

1 **Determinants and Clinical implications of Chromosomal Instability in Cancer**

2
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10 11 **Abstract**

12 One of the most common characteristics of cancer genomes is their aberrant architecture,
13 ranging from small insertions or deletions to large chromosomal alterations. Chromosomal
14 instability (CIN) underpins much of the intra-tumoural heterogeneity observed in cancers
15 and drives phenotypic adaptation during tumour evolution. There is an urgent need to
16 increase our efforts to target CIN as if it were a molecular entity. Indeed, CIN accelerates
17 drug resistance acquisition, tumour relapse and treatment failure that plagues current
18 therapies. Identifying novel strategies to modulate CIN and to exploit the fitness cost
19 associated with aneuploidy in cancer is therefore of paramount importance for the success
20 of cancer medicine. Modern sequencing and analytical methods greatly facilitate the
21 cataloguing of somatic copy number alterations (SCNAs) and offer new possibilities to better
22 exploit the dynamic process of CIN. Here we will review the principles governing CIN
23 propagation in cancer, how CIN may impact on immune checkpoint blockade therapy and
24 survey vulnerabilities associated with CIN that could offer therapeutic opportunities.

25 26 **Introduction**

27 Aneuploidy is one of the most striking and widespread features of human cancers, with the
28 vast majority of tumours displaying various types of SCNAs including segmental
29 aneuploidies, focal events, or whole-chromosome aneuploidies. Considering only the most
30 frequent cancers, approximately 60% of lung tumors, 60-80% of breast tumors, 70% of
31 colorectal tumors and 30 % of prostate tumors deviate from a diploid karyotype¹⁻⁶. Tumours
32 that do not feature gross aneuploidy often display hypermutation due to mismatch repair
33 deficiency or POLE/POLD mutations, which may reflect the limits that cancer cells can
34 handle in terms of genetic instability⁷. CIN refers to the ongoing acquisition of genomic
35 alterations that can involve gain or loss of whole-chromosomes (w-CIN) or structural
36 aberrations (s-CIN), which range from point mutations to small-scale genomic alterations
37 and gross chromosomal rearrangements. However, aneuploidy (an aberrant genomic state)
38 and CIN (the property of displaying a high rate of genomic changes) may differ in their
39 prognostic value, a distinction that warrants careful investigation. In this review, we will
40 discuss how CIN impacts upon tumour evolution, provide an overview of the causes of CIN
41 in cancer with an emphasis on the mechanisms enabling CIN propagation, and strategies to
42 target CIN in cancer.

43 44 **CIN:opening Pandora's box**

45
46 *Mitotic causes of CIN*

1 CIN cells acquire a high rate of SCNAs during cancer cell proliferation, creating genetic
2 heterogeneity within the population. A myriad of defects can result in frequent
3 missegregation of chromosomes during cell division. These mechanisms and their causative
4 role in cancer have been reviewed in detail previously^{8,9}. They include defects that directly
5 impinge on the chromosome segregation machinery, such as altered microtubule spindle
6 dynamics, mechanisms required to correct erroneous microtubule-kinetochore
7 attachments, and defects affecting the mitotic checkpoint or sister-chromatid cohesion⁸⁻¹⁴.

8 Supernumerary centrosomes are frequent in cancer and threaten genome stability
9 by increasing the probability of creating merotelic attachments, a type of microtubule-
10 kinetochore attachment defects that does not trigger the mitotic checkpoint^{12,15}. Failure to
11 cluster extra centrosomes into two poles leads to a multipolar division, most likely lethal
12 due to an excessive loss of chromosomes¹⁵⁻¹⁸ (Figure 1).

13 Genome-doubling or tetraploidization, which may arise from a cell division failure or
14 endoreplication (re-replication without intervening mitosis) amongst several mechanisms¹⁹,
15 directly impair chromosome segregation fidelity during ensuing divisions due to the
16 presence of extra centrosomes^{15,20} (Figure 1). Tetraploidization is not only linked to cancer
17 development but is also part of the normal development program of differentiated cell
18 types such as hepatocytes, or megakaryocyte and placental trophoblasts which can become
19 highly polyploid. In addition, tetraploidy is found in ageing tissues and in response to various
20 stresses^{21,22}. Genome doubling is a frequent feature of human cancers, reported in over 40%
21 of lung, head and neck, breast, bladder, colorectal, oesophageal and ovarian cancers^{2,23,24}.
22 Of note, sequencing-based studies can identify tumors that have undergone WGD during
23 their development, even if the ploidy is no longer tetraploid at diagnosis due to
24 chromosome losses. This explains the possible discrepancy with cytometry-based studies
25 where estimates are based on cells carrying an exact tetraploid DNA content. For example,
26 computational approaches using genomics estimated that over 50% of breast cancer² had
27 undergone WGD, while a large scale cytometry-based study detected tetraploid cells in 32%
28 of tumors²⁵. Genomics studies suggest that genome-doubling is a relatively early event in
29 the evolution of several cancers and precedes the acquisition of additional SCNAs and
30 subclonal expansion^{23,24,26,27}. Tetraploid cells have also been detected in pre-malignant
31 lesions in oesophageal, cervical, breast and head and neck cancers^{25,28-30}. Genome doubling
32 could therefore represent the CIN-initiating event in an important proportion of human
33 cancers.

34 *Structural defects trigger CIN*

36 Aneuploid tumours almost invariably display both numerical and structural chromosomal
37 aberrations. Pre-mitotic defects such as replication stress can generate chromosome fusions
38 resulting in dicentric chromosomes (telomere fusion for example) and acentric chromosome
39 fragments, both of which may be randomly distributed to daughter cells³¹. DNA bridges
40 from dicentric chromosomes can also physically prevent cell division and generate
41 tetraploid cells which are inherently prone to CIN³²⁻³⁵. Under-replicated regions may also
42 prevent the physical separation of chromosomes during mitosis, leading to aneuploidy³⁶.
43 Numerical chromosomal aberrations can be symptomatic of DNA replication stress without
44 underlying defects in the chromosome segregation machinery. Replication stress therefore
45 provides an alternative route to generate complex karyotypes through the uneven
46 distribution of damaged genetic material during division.

1 *wCIN*, *sCIN* and nuclear envelope defects

2 Recent studies indicate that missegregated chromosomes are prone to accumulate
3 mutations and structural defects. For example, mitotic errors can result in lagging
4 chromosomes during the partitioning of DNA into daughter cells, which may become
5 trapped during cell division or isolated and form micronuclei. Both situations create a
6 context whereby the DNA may sustain extensive DNA damage and chromosomal
7 rearrangements including chromothripsis^{37,38}. Interestingly, micronuclei and DNA bridges
8 both display nuclear envelope (NE) disruption and therefore loose compartmentalisation
9 with the cytoplasm, potentially exposing DNA to reactive oxygen species and cytoplasmic
10 enzymes. In micronuclei, aberrant DNA replication correlates with NE collapse, the massive
11 accumulation of DNA damage and chromothripsis³⁷⁻³⁹. Importantly these observations are
12 not limited to *in vitro* analyses, as micronuclei displaying disrupted NE and DNA damage
13 accumulation could readily be found on NSCLC paraffin samples³⁹.

14 NE integrity is also lost when dicentric chromosomes create ultrafine bridges, which
15 can also lead to chromothripsis and hyper-mutation (kataegis) of localized chromatin
16 regions⁴⁰. NE loss exposes ultrafine bridges to a cytoplasmic nuclease creating single
17 stranded DNA, the substrate for mutagenic APOBEC3 enzymes, which could explain the
18 APOBEC mutational signature often found near rearrangement breakpoints^{40,41}. The
19 physical yet often transient isolation of DNA during CIN may contribute to the highly
20 localized nature of APOBEC-driven mutations in cancer, as well as its appearance following
21 the onset of CIN during tumour evolution⁴²⁻⁴⁴.

22 Interestingly, cell migration through tight spaces and excessive cytoskeletal forces
23 exert pressure onto the nucleus, leading to NE rupture, chromatin extrusion, DNA damage⁴⁵⁻
24 ⁴⁷ and karyotypic abnormalities⁴⁷. The process of epithelial-to-mesenchymal transition
25 induced by TGF- β often associated with metastasis, was also linked to chromosomal
26 instability and NE defects⁴⁸. Physical constraints and paracrine effects associated with
27 cancer cell dissemination therefore provide additional routes to genomic instability.

28

29 *Cancer cell-extrinsic causes of CIN*

30 Additional cell-extrinsic or non-genetic causes of CIN have also been proposed, besides
31 mechanical forces upon the nucleus and paracrine induction of EMT described earlier.
32 Glucose deprivation, hypoxia or acidification of the extracellular milieu, which mimic
33 properties of the tumour microenvironment, induce genomic instability and aneuploidy^{49,50}.
34 Entosis, the process of cell engulfment by another cell, causes tetraploid and CIN by
35 blocking division of the host cell, has been reported to be present at low frequency in
36 human tumour specimens^{51,52}.

37

38 In summary, chromosome segregation errors can potentially trigger a chain of events
39 resulting in extensive numerical and structural chromosomal aberration, and cause
40 mutation acquisition. Indeed, there are numerous examples showing that aneuploidy itself
41 can be a trigger for further chromosomal instability and rearrangements⁵³⁻⁵⁵. Aneuploid and
42 tetraploid cells evolve to gradually accumulate further whole-chromosome and segmental
43 aberrations with time^{20,24,34,54-56}. Consequently, even infrequent missegregation events in
44 cancer cells could induce a leap in cell fitness by causing profound copy-number changes
45 and acquire point mutations.

46

47 **Accelerated evolution through CIN and therapy resistance**

1 CIN provides an efficient means to respond to various selective pressures, as exemplified by
2 experimental data in various organisms⁵⁶⁻⁵⁹. Rare clones within karyotypically
3 heterogeneous populations will often outcompete other cells only when facing selective
4 pressure⁵⁷, and tetraploidization in particular facilitates the rapid acquisition of copy-
5 number alterations and mutations in response to stressful conditions, leading to increased
6 fitness^{56,58}. CIN and tetraploidization also confer multidrug resistance, including for some of
7 the most commonly used chemotherapeutic drugs^{60,61}. Oncogene addiction, the basic
8 principle for the efficacy of targeted therapies⁶², can be circumvented by ongoing CIN.
9 Elegant experiments using inducible mouse models showed that CIN (driven by MAD2
10 overexpression), when combined with KRAS^{G12D} or HER2 oncogenes, consistently contribute
11 to bypass oncogene addiction upon oncogene withdrawal and facilitates tumour relapse
12 and persistence^{63,64}. CIN thus offer an escape mechanism following targeted therapy, and
13 suggests that the loss of driver oncogenic mutations from a copy-number event would not
14 be as deleterious in cancer cells with ongoing CIN. This represents a conceivable scenario
15 since ongoing CIN contributes to mutational heterogeneity by causing the loss of
16 chromosomal regions previously harboring clonal mutations, which has been observed in
17 lung⁶⁵ and breast²⁶ cancers.

18 CIN is also an important driver of parallel evolution. In NSCLC, focal amplification of
19 driver genes takes place from different alleles in different tumour subclones, a process
20 termed mirrored subclonal allelic imbalance, indicative of ongoing CIN⁶⁵. Comparison of
21 SCNAs in circulating tumour cells (CTCs) and metastatic tumours also revealed convergence
22 towards common SCNA in patients from various cancer types⁶⁶. Of note, neither studies
23 observed convergence at the mutational level, suggesting CIN allows more rapid selection of
24 driver events than other mutagenic processes in some cancers. Convergence at the copy-
25 number level involving LOH or oncogene amplification have been reported in high-grade
26 ovarian cancer⁶⁷. The emergence of resistance during therapy can also proceed through
27 parallel convergence. Resistance to ERK-inhibition can occur through parallel amplification
28 of BRAF in divergent clones⁵², while resistance to a high dose of a PI3K α inhibitor arose
29 through parallel convergence on PTEN loss^{68,69}. CIN therefore allows cells to explore
30 evolutionary trajectories during tumour evolution and adapt to therapy which underlies
31 treatment failure. Radiation therapy⁷⁰, as well as many of the most commonly used
32 chemotherapeutic drugs induce chromosomal instability *in vitro*^{71,72}. CIN induction was
33 observed for several classes of anticancer compounds targeting microtubules (Taxol), DNA
34 damage response pathways (PARP and topoisomerase inhibitors) as well as DNA
35 intercalating agents (cisplatin) or nucleoside analogues (Gemcitabine). Notably, in some
36 cases CIN induction was exacerbated when using drug combinations below their respective
37 IC50 values⁷¹. The efficacy of several drug used as standard of care could be linked to their
38 common effect of driving excessive genomic instability in cancer cells. Based on this
39 interpretation, what would make cancer cells exquisitely sensitive to several of these
40 compounds is not their faster proliferation rate but rather the loss of various checkpoints,
41 causing them to acquire additional SCNAs beyond a threshold compatible with cell survival
42 (see *CIN Attenuation* section below).

43

44 **Mechanisms enabling CIN propagation**

45

46 *Aneuploidy tolerance*

1 A fundamental difference between normal and transformed cells is in their ability to cope
2 with genetic imbalances. The deleterious impact of aneuploidy on cellular proliferation has
3 been documented for many cell types⁷³. For example, in the haematopoietic compartment,
4 aneuploid cells are outcompeted due to slower proliferation⁷⁴. Aneuploidy also impairs
5 organismal development and is the main cause of spontaneous abortions in humans, where
6 most constitutive aneuploidies are embryonic lethal with trisomy 21 being a rare
7 exception⁷⁵. Aneuploidy has profound consequences of gene dosage by causing imbalances
8 in the expression of hundreds to thousands of genes residing on the extra chromosome(s)⁷⁶.
9 This results in a number of aneuploidy-associated stresses that impair overall cellular fitness
10 by causing metabolic changes and impacting on the protein turnover machinery⁷⁷⁻⁷⁹.
11 Chromosome gains appear to be particularly detrimental to cell proliferation and tumours
12 more frequently harbour chromosome losses than gains⁵⁴. Therefore, it appears that the
13 aneuploid state itself is not sufficient to transform normal cells and in fact aneuploid cells
14 are largely under negative selection pressure^{54,74}. Then how to reconcile this observation
15 with the often-reported high proliferation rate of aneuploid cancer cells? As discussed
16 below, aneuploidy tolerance mechanisms associated with cell transformation are thought to
17 enable CIN propagation. There may also be an overestimation regarding the hyper-
18 proliferative feature of cancer cells, symptomatic of *in vitro* analyses. The proliferation rate
19 of primary human tumours based on radiographic measurements or derived from tumor
20 marker levels, suggest their doubling times range from 30 days to several months (reviewed
21 in⁸⁰). These measurements reflect a combination of cellular proliferation and other factors
22 acting upon cancer cells' fitness such as immuno-editing, which may mask their actual
23 proliferative rate. Potential doubling time (Tpot) estimations derived from BrdU
24 incorporation measurements, also suggest relatively slow doubling times from 1 to 2 days in
25 head and neck cancer^{81,82}, 4.5 days in colorectal cancer⁸³ and 12.5 to 28 days in breast
26 cancer^{84,85}. Intravital imaging in immunocompromised mice also shows that cancer cell lines
27 proliferate significantly more slowly *in vivo* than they do in cell culture⁸⁶. The ability of
28 cancer cells to proliferate *despite* aneuploidy, even at a slow rate, might be a crucial CIN
29 determinant more physiologically relevant for tumour evolution.

30 What might then enable cancer cells to tolerate aneuploidy? Genetic alterations that
31 improve protein turnover, hence alleviate proteotoxic stress, were reported to improve the
32 fitness of aneuploid cells^{87,88}. *TP53* disruption was proposed as an important mechanism
33 enabling the propagation of chromosomal instability *in vitro* and in mouse models. In CIN+
34 colorectal cancers for example, *TP53* and Adenomatous polyposis coli (*APC*) mutations, are
35 the most significantly associated alterations^{20,31,89-91}. Recent studies suggest that *p53* does
36 not invariably arrest cells following chromosome missegregation, and some aneuploidies
37 can be propagated in a *p53*-proficient background^{92,93}. One hypothesis is that *p53* does not
38 detect whole-chromosome aneuploidies *per se*, but some aneuploidies (involving specific
39 chromosomes, or a combination thereof) generate a level of stress sufficient to induce *p53*
40 stabilisation⁹². On the other hand, the propagation of structural aberrations seems
41 exquisitely dependent on *p53* disruption and linked with the acquisition of complex
42 karyotypes^{92,93}. *P53* stabilisation following chromosome missegregation has been linked to
43 DNA damage resulting from the entrapment of the chromosome during cytokinesis or from
44 the aberrant DNA replication and genomics rearrangements occurring within micronuclei⁹⁴.
45 The requirement for *TP53* pathway disruption for CIN propagation may therefore be
46 intimately linked to the co-occurrence of DNA damage at sites of chromosomal
47 rearrangements that link numerical and structural aneuploidies.

1 However, classical DNA damage response signalling cannot completely explain CIN-
2 induced p53 stabilisation, and in some experimental conditions stabilisation occurs without
3 p53 phosphorylation at sites associated with DNA damage and cell cycle arrest cannot be
4 reverted by ATM inhibitors⁹⁵. We recently identified Caspase-2 (CASP2) as an upstream
5 regulator of p53 following chromosome missegregation⁹⁶. CASP2 was found to cleave
6 MDM2 in response to chromosome missegregation, known to disrupt MDM2's ability to
7 ubiquitinate p53 and targeting it for proteasomal degradation⁹⁷. In colorectal cell lines, the
8 CASP2 steady-state level was found to require BCL9L, which acts as a beta-catenin co-factor
9 for CASP2 transcription. BCL9L mutational inactivation or CASP2 downregulation both
10 conferred tolerance to chromosomal instability⁹⁶. Importantly, reduced CASP2 levels also
11 improved CIN tolerance in p53-deficient cells, by impairing generation of the pro-apoptotic
12 product tBID. It remains unclear why CASP2 becomes active after chromosome
13 missegregation. Several pathways therefore converge onto p53 and the apoptotic
14 machinery to control CIN tolerance in cancer cells, determined by the ability of cells to cope
15 with global transcriptional and metabolic changes and ongoing genomic rearrangements.

16 17 *Tolerance to Genome-Doubling*

18 Genome-doubled cells appear inherently more tolerant to the gain or loss of whole
19 chromosomes, possibly because the impact on overall gene expression is less than when it
20 occurs in a diploid cell^{24,98}. The greater ability of genome-doubled cells to buffer the
21 negative impact of protein imbalances associated with aneuploidy, and their propensity to
22 CIN due to the presence of extra chromosome(s), may explain why genome-doubling is such
23 a common precursor of CIN in cancer appearing early in tumour development^{23,24,27} (Figure
24 1). However the propagation of genome-doubled cells immediately following cell division
25 failure is limited at least in part through a p53-mediated G1 arrest⁹⁹, which may explain why
26 TP53 mutations are more frequent in genome-doubled tumours and occur prior to genome-
27 doubling¹⁰⁰. Activation of the Hippo pathway in response to an increase in centrosomes and
28 microtubule nucleation was shown to contribute to p53 stabilisation¹⁰¹. However, TP53
29 disruption is not an obligatory step for the expansion of genome-doubled cells, and several
30 mechanisms have been described allowing the bypass of TP53 activation. Growth factor
31 signalling for example, promotes proliferation of tetraploid cells despite engagement of the
32 p53-p21 axis¹⁰¹. This may be achieved in cancer through activating mutations in *PIK3CA*
33 (encoding the p110 α catalytic subunit PI3K), which were shown to confer tolerance to
34 genome doubling (Martin-Berenjeno et al 2017, in press). In breast cancer, *PIK3CA*^{H1047R}
35 mutations are predominantly clonal and occur prior to genome-doubling (Martin-Berenjeno
36 et al. 2017, in press), with a similar association observed in colorectal adenocarcinoma
37 (*PIK3CA* mutation)²⁷ and lung squamous-cell carcinoma (chromosome 3q amplification,
38 which harbours the *PIK3CA* locus)⁶⁵. Finally, overexpression of D-type cyclins, which link
39 mitogenic signalling to cell cycle progression¹⁰², allow the circumvention of a G1-arrest
40 following tetraploidization by quenching p21 resulting from p53 transcriptional activation,
41 preventing it from exerting its anti-proliferative function^{103,104}. An important mechanism by
42 which PI3K/AKT and ERK signalling contribute to bypass p53 stabilisation may be through
43 the up-regulation of D-type cyclins¹⁰⁵.

44 45 *CIN Attenuation*

46 Cahill and colleagues proposed that genomic instability may contribute to tumour
47 development only if it does not exceed a threshold beyond which it generates unviable

1 karyotypes. It follows the same principles observed in bacterial genetics and virology where
2 an excessive mutator phenotype has catastrophic consequences¹⁰⁶. This concept is
3 supported by the finding that high SCNA burden and greater intratumour heterogeneity
4 prior to therapy are associated with improved overall survival, while tumours with
5 intermediate levels display a poor clinical outcome¹⁰⁷⁻¹⁰⁹. Accordingly, CIN can be either
6 oncogenic or tumour suppressive in mouse models according to the level of instability,
7 which is affected by the genetic context and the tissue^{110,111}. Elevating chromosome
8 missegregation rates increases cell death in various cancer cell lines and reduces their
9 tumorigenic potential^{112,113}. In addition, the efficacy of some cancer treatments that induce
10 CIN such as taxol and radiation, is improved in cells where the basal rate of chromosomal
11 instability is higher^{70,112,114}.

12 The requirement to reach an equilibrium of low CIN may explain the scarcity of
13 mutations in genes whose disruption robustly induce CIN experimentally, since those would
14 essentially be under negative selection. Analogous to Muller's ratchet principle that links
15 mutation acquisition and species extinction, the accumulation of genomic alterations during
16 tumour evolution may gradually increase CIN and lead to cancer cell death. It is thus
17 possible that alterations that limit CIN might be selected for during cancer progression.
18 Aneuploidy tolerance, although essential for CIN propagation, leaves cells vulnerable to
19 extreme karyotypic changes, raising the question whether CIN levels can be modulated
20 during tumour development to mitigate the impact associated with excessive instability.

21 We reported recently that deleterious mutations in various subunits of the
22 Anaphase-Promoting Complex/Cyclosome (APC/C) are selected for in cancer, and showed
23 that monoallelic inactivation of various subunits significantly reduced the rate of
24 endogenous segregation errors in cancer cell lines⁹⁵. APC/C dysfunction delayed mitotic
25 progression only by 5 to 10 minutes, which was sufficient to significantly improve
26 chromosome segregation fidelity, the fitness of tetraploid cells and reduce the frequency of
27 merotelic attachment errors, considered a main cause of w-CIN¹² (Figure 1). Although
28 cancer cells divide much less frequently *in vivo* than *in vitro*, intravital imaging studies
29 suggest that the total duration of the mitotic phase itself is unchanged *in vivo* (~1h)⁸⁶,
30 similar to that reported for various cell types in mouse embryos¹¹⁵. Pharmacological *in vitro*
31 induction of extreme CIN rapidly selected for cells with APC/C mutations or reduced activity,
32 translating into a 10-minute mitotic delay. The plasticity in mitotic duration, which merely
33 affects the overall proliferation rate, offers an effective mechanism to attenuate many CIN-
34 causing defects⁹⁵. Delaying mitotic progression also improves tetraploid cell fitness by
35 facilitating centrosome clustering which reduces the frequency of unviable multipolar
36 divisions^{16,95,116}. Mitotic biomarkers such as MPM-2 and phospho-Histone H3 may therefore
37 not be optimal to determine the proliferation index on fixed samples. Secondary alterations
38 that improve cell fitness by reducing CIN may therefore be acquired during tumour
39 evolution. Mild alterations in mitotic duration due to genetic or epigenetic regulation of
40 critical mitotic regulators may provide an effective mechanism to fine-tune the level of CIN
41 to optimise cancer cell fitness.

42 A crucial determinant for CIN propagation therefore relies on the capacity of cancer
43 cells to tolerate a given rate of instability, and disruption of this equilibrium is likely to
44 impair cell fitness (Figure 2). For example many cancer cell lines that display a stable
45 karyotype missegregate chromosomes at non-negligible frequencies, yet these events are
46 not tolerated leading to cell death and clearance of aneuploid cells⁹⁶ (Figure 2). For
47 example, APC-mutated organoids show a high rate of segregation errors (and would appear

1 aneuploid by FISH), but the aneuploid progeny is not propagated efficiently and the
2 population does not become fully aneuploid unless TP53 is disrupted⁹¹. Reduced instability
3 in evolved tetraploid cells can also be achieved by eliminating the extra centrosomes^{15,104}.
4 Buffering CIN rates is also a recurrent observation upon mathematical modelling the
5 evolutionary dynamics of genetically unstable populations, and cell fitness is improved
6 when CIN rates are reduced¹¹⁷⁻¹¹⁹. Identifying additional mechanisms driving CIN adaptation
7 and tolerance may therefore reveal new strategies to target CIN therapeutically.

9 **Interplay between immunosurveillance and CIN**

10 A complex picture is emerging whereby CIN could impact on cancer cell recognition by the
11 immune system in multiple and opposing ways.

12 Immune evasion may be particularly crucial for chromosomally-unstable tumours
13 since the genomic alterations and stresses associated with aneuploidy may increase their
14 immunogenicity. A recent analysis of 5,255 tumours and normal samples from TCGA
15 revealed that high level segmental or whole-chromosome SCNAs in tumours correlate with
16 reduced expression of gene signatures associated with adaptive immunity and cytotoxic
17 CD8⁺ T-cell/NK cells, suggestive of reduced immune infiltration¹²⁰. Although these
18 observations remain to be validated *in vivo*, it supports the notion that the tumour
19 microenvironment of highly aneuploidy tumours is immunosuppressive, which is supported
20 by a lower frequency of neoantigen editing in CRC¹²⁰.

21 General features shared by CIN cells may constitute an immunogenic trigger. This
22 effect may in part be driven by endoplasmic reticulum-associated stress in polyploid cells
23 resulting in extracellular exposure of calreticulin and recognition by cytotoxic T-cells and NK
24 cells^{121,122}. Pharmacological induction of CIN using an Mps1 inhibitor induced a pro-
25 inflammatory gene signature, increased cytokine secretion, cell surface expression of NK-
26 activating ligands and efficient clearance by NK92 cells in co-culture assays⁹³. In mice,
27 combining an Mps1 inhibitor with anti-PD1 therapy potentiated tumour regression,
28 although it is unclear if immunogenicity was triggered by apoptotic cell death or by a feature
29 of highly aneuploid cells caused by Mps1 inhibition¹²³. Defects in nuclear envelope integrity
30 from micronuclei DNA, chromatin bridges or during cell migration, were recently shown to
31 allow DNA recognition by cytosolic cyclic GMP-AMP synthase (cGAS), a crucial sensor of
32 double-stranded DNA that mediates type I interferon immune responses^{124,125}. This led to a
33 pro-inflammatory program downstream of STING (stimulator of interferon genes), known to
34 promote anticancer T-cell responses¹²⁶. ER-stress and transient cytosolic DNA exposure
35 associated with CIN are two mechanisms that may trigger a cell-intrinsic innate immune
36 reaction against chromosomally unstable cells.

37 Alternatively, CIN could generate tumour-specific neoantigens, which are targeted
38 by activated T-cells in response to checkpoint blockade¹²⁷ or during adoptive T-cell
39 therapy¹²⁸. The efficacy of immune checkpoint blockade therapy has been associated with a
40 high mutational burden from non-synonymous single-nucleotide variants (nsSNVs, causing
41 single amino-acid substitutions), such as reported in melanoma, NSCLC, and cancers with
42 DNA mismatch-repair deficiency¹²⁹. As discussed earlier, CIN cells are prone to accumulate
43 mutations, but this is unlikely to significantly increase nsSNV burden. Genomic
44 rearrangements associated with CIN on the other hand, especially chromothripsis and
45 chromoplexy¹³⁰, could potentially generate many frameshifts in a single catastrophic event.
46 By analysing tumour mutational spectra in a pan-cancer study, we found that frameshifts
47 may represent a strong trigger for antitumour T-cell reactivity¹³¹. Frameshifts result in the

1 expression of aberrant neopeptides of various lengths which, upon processing by antigen-
2 presenting cells, can potentially generate a much larger number of neoantigens compared
3 to point mutations. This may explain why renal clear cell carcinomas, which have a low
4 nsSNV burden but a high frameshift burden, respond to checkpoint inhibitor therapy¹³¹. The
5 contribution of complex rearrangements as a source of neopeptides, and their impact on
6 checkpoint inhibitor efficacy warrants further investigation.

7 However, ongoing CIN during checkpoint blockade therapy may also lead to
8 treatment failure. Indeed, checkpoint inhibitor resistance in NSCLC was recently linked to
9 the loss of reactive cancer neoantigens through loss-of-heterozygosity¹³². This may be
10 expected since CIN underpins the frequent loss of clonal mutations during NSCLC
11 evolution⁶⁵.

12 Further studies are needed to understand the global impact of CIN on
13 immunosurveillance, considering the metabolic stresses associated with aneuploidy, the
14 immunogenicity associated with segregation errors as well as CIN's impact on neoantigen
15 generation and elimination. While CIN induction *prior* to checkpoint blockade therapy may
16 improve response, it may prove crucial to mitigate CIN during treatment to avoid resistance
17 acquisition.

18 **Leveraging CIN for cancer treatment**

19 *Challenges in identifying CIN biomarkers*

20
21 A major limitation in our ability to specifically leverage CIN for prognostic and therapeutic
22 purposes is the current lack of biomarkers to adequately capture the dynamics of the CIN
23 phenotype, rather than the static nature of aneuploidy. DNA ploidy assessment using
24 image-based cytometry or flow cytometry efficiently detect severe aneuploidies and
25 tetraploidy, provide an indication of heterogeneity between tumour cells and are useful to
26 determine absolute copy-number from sequencing data¹³³. However, they lack resolution,
27 fail to detect s-CIN or low w-CIN rates especially in near-diploid samples. Nuclear
28 morphological defects on mitotic cells and micronuclei represent a surrogate for
29 segregation errors. Cytogenetics methods relying on analysis of metaphase cells cannot be
30 applied in a clinical setting, and FISH-based methods can only detect specific translocations
31 or measure centromeric modal deviation for limited number of chromosomes at once¹³⁴.
32 Copy-number analysis using array-CGH or DNA sequencing from bulk samples essentially
33 reveal clonally-selected alterations within any given tumour region, and fail to detect
34 heterogeneity. This is illustrated by the illusion of diploidy observed when analysing highly
35 aneuploid populations or after mixing defined aneuploid clones^{64,135}. All those methods
36 mostly report on the genomic complexity of cancer genomes, but not whether ongoing CIN
37 is at play, not whether errors are tolerated and propagated.

38
39 Multi-region sequencing provides further insight into CIN dynamics, enabling to
40 distinguish between clonal and subclonal SCNAs, and a high proportion of subclonal SCNAs
41 is therefore indicative of ongoing CIN during tumour evolution. In NSCLC, tumours where
42 the majority of SCNA events were subclonal displayed shorter disease-free survival, and
43 observation independent of clinical factors in a multivariate analysis⁶⁵. On the other hand, a
44 high proportion of subclonal mutations, indicative of ongoing mutagenesis, had no
45 prognostic value. CIN may therefore be a more important driver of cancer progression than
46 an increased mutation rate, a provocative thought that warrants further investigation.

1 Analysis of circulating tumour cells (CTCs) or tumour-derived cell-free DNA (cfDNA)
2 from liquid biopsies offers an amenable way to track SCNA evolution during cancer
3 progression and treatment^{66,136-138}. In particular low-coverage sequencing on CTCs provides
4 a non-invasive way to assess tumour heterogeneity at the single cell level to infer CIN⁶⁶.
5 Obviously, single cell genomics provides the ultimate level of resolution to fully appreciate
6 the extent of heterogeneity, and represents the most promising avenue to develop clinically
7 applicable biomarkers for CIN¹³⁹. Combining DNA image cytometry and multi-region
8 sequencing could be exploited to drive the identification of robust biomarkers capable of
9 capturing CIN dynamics particularly in samples with low cancer cell fraction, which is crucial
10 if we are to leverage CIN for stratification purposes or to exploit it for direct therapeutic
11 intervention.

12 13 *CIN in Clinical trials*

14 Considering the pervasiveness of CIN in cancer and the consequences of tumor
15 heterogeneity for cancer treatment, there is currently a very limited number of clinical trials
16 (reported on clinicaltrials.gov) that either directly investigate the impact of CIN, explore
17 ways to leverage CIN therapeutically or monitor CIN during disease progression or therapy.
18 One trial (NCT03096418) is directly investigating whether paclitaxel increases CIN levels in
19 breast tumors, as suggested from initial studies¹¹⁴, and whether breast cancers with CIN
20 may be more sensitive to further instability resulting from neoadjuvant therapy. In this
21 study, the level of aneuploidy and CIN will be measured by parallel methods including
22 whole-genome sequencing and FISH on independent core samples per biopsy. In addition,
23 clinical response will also be correlated with tumor levels of paclitaxel (measured by HPLC)
24 as well as proliferative (Ki-67) and mitotic (phospho-Histone H3) biomarkers. A recently
25 completed trial (NCT00512642) involving Lung Imaging Fluorescence Endoscopy (LIFE) to
26 detect early lung lesions in high risk patients involved the collection of analysis of p53 status
27 and genomic instability (aneuploidy) when lesions were found. Studying CIN in pre-
28 malignant and early disease could be further explored for specific cancer types, such as in a
29 current study investigating the correlation between ploidy and recurrence in early rectal
30 cancer (NCT03039595). Another interesting line of investigation worth exploring is to
31 examine the occurrence of CIN in resection margin as predictor of relapse, similar to what
32 has been done in a study of oral squamous cell cancer¹⁴⁰.

33 34 *Perspectives for Targeting CIN cancers*

35 Given the far-reaching consequences of CIN for treatment success and outcome, several
36 approaches have been explored to target CIN, taking advantage of features associated with
37 the aneuploid state or their capacity to sustain further instability.

38 Reducing fitness of aneuploid cells may be achieved using compounds that
39 exacerbate the proteotoxic stress (the Hsp90 inhibitor 17-AAG) and metabolic stress (the
40 AMPK agonist AICAR) associated with aneuploidy, which have shown some selectivity
41 against aneuploid and CIN cells^{87,141}. Aneuploid and CIN cell lines were recently found to
42 contain higher levels of ceramides, a class of pro-apoptotic sphingolipids synthesised on the
43 ER¹⁴², and consequently were more sensitive to pharmacological increase in ceramide
44 levels¹⁴³. This may explain the reported synergy between conditions that increase ceramide
45 levels and paclitaxel, which induces chromosome missegregation at clinically relevant
46 doses^{114,143-145}.

1 Increasing chromosome missegregation rates to generate unviable karyotypes is
2 another avenue actively explored. Several groups have developed Mps1 inhibitors aimed at
3 causing massive aneuploidy by ablating the mitotic checkpoint, which again seems to
4 synergise with paclitaxel^{112,123,146}. Identifying cancer types exquisitely sensitive to Mps1
5 inhibitors may prove challenging and relies on the premise that unwanted aneuploidy in
6 normal tissues would not be propagated. Mps1 inhibitor efficacy may therefore be
7 restricted to cancers where paclitaxel has proven effective. The success of Mps1 inhibitor
8 monotherapy may also be limited by the rapid acquisition of resistance as observed *in vitro*
9 through Mps1 mutations, APC/C dysfunction and aneuploidy tolerance acquisition^{95,96,147}.
10 Forcing cells with extra-centrosomes (such as genome-doubled cells) into a catastrophic
11 multipolar division, by preventing centrosome clustering, is also being explored for example
12 by targeting of the non-essential kinesin HSET^{15,16,148}. By accelerating mitosis, Mps1
13 inhibitors also impair efficient centrosome clustering and promote multipolarity^{16,95,116}.
14 Phase I studies are currently ongoing for Mps1 inhibitors (NCT02366949, NCT02138812,
15 NCT02792465).

16 Targeting tolerance mechanisms in combination with approaches aimed at
17 increasing CIN rates may represent an efficient way to limit resistance acquisition and
18 possibly improve response to DNA damaging agents that also drive excessive CIN. Targeting
19 pathways that converge onto p53 are particularly relevant, either by reactivating p53 in CIN
20 tumours, disrupting cyclin D-p21 interaction or by blocking signalling pathways that induce
21 tolerance. For example, low doses of PI3K α inhibitors which dampen the low-level pathway
22 activation upon oncogenic activation of *PIK3CA* may reduce CIN tolerance and tumour
23 heterogeneity, and limit the generation of drug-resistant clones.

24 Reducing tumour heterogeneity by directly suppressing chromosome missegregation
25 may be confounded by the complexity of the CIN phenotype in established tumours, and
26 CIN may only be temporarily reduced as was reported upon targeting a CIN-driving process
27 using an MCAK inhibitor¹⁴⁹.

28 Further studies are needed to understand the evolutionary trajectories of
29 heterogeneous CIN populations in response to various treatments, which may uncover new
30 targetable dependencies. A deeper understanding of the biological processes affecting the
31 fitness of CIN cells combined with the ongoing cataloguing of cancer mutations associated
32 with subclonal expansion may also identify additional druggable targets. In addition,
33 whether acute induction of extreme CIN will potentiate antitumour immune responses or
34 drive resistance to checkpoint blockade warrants further investigation.

35 Although the prognostic value of aneuploidy has been demonstrated for several
36 indications, deriving robust approaches to assess whether ongoing CIN is taking place within
37 a given near-diploid or aneuploid sample may be crucial to efficiently exploit it in a clinical
38 setting. Indeed, aneuploid cancer cells are not invariably chromosomally unstable and can
39 maintain a stable yet abnormal karyotype. Discriminating between CIN+ and CIN- regardless
40 of the ploidy status will potentially inform on the response to therapy and chances of
41 relapse.

42

43

44 **Conclusion**

45 Development of robust biomarkers capable of capturing CIN dynamics is crucial if we are to
46 leverage its potential for stratification purposes and to exploit it for direct therapeutic
47 intervention. Tackling CIN is essential for the success of personalised medicine, a problem

1 that is only just beginning to be understood from a therapeutic perspective. Great attention
2 has been given to the extremely diverse causes of chromosomal instability, but tolerance
3 mechanisms, ripe for exploitation, are starting to emerge as being crucial determinants for
4 its propagation.

5

6

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30

1 **FIGURE LEGENDS**

2

3 **FIGURE 1 : Merotelic, tetraploidy and CIN attenuation.**

4 A) Several types of mitotic defects can lead to chromosome missegregation. Illustrated are
5 merotelic attachments, whereby one of the sister chromatids is attached to opposite poles
6 (magenta). These errors are not detected by the mitotic checkpoint, hence mitosis proceed
7 without delay, resulting in lagging chromosome that can be missegregated to daughter cells.
8 Severe defects (excessive CIN) generates a high frequency of unviable aneuploid karyotype
9 that deviates greatly from a $2n$ diploid content ($2n \pm x$), due to the loss or gain of too many
10 chromosomes (red daughter cells).

11 B) Infrequent segregation errors involving fewer chromosomes likely generate viable
12 progeny (orange daughter cells), whose proliferation will then depend on various tolerance
13 mechanisms. The frequency of segregation errors can be attenuated by acquiring secondary
14 alteration that will improve mitotic fidelity. APC/C dysfunction is one mechanism allowing
15 cells to delay mitotic progression, giving more time for endogenous mechanisms to correct
16 attachment errors.

17 C) Supernumerary centrosomes in tetraploid cells ($4n$) frequently generate multipolar
18 spindles and merotelic attachment. Failure to cluster extra centrosomes into two poles will
19 lead to a multipolar division (4, or 3 daughter cells as shown here) with severe and random
20 chromosome losses ($4n - x$). The presence of extra centrosomes also greatly increases
21 merotelic.

22 D) Tetraploid cells avoid multipolar divisions by achieving centrosome clustering, which
23 requires the kinesin HSET. Tetraploids are believed to be more tolerant to segregation
24 errors because it has a milder impact on overall protein stoichiometry, compared to a
25 diploid cell. Delaying mitotic progression provides more time to achieve centrosome
26 clustering, and reduces the frequency of segregation errors, improving tetraploid cell fitness
27 and the propagation of a sustainable rate of CIN (yellow daughter cells).

28

29 **FIGURE 2 : Impact of CIN tolerance and attenuation on the propagation of cells with**
30 **complex karyotypes.**

31 A) Cells without CIN and functional stress response pathways will maintain a stable
32 karyotype. Rare stochastic segregation errors will be outcompeted but may persist.

33 B) CIN in the presence on functional stress response pathways including p53 will prevent the
34 propagation of cells with complex karyotypes. Only aneuploidies involving specific
35 chromosomes may be tolerated and will proliferate at a much slower rate.

36 C) CIN tolerance allows rare stochastic error from an otherwise karyotypically stable
37 population, to be efficiently propagated. Additional numerical and structural aberration
38 could be acquired and propagated.

39 D) Aneuploidy tolerance combined with high chromosomal instability will generate an
40 increasing number of cells with unviable karyotypes and is therefore tumour suppressive.

41 E) Alterations causing a transient or less penetrant CIN phenotype will reduce the frequency
42 of unviable karyotypes. CIN cells may also acquire secondary mutations to reduce the rate
43 and severity of chromosome segregation errors, improving fitness.

FIGURE 1

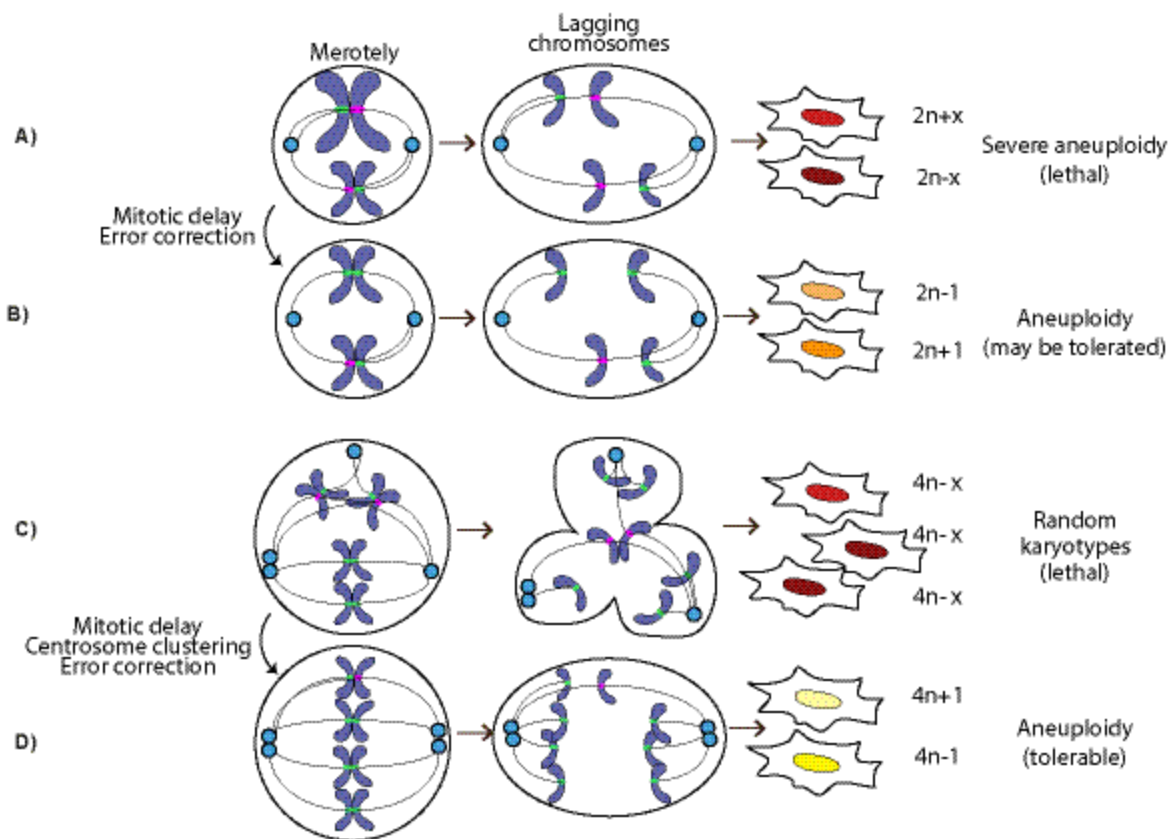
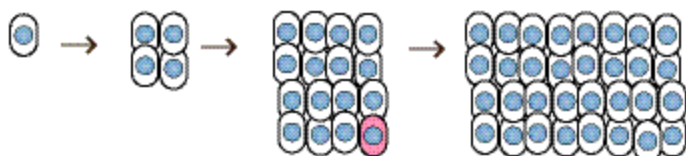


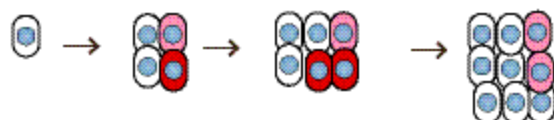
FIGURE 2

A) No CIN & Functional aneuploid stress response



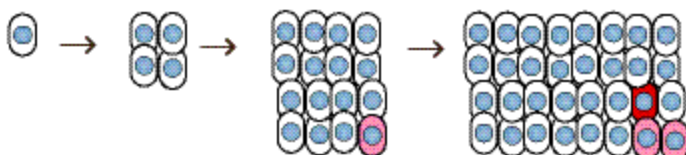
Rare Aneuploid persisters are outcompeted

B) CIN & Functional aneuploid stress response



Complex karyotypes and most aneuploidies are cleared from the population

C) No CIN + Tolerance to wCIN and sCIN



Stochastic segregation errors will be propagated potentially generating complex karyotypes

D) High CIN + Tolerance to wCIN and sCIN



Extreme CIN generates high frequency of unviable karyotypes.

E) "Optimal" CIN level & Tolerance to wCIN and sCIN



CIN attenuation generates fewer unviable cells and creates a highly heterogeneous population.



Stable Karyotype



Minimal karyotypic change Tolerated but at a fitness cost



Complex karyotype Restrained by multiple tolerance pathways



Extreme aneuploidy and SCNAs Unviable regardless of tolerance pathways