REPORT
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RESOLUTION OF THE DNA BRIDGES AND GENERATION OF GENOMIC INSTABILITY

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1. Project Summary

Chromosome bridges are common intermediaries in mechanisms that generate chromosomal instability. Although they are known to be involved in tumorigenesis and tumor progression, the processes governing the irresolution remain poorly understood. Diverse models have emerged regarding the irresolution; however, these models conflict with one another. Barbara McClintock proposed as early as 1941 that dicentric chromosomes form bridges and that these bridges can break during mitotic division (McClintock, 1941). Many years later, Janssen and colleagues suggested that chromatin pending segregation, such as that found in bridges and micronuclei, can be severed by compression forces during cytokinesis (Janssen et al., 2011). Finally, recent studies postulated that bridges do not break during mitosis but rather during interphase, either due to biochemical causes (Maciejowski et al., 2015) or mechanical causes (Umbreit et al., 2020). This research project 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 coordinated project, two research teams have participated, one led by Anna Genescà at Universitat Autònoma de Barcelona (UAB) and another led by Neil Ganem at Boston University (BU). We have used an experimental model based on the CRISPR/Cas9 technology to generate chromosome bridges with defined geometry under exquisite temporal control. Our joint study revealed that at the exit of mitosis, the resolution of chromosome bridges is intricately linked to the tension forces imposed by the microtubules of the mitotic spindle on the bridge kinetochores as 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 results in an increased frequency of chromosome bridges reaching 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 alternative mechanism for chromosome bridge resolution that operates during interphase relies on nuclear envelope rupture. Focal adhesions anchoring the bridge to the substrate combined with independent migration of daughter cells results in DNA breakage and nuclear envelope rupture, allowing action of the cytoplasmic

exonucleases, such as TREX1 for the resolution of chromosome bridges at this stage of the cell cycle. Altogether, the findings of this research project 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.

2. Results

2.1. Chromosome bridge resolution during mitosis

The results obtained by the UAB team 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 intact 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.

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 a subset of bridges is resolved during mitosis. Secondly, the mitotic cells examined frequently exhibited the DNA DSB marker γH2AX flanking disrupted chromatin of the bridges demonstrating that microscopically visible discontinuities 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 at the exit of mitosis the DNA flanking discontinuities in chromosome bridges 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 asserted by others, our observations firmly demonstrate the resolution of chromosome bridges during mitosis.

To further investigate the causes of chromosome bridge breakage during mitosis the UAB group employed CRISPR/Cas9 technology to induce DNA breaks at selected sites of the genome and thus generate chromosomal bridges with defined intercentromeric length in megabases. Using this approach, five different sgRNA cell lines were generated. We observed that the minimum separation between kinetochores required for bridge breakage was characteristic of each cell line and correlated with the length of their bridging chromatin. Consequently, a larger distance in base pairs between bridge centromeres increases the probability of the bridge remaining intact, leading to the birth of daughter cells with a bridge. Taken together, our results indicate that the breakage of chromosome bridges upon mitotic exit may be attributed to 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 leading to breakage. Furthermore, this tension is influenced by the distance between centromeres in base pairs. This correlation aligns with physical principles, as longer chromosome bridges would require greater separation to accumulate enough tension from microtubules attached to bridge kinetochores to break the bridge.

2.2. Unresolved chromosome bridges activate the p53 pathway but not the Hippo pathway

Cells with chromosome bridges often delay the completion of cytokinesis until the bridge is resolved; however, bridges that are not resolved in a timely manner promote cytokinetic furrow regression, cytokinesis failure, and tetraploidy. The Hippo tumor suppressor pathway is activated following cytokinesis failure and limits proliferation of the resulting pro-tumorigenic tetraploid cells. The BU team participating in this research project sought to determine whether unresolved chromosome bridges induce activation of the Hippo tumor suppressor pathway. Our data demonstrate that cells with chromosome bridges, either unbroken or broken, do not activate the Hippo pathway, as there is no difference in the nuclear: cytoplasmic ratio of YAP in these cells relative to controls.

The BU team also assessed whether cells with unbroken chromosome bridges activate the p53 pathway. It is well known that myriad cell stress, such as DNA damage and tetraploidy, stabilize p53 which leads to cell cycle arrest. We induced chromosome bridges using the system described above and then stained the resulting daughter cells for p53. Nuclear p53 levels were then quantified in cells with and without bridges. We found that unbroken chromosome bridges induce a statistically significant increase in p53 levels. These data indicate that unbroken bridges emit a stress signal to p53 that not depends on Hippo pathway activation.

2.3. RNAi screen to identify exo- and endonucleases that promote chromosome bridge breakage

We observed by live-cell imaging that chromosome bridges break in the following cell cycle after they are generated. One model posits that this breakage is due entirely to mechanical pulling forces from cells migrating apart from one another, which causes the bridge to stretch and ultimately break. However, a non-mutually exclusive model is that this breakage is also facilitated by the action of exo- and endonucleases in the cell, which gain access to the chromosomal DNA in the bridge following nuclear envelope rupture. To test this second model, the BU team performed a live-cell imaging-based siRNA screen targeting 38 human exo- and endonucleases. We hypothesized that specific exo- and endonucleases are important for promoting bridge breakage. If true, loss of these nucleases would be expected to significantly increase the amount of time it takes bridges to break, thus increasing the fraction of cells with chromosome bridges. Our data indicated that depletion of several nucleases significantly increased the fraction of cells with chromosome bridges, relative to controls.

Among all the nucleases tested, the endonuclease ANKLE1 was identified as potentially involved in the resolution of chromosome bridges since ANKLE1 inhibited cells showed and increased frequency of chromosome bridges in interphase cells after CRISPR/Cas9 induction as compared to the non-inhibited cells.

2.4. Chromosome bridge resolution during cytokinesis and early interphase

The distinct scenarios observed during mitosis and cytokinesis suggest that the causes of chromosome bridge breakage might be different. 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 cytokinesis, which includes specification of the cleavage plane, ingression of the cleavage furrow, formation of the midbody, and abscission. The endonuclease ANKLE1 might be responsible for chromosome bridge resolution during cytokinesis. Our conclusion is based on the observation that inhibition of ANKLE1 led to a higher frequency of cells reaching the interphase with chromosome bridges, indicating a role of ANKLE1 in chromosome bridge resolution prior to interphase. BU results align with those reported by other studies that suggested that the *C. elegans* ortholog of ANKLE1 resolve chromatin bridges (Hong et al., 2018), and that ANKLE1 is recruited at the midbody region to resolve trapped chromatin human cells (Jiang et al., 2023).Taken together, these findings strongly suggest that ANKLE1 can contribute to 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. A role for ANKLE1 during interphase is evident from the observation that the inhibition of ANKLE1 or impediment of its recruitment reduces the fraction of bridges that resolve in a short time and through a mechanism independent of NER.ANKLE1 localize 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.

2.5. Chromosome bridge resolution during interphase

Cells harboring chromosome bridges can progress through the interphase of the cell cycle and frequently exhibit a collapse of the nuclear envelope surrounding the bridge. 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, the bridges undergo NER, yet the cells continue migrating. Suddenly the bridge becomes discontinuous. Although NER precedes bridge breakage, the timing of NER occurrence

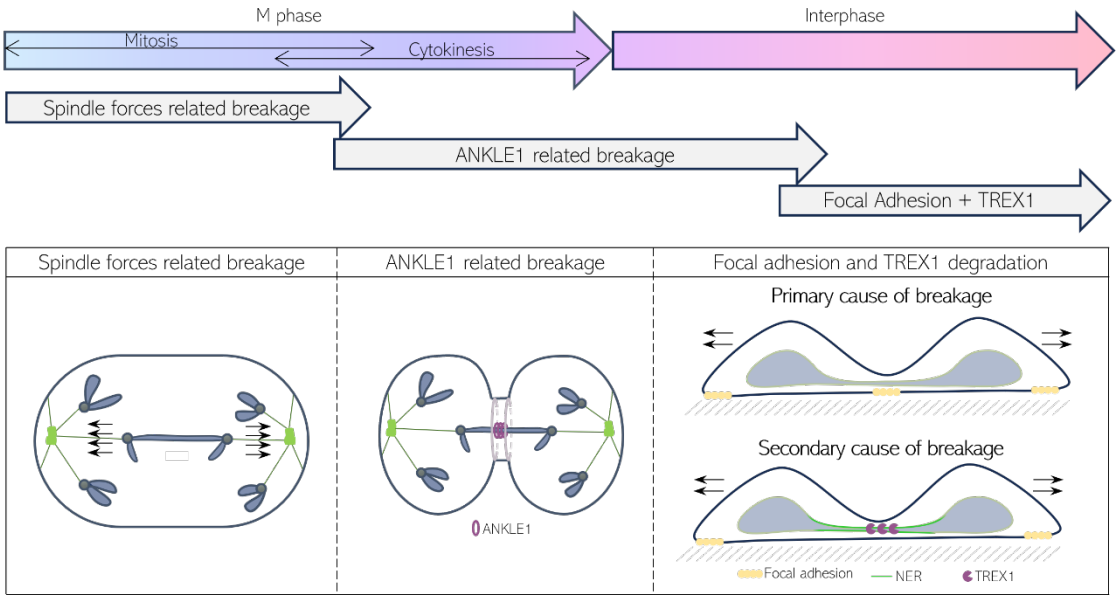
does not correlate with the moment of bridge breakage, indicating a potential facilitating role of NER in the breakage event.

During the interphase of the cell cycle, the resolution of chromosome bridges depends on their anchorage to the substrate through focal adhesions. This anchorage, in turn, triggers NER and permits the recruitment of TREX1, a cytoplasmic exonuclease. This conclusion is based on four observations. Firstly, chromosome bridges often display paxillin, a key component of focal adhesions, indicating their anchorage to the substrate. Secondly, by reducing focal adhesion strength through inhibition of myosin light chain phosphorylation, both NER and bridge resolution are delayed. This demonstrates that NER and chromosome bridge resolution depend on the adhesion of the daughter cells to the substrate. Thirdly, TREX1 is recruited only to bridges exhibiting NER, thereby supporting the notion 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. Our result show that bridges that exhibit bright BAF are more likely to have DSBs than basal-BAF bridges. Given the association between bright-BAF and focal adhesion, it can be inferred that focal adhesion may contribute to facilitate the initial DNA nick required for TREX1 activity. Taken together, our observations suggest that migration apart of cells with chromosome bridges along with the presence of focal adhesion beneath bridging chromatin, may facilitate NER and breakage of the DNA molecule within the bridge. These events are crucial for recruiting the exonuclease TREX1 and for enabling the digestion of DNA within the chromosome bridges.

In summary, the resolution of chromosome bridges during interphase arises from the synergy between mechanical and biochemical processes. Cells harboring chromosome bridges in interphase behave as two separate entities, migrating apart from each other yet connected by the bridge, often anchored to the substrate through focal adhesion. This combination of events initiates bridge resolution during interphase and triggers NER. Subsequently, TREX1 is recruited to the bridge to digest the DNA within the ruptured nuclear envelope. Importantly, this exonuclease necessitates an initial break, which could potentially result from either focal adhesions or alternative mechanisms.

2.6. Dynamics of chromosome bridge resolution: Towards an integrative model

The prevailing understanding held that bridge resolution is driven by a single mechanism, either directed by TREX1 (Maciejowski et al., 2015) or stretching forces applied by the cytoskeleton (Umbreit et al., 2020). However, the results presented in this dissertation demonstrate that chromosome bridges are resolved through diverse mechanisms at different stages of the cell cycle. Consequently, we perceive resolution of chromosome bridges as a multifaceted process. Considering that bridge resolution occurs throughout the cell cycle, each stage tailored by unique environmental conditions, it is unsurprising that we observe a variety of mechanisms governing bridge resolution (Figure 8). During mitosis, breakage occurs primarily due to mechanical factors, driven by tensile forces exerted by the mitotic spindle. Additionally, some bridges persist beyond telophase, and the ANKLE1 endonuclease recruited during cytokinesis degrades chromosome bridges, resulting in bridge breakage. Finally, bridges that have not resolved either during mitosis due to mechanical causes or afterwards due to biochemical mechanisms may have a new opportunity to resolve during interphase. Despite being connected by the nucleoplasmic bridge, cells born with unresolved chromosome bridges migrate independently away from each other. In these circumstances, we understand that the resolution of chromosome bridges 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, facilitate the entry of TREX enzyme to complete the bridge resolution.



3. Relevance and potential futures implications

Understanding the different mechanisms by which bridges resolve is crucial as the potential outcomes resulting from these resolution mechanisms can vary significantly. We speculate that at the cellular level, the extent of the DNA damage may vary depending on the mechanism involved. Processes that act in a localized manner, such as microtubule pulling forces or ANKLE1 activity, may likely result in less damage compared to mechanisms involving enzymes like TREX1, which degrade 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, potentially triggering 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 lacking 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. Therefore, chromosome bridges with heightened BAF or processed by TREX1 might also impede 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 research project contributes toward resolving long-standing debates surrounding the resolution of chromosome bridges. This study unveils a model for chromosome bridge resolution, shedding light on both mechanical and biochemical processes that operate in a cell cycle-dependent manner. These findings hold significant implications for understanding genomic instability in cancer cells, in which the presence of chromosome bridges may contribute to cycles of DNA alterations culminating in chromosomal instability.

4. Generated scientific publications

Publications directly derived from the research project funded by La Marató de TV3:

1. A matter of wrapper: Defects in the nuclear envelope of lagging and bridging chromatin threatens genome integrity. Rodriguez-Muñoz M, Anglada T, Genescà A. Semin Cell Dev Biol. 2022 Mar;123:124-130. doi: 10.1016

2. Breakage of CRISPR/Cas9-Induced Chromosome Bridges in Mitotic Cells. Rodriguez-Muñoz M, Serrat M, Soler D, Genescà A, Anglada T. Front Cell Dev Biol. 2021 Sep 28;9:745195. doi: 10.3389/fcell.2021.745195.

3. Role of H4K16 acetylation in 53BP1 recruitment to double-strand break sites in in vitro aged cells. González-Bermúdez L, Genescà A, Terradas M, Martín M. Biogerontology. 2022 Aug;23(4):499-514. doi: 10.1007/s10522-022-09979-6.
Manuscripts ready for submission:

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

5. Unveiling Chromosome Bridge Resolution beyond Mitosis: Contribution of Physical- and Biochemical-based mechanisms
Manuscripts in preparation:

6. Unresolved chromosome bridges activate the p53 pathway but not the Hippo pathway

Presentations at scientific conferences directly derived from the research project:

1. Resolution of DNA bridges: stage-dependent mechanisms. Rodríguez-Muñoz M, Serrat M, Soler D, Anglada T, Genescà A. VI Jornades de BioRecerca. Facultat de Biociències - Universitat Autònoma de Barcelona. June 2021

2. Breakage of chromosome bridges during mitosis: Relevance of mechanical stress. Rodríguez-Muñoz M, Serrat M, Anglada T, Genescà A. The Consequences of Aneuploidy Conference. Massachusetts, 11-16 Sept 2022

3. Breakage of chromosome bridges during mitosis: relevance of mechanical stress. Rodríguez-Muñoz M, Serrat M, Anglada T, Genescà A. VIII Jornades de BioRecerca. Facultat de Biociències – Universitat Autònoma de Barcelona. 15 June 2023

4. Multiwalled Carbon Nanotubes, a physical barrier to cell division. Pulido N, Rodríguez-Muñoz M, Anglada T, Genescà A. VIII Jornades de BioRecerca. Facultat de Biociències – Universitat Autònoma de Barcelona. 15 June 2023.