifs for DNA cleavage in vivo. Journal of Cell Science, 125(Pt 4), 1048–1057. https://doi.org/10.1242/JCS.098392

Burrell, R. A., McGranahan, N., Bartek, J., & Swanton, C. (2013). The causes and consequences of genetic heterogeneity in cancer evolution. Nature, 501(7467), 338–345. https://doi.org/10.1038/NATURE12625

C

Campbell, P. J., Getz, G., Korbel, J. O., Stuart, J. M., Jennings, J. L., Stein, L. D., Perry, M. D., Nahal-Bose, H. K., Ouellette, B. F. F., Li, C. H., Rheinbay, E., Nielsen, G. P., Sgroi, D. C., Wu, C. L., Faquin, W. C., Deshpande, V., Boutros, P. C., Lazar, A. J., Hoadley, K. A., … Zhang, J. (2020). Pan-cancer analysis of whole genomes. Nature 2020 578:7793, 578(7793), 82–93. https://doi.org/10.1038/s41586-020-1969-6

Carter, S. L., Cibulskis, K., Helman, E., McKenna, A., Shen, H., Zack, T., Laird, P. W., Onofrio, R. C., Winckler, W., Weir, B. A., Beroukhim, R., Pellman, D., Levine, D. A., Lander, E. S., Meyerson, M., & Getz, G. (2012). Absolute quantification of somatic DNA alterations in human cancer. Nature Biotechnology, 30(5), 413–421. https://doi.org/10.1038/NBT.2203

Caruso, R. A., Fedele, F., Consolo, P., Luigiano, C., Venuti, A., & Cavallari, V. (2008). Abnormal nuclear structures (micronuclei, nucleoplasmic bridges, and nuclear buds) in a pleomorphic giant cell carcinoma of the stomach. Ultrastructural Pathology, 32(1), 11–15. https://doi.org/10.1080/01913120701829319

Childs, B. G., Baker, D. J., Kirkland, J. L., Campisi, J., & van Deursen, J. M. (2014). Senescence and apoptosis: dueling or complementary cell fates? EMBO Reports, 15(11), 1139–1153. https://doi.org/10.15252/EMBR.201439245

Cortés-Ciriano, I., Lee, J. J. K., Xi, R., Jain, D., Jung, Y. L., Yang, L., Gordenin, D., Klimczak, L. J., Zhang, C. Z., Pellman, D. S., Akdemir, K. C., Alvarez, E. G., Baez-Ortega, A., Beroukhim, R., Boutros, P. C., Bowtell, D. D. L., Brors, B., Burns, K. H., Campbell, P. J., … von Mering, C. (2020). Comprehensive analysis of chromothripsis in 2,658 human cancers using whole-genome sequencing. Nature Genetics 2020 52:3, 52(3), 331–341. https://doi.org/10.1038/s41588-019-0576-7

Crasta, K., Ganem, N. J., Dagher, R., Lantermann, A. B., Ivanova, E. V., Pan, Y., Nezi, L., Protopopov, A., Chowdhury, D., & Pellman, D. (2012). DNA breaks and chromosome pulverization from errors in mitosis. Nature, 482(7383), 53–58. https://doi.org/10.1038/NATURE10802

Cuadrado, A., & Losada, A. (2020). Specialized functions of cohesins STAG1 and STAG2 in 3D genome architecture. Current Opinion in Genetics & Development, 61, 9–16. https://doi.org/10.1016/J.GDE.2020.02.024

D

De Koninck, M., & Losada, A. (2016). Cohesin Mutations in Cancer. Cold Spring Harbor Perspectives in Medicine, 6(12). https://doi.org/10.1101/CSHPERSPECT.A026476

Decout, A., Katz, J. D., Venkatraman, S., & Ablasser, A. (2021). The cGAS-STING pathway as a therapeutic target in inflammatory diseases. Nature Reviews. Immunology, 21(9), 548–569. https://doi.org/10.1038/S41577-021-00524-Z

Dhital, B., & Rodriguez-Bravo, V. (2023). Mechanisms of chromosomal instability (CIN) tolerance in aggressive tumors: surviving the genomic chaos. Chromosome Research : An International Journal on the Molecular, Supramolecular and Evolutionary Aspects of Chromosome Biology, 31(2). https://doi.org/10.1007/S10577-023-09724-W

Du, J. min, Qian, M. jia, Yuan, T., Chen, R. han, He, Q. jun, Yang, B., Ling, Q., & Zhu, H. (2022). cGAS and cancer therapy: a double-edged sword. Acta Pharmacologica Sinica 2021 43:9, 43(9), 2202–2211. https://doi.org/10.1038/s41401-021-00839-6

E

Engeland, K. (2022). Cell cycle regulation: p53-p21-RB signaling. Cell Death & Differentiation 2022 29:5, 29(5), 946–960. https://doi.org/10.1038/s41418-022-00988-z

Erhardt, K., Silfversward, C., & Auer, G. (1989). Nuclear DNA content and nuclear atypia. Relation to survival in patients with breast adenocarcinoma and serious ovarian tumors. Anticancer Research, 9(5), 1325–1330. https://europepmc.org/article/med/2589823

F

Feijoo, P., Dominguez, D., Tusell, L., & Genesca, A. (2014). Telomere-dependent genomic integrity: evolution of the fusion-bridge-breakage cycle concept. Current Pharmaceutical Design, 20(41), 6375–6385. https://doi.org/10.2174/1381612820666140630085416

Fenech, M., & Bonassi, S. (2011). The effect of age, gender, diet and lifestyle on DNA damage measured using micronucleus frequency in human peripheral blood lymphocytes. Mutagenesis, 26(1), 43–49. https://doi.org/10.1093/MUTAGE/GEQ050

Finardi, A., Massari, L. F., & Visintin, R. (2020). Anaphase Bridges: Not All Natural Fibers Are Healthy. Genes, 11(8), 1–29. https://doi.org/10.3390/GENES11080902

Fito-Lopez, B., Salvadores, M., Alvarez, M. M., & Supek, F. (2023). Prevalence, causes and impact of TP53-loss phenocopying events in human tumors. BMC Biology, 21(1), 1–25. https://doi.org/10.1186/S12915-023-01595-1/FIGURES/5

Flynn, P. J., Koch, P. D., & Mitchison, T. J. (2021). Chromatin bridges, not micronuclei, activate cGAS after drug-induced mitotic errors in human cells. Proceedings of the National Academy of Sciences of the United States of America, 118(48), e2103585118. https://doi.org/10.1073/PNAS.2103585118/SUPPL_FILE/PNAS.2103585118.SAPP.PDF

Freeman, A. D. J., Declais, A. C., Wilson, T. J., & Lilley, D. M. J. (2023). Biochemical and mechanistic analysis of the cleavage of branched DNA by human ANKLE1. Nucleic Acids Research, 51(11), 5743–5754. https://doi.org/10.1093/NAR/GKAD416

G

Gaillard, H., García-Muse, T., & Aguilera, A. (2015). Replication stress and cancer. Nature Reviews. Cancer, 15(5), 276–280. https://doi.org/10.1038/NRC3916

Gallina, I., Christiansen, S. K., Pedersen, R. T., Lisby, M., & Oestergaard, V. H. (2016). TopBP1-mediated DNA processing during mitosis. Cell Cycle (Georgetown, Tex.), 15(2), 176–183. https://doi.org/10.1080/15384101.2015.1128595

Ganem, N. J., Cornils, H., Chiu, S. Y., O'Rourke, K. P., Arnaud, J., Yimlamai, D., Théry, M., Camargo, F. D., & Pellman, D. (2014). Cytokinesis Failure Triggers Hippo Tumor Suppressor Pathway Activation. Cell, 158(4), 833. https://doi.org/10.1016/J.CELL.2014.06.029

Ganem, N. J., Godinho, S. A., & Pellman, D. (2009). A mechanism linking extra centrosomes to chromosomal instability. Nature 2009 460:7252, 460(7252), 278–282. https://doi.org/10.1038/nature08136

Ganem, N. J., Storchova, Z., & Pellman, D. (2007). Tetraploidy, aneuploidy and cancer. Current Opinion in Genetics & Development, 17(2), 157–162. https://doi.org/10.1016/J.GDE.2007.02.011

Gisselsson, D., Björk, J., Höglund, M., Mertens, F., Dal Cin, P., Åkerman, M., & Mandahl, N. (2001). Abnormal nuclear shape in solid tumors reflects mitotic instability. The American Journal of Pathology, 158(1), 199–206. https://doi.org/10.1016/S0002-9440(10)63958-2

Gisselsson, D., Jonson, T., Yu, C., Martins, C., Mandahl, N., Wiegant, J., Jin, Y., Mertens, F., & Jin, C. (2002). Centrosomal abnormalities, multipolar mitoses, and chromosomal instability in head and neck tumours with dysfunctional telomeres. British Journal of Cancer 2002 87:2, 87(2), 202–207. https://doi.org/10.1038/sj.bjc.6600438

Giunta, S., Belotserkovskaya, R., & Jackson, S. P. (2010). DNA damage signaling in response to double-strand breaks during mitosis. The Journal of Cell Biology, 190(2), 197. https://doi.org/10.1083/JCB.200911156

Giunta, S., & Jackson, S. P. (2011). Give me a break, but not in mitosis: The mitotic DNA damage response marks DNA double strand breaks with early signaling events. Cell Cycle, 10(8), 1215–1221. https://doi.org/10.4161/CC.10.8.15334

Gizem Sonugür, F., & Akbulut, H. (2019). The Role of Tumor Microenvironment in Genomic Instability of Malignant Tumors. Frontiers in Genetics, 10(OCT). https://doi.org/10.3389/FGENE.2019.01063

Gkretsi, V., & Stylianopoulos, T. (2018). Cell Adhesion and Matrix Stiffness: Coordinating Cancer Cell Invasion and Metastasis. Frontiers in Oncology, 8(MAY), 145. https://doi.org/10.3389/FONC.2018.00145

Griffin, C. S. (2002). Aneuploidy, centrosome activity and chromosome instability in cells deficient in homologous recombination repair. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 504(1–2), 149–155. https://doi.org/10.1016/S0027-5107(02)00088-X

Griffin, C. S., Simpson, P. J., Wilson, C. R., & Thacker, J. (2000). Mammalian recombination-repair genes XRCC2 and XRCC3 promote correct chromosome segregation. Nature Cell Biology, 2(10), 757–761. https://doi.org/10.1038/35036399

Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H., & De Lange, T. (1999). Mammalian telomeres end in a large duplex loop. Cell, 97(4), 503–514. https://doi.org/10.1016/S0092-8674(00)80760-6

Groelly, F. J., Fawkes, M., Dagg, R. A., Blackford, A. N., & Tarsounas, M. (2023). Targeting DNA damage response pathways in cancer. Nature Reviews. Cancer, 23(2), 78–94. https://doi.org/10.1038/S41568-022-00535-5

Guacci, V., Koshland, D., & Strunnikov, A. (1997). A Direct Link between Sister Chromatid Cohesion and Chromosome Condensation Revealed through the Analysis of MCD1 in S. cerevisiae. Cell, 91(1), 47. https://doi.org/10.1016/S0092-8674(01)80008-8

Guey, B., Wischnewski, M., Decout, A., Makasheva, K., Kaynak, M., Saka, M. S., Fierz, B., & Ablasser, A. (2020). BAF restricts cGAS on nuclear DNA to prevent innate immune activation. Science (New York, N.Y.), 369(6505), 823–828. https://doi.org/10.1126/SCIENCE.AAW6421

H

Halfmann, C. T., Sears, R. M., Katiyar, A., Busselman, B. W., Aman, L. K., Zhang, Q., O'Bryan, C. S., Angelini, T. E., Lele, T. P., & Roux, K. J. (2019). Repair of nuclear ruptures requires barrier-to-autointegration factor. The Journal of Cell Biology, 218(7), 2136–2149. https://doi.org/10.1083/JCB.201901116

Hammel, M., Yu, Y., Mahaney, B. L., Cai, B., Ye, R., Phipps, B. M., Rambo, R. P., Hura, G. L., Pelikan, M., So, S., Abolfath, R. M., Chen, D. J., Lees-Miller, S. P., & Tainer, J. A. (2010). Ku and DNA-dependent protein kinase dynamic conformations and assembly regulate DNA binding and the initial non-homologous end joining complex. The Journal of Biological Chemistry, 285(2), 1414–1423. https://doi.org/10.1074/JBC.M109.065615

Hanahan, D. (2022). Hallmarks of Cancer: New Dimensions. Cancer Discovery, 12(1), 31–46. https://doi.org/10.1158/2159-8290.CD-21-1059

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. Cell, 100(1), 57–70. https://doi.org/10.1016/S0092-8674(00)81683-9

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. Cell, 144(5), 646–674. https://doi.org/10.1016/J.CELL.2011.02.013

Hatch, E. M., Fischer, A. H., Deerinck, T. J., & Hetzer, M. W. (2013). Catastrophic Nuclear Envelope Collapse in Cancer Cell Micronuclei. Cell, 154(1), 47. https://doi.org/10.1016/j.cell.2013.06.007

Hatch, E. M., & Hetzer, M. W. (2016). Nuclear envelope rupture is induced by actin-based nucleus confinement. Journal of Cell Biology, 215(1), 27–36. https://doi.org/10.1083/JCB.201603053/VIDEO-7

Hayflick, L. (1965). THE LIMITED IN VITRO LIFETIME OF HUMAN DIPLOID CELL STRAINS. Experimental Cell Research, 37(3), 614–636. https://doi.org/10.1016/0014-4827(65)90211-9

Her, J., & Bunting, S. F. (2018). How cells ensure correct repair of DNA double-strand breaks. The Journal of Biological Chemistry, 293(27), 10502–10511. https://doi.org/10.1074/JBC.TM118.000371

Holland, A. J., & Cleveland, D. W. (2012). Losing balance: The origin and impact of aneuploidy in cancer. EMBO Reports, 13(6), 501–514. https://doi.org/10.1038/EMBOR.2012.55

J

Jackson, S. P., & Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature, 461(7267), 1071–1078. https://doi.org/10.1038/NATURE08467

Janssen, A., Van Der Burg, M., Szuhai, K., Kops, G. J. P. L., & Medema, R. H. (2011). Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. Science (New York, N.Y.), 333(6051), 1895–1898. https://doi.org/10.1126/SCIENCE.1210214

Jiang, H., Kong, N., Liu, Z., West, S. C., & Chan, Y. W. (2023). Human Endonuclease ANKLE1 Localizes at the Midbody and Processes Chromatin Bridges to Prevent DNA Damage and cGAS-STING Activation. Advanced Science (Weinheim, Baden-Wurttemberg, Germany), 10(12). https://doi.org/10.1002/ADVS.202204388

Jin, Y., Stewénius, Y., Lindgren, D., Frigyesi, A., Calcagnile, O., Jonson, T., Edqvist, A., Larsson, N., Lundberg, L. M., Chebil, G., Liedberg, F., Gudjonsson, S., Månsson, W., Höglund, M., & Gisselsson, D. (2007). Distinct mitotic segregation errors mediate chromosomal instability in aggressive urothelial cancers. Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 13(6), 1703–1712. https://doi.org/10.1158/1078-0432.CCR-06-2705

K

Kaitna, S., Pasierbek, P., Jantsch, M., Loidl, J., & Glotzer, M. (2002). The aurora B kinase AIR-2 regulates kinetochores during mitosis and is required for separation of homologous Chromosomes during meiosis. Current Biology : CB, 12(10), 798–812. https://doi.org/10.1016/S0960-9822(02)00820-5

Kandoth, C., McLellan, M. D., Vandin, F., Ye, K., Niu, B., Lu, C., Xie, M., Zhang, Q., McMichael, J. F., Wyczalkowski, M. A., Leiserson, M. D. M., Miller, C. A., Welch, J. S., Walter, M. J., Wendl, M. C., Ley, T. J., Wilson, R. K., Raphael, B. J., & Ding, L. (2013). Mutational landscape and significance across 12 major cancer types. Nature 2013 502:7471, 502(7471), 333–339. https://doi.org/10.1038/nature12634

Karlseder, J., Smogorzewska, A., & De Lange, T. (2002). Senescence induced by altered telomere state, not telomere loss. Science (New York, N.Y.), 295(5564), 2446–2449. https://doi.org/10.1126/SCIENCE.1069523

Kelleher, C., Kurth, I., & Lingner, J. (2005). Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. Molecular and Cellular Biology, 25(2), 808–818. https://doi.org/10.1128/MCB.25.2.808-818.2005

Krupina, K., Goginashvili, A., & Cleveland, D. W. (2021). Causes and consequences of micronuclei. Current Opinion in Cell Biology, 70, 91–99. https://doi.org/10.1016/J.CEB.2021.01.004

Kujirai, T., Zierhut, C., Takizawa, Y., Kim, R., Negishi, L., Uruma, N., Hirai, S., Funabiki, H., & Kurumizaka, H. (2020a). Structural basis for the inhibition of cGAS by nucleosomes. Science (New York, N.Y.), 370(6515). https://doi.org/10.1126/SCIENCE.ABD0237

Kujirai, T., Zierhut, C., Takizawa, Y., Kim, R., Negishi, L., Uruma, N., Hirai, S., Funabiki, H., & Kurumizaka, H. (2020b). Structural basis for the inhibition of cGAS by nucleosomes. Science (New York, N.Y.), 370(6515), 455. https://doi.org/10.1126/SCIENCE.ABD0237

L

Lecona, E., & Fernández-Capetillo, O. (2014). Replication stress and cancer: it takes two to tango. Experimental Cell Research, 329(1), 26–34. https://doi.org/10.1016/J.YEXCR.2014.09.019

Leibowitz, M. L., Zhang, C. Z., & Pellman, D. (2015). Chromothripsis: A New Mechanism for Rapid Karyotype Evolution. Annual Review of Genetics, 49, 183–211. https://doi.org/10.1146/ANNUREV-GENET-120213-092228

Leimbacher, P. A., Jones, S. E., Shorrocks, A. M. K., de Marco Zompit, M., Day, M., Blaauwendraad, J., Bundschuh, D., Bonham, S., Fischer, R., Fink, D., Kessler, B. M., Oliver, A. W., Pearl, L. H., Blackford, A. N., & Stucki, M. (2019). MDC1 Interacts with TOPBP1 to Maintain Chromosomal Stability during Mitosis. Molecular Cell, 74(3), 571-583.e8. https://doi.org/10.1016/J.MOLCEL.2019.02.014

Levine, A. J., & Oren, M. (2009). The first 30 years of p53: growing ever more complex. Nature Reviews. Cancer, 9(10), 749–758. https://doi.org/10.1038/NRC2723

Li, M., Fang, X., Wei, Z., York, J. P., & Zhang, P. (2009). Loss of spindle assembly checkpoint–mediated inhibition of Cdc20 promotes tumorigenesis in mice. The Journal of Cell Biology, 185(6), 983. https://doi.org/10.1083/JCB.200904020

Lindahl, T., & Barnes, D. E. (2000). Repair of endogenous DNA damage. Cold Spring Harbor Symposia on Quantitative Biology, 65, 127–133. https://doi.org/10.1101/SQB.2000.65.127

Lingle, W. L., Barrett, S. L., Negron, V. C., D'Assoro, A. B., Boeneman, K., Liu, W., Whitehead, C. M., Reynolds, C., & Salisbury, J. L. (2002). Centrosome amplification drives chromosomal instability in breast tumor development. Proceedings of the National Academy of Sciences of the United States of America, 99(4), 1978–1983. https://doi.org/10.1073/PNAS.032479999

Litwin, I., Pilarczyk, E., & Wysocki, R. (2018). The Emerging Role of Cohesin in the DNA Damage Response. Genes, 9(12). https://doi.org/10.3390/GENES9120581

Liu, S., Kwon, M., Mannino, M., Yang, N., Renda, F., Khodjakov, A., & Pellman, D. (2018). Nuclear envelope assembly defects link mitotic errors to chromothripsis. Nature, 561(7724), 551–555. https://doi.org/10.1038/S41586-018-0534-Z

Liu, S., & Pellman, D. (2020). The coordination of nuclear envelope assembly and chromosome segregation in metazoans. Nucleus (Austin, Tex.), 11(1), 35–52. https://doi.org/10.1080/19491034.2020.1742064

Loayza, D., & De Lange, T. (2003). POT1 as a terminal transducer of TRF1 telomere length control. Nature, 423(6943), 1013–1018. https://doi.org/10.1038/NATURE01688

López-García, C., Sansregret, L., Domingo, E., McGranahan, N., Hobor, S., Birkbak, N. J., Horswell, S., Grönroos, E., Favero, F., Rowan, A. J., Matthews, N., Begum, S., Phillimore, B., Burrell, R., Oukrif, D., Spencer-Dene, B., Kovac, M., Stamp, G., Stewart, A., … Swanton, C. (2017). BCL9L Dysfunction Impairs Caspase-2 Expression Permitting Aneuploidy Tolerance in Colorectal Cancer. Cancer Cell, 31(1), 79. https://doi.org/10.1016/J.CCELL.2016.11.001

Lukow, D. A., Sausville, E. L., Suri, P., Chunduri, N. K., Wieland, A., Leu, J., Smith, J. C., Girish, V., Kumar, A. A., Kendall, J., Wang, Z., Storchova, Z., & Sheltzer, J. M. (2021). Chromosomal instability accelerates the evolution of resistance to anti-cancer therapies. Developmental Cell, 56(17), 2427-2439.e4. https://doi.org/10.1016/J.DEVCEL.2021.07.009

M

Maciejowski, J., & De Lange, T. (2017). Telomeres in cancer: tumour suppression and genome instability. Nature Reviews. Molecular Cell Biology, 18(3), 175–186. https://doi.org/10.1038/NRM.2016.171

Maciejowski, J., Li, Y., Bosco, N., Campbell, P. J., & De Lange, T. (2015). Chromothripsis and kataegis induced by telomere crisis. Cell, 163(7), 1641. https://doi.org/10.1016/J.CELL.2015.11.054

MacKenzie, K. J., Carroll, P., Martin, C. A., Murina, O., Fluteau, A., Simpson, D. J., Olova, N., Sutcliffe, H., Rainger, J. K., Leitch, A., Osborn, R. T., Wheeler, A. P., Nowotny, M., Gilbert, N., Chandra, T., Reijns, M. A. M., & Jackson, A. P. (2017). cGAS surveillance of micronuclei links genome instability to innate immunity. Nature, 548(7668), 461. https://doi.org/10.1038/NATURE23449

Makarov, V. L., Hirose, Y., & Langmore, J. P. (1997). Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. Cell, 88(5), 657–666. https://doi.org/10.1016/S0092-8674(00)81908-X

Martín, M., Genescà, A., Latre, L., Jaco, I., Taccioli, G. E., Egozcue, J., Blasco, M. A., Iliakis, G., & Tusell, L. (2005). Postreplicative joining of DNA double-strand breaks causes genomic instability in DNA-PKcs-deficient mouse embryonic fibroblasts. Cancer Research, 65(22), 10223–10232. https://doi.org/10.1158/0008-5472.CAN-05-0932

Martín, M., Terradas, M., Tusell, L., & Genescà, A. (2012). ATM and DNA-PKcs make a complementary couple in DNA double strand break repair. Mutation Research/Reviews in Mutation Research, 751(1), 29–35. https://doi.org/10.1016/J.MRREV.2011.12.006

Martincorena, I., & Campbell, P. J. (2015). Somatic mutation in cancer and normal cells. Science (New York, N.Y.), 349(6255), 1483–1489. https://doi.org/10.1126/SCIENCE.AAB4082

Martino, F., Perestrelo, A. R., Vinarský, V., Pagliari, S., & Forte, G. (2018). Cellular mechanotransduction: From tension to function. Frontiers in Physiology, 9(JUL), 378185. https://doi.org/10.3389/FPHYS.2018.00824/BIBTEX

McClintock, B. (1941). The Stability of Broken Ends of Chromosomes in Zea Mays. Genetics, 26(2), 234. https://doi.org/10.1093/GENETICS/26.2.234

Merino-Casallo, F., Gomez-Benito, M. J., Hervas-Raluy, S., & Garcia-Aznar, J. M. (2022). Unravelling cell migration: defining movement from the cell surface. Cell Adhesion & Migration, 16(1), 25–64. https://doi.org/10.1080/19336918.2022.2055520

Michaelis, C., Ciosk, R., & Nasmyth, K. (1997). Cohesins: Chromosomal Proteins that Prevent Premature Separation of Sister Chromatids. Cell, 91(1), 35–45. https://doi.org/10.1016/S0092-8674(01)80007-6

Michel, L. S., Liberal, V., Chatterjee, A., Kirchwegger, R., Pasche, B., Gerald, W., Dobles, M., Sorger, P. K., Murty, V. V. V. S., & Benezra, R. (2001). MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. Nature, 409(6818), 355–359. https://doi.org/10.1038/35053094

Mohr, L., Toufektchan, E., von Morgen, P., Chu, K., Kapoor, A., & Maciejowski, J. (2021). ER-directed TREX1 limits cGAS activation at micronuclei. Molecular Cell, 81(4), 724-738.e9. https://doi.org/10.1016/J.MOLCEL.2020.12.037

N

Neben, K., Glesecke, C., Schweizer, S., Ho, A. D., & Krämer, A. (2003). Centrosome aberrations in acute myeloid leukemia are correlated with cytogenetic risk profile. Blood, 101(1), 289–291. https://doi.org/10.1182/BLOOD-2002-04-1188

Negrini, S., Gorgoulis, V. G., & Halazonetis, T. D. (2010). Genomic instability — an evolving hallmark of cancer. Nature Reviews Molecular Cell Biology 2010 11:3, 11(3), 220–228. https://doi.org/10.1038/nrm2858

Nilsson, J., Yekezare, M., Minshull, J., & Pines, J. (2008). The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction. Nature Cell Biology, 10(12), 1411–1420. https://doi.org/10.1038/NCB1799

Normand, G., & King, R. W. (2010). Understanding Cytokinesis Failure. Advances in Experimental Medicine and Biology, 676, 27. https://doi.org/10.1007/978-1-4419-6199-0_3

Norppa, H., Bonassi, S., Hansteen, I. L., Hagmar, L., Strömberg, U., Rössner, P., Boffetta, P., Lindholm, C., Gundy, S., Lazutka, J., Cebulska-Wasilewska, A., Fabiánová, E., Šrám, R. J., Knudsen, L. E., Barale, R., & Fucic, A. (2006). Chromosomal aberrations and SCEs as biomarkers of cancer risk. Mutation Research, 600(1–2), 37–45. https://doi.org/10.1016/J.MRFMMM.2006.05.030

O

Otsuka, S., Bui, K. H., Schorb, M., Julius Hossain, M., Politi, A. Z., Koch, B., Eltsov, M., Beck, M., & Ellenberg, J. (2016). Nuclear pore assembly proceeds by an inside-out extrusion of the nuclear envelope. ELife, 5(September2016). https://doi.org/10.7554/ELIFE.19071

P

Pampalona, J., Roscioli, E., Silkworth, W. T., Bowden, B., Genescà, A., Tusell, L., & Cimini, D. (2016). Chromosome Bridges Maintain Kinetochore-Microtubule Attachment throughout Mitosis and Rarely Break during Anaphase. PloS One, 11(1). https://doi.org/10.1371/JOURNAL.PONE.0147420

Papanicolaou, G., and, H. T.-A. J. of O., & 1941, undefined. (1941). The diagnostic value of vaginal smears in carcinoma of the uterus. Medicine.Mcgill.CaGN Papanicolaou, HF TrautAmerican Journal of Obstetrics and Gynecology, 1941•medicine.Mcgill.Ca. https://doi.org/10.1016/S0002-9378(16)40621-6

Pickett-Heaps, J. D., Tippit, D. H., Cohn, S. A., & Spurck, T. P. (1986). Microtubule dynamics in the spindle. Theoretical aspects of assembly/disassembly reactions in vivo. Journal of Theoretical Biology, 118(2), 153–169. https://doi.org/10.1016/S0022-5193(86)80131-X

Przanowski, P., Przanowska, R. K., & Guertin, M. J. (2023). ANKLE1 cleaves mitochondrial DNA and contributes to cancer risk by promoting apoptosis resistance and metabolic dysregulation. Communications Biology 2023 6:1, 6(1), 1–13. https://doi.org/10.1038/s42003-023-04611-w

R

Rodriguez-Bravo, V., Maciejowski, J., Corona, J., Buch, H. K., Collin, P., Kanemaki, M. T., Shah, J. V., & Jallepalli, P. V. (2014). Nuclear pores protect genome integrity by assembling a premitotic and Mad1-dependent anaphase inhibitor. Cell, 156(5), 1017. https://doi.org/10.1016/J.CELL.2014.01.010

Rodriguez-Muñoz, M., Serrat, M., Soler, D., Genescà, A., & Anglada, T. (2021). Breakage of CRISPR/Cas9-Induced Chromosome Bridges in Mitotic Cells. Frontiers in Cell and Developmental Biology, 9. https://doi.org/10.3389/FCELL.2021.745195

Rogakou, E. P., Boon, C., Redon, C., & Bonner, W. M. (1999). Megabase chromatin domains involved in DNA double-strand breaks in vivo. The Journal of Cell Biology, 146(5), 905–915. https://doi.org/10.1083/JCB.146.5.905

Roylance, R., Endesfelder, D., Gorman, P., Burrell, R. A., Sander, J., Tomlinson, I., Hanby, A. M., Speirs, V., Richardson, A. L., Birkbak, N. J., Eklund, A. C., Downward, J., Kschischo, M., Szallasi, Z., & Swanton, C. (2011). Relationship of extreme chromosomal instability with long-term survival in a retrospective analysis of primary breast cancer. Cancer Epidemiology, Biomarkers & Prevention : A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology, 20(10), 2183–2194. https://doi.org/10.1158/1055-9965.EPI-11-0343

S

Saldivar, J. C., Cortez, D., & Cimprich, K. A. (2017). The essential kinase ATR: ensuring faithful duplication of a challenging genome. Nature Reviews Molecular Cell Biology 2017 18:10, 18(10), 622–636. https://doi.org/10.1038/nrm.2017.67

Samson, N., & Ablasser, A. (2022). The cGAS–STING pathway and cancer. Nature Cancer 2022 3:12, 3(12), 1452–1463. https://doi.org/10.1038/s43018-022-00468-w

Samwer, M., Schneider, M. W. G., Hoefler, R., Schmalhorst, P. S., Jude, J. G., Zuber, J., & Gerlich, D. W. (2017). DNA Cross-Bridging Shapes a Single Nucleus from a Set of Mitotic Chromosomes. Cell, 170(5), 956-972.e23. https://doi.org/10.1016/J.CELL.2017.07.038

Sansregret, L., Vanhaesebroeck, B., & Swanton, C. (2018). Determinants and clinical implications of chromosomal instability in cancer. Nature Reviews. Clinical Oncology, 15(3), 139–150. https://doi.org/10.1038/NRCLINONC.2017.198

Saxena, S., & Zou, L. (2022). Hallmarks of DNA replication stress. Molecular Cell, 82(12), 2298–2314. https://doi.org/10.1016/J.MOLCEL.2022.05.004

Scholey, J. M., Brust-Mascher, I., & Mogilner, A. (2003). Cell division. Nature, 422(6933), 746–752. https://doi.org/10.1038/NATURE01599

Schwertman, P., Bekker-Jensen, S., & Mailand, N. (2016). Regulation of DNA double-strand break repair by ubiquitin and ubiquitin-like modifiers. Nature Reviews. Molecular Cell Biology, 17(6), 379–394. https://doi.org/10.1038/NRM.2016.58

Sharpless, N. E., Ramsey, M. R., Balasubramanian, P., Castrillon, D. H., & DePinho, R. A. (2004). The differential impact of p16INK4a or p19ARF deficiency on cell growth and tumorigenesis. Oncogene, 23(2), 379–385. https://doi.org/10.1038/sj.onc.1207074

Shay, J. W., & Bacchetti, S. (1997). A survey of telomerase activity in human cancer. European Journal of Cancer (Oxford, England : 1990), 33(5), 787–791. https://doi.org/10.1016/S0959-8049(97)00062-2

Shay, J. W., & Wright, W. E. (2000). Hayflick, his limit, and cellular ageing. Nature Reviews. Molecular Cell Biology, 1(1), 72–76. https://doi.org/10.1038/35036093

Shen, Z. (2011). Genomic instability and cancer: an introduction. Journal of Molecular Cell Biology, 3(1), 1–3. https://doi.org/10.1093/JMCB/MJQ057

Silkworth, W. T., Nardi, I. K., Scholl, L. M., & Cimini, D. (2009). Multipolar Spindle Pole Coalescence Is a Major Source of Kinetochore Mis-Attachment and Chromosome Mis-Segregation in Cancer Cells. PLoS ONE, 4(8), 6564. https://doi.org/10.1371/JOURNAL.PONE.0006564

Singh, I., & Lele, T. P. (2022). Nuclear morphological abnormalities in cancer – a search for unifying mechanisms. Results and Problems in Cell Differentiation, 70, 443. https://doi.org/10.1007/978-3-031-06573-6_16

Siri, S. O., Martino, J., & Gottifredi, V. (2021). Structural Chromosome Instability: Types, Origins, Consequences, and Therapeutic Opportunities. Cancers, 13(12). https://doi.org/10.3390/CANCERS13123056

Sjöblom, T., Jones, S., Wood, L. D., Parsons, D. W., Lin, J., Barber, T. D., Mandelker, D., Leary, R. J., Ptak, J., Silliman, N., Szabo, S., Buckhaults, P., Farrell, C., Meeh, P., Markowitz, S. D., Willis, J., Dawson, D., Willson, J. K. V., Gazdar, A. F., … Velculescu, V. E. (2006). The consensus coding sequences of human breast and colorectal cancers. Science (New York, N.Y.), 314(5797), 268–274. https://doi.org/10.1126/SCIENCE.1133427

Song, J., Freeman, A. D. J., Knebel, A., Gartner, A., & Lilley, D. M. J. (2020). Human ANKLE1 Is a Nuclease Specific for Branched DNA. Journal of Molecular Biology, 432(21), 5825–5834. https://doi.org/10.1016/J.JMB.2020.08.022

Soto, M., García-Santisteban, I., Krenning, L., Medema, R. H., & Raaijmakers, J. A. (2018). Chromosomes trapped in micronuclei are liable to segregation errors. Journal of Cell Science, 131(13). https://doi.org/10.1242/JCS.214742

Soto, M., Raaijmakers, J. A., Bakker, B., Spierings, D. C. J., Lansdorp, P. M., Foijer, F., & Medema, R. H. (2017). p53 Prohibits Propagation of Chromosome Segregation Errors that Produce Structural Aneuploidies. Cell Reports, 19(12), 2423–2431. https://doi.org/10.1016/J.CELREP.2017.05.055

Steigemann, P., Wurzenberger, C., Schmitz, M. H. A., Held, M., Guizetti, J., Maar, S., & Gerlich, D. W. (2009). Aurora B-mediated abscission checkpoint protects against tetraploidization. Cell, 136(3), 473–484. https://doi.org/10.1016/J.CELL.2008.12.020

Stephens, P. J., Greenman, C. D., Fu, B., Yang, F., Bignell, G. R., Mudie, L. J., Pleasance, E. D., Lau, K. W., Beare, D., Stebbings, L. A., McLaren, S., Lin, M. L., McBride, D. J., Varela, I., Nik-Zainal, S., Leroy, C., Jia, M., Menzies, A., Butler, A. P., … Campbell, P. J. (2011). Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell, 144(1), 27–40. https://doi.org/10.1016/J.CELL.2010.11.055

Stucki, M., Clapperton, J. A., Mohammad, D., Yaffe, M. B., Smerdon, S. J., & Jackson, S. P. (2005). MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. Cell, 123(7), 1213–1226. https://doi.org/10.1016/J.CELL.2005.09.038

T

Terradas, M., Martín, M., Hernández, L., Tusell, L., & Genescà, A. (2012). Nuclear envelope defects impede a proper response to micronuclear DNA lesions. Mutation Research, 729(1–2), 35–40. https://doi.org/10.1016/J.MRFMMM.2011.09.003

Terradas, M., Martín, M., Tusell, L., & Genescà, A. (2010). Genetic activities in micronuclei: is the DNA entrapped in micronuclei lost for the cell? Mutation Research, 705(1), 60–67. https://doi.org/10.1016/J.MRREV.2010.03.004

Thompson, S. L., & Compton, D. A. (2011). Chromosome missegregation in human cells arises through specific types of kinetochore-microtubule attachment errors. Proceedings of the National Academy of Sciences of the United States ofAmerica,108(44),17974–17978. https://doi.org/10.1073/PNAS.1109720108/SUPPL_FILE/PNAS.201109720SI.PDF

Trojani, M., Contesso, G., Coindre, J. M., Rouesse, J., Bui, N. B., De Mascarel, A., Goussot, J. F., David, M., Bonichon, F., & Lagarde, C. (1984). Soft-tissue sarcomas of adults; study of pathological prognostic variables and definition of a histopathological grading system. International Journal of Cancer, 33(1), 37–42. https://doi.org/10.1002/IJC.2910330108

Tsegay, P. S., Lai, Y., & Liu, Y. (2019). Replication Stress and Consequential Instability of the Genome and Epigenome. Molecules (Basel, Switzerland), 24(21). https://doi.org/10.3390/MOLECULES24213870

Tucker, J. B., Bonema, S. C., García-Varela, R., Denu, R. A., Hu, Y., McGregor, S. M., Burkard, M. E., & Weaver, B. A. (2023). Misaligned Chromosomes are a Major Source of Chromosomal Instability in Breast Cancer. Cancer Research Communications, 3(1), 54. https://doi.org/10.1158/2767-9764.CRC-22-0302

Turajlic, S., Sottoriva, A., Graham, T., & Swanton, C. (2019). Resolving genetic heterogeneity in cancer. Nature Reviews. Genetics, 20(7), 404–416. https://doi.org/10.1038/S41576-019-0114-6

U

Umbreit, N. T., Zhang, C. Z., Lynch, L. D., Blaine, L. J., Cheng, A. M., Tourdot, R., Sun, L., Almubarak, H. F., Judge, K., Mitchell, T. J., Spektor, A., & Pellman, D. (2020). Mechanisms generating cancer genome complexity from a single cell division error. Science (New York, N.Y.), 368(6488). https://doi.org/10.1126/SCIENCE.ABA0712

V

Vacher-Lavenu, M. C., Le Tourneau, A., Duvillard, P., Godefroy, N., & Pinel, M. C. (1993). Pathological classification and grading of primary ovarian carcinoma: experience of the ARTAC ovarian study group. Bulletin Du Cancer, 80(2), 135–141. https://europepmc.org/article/med/8173164

Vietri, M., Schultz, S. W., Bellanger, A., Jones, C. M., Petersen, L. I., Raiborg, C., Skarpen, E., Pedurupillay, C. R. J., Kjos, I., Kip, E., Timmer, R., Jain, A., Collas, P., Knorr, R. L., Grellscheid, S. N., Kusumaatmaja, H., Brech, A., Micci, F., Stenmark, H., & Campsteijn, C. (2020). Unrestrained ESCRT-III drives micronuclear catastrophe and chromosome fragmentation. Nature Cell Biology, 22(7), 856–867. https://doi.org/10.1038/S41556-020-0537-5

W

Wang, Y., Sharpless, N., & Chang, S. (2013). p16INK4a protects against dysfunctional telomere–induced ATR-dependent DNA damage responses. The Journal of Clinical Investigation, 123(10), 4489. https://doi.org/10.1172/JCI69574

WHO. (2020). Cancer. https://www.who.int/news-room/fact-sheets/detail/cancer

Williams, A. B., & Schumacher, B. (2016). p53 in the DNA-Damage-Repair Process. Cold Spring Harbor Perspectives in Medicine, 6(5). https://doi.org/10.1101/CSHPERSPECT.A026070

Wood, L. D., Parsons, D. W., Jones, S., Lin, J., Sjöblom, T., Leary, R. J., Shen, D., Boca, S. M., Barber, T., Ptak, J., Silliman, N., Szabo, S., Dezso, Z., Ustyanksky, V., Nikolskaya, T., Nikolsky, Y., Karchin, R., Wilson, P. A., Kaminker, J. S., … Vogelstein, B. (2007). The genomic landscapes of human breast and colorectal cancers. Science (New York, N.Y.), 318(5853), 1108–1113. https://doi.org/10.1126/SCIENCE.1145720

X

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., & Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. Nature, 366(6456), 701–704. https://doi.org/10.1038/366701A0

Y

Yamamoto, A., & Capranico, G. (2021). Shake It Off: The Elimination of Erroneous Kinetochore-Microtubule Attachments and Chromosome Oscillation. International Journal of Molecular Sciences 2021, Vol. 22, Page 3174, 22(6), 3174. https://doi.org/10.3390/IJMS22063174

Young, A. M., Gunn, A. L., & Hatch, E. M. (2020). BAF facilitates interphase nuclear membrane repair through recruitment of nuclear transmembrane proteins. Molecular Biology of the Cell, 31(15), 1551–1560. https://doi.org/10.1091/MBC.E20-01-0009

Z

Zeman, M. K., & Cimprich, K. A. (2014). Causes and consequences of replication stress. Nature Cell Biology, 16(1), 2–9. https://doi.org/10.1038/NCB2897

Zerbib, J., Ippolito, M. R., Eliezer, Y., Feudis, G. De, Reuveni, E., Kadmon, A. S., Martin, S., Viganò, S., Leor, G., Berstler, J., Laue, K., Cohen-Sharir, Y., Scorzoni, S., Vazquez, F., Ben-David, U., & Santaguida, S. (2023). Human aneuploid cells depend on the RAF/MEK/ERK pathway for overcoming increased DNA damage. BioRxiv, 2023.01.27.525822. https://doi.org/10.1101/2023.01.27.525822

Zhang, C. Z., Spektor, A., Cornils, H., Francis, J. M., Jackson, E. K., Liu, S., Meyerson, M., & Pellman, D. (2015). Chromothripsis from DNA damage in micronuclei. Nature, 522(7555), 179–184. https://doi.org/10.1038/NATURE14493

Zhang, & Kschischo, M. (2022). Distinct and Common Features of Numerical and Structural Chromosomal Instability across Different Cancer Types. Cancers, 14(6). https://doi.org/10.3390/CANCERS14061424

Zhou, W., Mohr, L., Maciejowski, J., & Kranzusch, P. J. (2021). cGAS phase separation inhibits TREX1-mediated DNA degradation and enhances cytosolic DNA sensing. Molecular Cell, 81(4), 739. https://doi.org/10.1016/J.MOLCEL.2021.01.024

Zierhut, C., Yamaguchi, N., Paredes, M., Luo, J. D., Carroll, T., & Funabiki, H. (2019). The Cytoplasmic DNA Sensor cGAS Promotes Mitotic Cell Death. Cell, 178(2), 302-315.e23. https://doi.org/10.1016/J.CELL.2019.05.035

Zlopasa, L., Brachner, A., & Foisner, R. (2016). Nucleo-cytoplasmic shuttling of the endonuclease ankyrin repeats and LEM domain-containing protein 1 (Ankle1) is mediated by canonical nuclear export- and nuclear import signals. BMC Cell Biology, 17(1). https://doi.org/10.1186/S12860-016-0102-Z

Zou, L., & Elledge, S. J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science (New York, N.Y.), 300(5625), 1542–1548. https://doi.org/10.1126/SCIENCE.1083430