Look-Seq

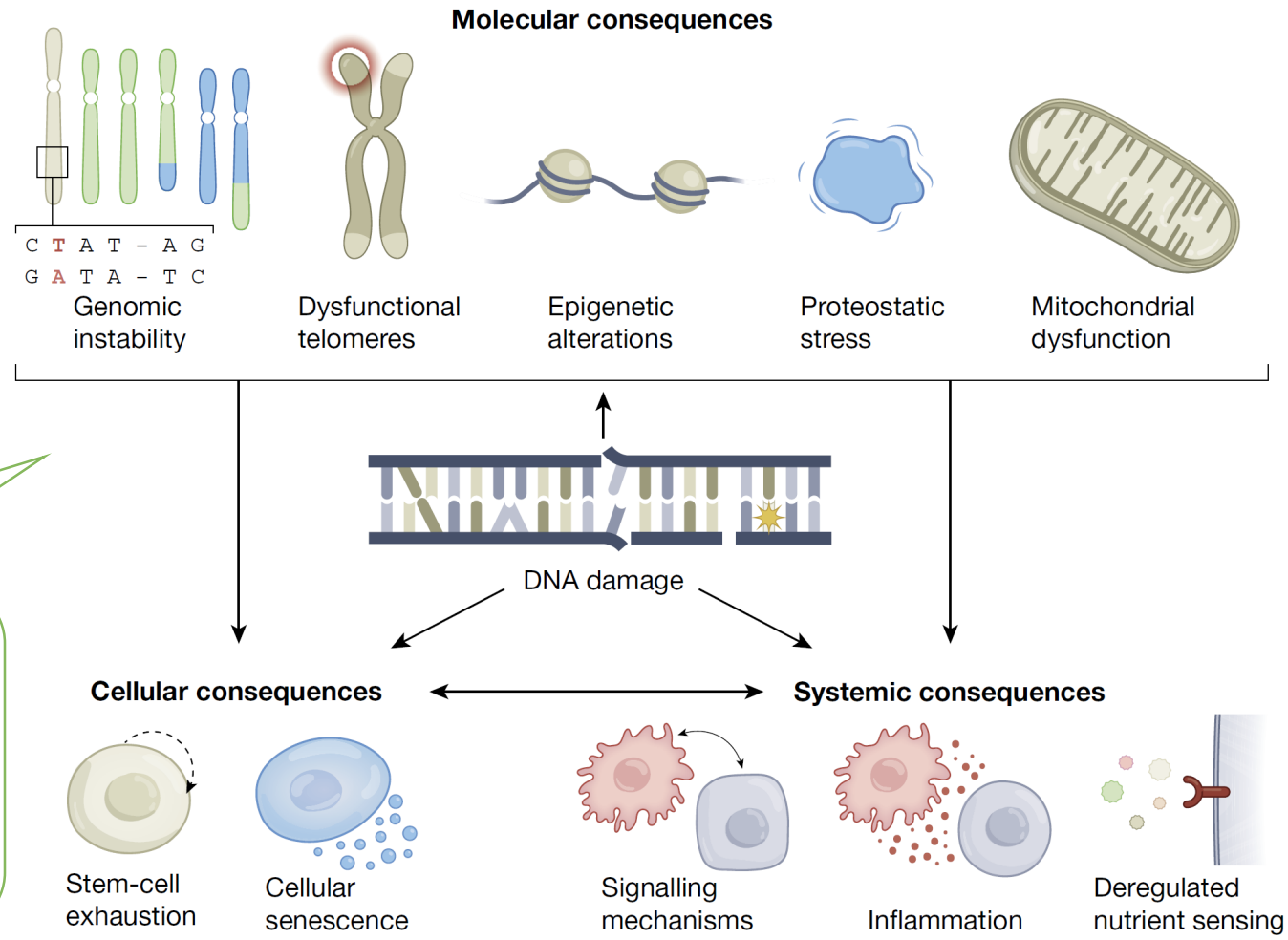
Watching genomic catastrophe unfold

Technical Journal Club

Lukas Frick

25 May 2021

DNA damage
is a root cause
of both cancer
and **ageing**



Review

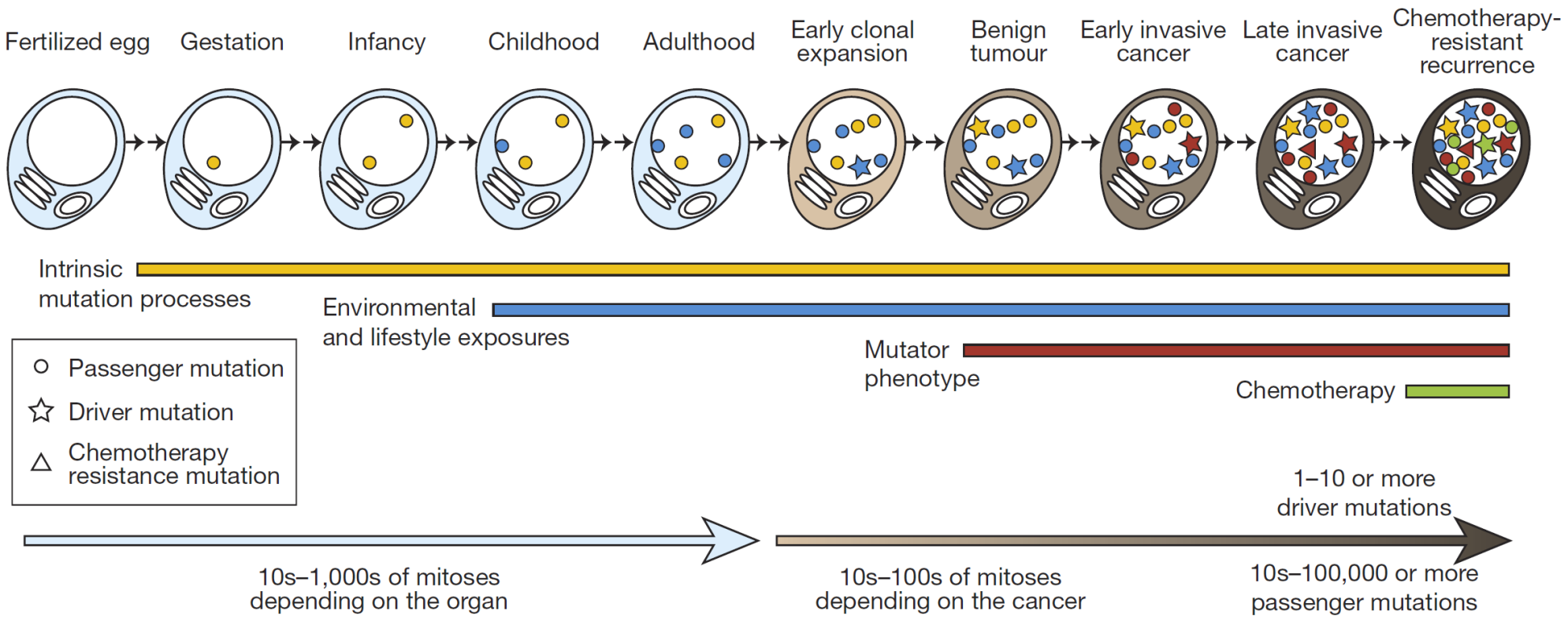
The central role of DNA damage in the ageing process

Björn Schumacher[1,2✉], Joris Pothof[3], Jan Vijg[4,5] & Jan H. J. Hoeijmakers[1,2,3,6]

Nature, 2021

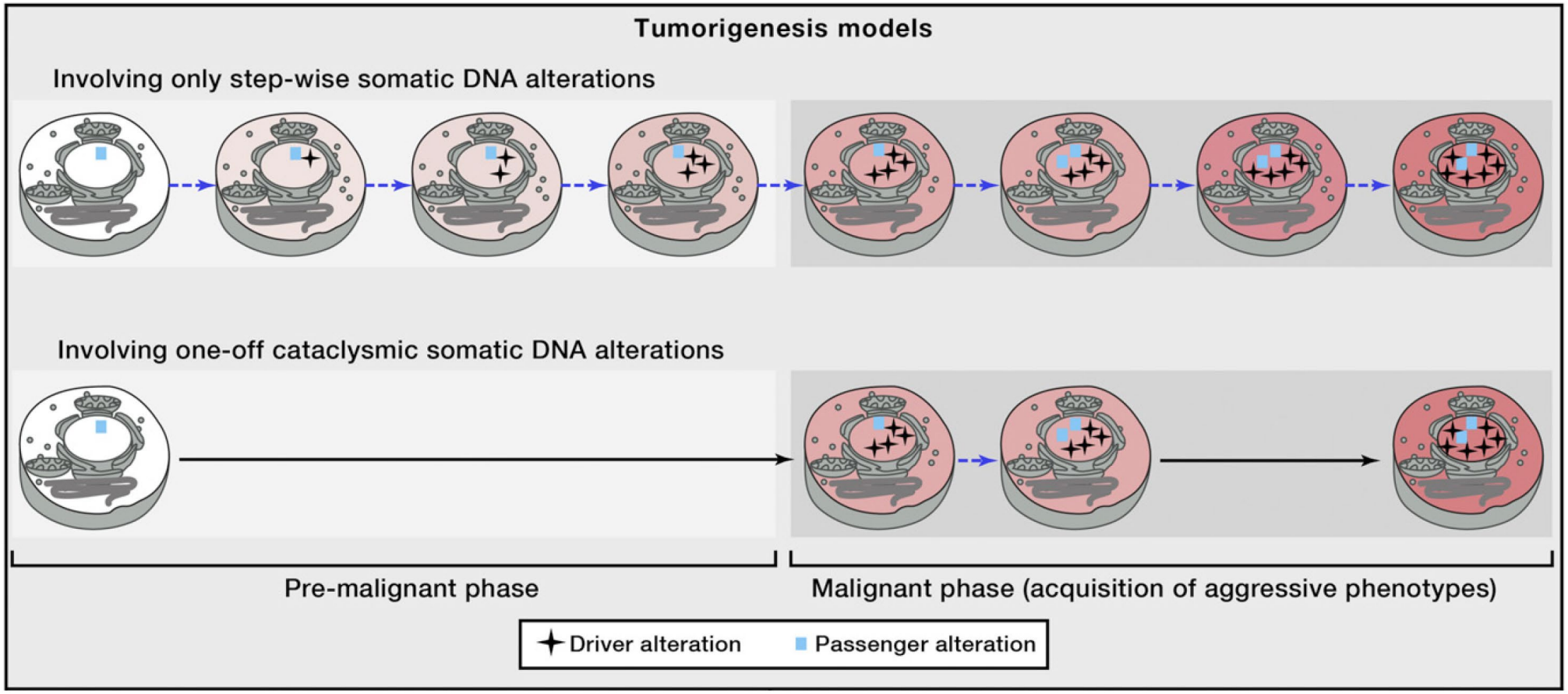
In the conventional view, somatic mutations accumulate **gradually.**

In rare instances, this leads to cancer.



The Cancer Genome. Stratton et al., *Nature*, 2009

In the early 2010s, evidence emerged that cancers can acquire many mutations at once – in a “genomic catastrophe”



Criteria for Inference of Chromothripsis in Cancer Genomes, Korbel and Campbell, *Cell*, 2013

In 2011, a group of researchers performed next-generation sequencing of 11 chronic lymphocytic leukaemia (CLL) patients.

One patient showed an unusual, clustered pattern of mutations.

This led to the discovery of a new phenomenon: **chromothripsis**.

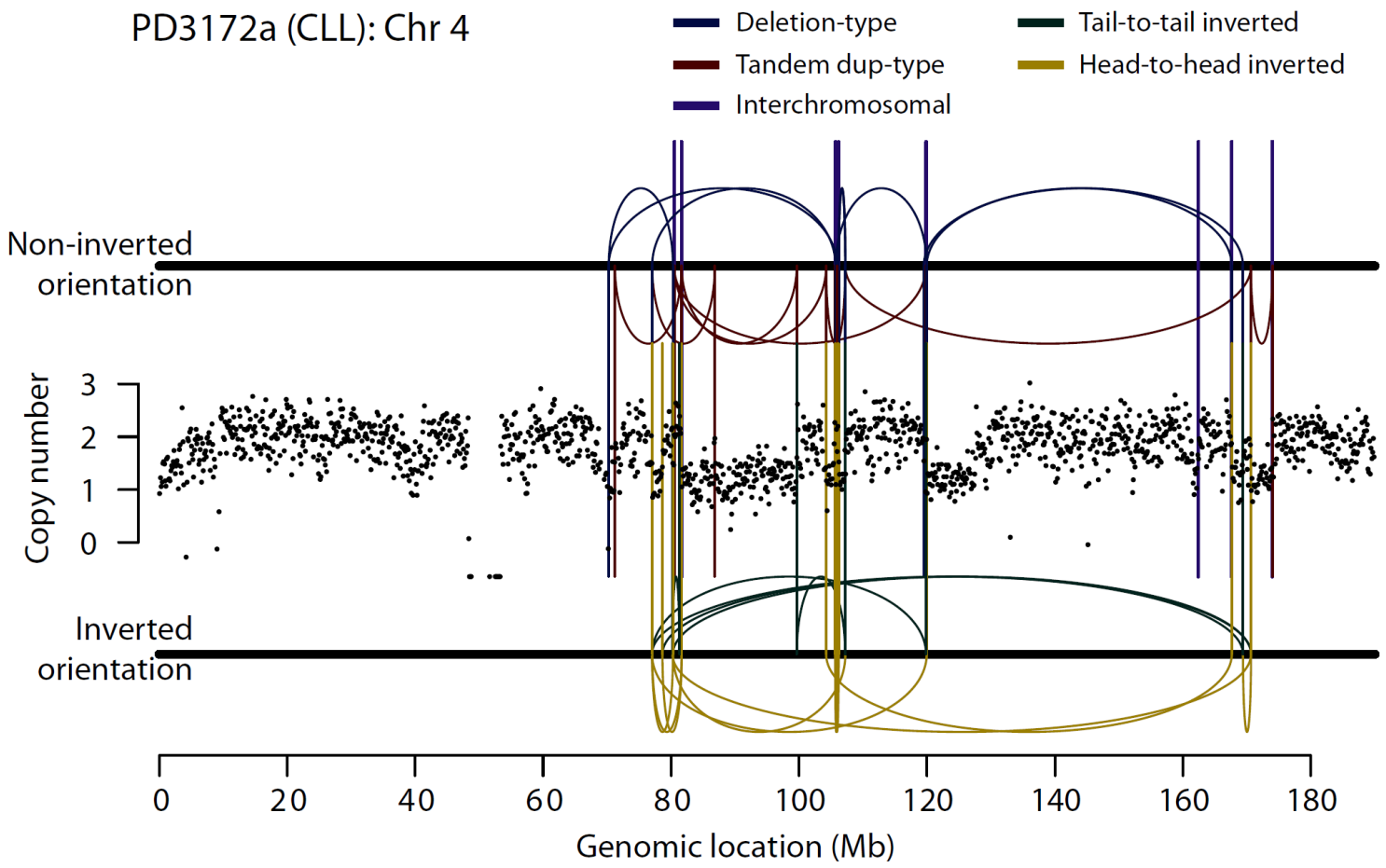
Massive Genomic Rearrangement Acquired in a Single Catastrophic Event during Cancer Development

Philip J. Stephens,[1] Chris D. Greenman,[1] Beiyuan Fu,[1] Fengtang Yang,[1] Graham R. Bignell,[1] Laura J. Mudie,[1] Erin D. Pleasance,[1] King Wai Lau,[1] David Beare,[1] Lucy A. Stebbings,[1] Stuart McLaren,[1] Meng-Lay Lin,[1] David J. McBride,[1] Ignacio Varela,[1] Serena Nik-Zainal,[1] Catherine Leroy,[1] Mingming Jia,[1] Andrew Menzies,[1] Adam P. Butler,[1] Jon W. Teague,[1] Michael A. Quail,[1] John Burton,[1] Harold Swerdlow,[1] Nigel P. Carter,[1] Laura A. Morsberger,[2] Christine Iacobuzio-Donahue,[2] George A. Follows,[3] Anthony R. Green,[3,4] Adrienne M. Flanagan,[5,6] Michael R. Stratton,[1,7] P. Andrew Futreal,[1] and Peter J. Campbell[1,3,4,*]

Cell, 2011

The authors coined the term from the Greek words:

- *chromos* = "color" (→ chromosome)
- *thripsis* = "**shattering**"



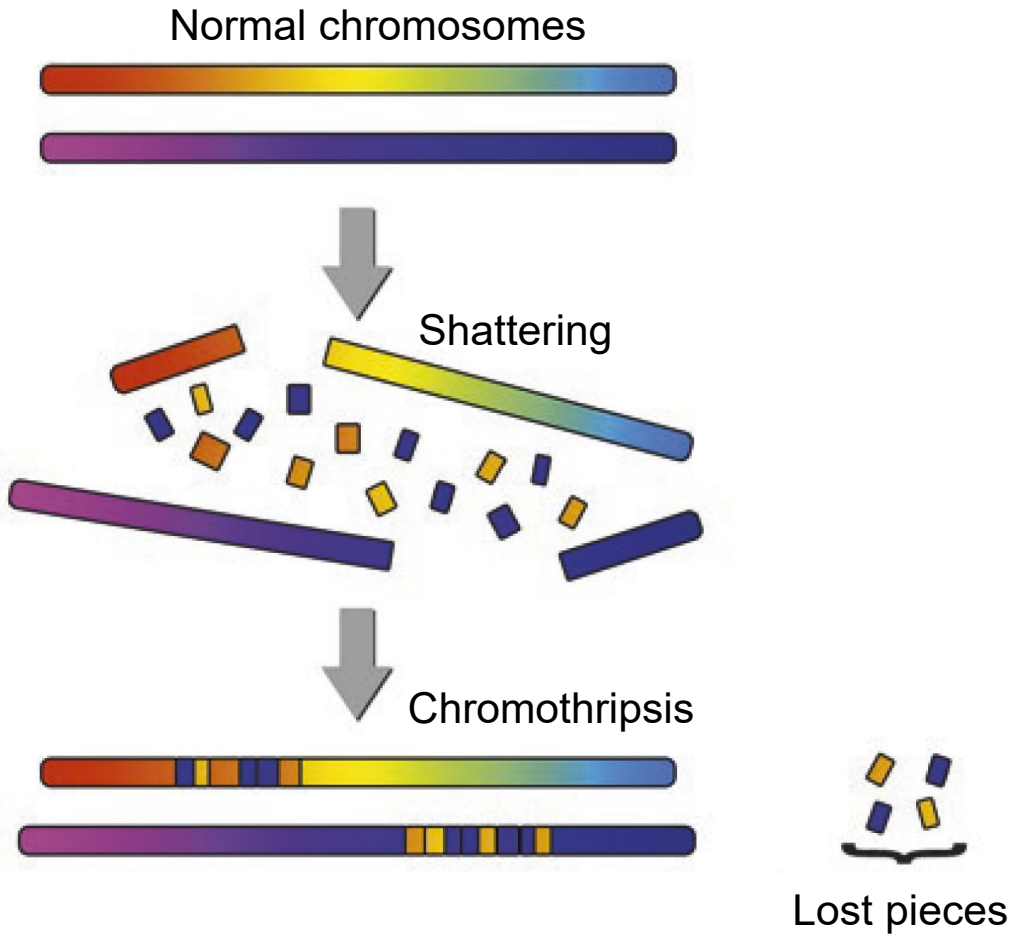
Data from paired-end Illumina DNA sequencing.

Stephens et al., Cell, 2011

- The patient had very many (42!) genomic **rearrangements** involving a **single chromosomal arm** (4q).
- **Copy number oscillated** back and forth (between one and two copies of the DNA segment).

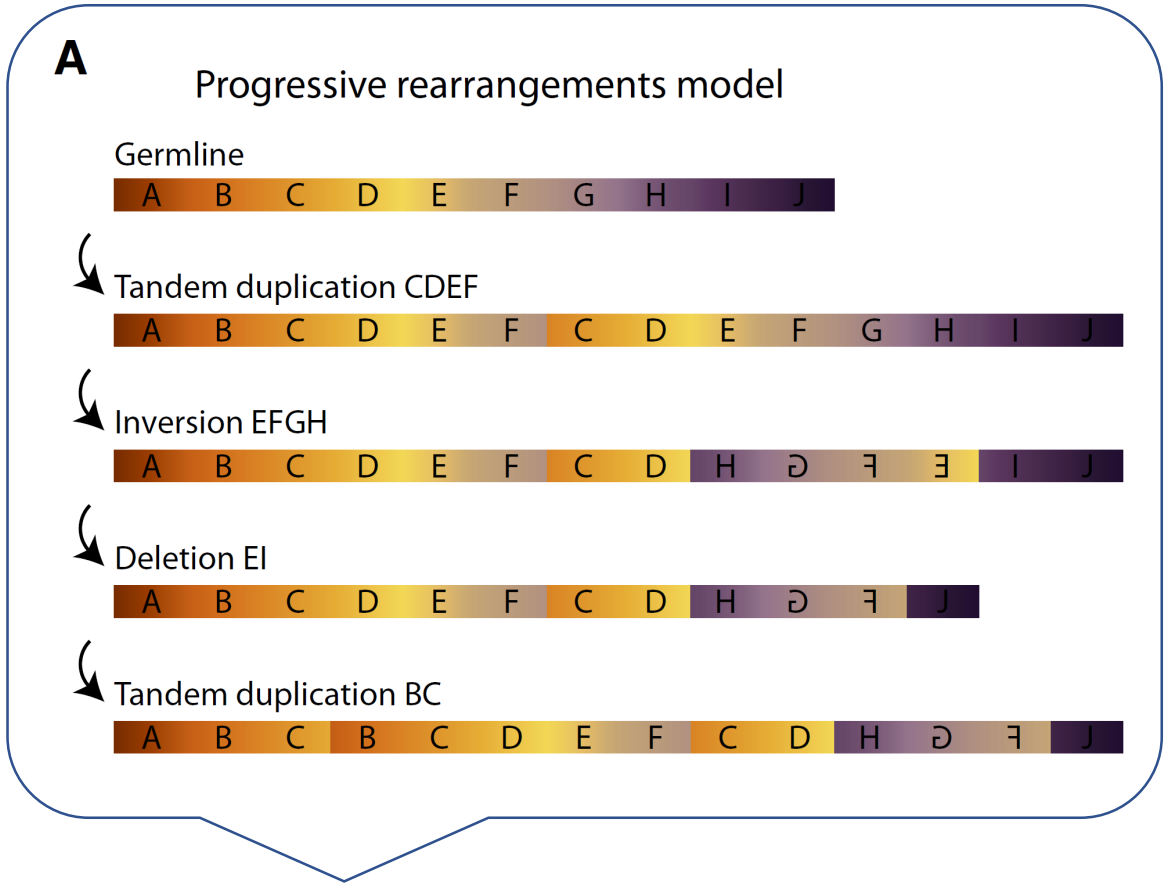
Chromothripsis is the **extensive re-arrangement of one or a few chromosomes**.

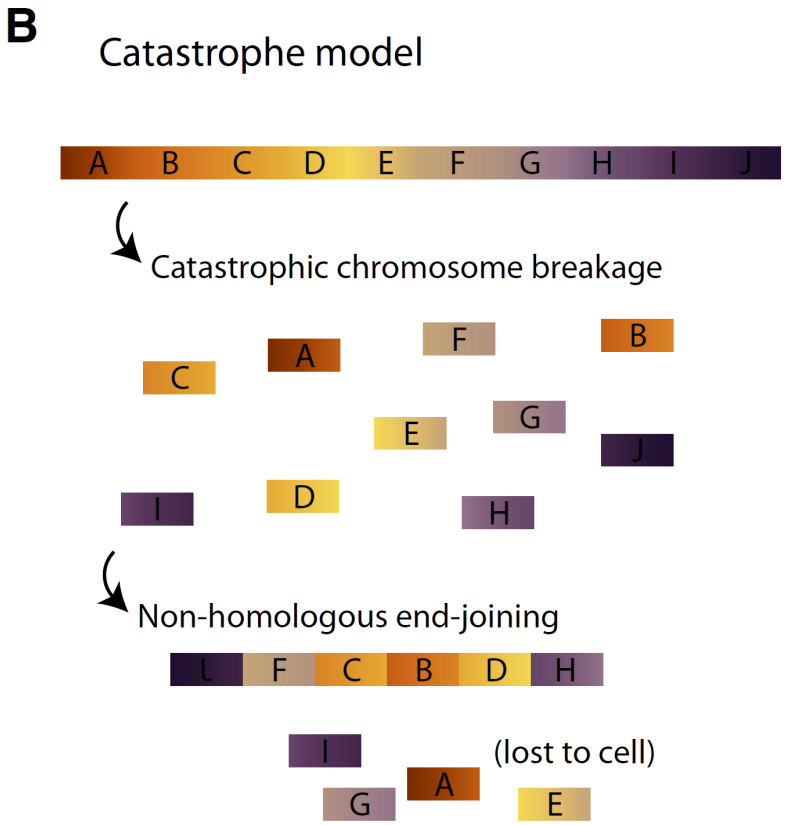
It results in a characteristic copy number pattern.



Chromothripsis: Methods and Protocols, 2018

How could the authors claim that all these changes occurred in a **single event**?





Couldn't it have happened in a step-wise fashion… ?

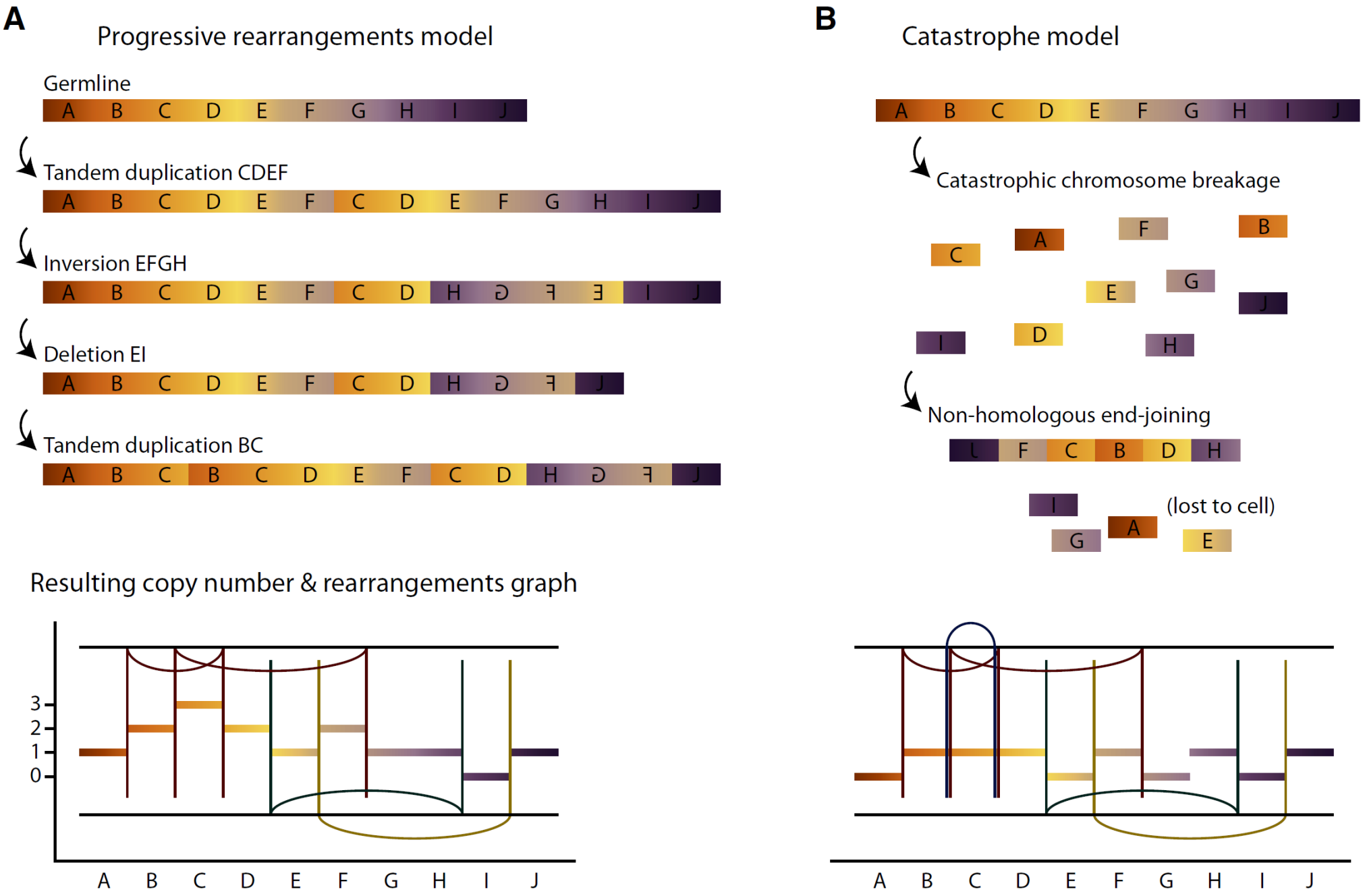
Stephens et al., Cell, 2011

Arguments for a single catastrophic event:

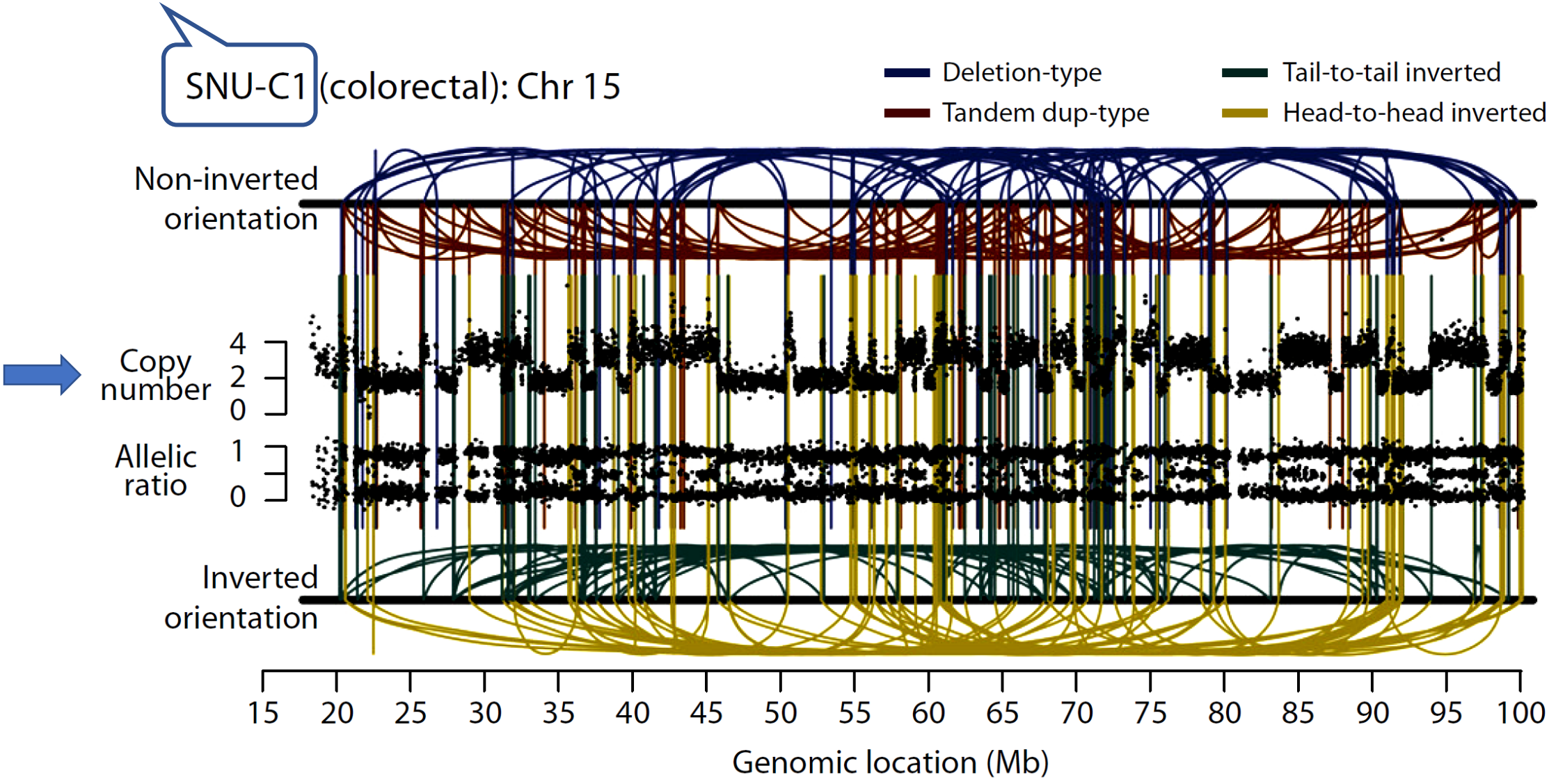
1. Only **two copy number states** are observed.
   - If there were many amplifications and deletions of small segments, it's extremely unlikely that none of them overlap (and lead to 0-3 or more copies).

2. In those regions with higher copy number, **heterozygosity is always preserved**.
   - For those pieces that are not lost, both a maternal and paternal copy are present.
   - This is unlikely if there are many independent amplifications and deletions. We would expect some loss of heterozygosity.

Stephens et al., Cell, 2011


A
Progressive rearrangements model
Germline
A B C D E F G H I J
Tandem duplication CDEF
A B C D E F C D E F G H I J
Inversion EFGH
A B C D E F C D H G F E I J
Deletion EI
A B C D E F C D H G F J
Tandem duplication BC
A B C B C D E F C D H G F J
B
Catastrophe model
A B C D E F G H I J
Catastrophic chromosome breakage
C A F B E G J D I H
Non-homologous end-joining
L F C B D H
I A G E
(lost to cell)
Resulting copy number & rearrangements graph
3
2
1
0
A B C D E F G H I J
A B C D E F G H I J

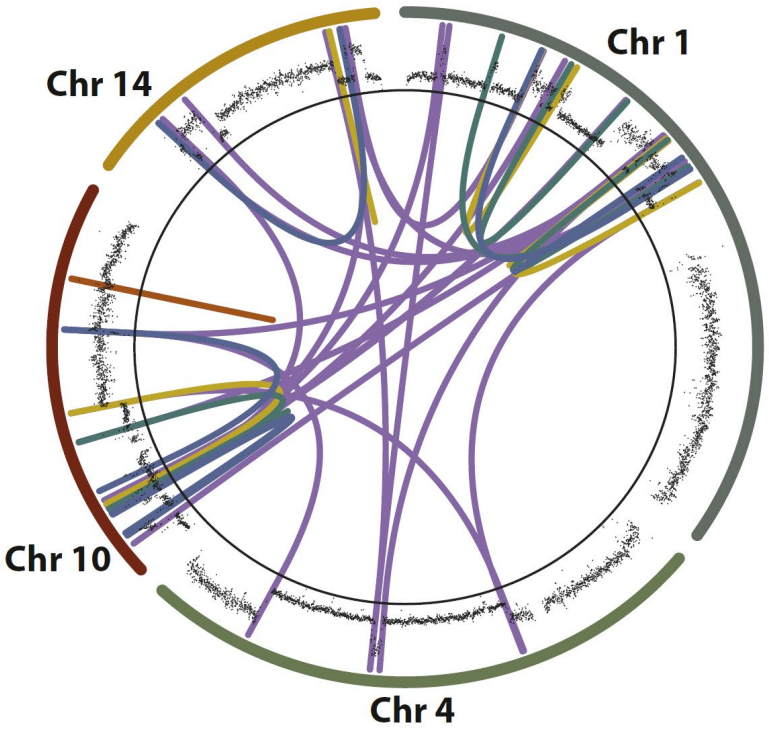
polyploid cell line



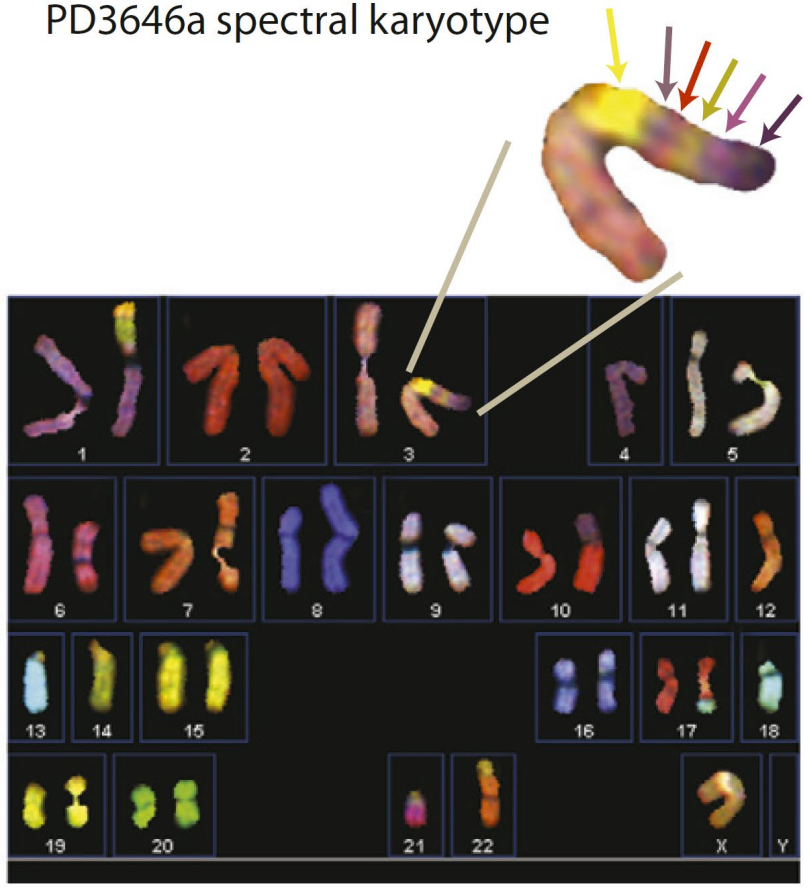
Stephens et al., Cell, 2011

With the technology of the time, chromothripsis was detectable in ~3% of cancers and cancer cell lines.

PD3646a (pancreatic)



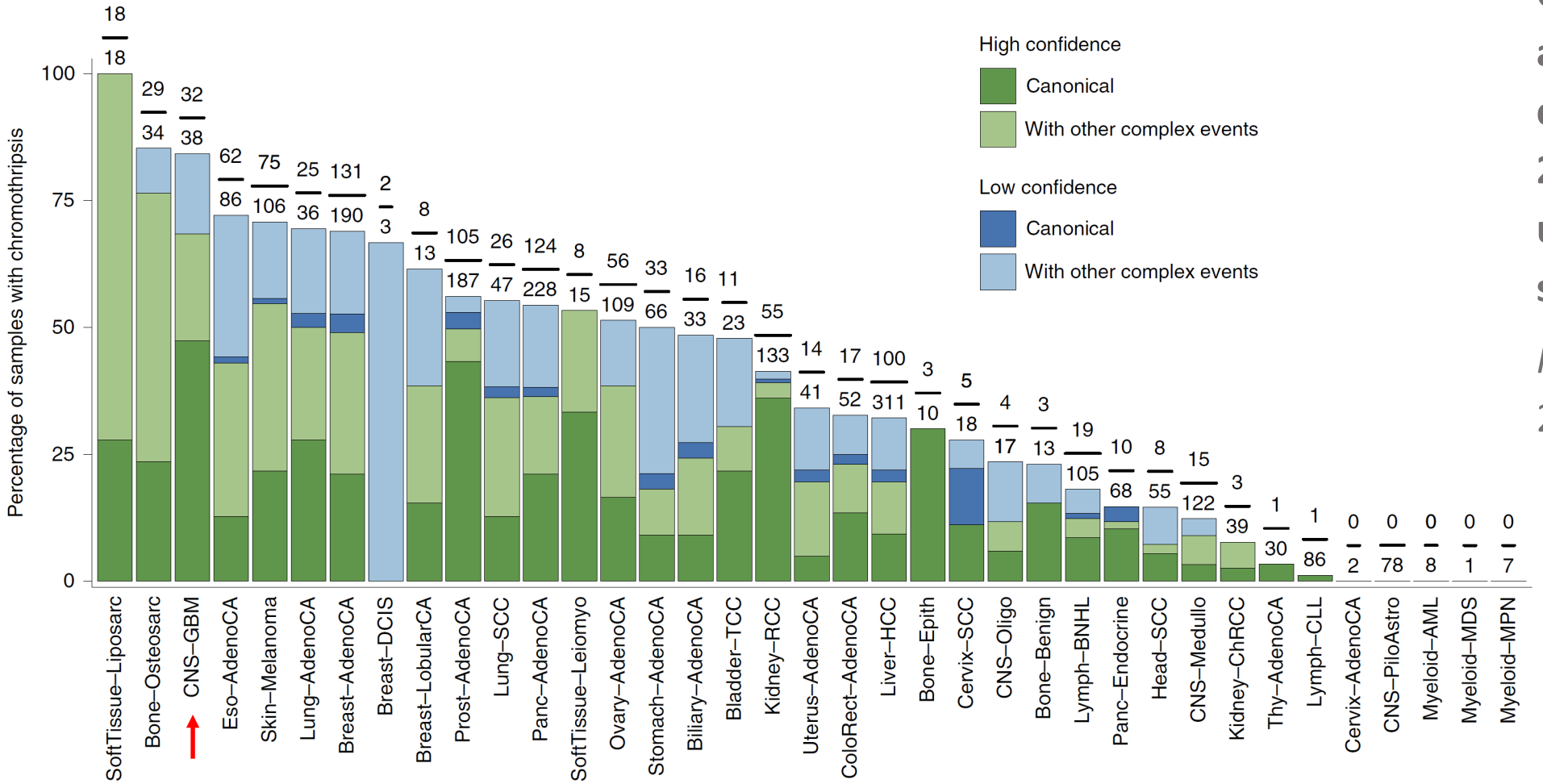
PD3646a spectral karyotype



Chromothripsis is always **localized**.

Stephens et al., Cell, 2011

A recent genomic meta-analysis found chromothripsis in over 50% of patients in many cancer types

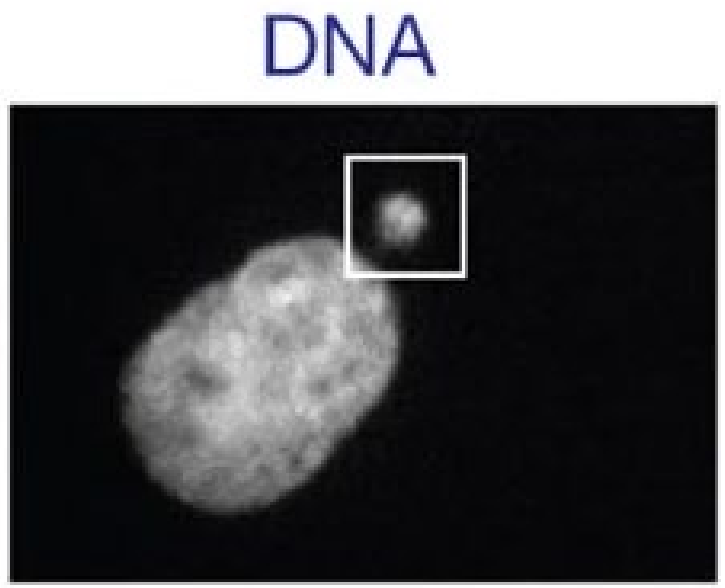


Comprehensive analysis of chromothripsis in 2,658 human cancers using whole-genome sequencing

Nature Genetics, 2020

Chromothripsis has also been identified as a cause of congenital disease.

A 2012 paper first discussed a potential link between chromothripsis and **micronuclei** (but conclusive evidence was not provided).



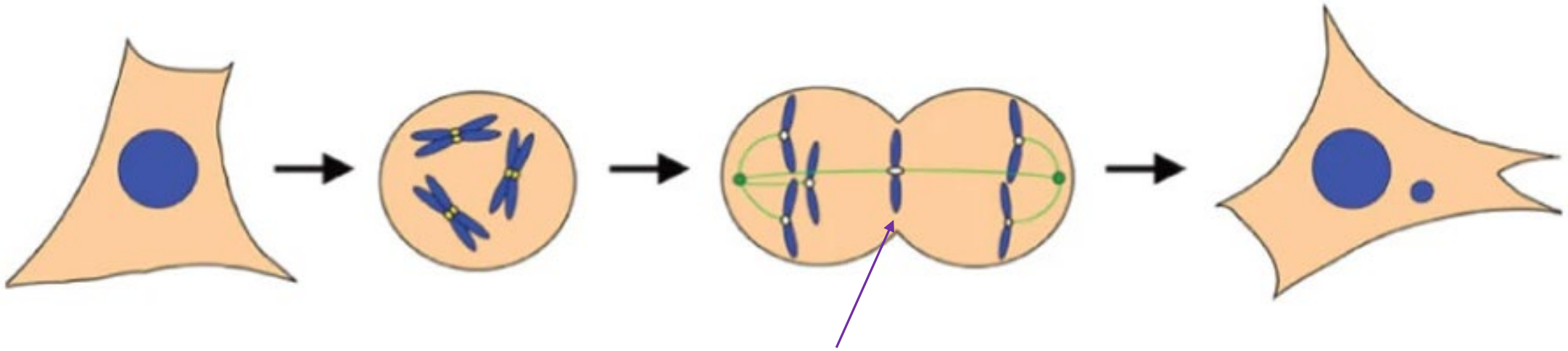
ARTICLE

DNA breaks and chromosome pulverization from errors in mitosis

Karen Crasta[1,2,3], Neil J. Ganem[1,2,3], Regina Dagher[1,2,3], Alexandra B. Lantermann[1], Elena V. Ivanova[4], Yunfeng Pan[5], Luigi Nezi[1], Alexei Protopopov[4], Dipanjan Chowdhury[5] & David Pellman[1,2,3]

A micronucleus is a small, separated nucleus-like structure that contains a chromosome or a fragment of a chromosome.

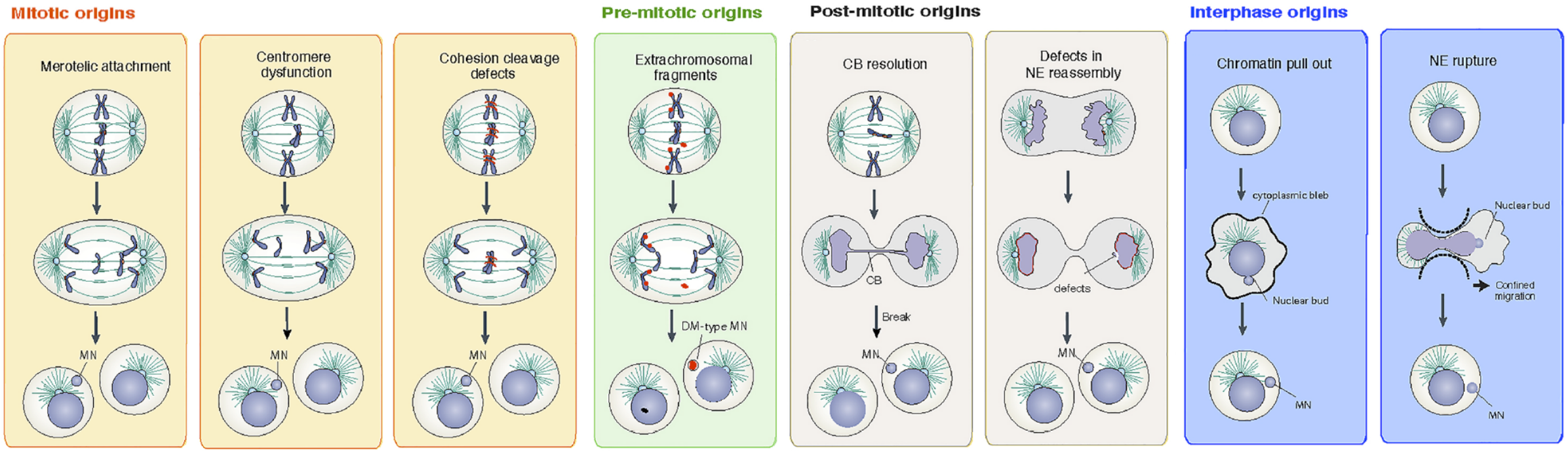
A micronucleus can form when a chromosome is left behind during mitosis (e.g. due to a defect in mitotic spindle attachment).



Krasta et al, Nature, 2012

Diverse mechanisms can lead to the genesis of micronuclei:

- Defects in nuclear membrane formation
- Budding from the nucleus during interphase
- …

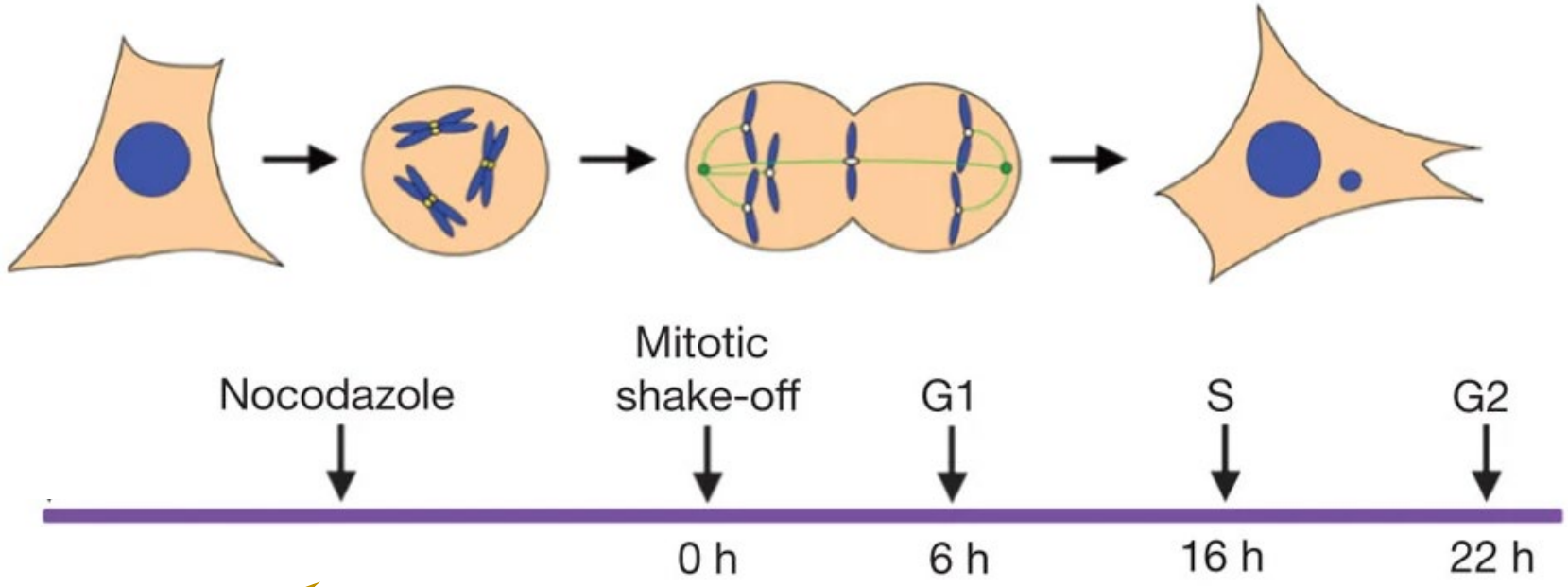


The molecular origins and pathophysiological consequences of micronuclei: New insights into an age-old problem. Guo et al., 2019

DNA breaks and chromosome pulverization from errors in mitosis

Nature, 2012

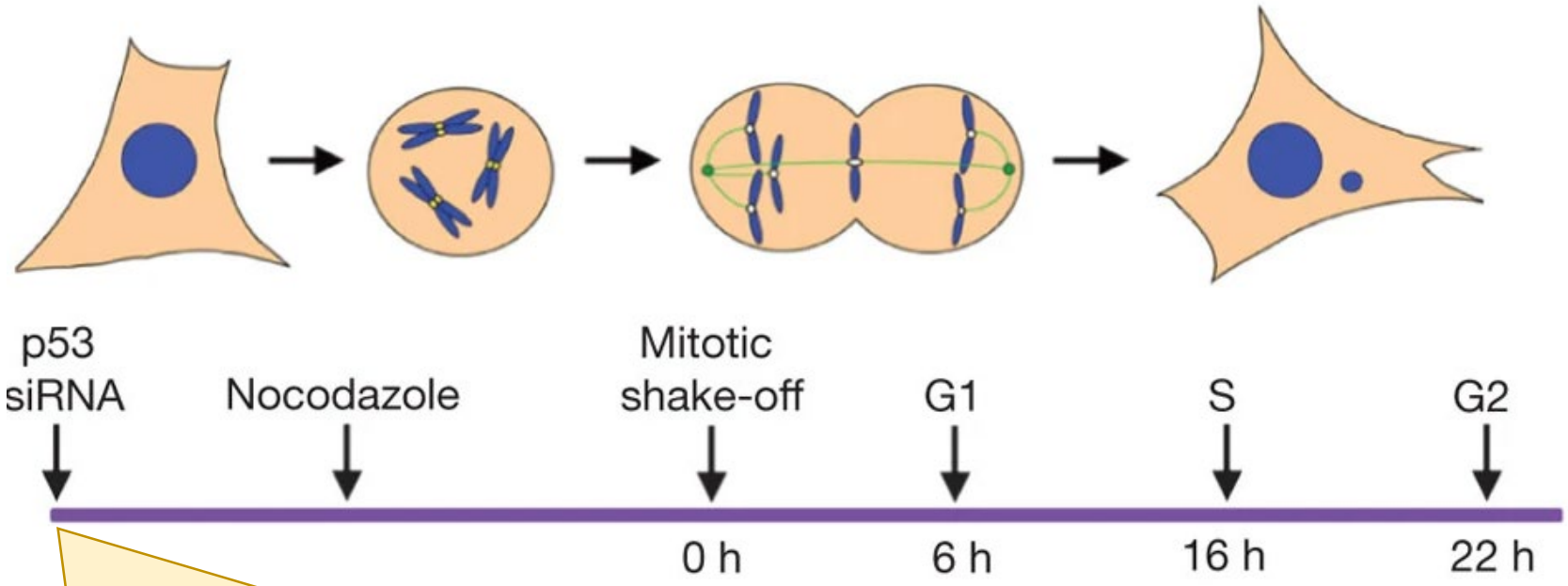
Micronuclei were induced in **cell-cycle synchronized cells** using a nocodazole release procedure.



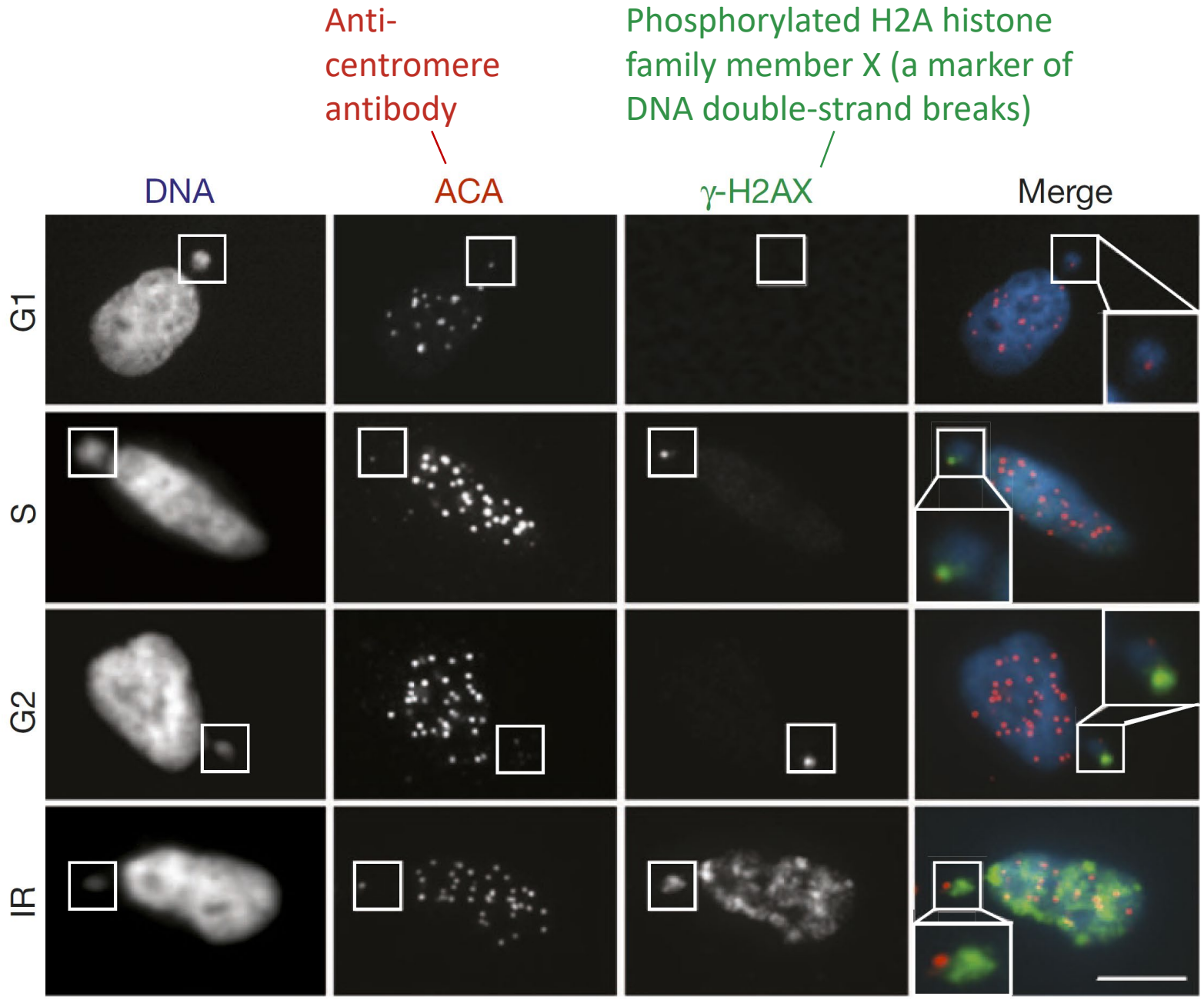
Cells are treated with nocodazole, which inhibits microtubule polymerization and causes **mitotic arrest.**

After 6h, mitotic cells are selected by shaking (they detach more easily), washed 3×, and re-plated.

After release from nocodazole, mitosis continues, but **spindle defects occur** (lagging chromosomes, incorrect attachments) which lead to **micronuclei in 10%** of cells.



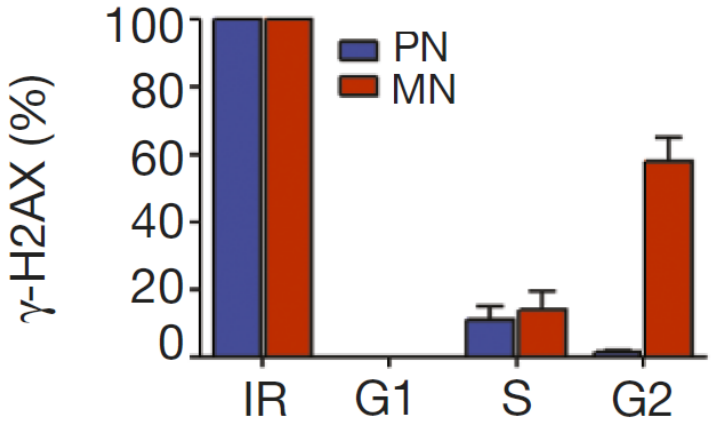
Telomerase-immortalized RPE-1 cells were used (= Retinal Pigment Epithelium 1; a genomically stable human cell line).
Aneuploidy can sometimes trigger a p53-dependent G1 checkpoint arrest. To prevent this, and ensure progression through the cell cycle, **p53 was knocked down.**



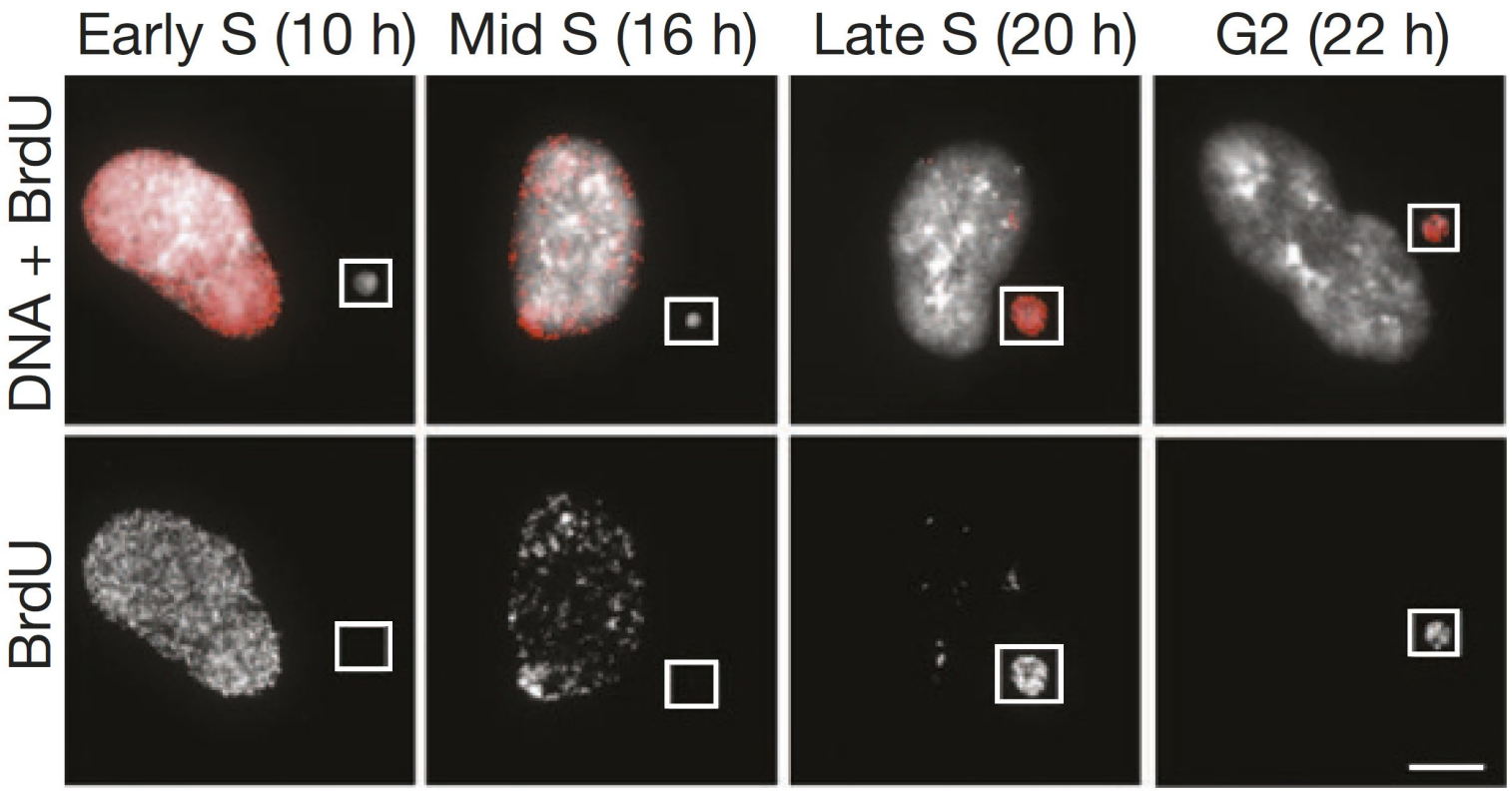
Crasta et al., Nature, 2012

DNA in micronuclei seems to become **damaged**.

This occurs **after** DNA replication (**in S/G2 phase** of the cell cycle).



Defective replication in micronuclei may explain the DNA damage.

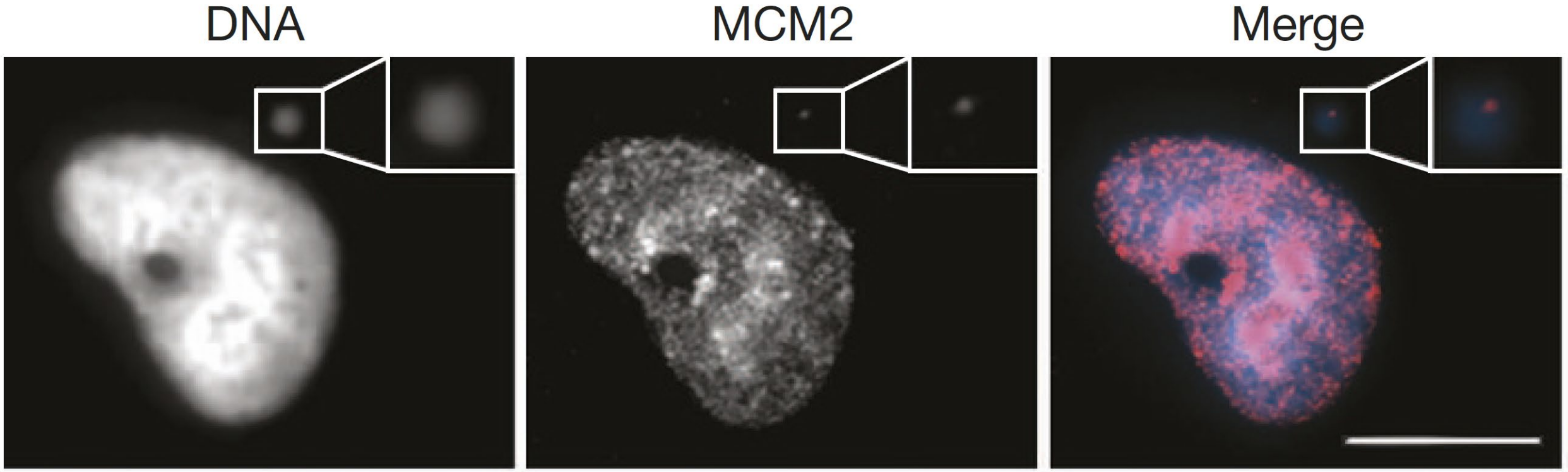


DNA synthesis in micronuclei is delayed and extends into G2 phase!

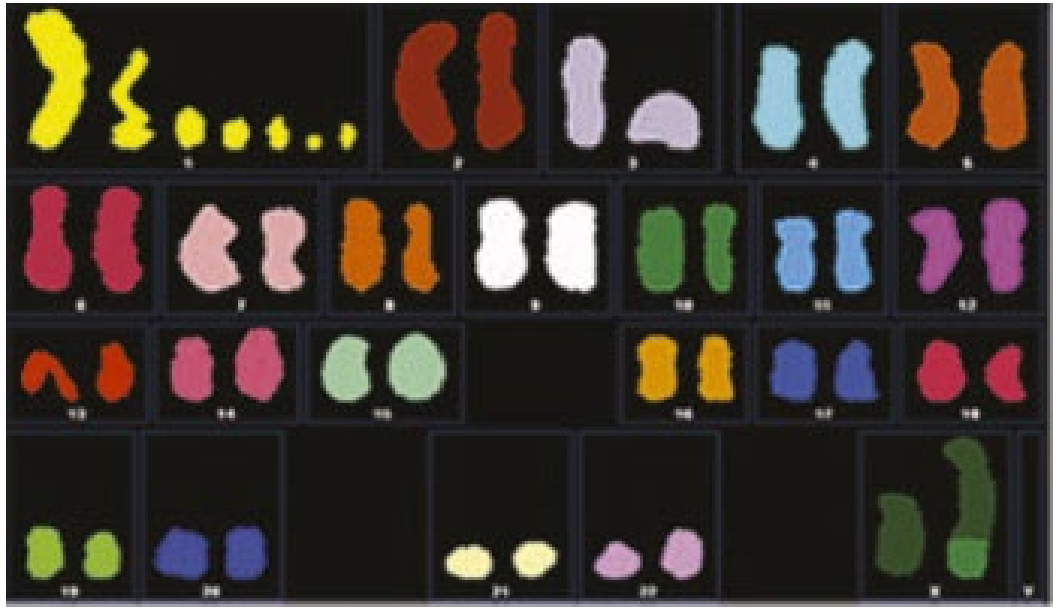
Bromodeoxyuridine (**BrdU**) is a thymidine analog.

It is incorporated into the DNA, and labels actively replicating cells.

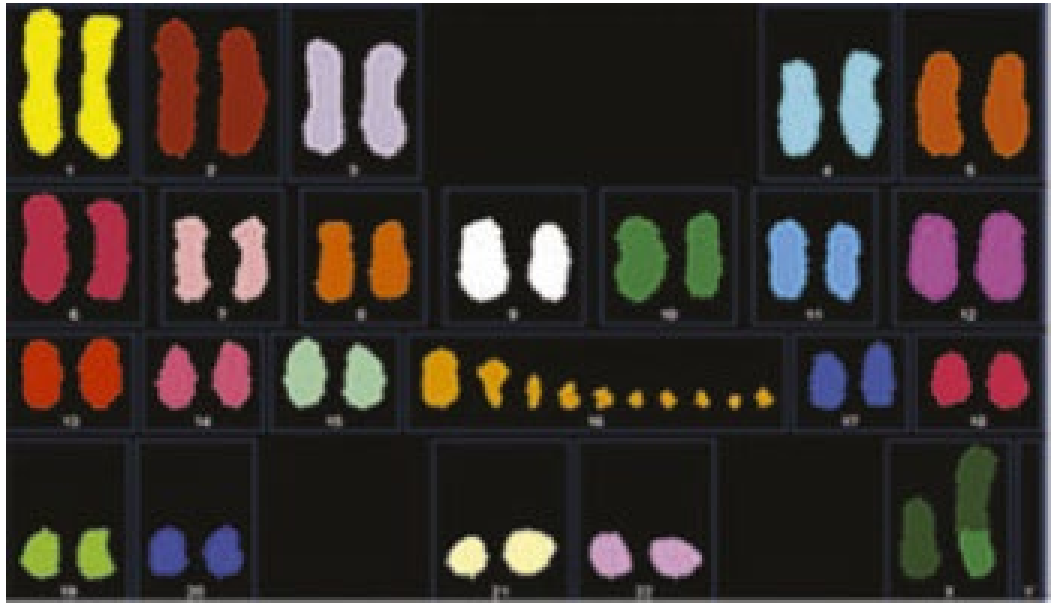
Crucial DNA replication factors like the MCM2 helicase are not recruited properly to micronuclei.



Incomplete replication in micronuclei led to **DNA breaks** and chromosome fragmentation!

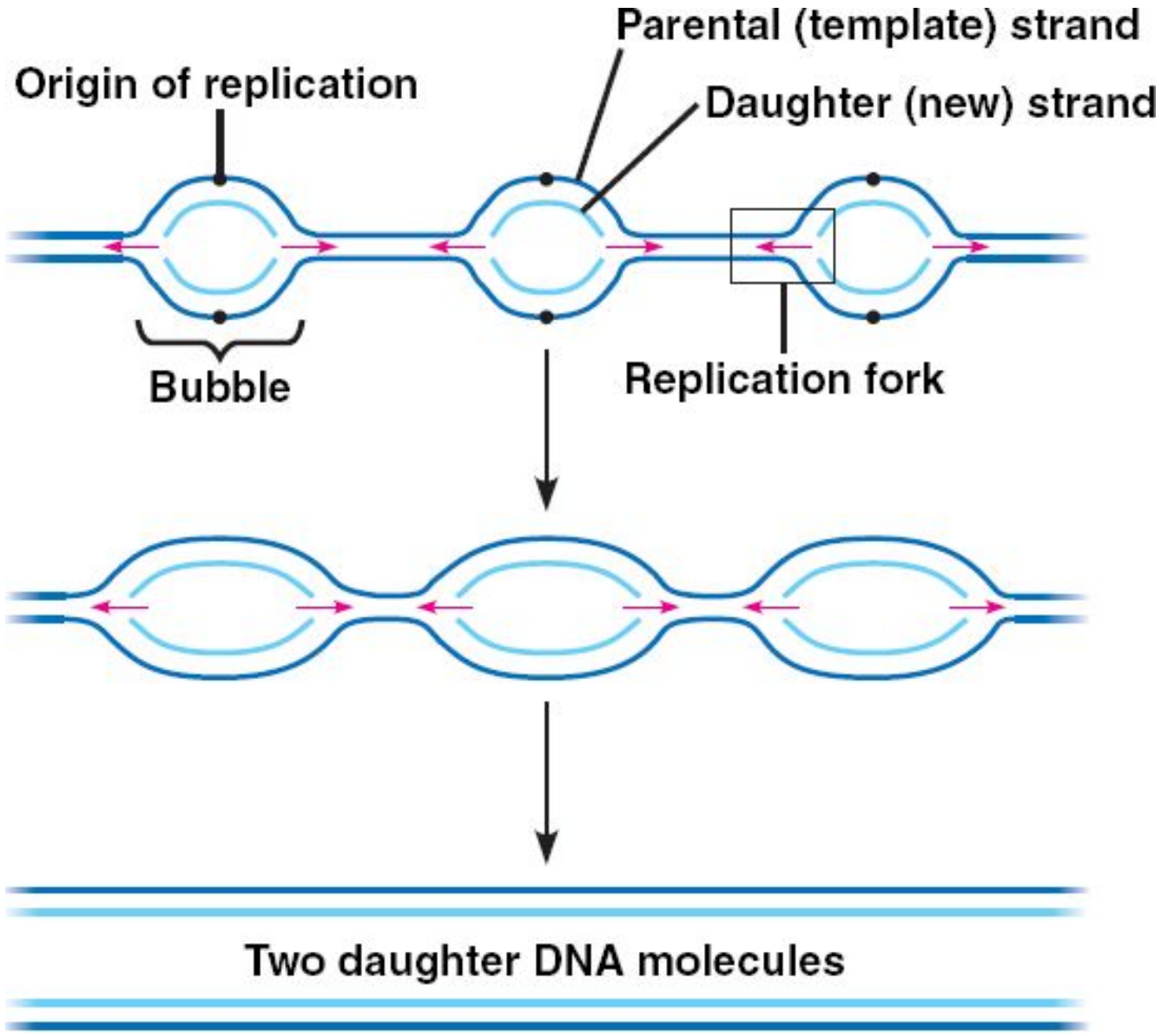


Pulverization of chromosome 1



Pulverization of chromosome 16

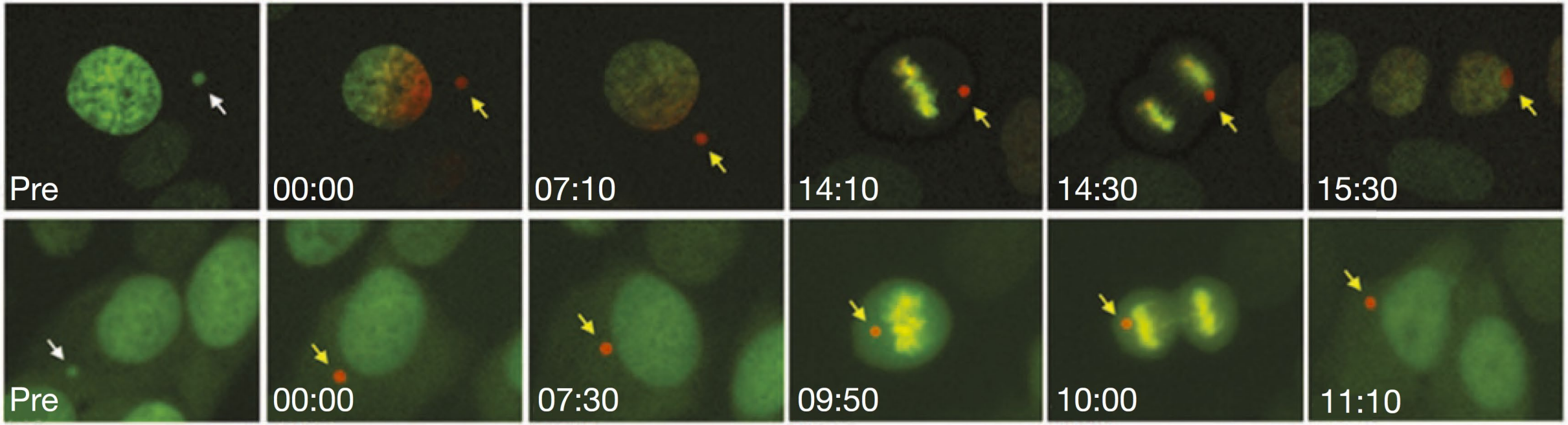
Pulverized chromosomes were seen in 7.6% of nocodazole-treated cells.



http://bio1151.nicerweb.com/Locked/media/ch16/init.html

- Eukaryotic cells have multiple origins of replication.
- If replication is incomplete, some of the replication forks have not yet met.
- These areas can **break during compaction** of the chromosomes before mitosis.

After mitosis, micronuclei have two fates: They can **persist** as micronuclei, or they can be **re-integrated** into the main nucleus (38% of cell divisions).



Red: Histone 2B, fused with tandem repeats of Kaede.

Kaede is a photoconvertible fluorescent protein, originally isolated from a stony coral.

When irradiated with UV light, it permanently converts from green to red fluorescence.

The authors discuss a potential mechanism for chromothripsis:

- **Chromosomes** that are segregated into micronuclei **become pulverized** during the next cell cycle.
- Some of these chromosomes are re-integrated into the nucleus.
- (The fragments are re-joined by DNA repair mechanisms.)

ARTICLE

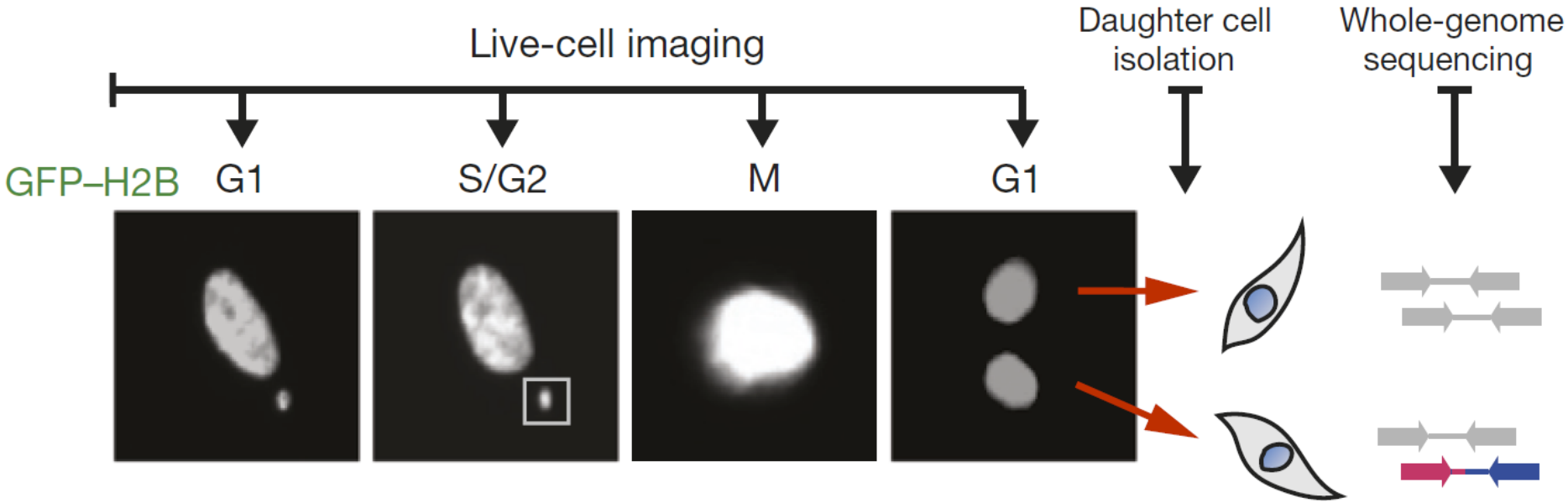
Chromothripsis from DNA damage in micronuclei

Cheng-Zhong Zhang[1,2,3,4]*, Alexander Spektor[2,4,5]*, Hauke Cornils[2,4]*, Joshua M. Francis[1,3]*, Emily K. Jackson[2,4,6], Shiwei Liu[2,4], Matthew Meyerson[1,3,7,8] & David Pellman[2,3,4,6]

Nature, 2015

The authors introduce the Look-Seq strategy:

1. Individual cells are characterized by live-cell microscopy.
2. These cells, or their daughter cells, are then analysed using single-cell whole-genome sequencing.

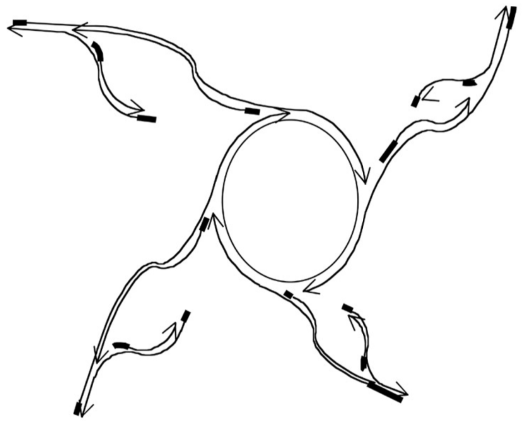


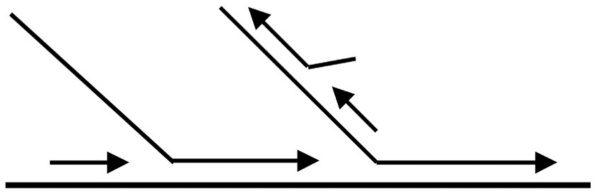
- Whole-genome sequencing of individual cells requires whole genome amplification (WGA).

  → This produces many copies from a single original molecule of DNA.

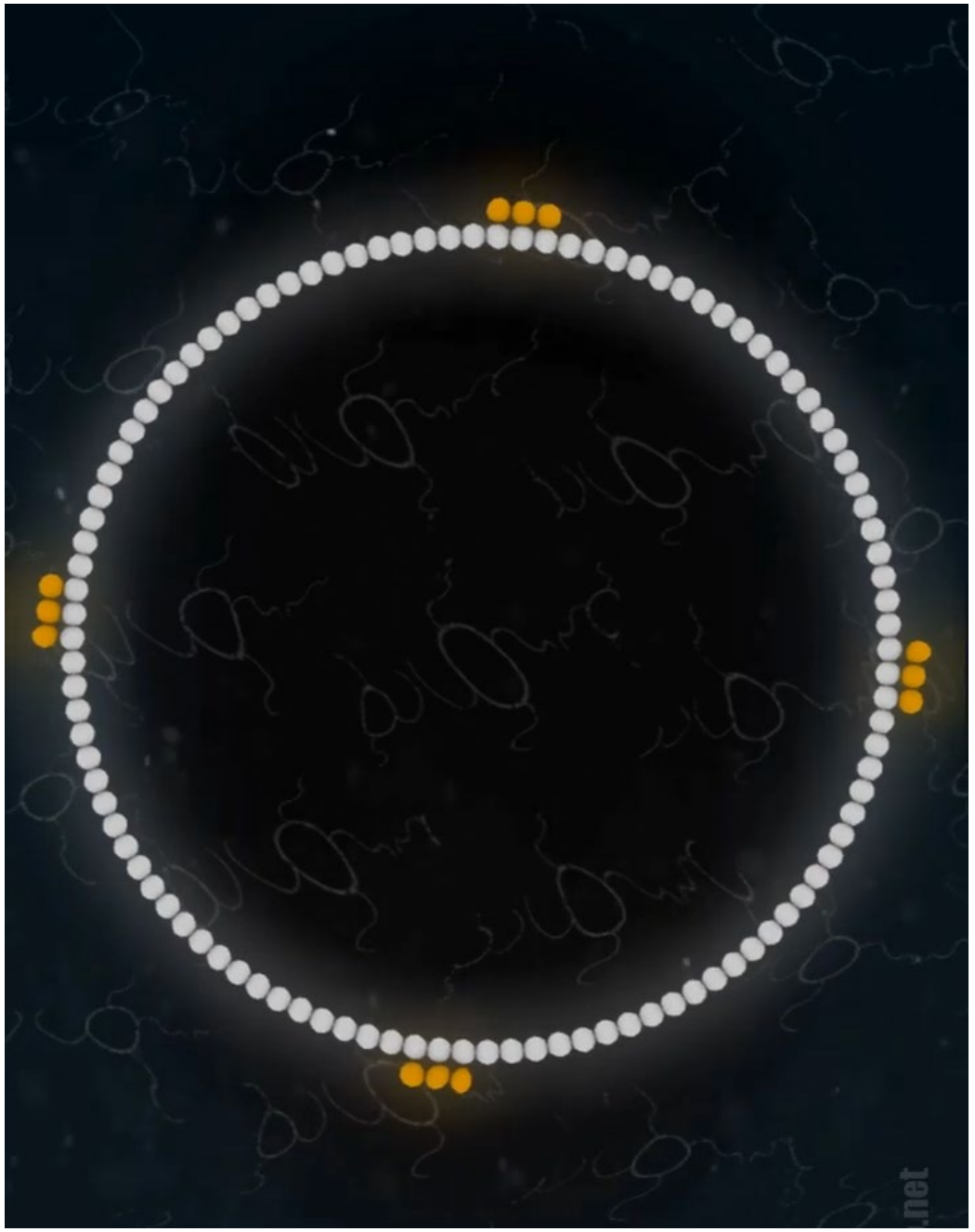
  → Standard DNA sequencing can then be performed.

- Multiple WGA methods have been developed. The authors chose multiple displacement amplification (MDA).





Dean et al.,
PNAS, 2002



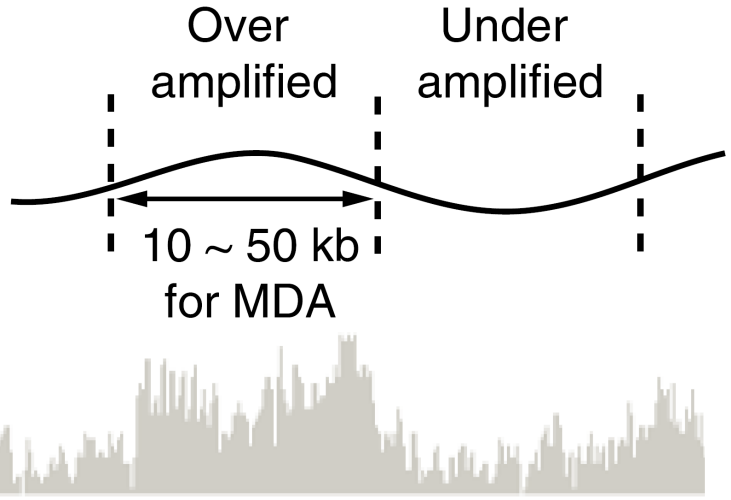
www.youtube.com/watch?v=CaFq9cnfTZI

Multiple displacement amplification (MDA)

- Uses random hexameric primers
- DNA synthesis by Φ29 polymerase
  - High fidelity (3’-5' proofreading activity, error rate < 1 in a million)
  - Produces amplicons 10-50 kb in length
- When the polymerase reaches the next primer site, the strand is displaced
  → more template becomes available
- Constant temperature (30°C for 8h)

- An advantage of MDA is that its amplification bias is **random**.
  - Some regions are amplified more than others, and some are lost completely.
  - This noise occurs during early amplification steps.
  - However, it differs randomly from cell to cell.
  - (In contrast, PCR-based WGA methods can introduce sequence-specific bias.)

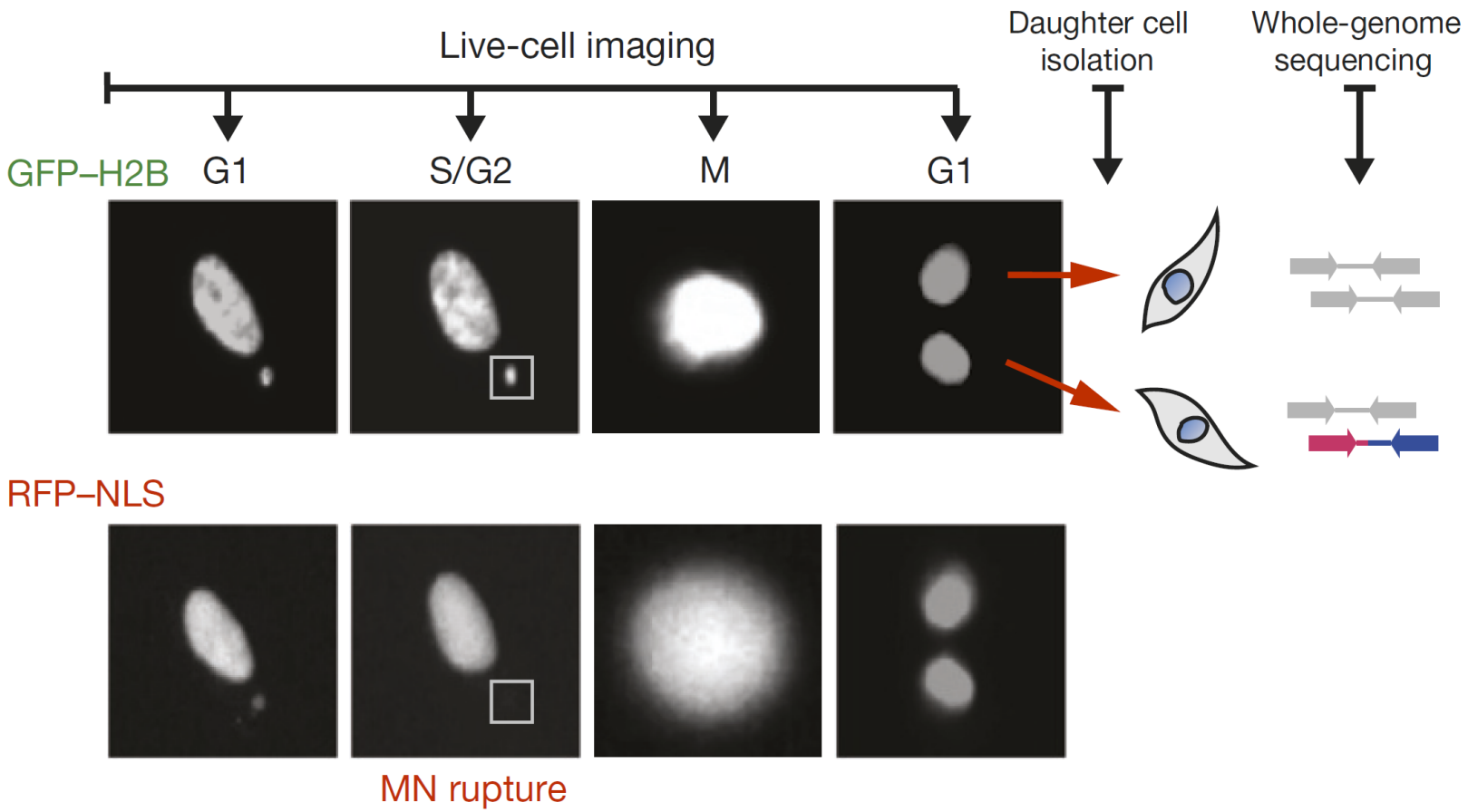
- Due to amplification errors, single-cell DNA sequencing cannot achieve perfect coverage of the genome.
- It cannot be used to reliably genotype a specific locus!



Calibrating genomic and allelic coverage bias in single-cell sequencing. Zhang et al., *Nature Communications*, 2015

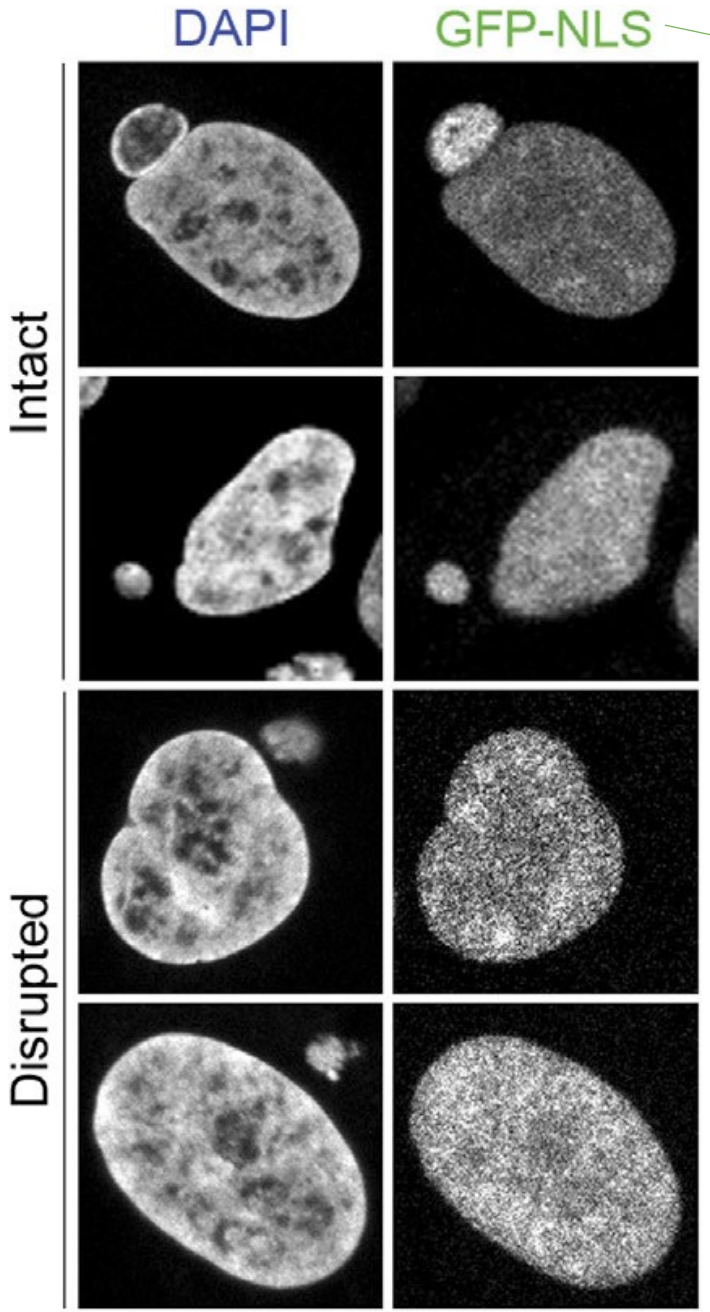
Inclusion criteria for Look-Seq

1. The mother cell had a micronucleus.
2. Neither daughter cell has a micronucleus (→ re-incorporation)
3. The micronucleus ruptured after entry into S phase.



Single cells were sorted into 384-well plates by FACS.

After mitosis, daughter cells were separated by limiting dilution.



GFP with a nuclear localization signal

Another group had proposed a classification into “intact micronuclei” or “disrupted micronuclei”, where the nuclear membrane had ruptured.

After rupture, localization of nuclear proteins to the micronucleus (and exclusion of cytoplasmic proteins) is no longer maintained.

Catastrophic Nuclear Envelope Collapse in Cancer Cell Micronuclei

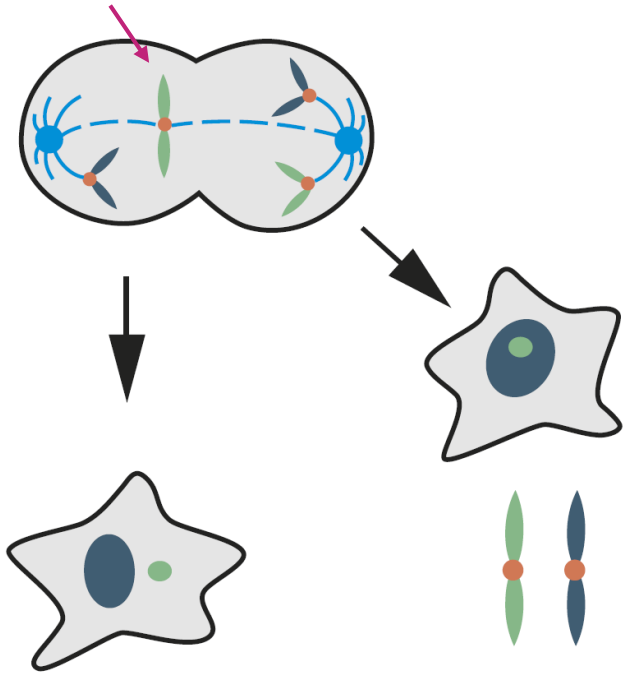
Emily M. Hatch,[1] Andrew H. Fischer,[2] Thomas J. Deerinck,[3] and Martin W. Hetzer[1,*]

Cell, 2013

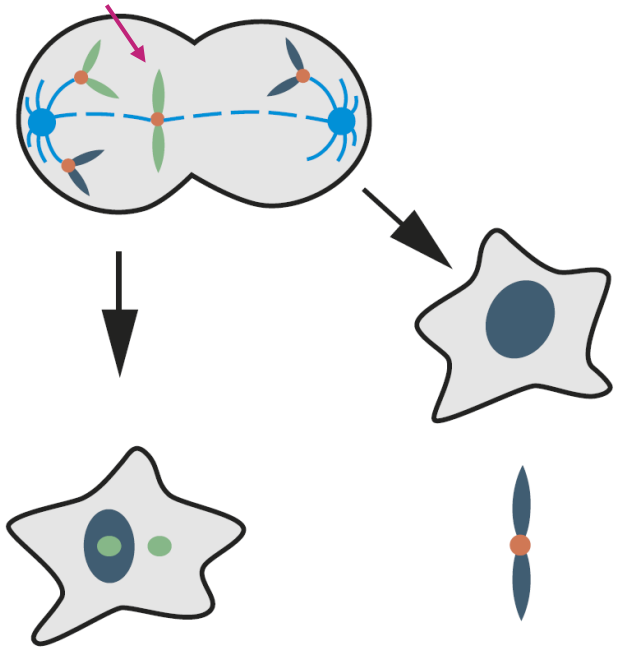
- The authors sequenced 9 pairs of daughter cells from micronucleated mother cells, and 10 pairs of control cells.
- What do we expect to see?

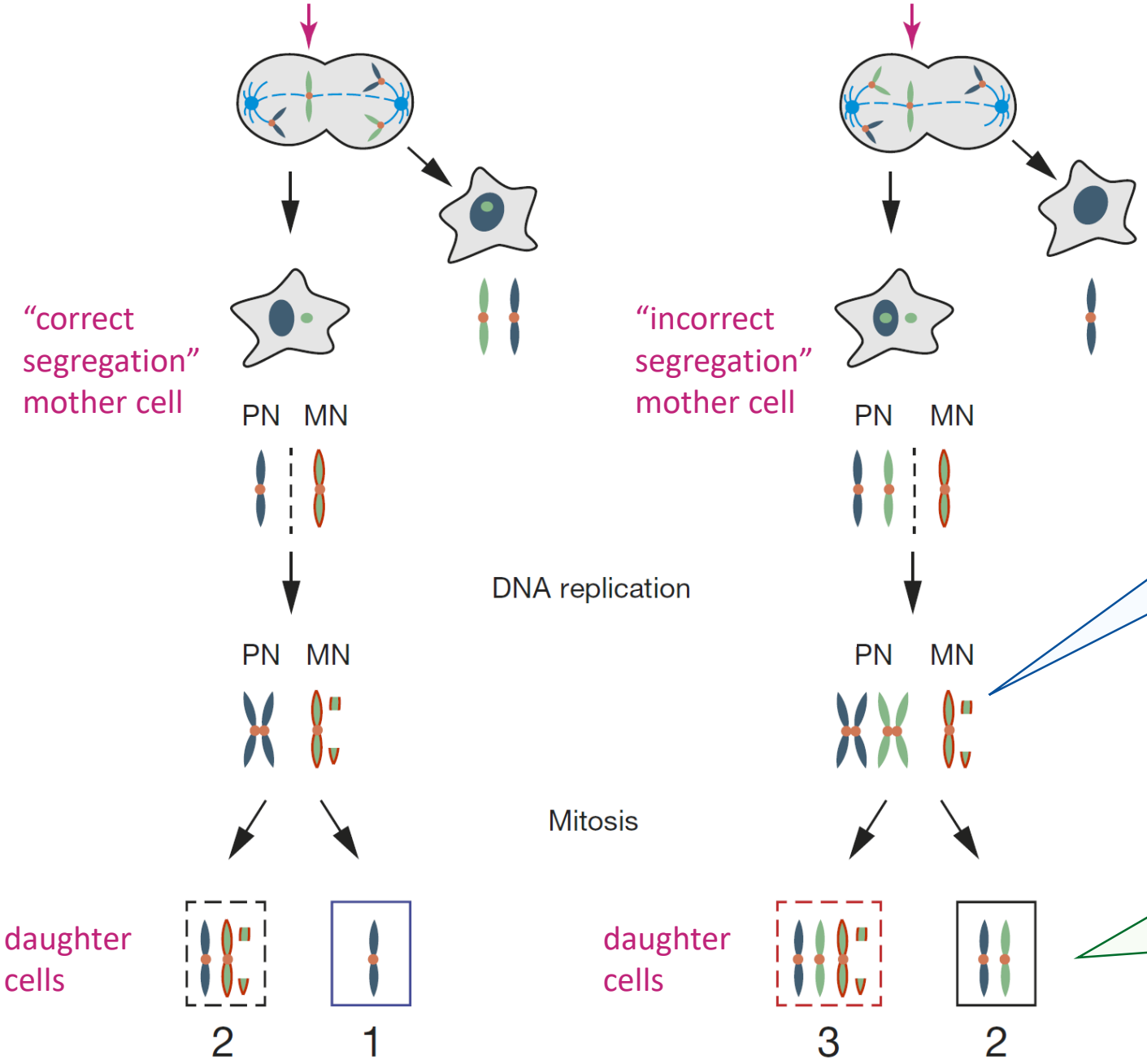
There are two types of micronucleated mother cells...

The lagging chromosome was partitioned into the correct cell.



The lagging chromosome ends up in the wrong cell (→ trisomy)

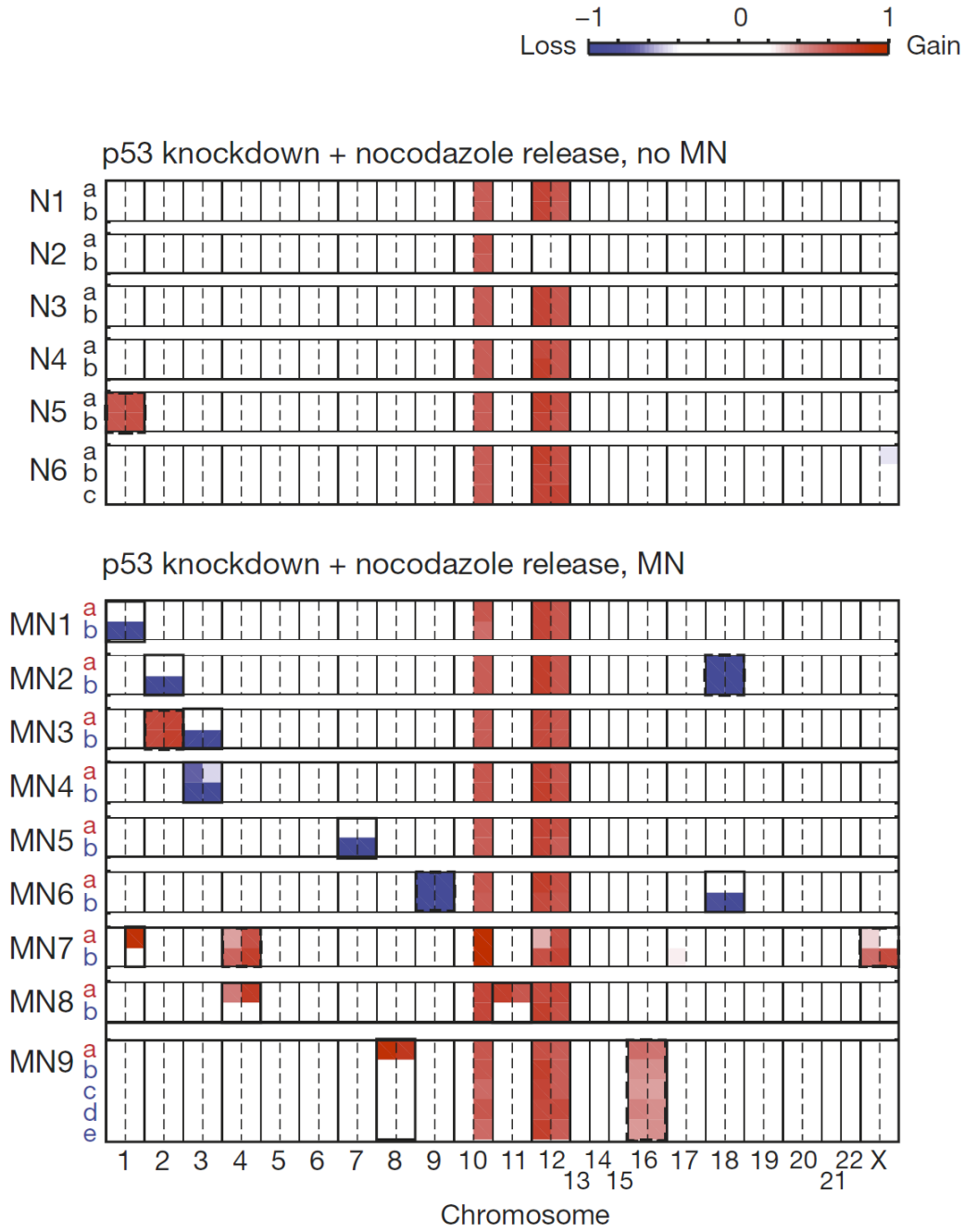




Replication is defective in micronuclei. The chromosome in the micronucleus is incompletely replicated – only small fragments are generated.

If a chromosome was included in a micronucleus, we expect a **copy number asymmetry** in the daughter cells (either ~2:1 or ~3:2 ratio).

The copy number **asymmetry between daughter cells** allowed the authors to **clearly identify** which chromosome had been inside the micronucleus!



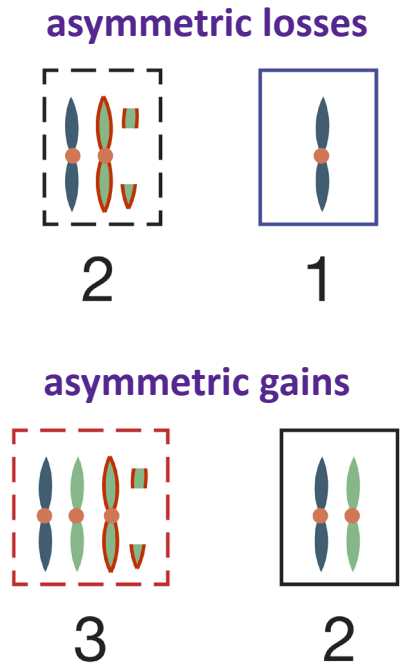
DNA copy number heatmap.

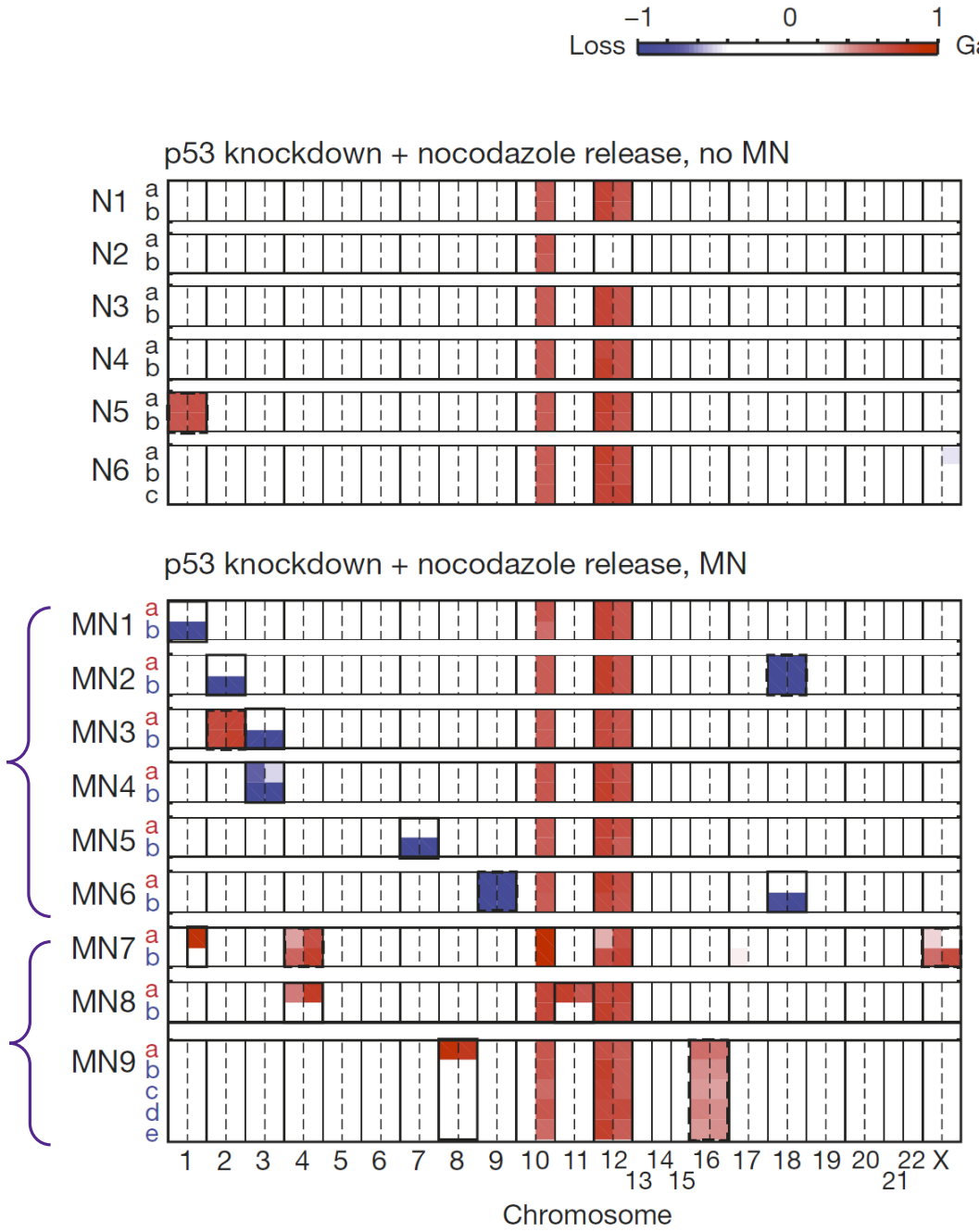
The two daughter cells are labelled a & b.

In control cells, we can observe the known gains of Chrs. 10 and 11 of the RPE-1 cell line.

There were also occasional subclonal alterations (in both daughters).

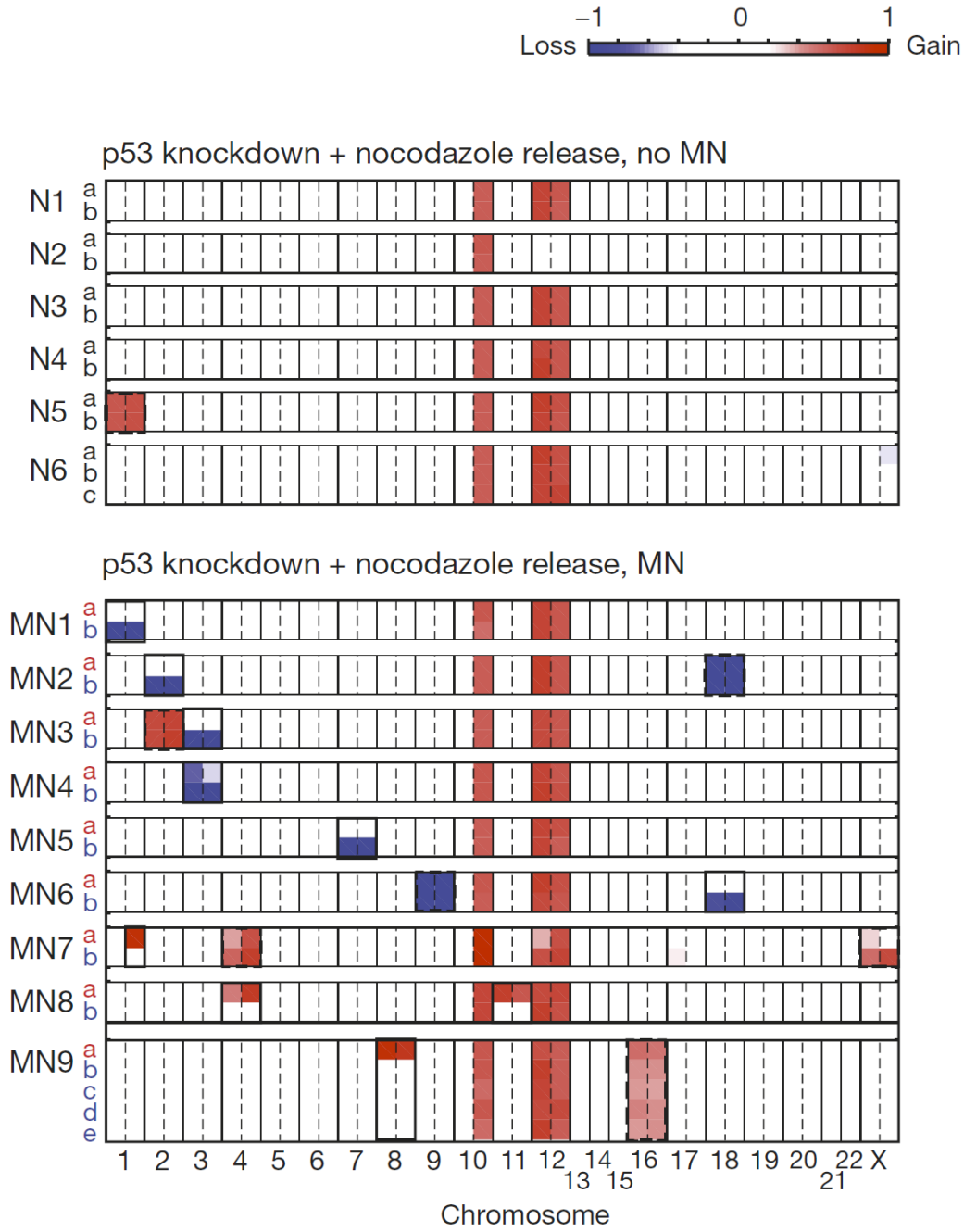
In contrast, **all 9 daughter pairs** from micronucleated mother cells showed **asymmetries**!





MN1 → Chromosome 1 had been inside the micronucleus

MN8 → Chromosomes 4 and 11 had been inside the micronucleus

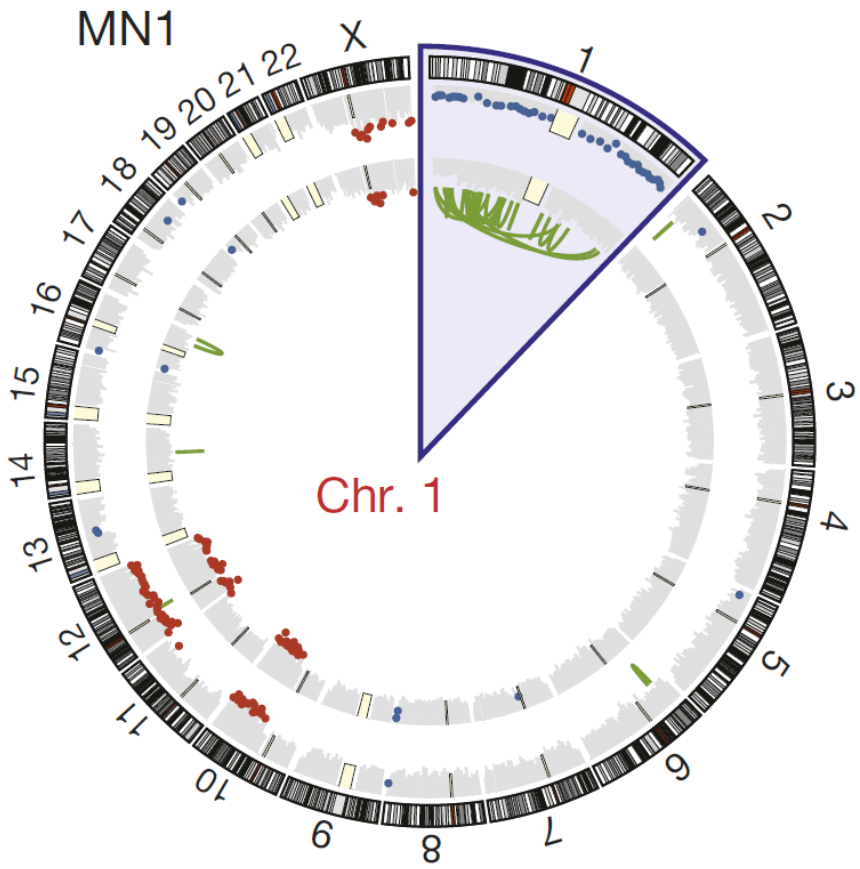


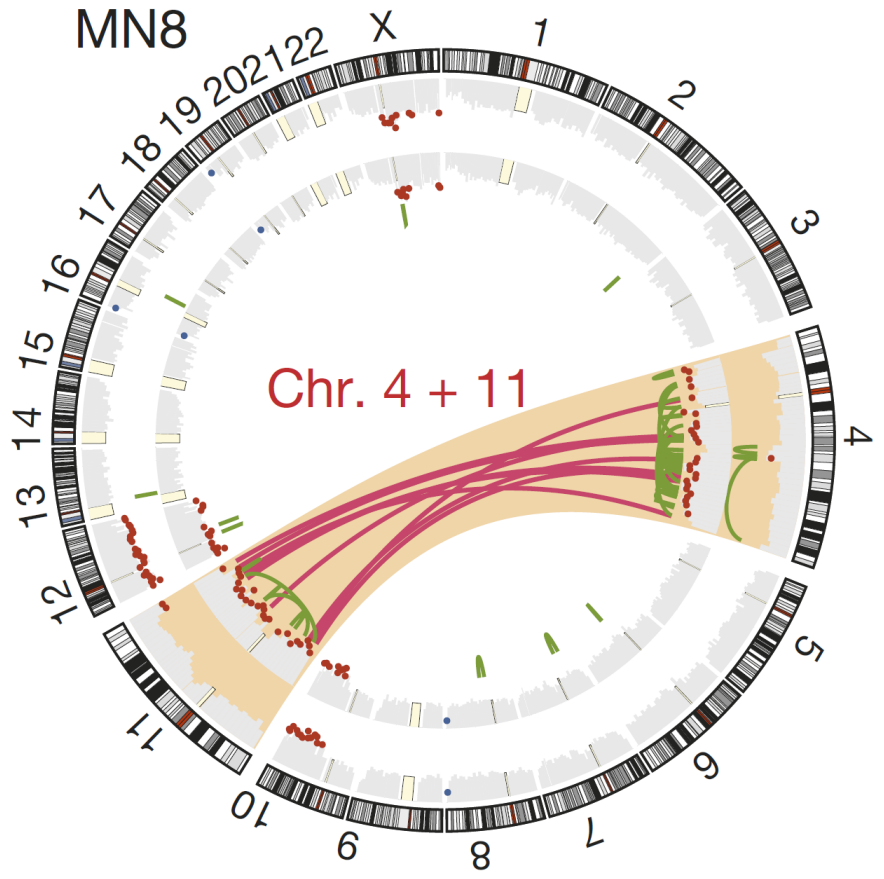
- The authors next checked for the characteristic chromosomal rearrangements of chromothripsis.
- Unfortunately, MDA (for whole genome amplification) also generates pseudo-rearrangements, due to template-switching of the polymerase.
  - Mostly short-range inversions → excluded from the analysis
- They developed statistical methods to test for significant **enrichment of re-arrangements along a chromosomal arm**.

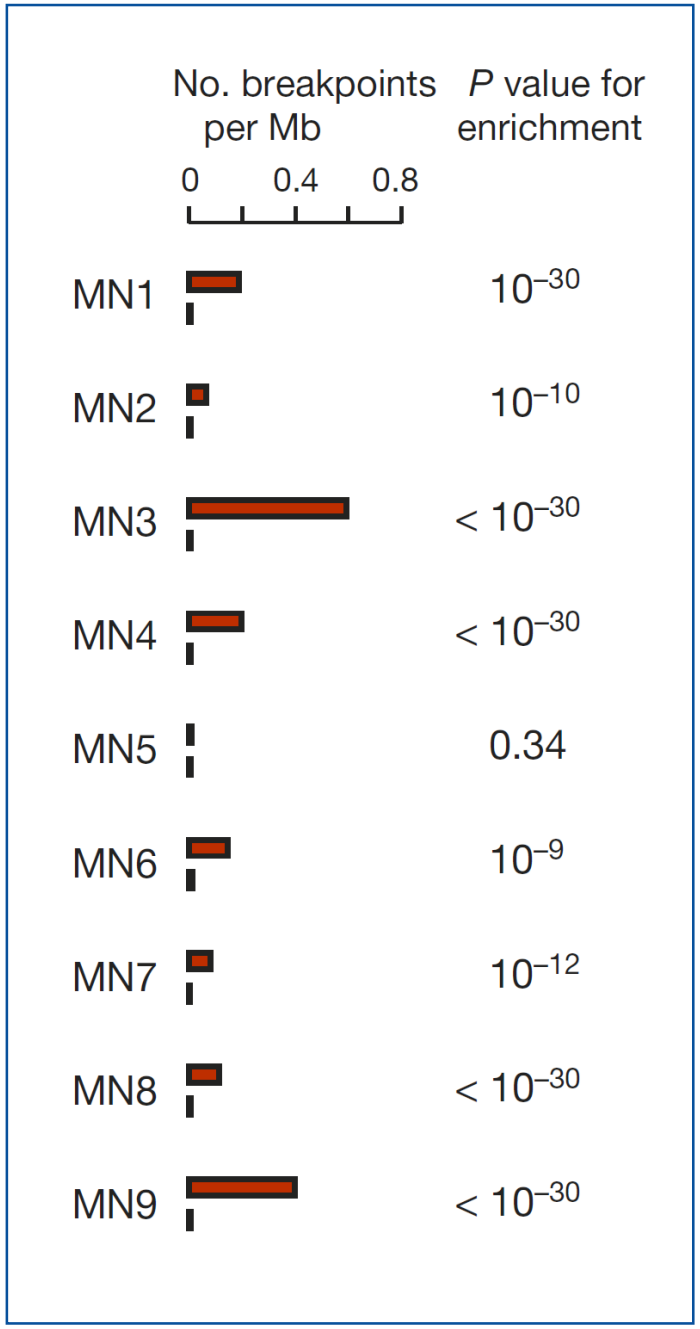
  → Distinguish chromothripsis from artefacts

Using Look-Seq, the authors could confirm the characteristic long-range rearrangements typical of chromothripsis.

They were restricted to the micronucleated chromosomes!







← In 8 out of 9 analysed cells, micronucleation led to chromothripsis!

The authors also confirmed the other cardinal features of chromothripsis:

- Oscillation between two copy number states
- Preservation of both the paternal and maternal alleles in regions of higher copy number

Conclusions

ARTICLE

Chromothripsis from DNA damage in micronuclei

- In their model, chromosomes in micronuclei are severely under-replicated. Also, there is accumulation of DNA damage during S/G2 phase.
- In those cases where the micronucleated chromosome is re-integrated in the next cell division, chromothripsis is an **extremely common** occurrence.
- Thus, chromothripsis occurs in micronuclei. (However, other pathways to chromothripsis may yet be found.)
- The formation of micronuclei may directly contribute to genomic instability and cancer.

RESEARCH ARTICLE

Mechanisms generating cancer genome complexity from a single cell division error

Neil T. Umbreit[1,2,3]*†, Cheng-Zhong Zhang[4,5]*†, Luke D. Lynch[2,3]‡, Logan J. Blaine[2,3]‡, Anna M. Cheng[1,2,3], Richard Tourdot[4,5], Lili Sun[6], Hannah F. Almubarak[4,5], Kim Judge[7], Thomas J. Mitchell[7,8], Alexander Spektor[2,3,9], David Pellman[1,2,3]†

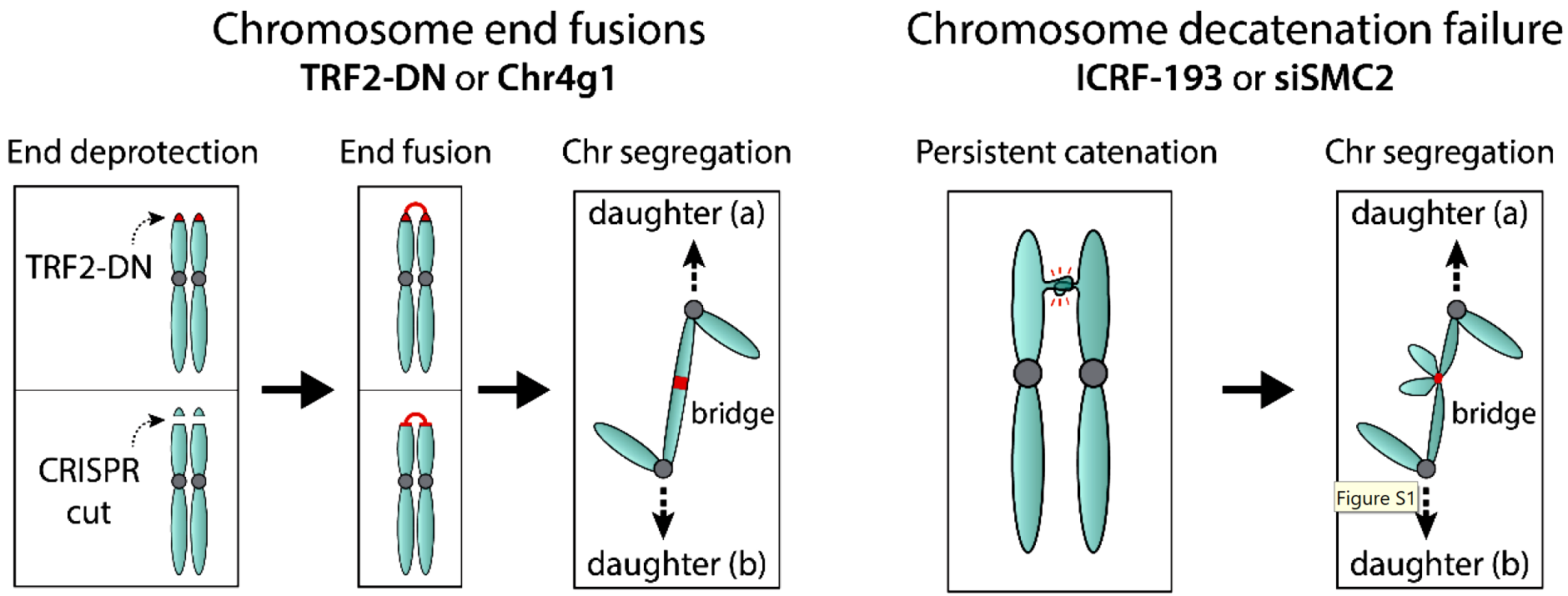
Science, 2020

The authors demonstrate that by inducing **chromosome bridges**, they can initiate a cascade of events (including micronucleus formation) that leads to **ongoing genomic instability** in progeny cells.

Chromosome bridges were artificially induced in 4 different ways

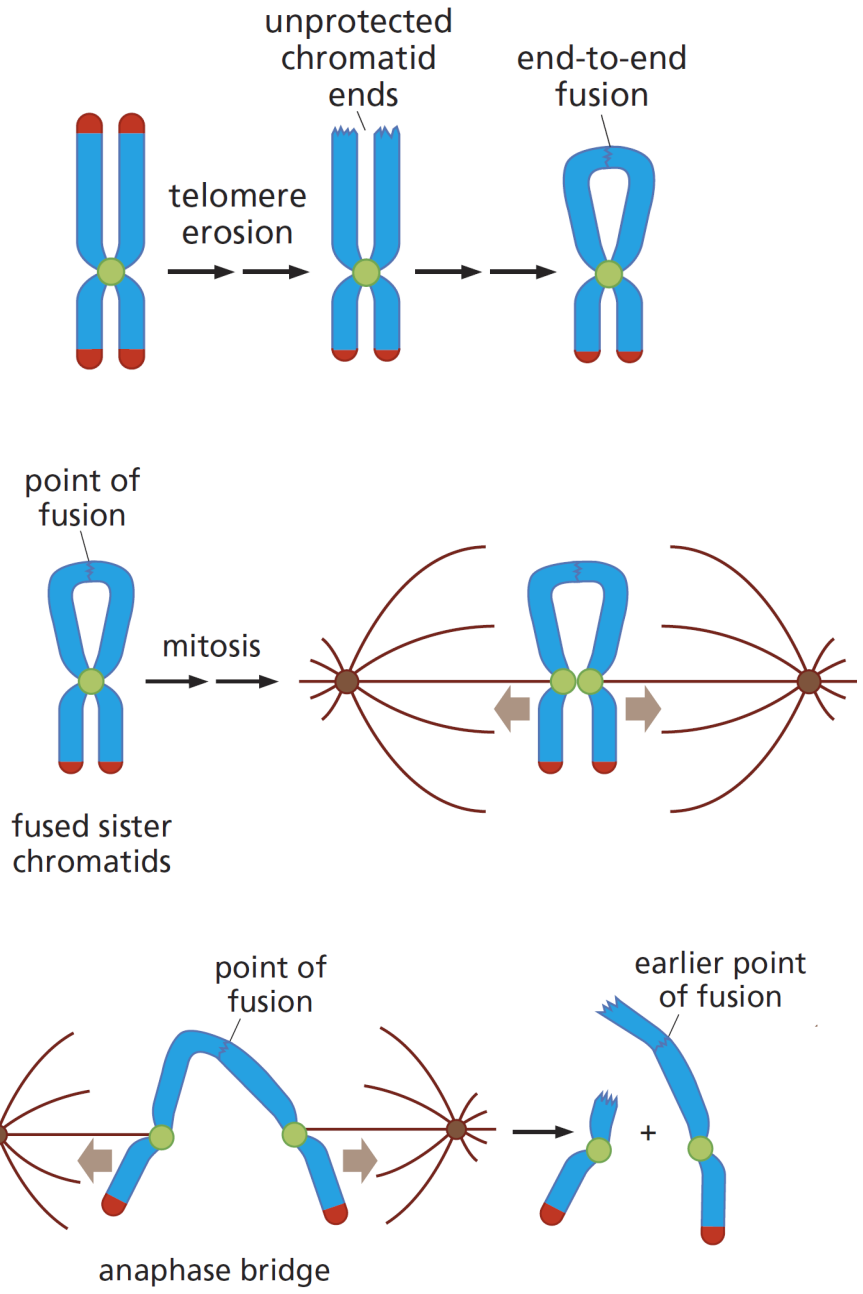
Dominant negative variant of TRF2 (telomeric repeat-binding factor 2)

CRISPR/Cas9 cut near the end of Chr4



Low-dose topoisomerase II inhibition

Partial knockdown of condensin



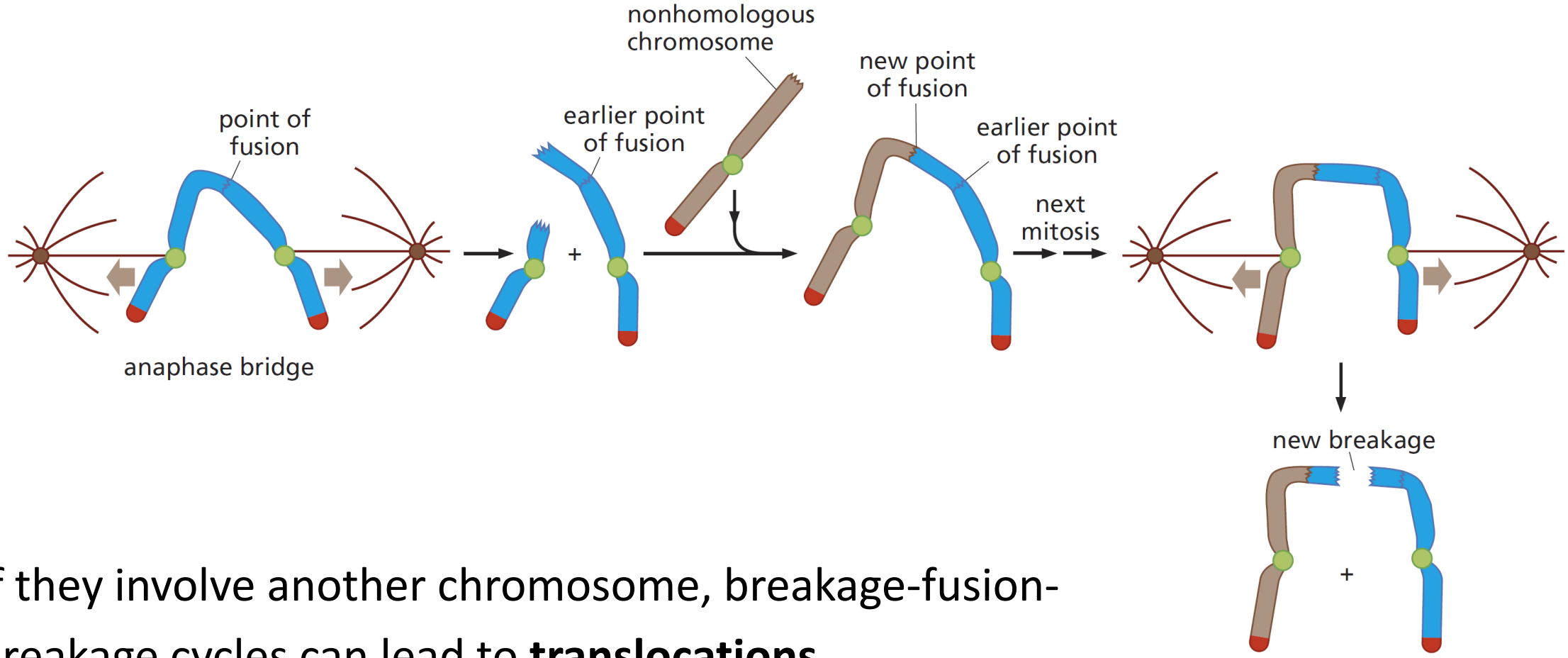
Weinberg's *The Biology of Cancer*, 2e

Telomere erosion leads to chromosome bridge formation.

During progression of mitosis, the two chromatids are pulled apart.

There is a break in a new location. The cycle continues.

⟶ Breakage-fusion-breakage cycles



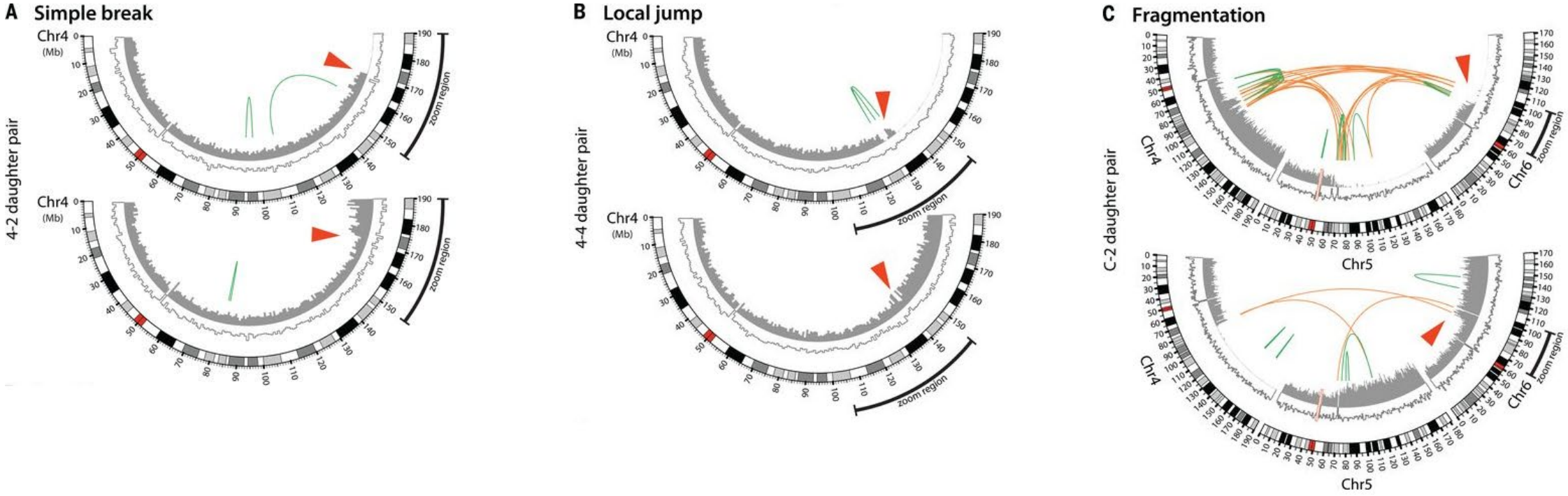
If they involve another chromosome, breakage-fusion-breakage cycles can lead to **translocations**.

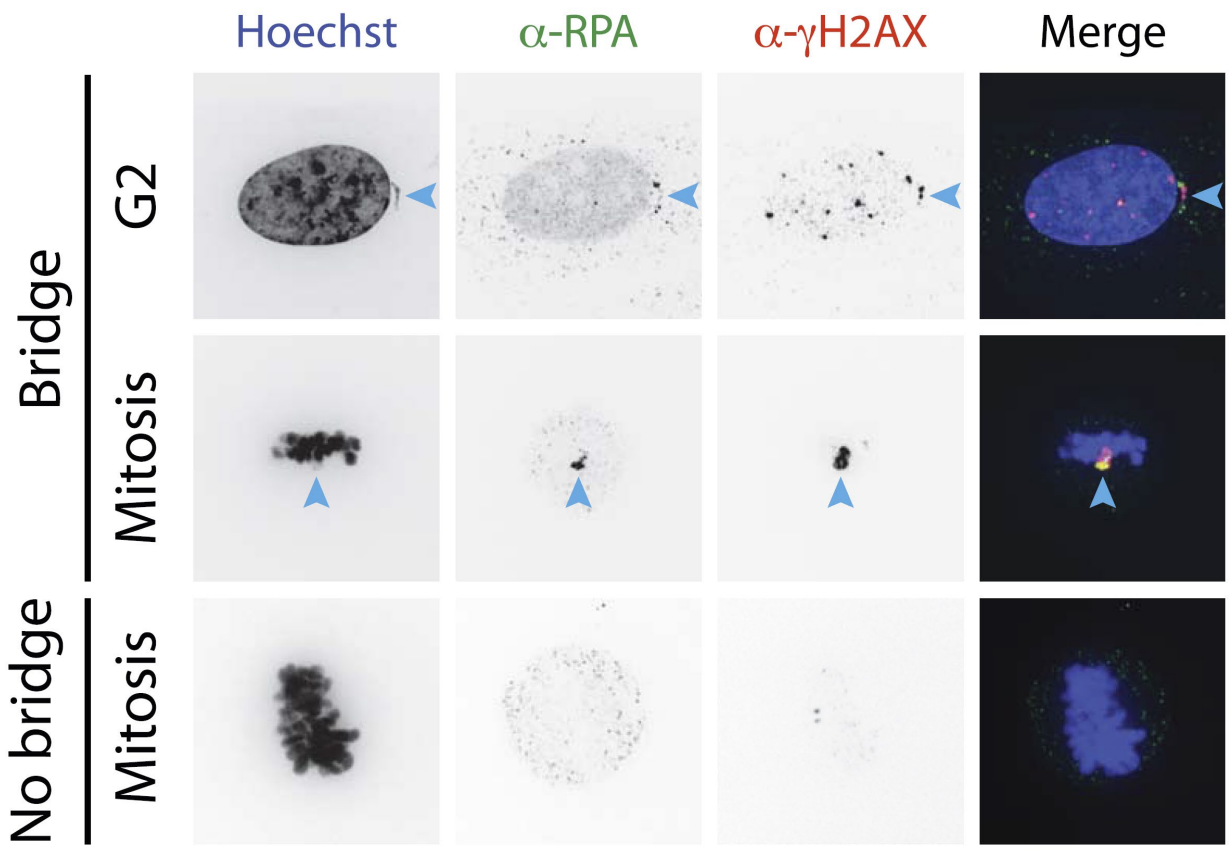
Amplifications or deletions near the breakpoints are common.

Chromothripsis has *not* been described.

Look-Seq was done to analyse the immediate consequences of chromosome bridge formation in daughter cells.

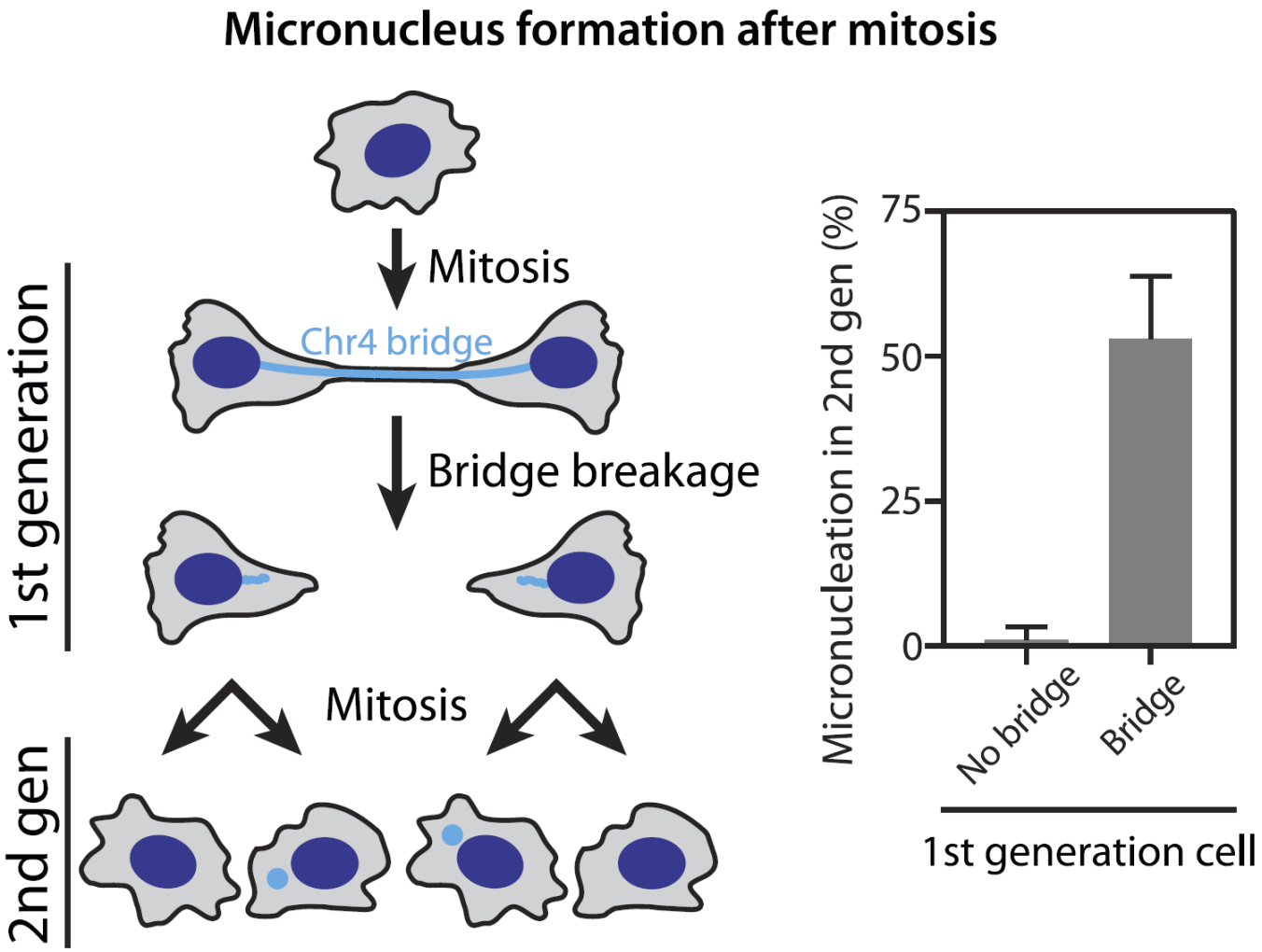
In different replicate cells, a spectrum of changes was seen, from simple rearrangements to complex chromothripsis-like states.





Similar to micronuclei, DNA in chromosomal bridges showed evidence of defective replication and accumulation of DNA damage.

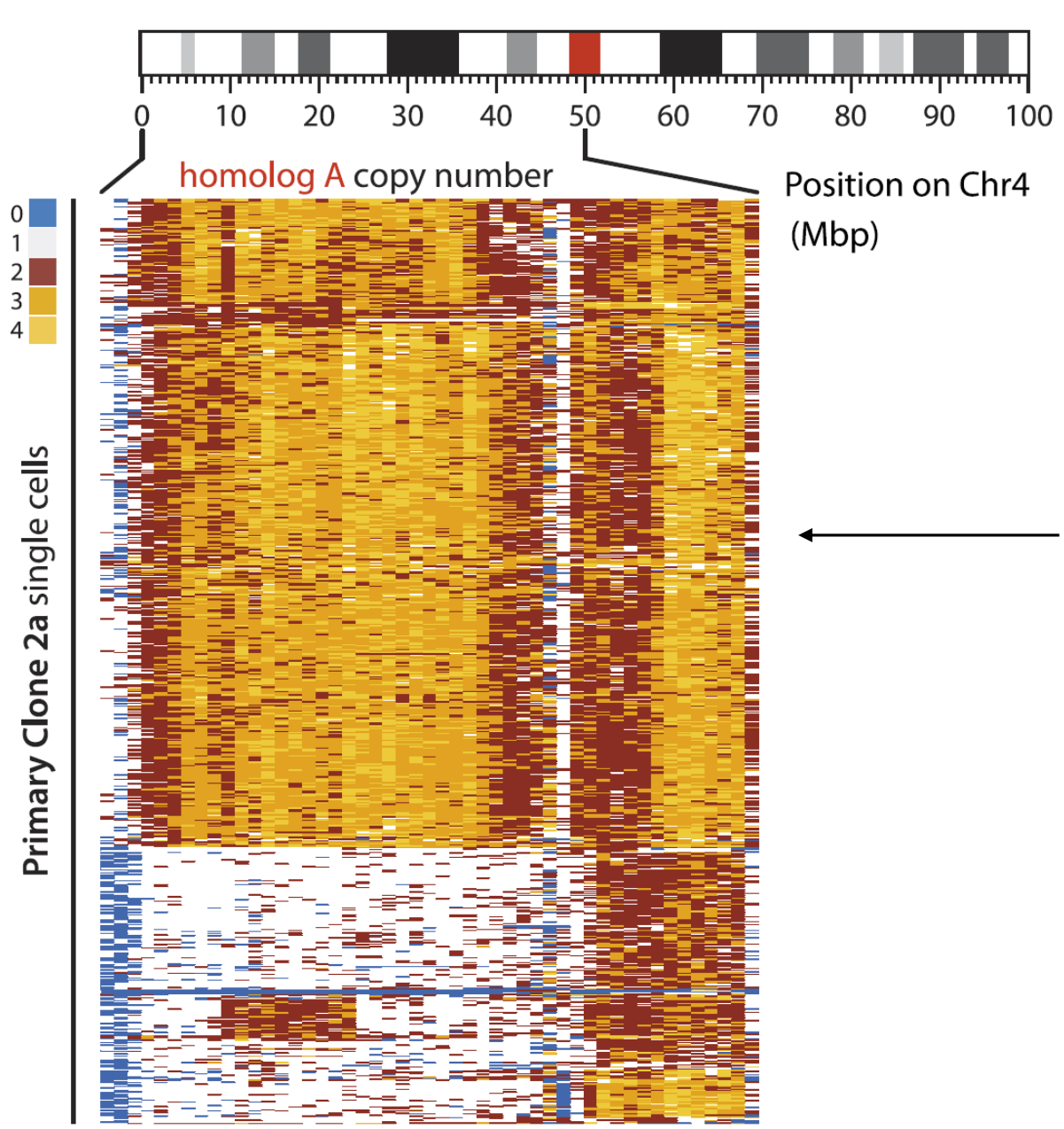
The authors speculate that both chromosomal bridges and micronuclei share deficiencies in the nuclear membrane that make regular DNA synthesis impossible.



Out of cells that formed bridges, **micronuclei formed in ~50%** after the next mitosis.

In those cells where bridges were induced using CRISPR, the micronuclei usually contained the targeted chromosome, Chr4 (in 80% of cells analyzed by FISH.)

→ We expect further genomic instability and chromothripsis!



Copy number heatmap for Chr4p with 637 single cells.

- After induction of bridge formation on Chr4, one of the cells was allowed to continue to divide.
- Its descendants showed ongoing genetic diversification.
- Divergent sub-clones are seen in single-cell DNA sequencing data.
- Ongoing chromosome bridge and micronucleus formation was also seen.
- A single event may trigger cancer genome evolution!

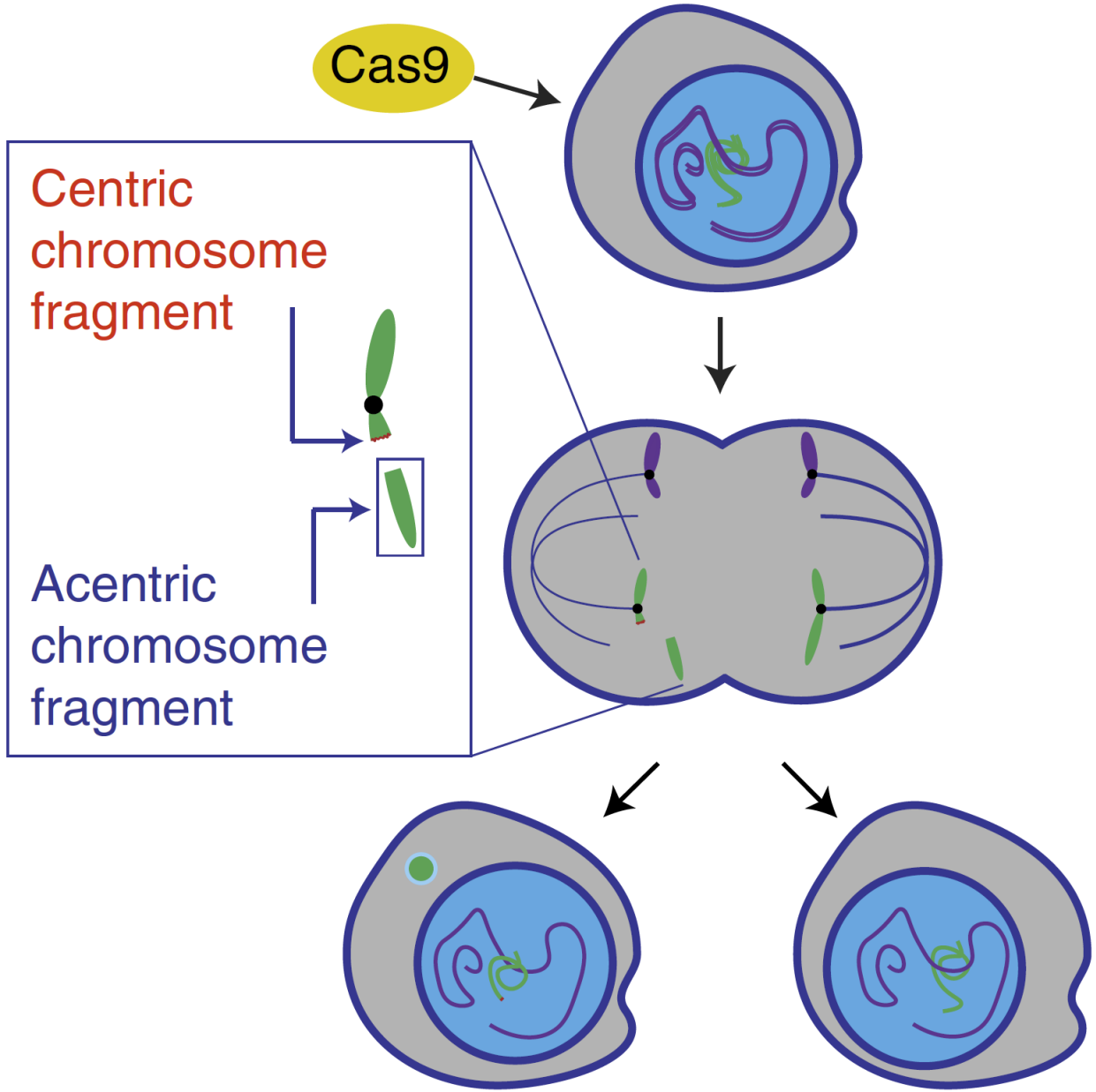
nature genetics

ARTICLES

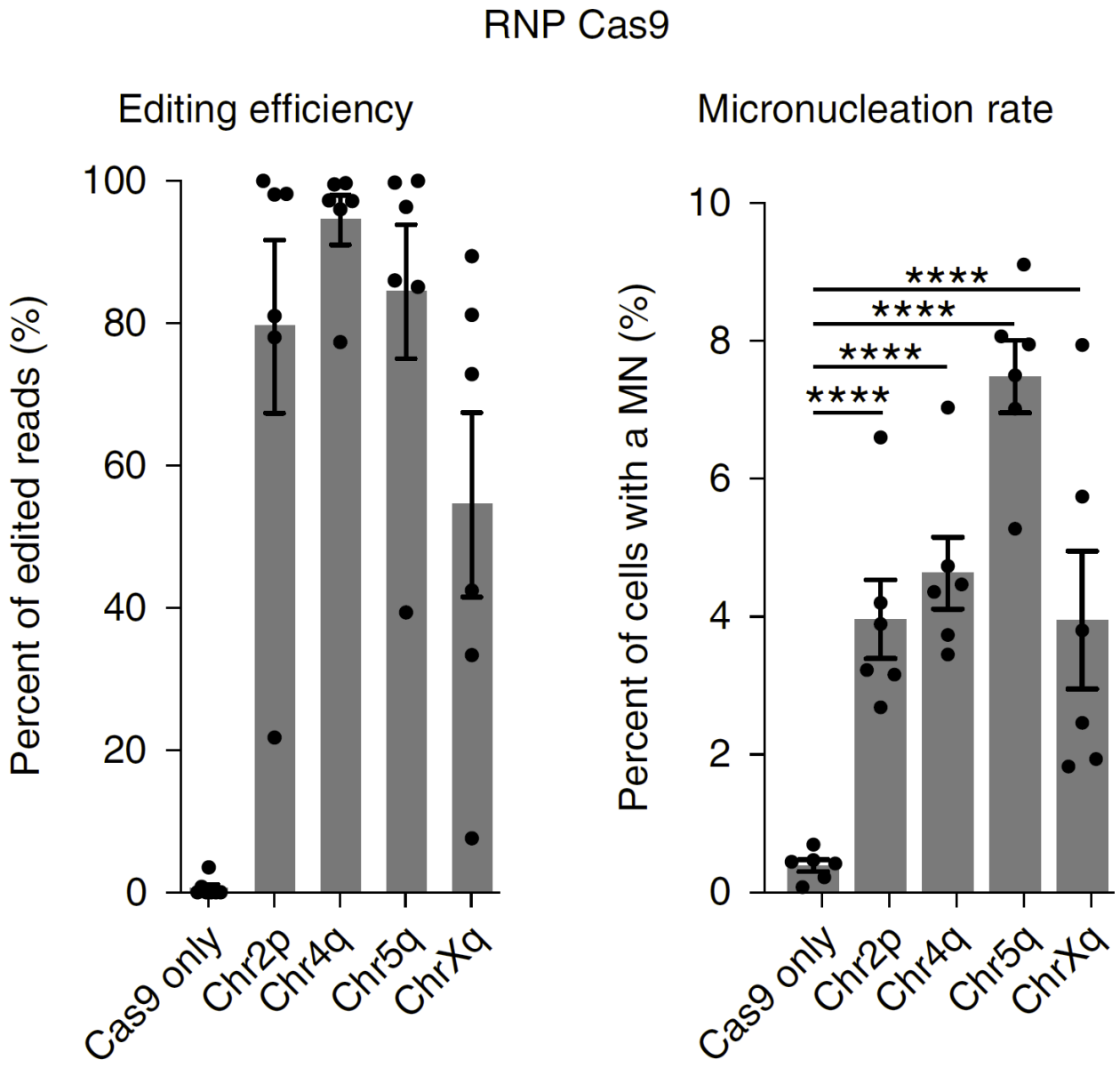
Chromothripsis as an on-target consequence of CRISPR–Cas9 genome editing

Mitchell L. Leibowitz[1,2,3,8], Stamatis Papathanasiou[2,3,8], Phillip A. Doerfler[4], Logan J. Blaine[2,3], Lili Sun[5], Yu Yao[4], Cheng-Zhong Zhang[6,7], Mitchell J. Weiss[4] ✉ and David Pellman[1,2,3] ✉

April 2021



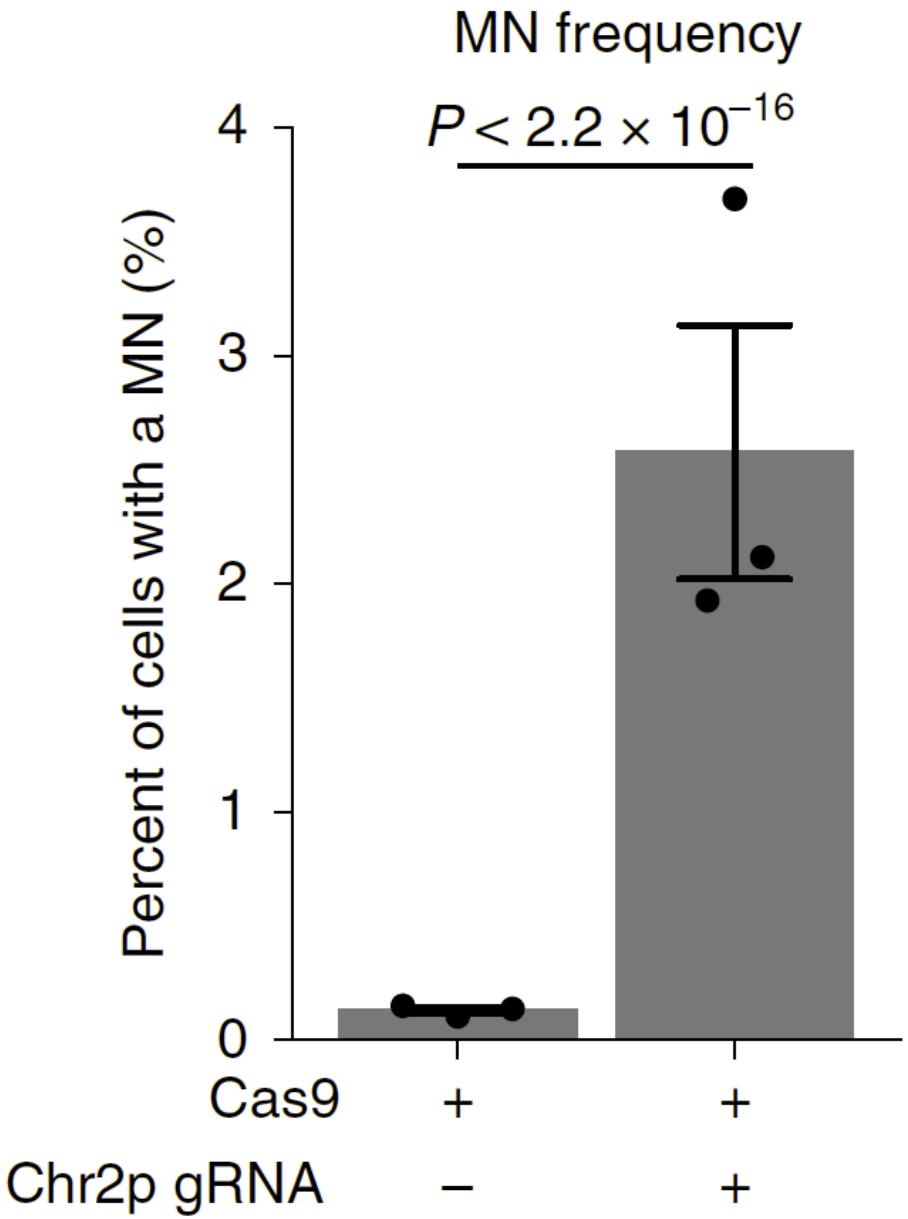
Acentric chromosome fragments are often incorporated into micronuclei.



Four single guide RNAs (targeting a unique site in the genome) were tested.

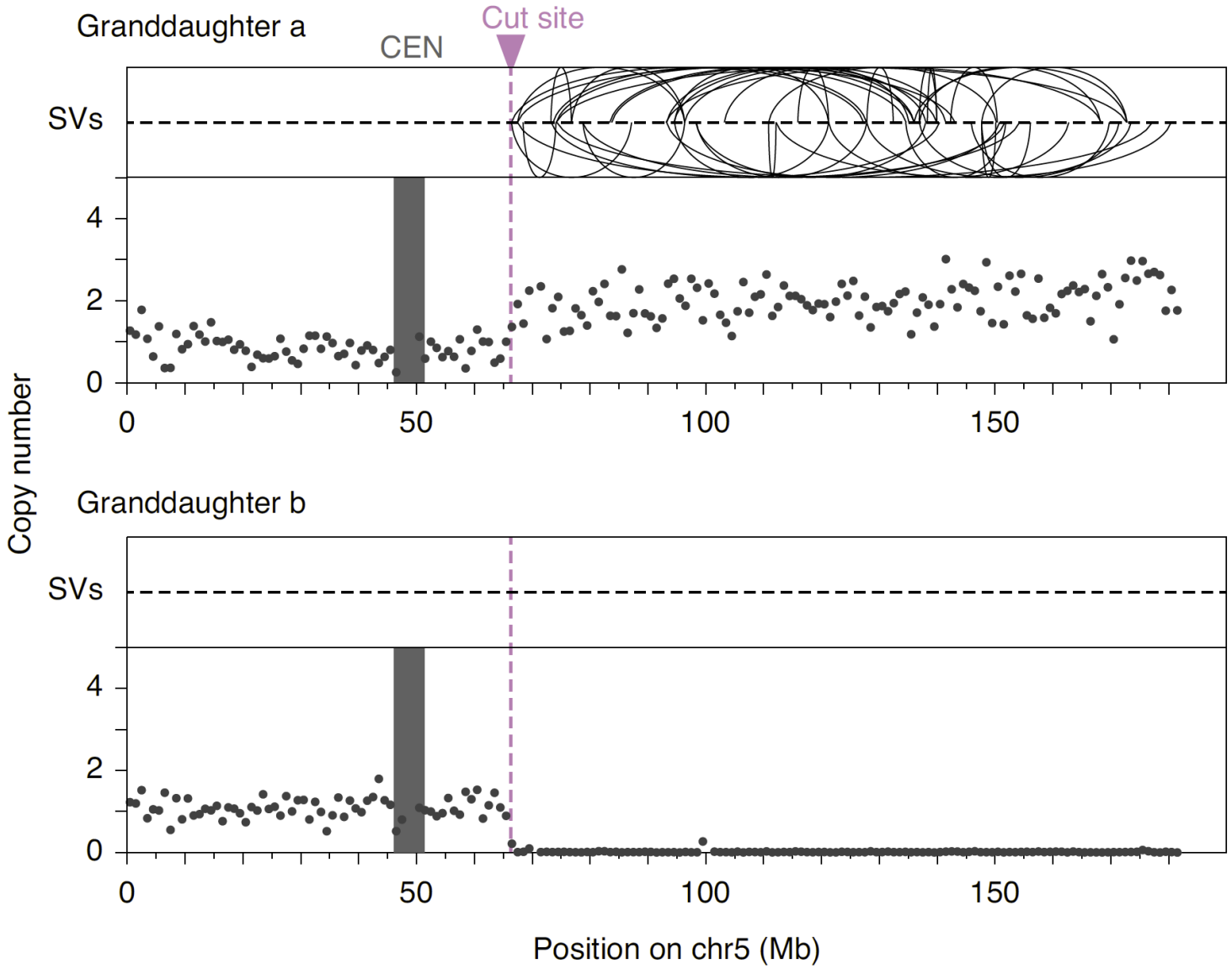
They caused a **10-20× fold increase in micronucleus formation over baseline!**

This occurred during a single cell division. Cells were cell-cycle synchronized using a serum-starve-and-release protocol, then transfected.



Micronucleus formation was also seen after CRISPR-Cas9 editing of human hematopoietic stem cells.

A locus was chosen (targeting *BCL11A* on Chr2) that is a potential target for gene therapy for β-thalassemia.



Look-Seq shows evidence of **chromothripsis** downstream of the CRISPR cut site.

A micronucleus had formed, and been re-incorporated into a grand-daughter cell.

Conclusions

nature genetics ARTICLES

Chromothripsis as an on-target consequence of CRISPR–Cas9 genome editing

- A number of recent studies have shown that CRISPR-Cas9-induced double-strand breaks can have unintended **on-target effects** (e.g. large deletions and recombinations).
  - These are often missed, unless single-cell whole-genome sequencing is performed. (Large deletions will delete PCR primer-binding sites → the mutation becomes invisible.)
- The induction of micronuclei, and consequently chromothripsis, is a potential mechanism.
- Actively dividing cells treated with CRISPR-Cas9 can be monitored for micronuclei.
- This has implications for gene therapy. Might rare instances of chromothripsis in CRISPR-edited stem cells lead to leukaemia?

Thank you for your attention!