ON THE ROAD TO CANCER: ANEUPLOIDY AND THE MITOTIC CHECKPOINT

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Abstract | Abnormal chromosome content — also known as aneuploidy — is the most common characteristic of human solid tumours. It has therefore been proposed that aneuploidy contributes to, or even drives, tumour development. The mitotic checkpoint guards against chromosome mis-segregation by delaying cell-cycle progression through mitosis until all chromosomes have successfully made spindle-microtubule attachments. Defects in the mitotic checkpoint generate aneuploidy and might facilitate tumorigenesis, but more severe disabling of checkpoint signalling is a possible anticancer strategy.

ADENOMATOSIS POLYPOSIS COLI
Tumour-suppressor protein that, in a mutated, defective form, causes familial adenomatous polyposis (FAP), a rare hereditary disease in which patients have thousands of colorectal polyps that develop into tumours. Most sporadic colorectal tumours harbour mutations in both *APC* alleles.

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The mechanisms by which chromosomes are equally separated between two daughter cells have been a challenge to understand ever since Theodor Boveri (in the footsteps of Walther Flemming[1]) showed the dramatic synchronous separation of chromosomes during the first mitotic divisions of fertilized sea urchin eggs over 100 years ago[2]. Boveri further described the detrimental effect of unequal segregation of chromosomes on these cells and their progeny[2]. After von Hansemann had observed many abnormal mitotic figures in samples from various carcinomas in 1890 (REF. 3), it was Boveri, again, who postulated that such misdistribution of chromosomes might be a cause for tumour development and birth defects[4]. Indeed, it now seems that all human aneuploidies (cells that have a chromosome number other than 46) that occur during development result in embryonic lethality — except certain combinations of sex chromosomes and trisomies 13, 18 and 21, which lead to severe birth defects. In addition, most solid tumour cells are aneuploid[5] and various cancer cell lines show 'chromosomal instability' (CIN), meaning that they frequently lose and gain whole chromosomes during divisions[6].

The cause of these observed chromosome imbalances is unknown, but will probably be found in defects in the processes that control chromosome segregation during mitosis (BOX 1). One of these processes is the mitotic checkpoint (also known as the spindle assembly checkpoint) — a complex signalling cascade that is essential for the survival of human cells[7,8]. Evidence indicates that mitotic checkpoint defects contribute to tumorigenesis. However, attacking the machinery that is responsible for chromosome segregation is one of the most successful strategies of clinical chemotherapy. So, gaining a better understanding of mitotic entry, progression and exit is essential, not only for uncovering the causes of CIN, but also for the design of more effective drugs to destroy tumour cells. In this review, we will discuss the mitotic checkpoint as one of the possible causes of CIN in tumour development, and the potential of targeting this checkpoint signalling pathway as a strategy for clinical anticancer therapies.

Aneuploidy and cancer

Since the predictions of Boveri[2,4], it has become clear that most solid tumours are not only aneuploid but have also acquired a number of mutations in oncogenes and tumour-suppressor genes such as *KRAS*, *TP53* (tumour protein p53), *RB1* (retinoblastoma 1), *PTEN* (phosphatase and tensin homologue), *APC* (ADENOMATOUS POLYPOSIS COLI), *BRCA1* (breast cancer 1) and others. This has fuelled the debate over whether aneuploidy is an essential contributor to, or merely a

Summary

- Aneuploidy, or abnormal chromosome content, is the most common characteristic of human solid tumours. Aneuploidy might contribute to tumour formation and is associated with acquired resistance to some chemotherapeutics.
- Tumour cells become aneuploid as a result of aberrant mitotic divisions. These aberrant divisions are caused by divisions with a multipolar spindle as a result of previous defects in cytokinesis or centrosome amplification, by defects in chromosome cohesion, by spindle attachment defects, or by impairment of the mitotic checkpoint response.
- The mitotic checkpoint is a signalling cascade that arrests the cell cycle in mitosis when even a single chromosome is not properly attached to the mitotic spindle. This arrest is achieved by inhibiting the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that is essential for mitotic progression.
- Many tumour cells have a diminished, but not absent, mitotic checkpoint response. Mouse models in which mitotic checkpoint signalling is decreased show an increase in spontaneous or carcinogen-induced tumour formation.
- Mutations in mitotic checkpoint genes themselves are not a common mechanism of checkpoint impairment in human tumour cells.
- Mitotic checkpoint impairment and aneuploidy in human tumour cells are often associated with changes in the protein levels of mitotic checkpoint proteins. In some tumour cells, these changes occur through altered transcriptional regulation by tumour suppressors or oncogene products.
- Complete inactivation of mitotic checkpoint signalling causes cell-autonomous lethality. Drugs that specifically and efficiently interfere with mitotic checkpoint signalling could therefore be useful as anticancer agents.

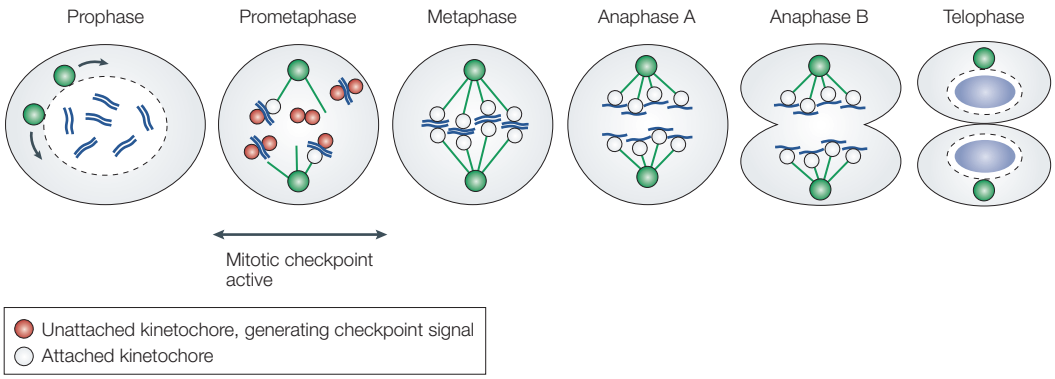
remnant of, oncogenic transformation (for example, see REF. 9). The products of most mutated oncogenes or tumour-suppressor alleles have effects on cellular proliferation and survival. However, what is the reason, if any, that aneuploidy is the most commonly observed genetic alteration in many types of tumours[5]? One possibility is that the ability of a population of cells to redistribute whole chromosomes facilitates tumorigenesis by increasing the chance of LOSS OF HETEROZYGOSITY (LOH) of a tumour-suppressor gene. Or this ability might, in effect, amplify an oncogene by duplicating the chromosome that harbours the mutated allele.

One example of the effects of LOH is the high incidence of loss of chromosome 10 in glioblastoma[10], often resulting in the inactivation of the *PTEN* tumour-suppressor gene. The protein product of *PTEN* regulates proliferation and survival by counteracting phosphatidylinositol 3-kinase (PI3K) activity. CIN might also contribute to tumour formation, without the aid of additional mutations, when the loss of a single allele of a tumour suppressor leads to its haploinsufficiency, as is the case for *CDKN1B* (cyclin-dependent kinase inhibitor 1B), *TP53*, *ARF* or *PTEN*. Mice that are heterozygous for these alleles are more prone to, or display accelerated, tumour development in certain tissues[11]. Additionally, CIN might increase the rate of Darwinian adaptation to changing intracellular and extracellular environments. In this way, CIN is thought to contribute to cellular resistance to chemotherapeutic drugs such as imatinib (Glivec) or 5-fluorouracil[12,13]. CIN might also mediate the development of multidrug resistance in a population of tumour cells that have been targeted with drugs such as etoposide, cisplatin, doxorubicin or paclitaxel. A causal role for aneuploidy in tumorigenesis has not

LOSS OF HETEROZYGOSITY
Following acquisition of a deleterious mutation in one of the two copies of a specific gene, loss of heterozygosity occurs from subsequent loss of, or mutation in, the normal allele.

Box 1 | **The phases of mitosis**

Mitosis is divided into six phases. At prophase, chromosome condensation initiates, the duplicated centrosomes (indicated by green circles in the figure) separate, and some mitotic checkpoint proteins, including BUB1 and BUBR1, are recruited to kinetochores. With nuclear envelope disassembly (indicated by the dashed circle) at entry to prometaphase, the chromosomes spill into the cytoplasm, and the mitotic checkpoint is activated at every unattached kinetochore (red circle; clear circles indicate attached kinetochores). Microtubule capture at both kinetochores of a duplicated chromatid pair results in checkpoint silencing and chromosome alignment to a midzone using a combination of microtubule-motor activities and microtubule dynamics. After capture and congression of the final sister chromatid pair (metaphase) and the turnover of the previously produced inhibitor that sends a 'wait anaphase' signal, anaphase is initiated. During anaphase A the duplicated chromosome pairs are pulled apart. Then, during the subsequent anaphase B, the spindle elongates, further separating the sister chromatids, and invaginations of the plasma membrane around the spindle midzone become apparent. At the end of telophase, the chromatin decondenses and the nuclear envelope reforms (dashed circle) while cytokinesis is completed.



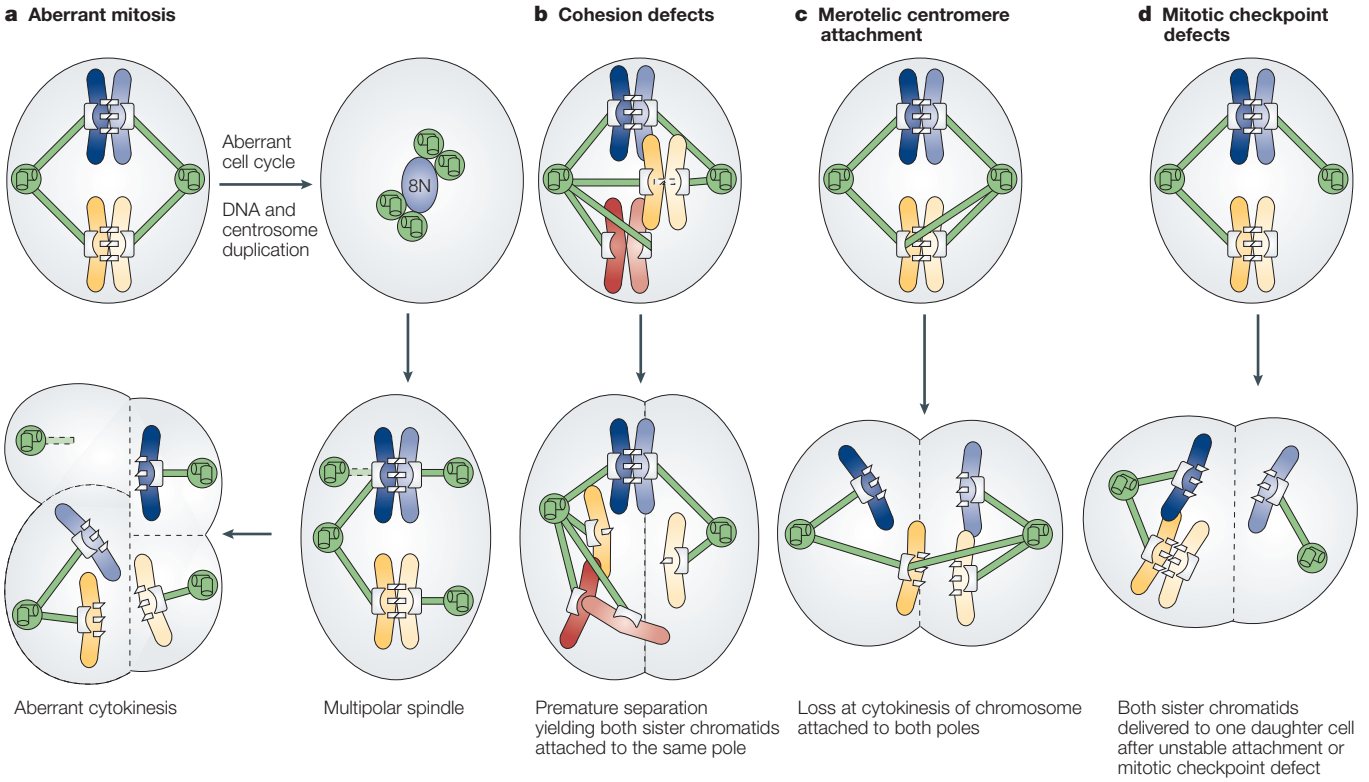


Figure 1 | **The roads to aneuploidy.** Losses and gains of whole chromosomes can occur in several ways. **a** | Aberrant mitosis can cause chromosome loss. Multipolar spindles and chromosome mis-segregation can result from too many centrosomes, produced by a previous skipping of cytokinesis, resulting in polyploidization (8N) or errors of centrosome duplication. **b** | Cohesion defects can cause chromosome loss. Premature loss of cohesion between sister chromatids can yield aberrant segregation with both copies distributed to the same daughter cell. Persistant cohesion between chromosomes in anaphase will result in similar errors. **c** | Attachment of a kinetochore to microtubules from both poles (referred to as merotelic attachment) can cause chromosome loss. **d** | Mitotic checkpoint defects can cause chromosome loss. Unstable microtubule capture can cause chromosome mis-segregation. Weakened mitotic checkpoint signalling by one or more unattached kinetochores does not generate a sufficiently high level of the 'wait anaphase' signal to prevent anaphase onset in the presence of unattached chromosomes.

been established but, regardless of the exact role of CIN in tumour development or plasticity, finding ways to inhibit it (or even hyper-induce it, as discussed later) could have significant implications for anticancer therapies.

Many roads to aneuploidy

Aneuploidy describes a state of abnormal chromosome number and content. Some tumours might, in fact, be stably aneuploid, meaning that some event during the evolution of the tumour cell caused chromosomal redistribution that conferred a proliferative advantage. In most cases, however, it probably reflects underlying CIN, where chromosome content is constantly reshuffled, as was shown for several colorectal cancer cell lines[6]. Aneuploidy or CIN can arise by several means. For this review, we narrow the definition of aneuploidy to losses and gains of whole chromosomes, excluding deletions, translocations, end-to-end fusions, and so on.

Aneuploidy can occur as a result of aberrant mitotic divisions that create cells that enter the subsequent mitosis with multipolar spindles (FIG. 1a). Such aberrant mitoses can be caused by polyploidization, which arises via previous cytokinesis defects, cell–cell fusion or from cells skipping mitosis altogether (endoreduplication)[14]. It can also be caused by defects in duplication, maturation or segregation of centrosomes[15]. In all of these cases, the divisions with a multipolar mitotic spindle that follow such events probably produce aneuploid daughter cells (FIG. 1a). Underlying genetic causes, either through polyploidization or direct effects on the centrosome cycle, include amplification of STK15/aurora kinase A[16], as well as inactivation of the tumour suppressors p53, RB (retinoblastoma protein) and BRCA1 (REFS 17,18).

Chromosome cohesion defects might also contribute to aneuploidy in human cancer cells (FIG. 1b). Resolution of sister-chromatid cohesion at the onset of anaphase depends on separase, a protease that is inhibited by securin (also identified as the pituitary tumour transforming gene 1, PTTG1 (REF. 19)). Inactivation of the securin or separase homologues in budding yeast (Pds1p and Esp1p, respectively) or fission yeast (Cut2p and Cut1p, respectively) results in chromosome loss[20–22]. More importantly, human cancer cells in which securin was removed by homologous recombination show high

levels of CIN[23] and (paradoxically) cells that stably overexpress securin cause tumours in NUDE MICE[19,24]. There is also a reported correlation between securin levels and the invasiveness of pituitary tumours[25].

Third, aneuploidy can arise by improper attachments of chromosomes to spindle microtubules (FIG. 1c,d). Merotelic attachments, in which one KINETOCHORE is simultaneously attached to microtubules emanating from both poles, might be an important cause of aneuploidy[26]. An increase in such attachments was recently observed following inhibition of an attachment-error-correction mechanism that includes the aurora kinase B, borealin, survivin and inner centromere protein (INCENP)[27]. In colorectal cancers (~90% of which show CIN), an important cause of aneuploidy might be mutations in *APC*[28,29]. Truncated forms of APC can kill two birds with one stone — not only are they responsible for increased cell proliferation, owing to a failure to degrade β-catenin[30], but they also cause CIN by affecting the stability of chromosome–microtubule attachments[28,29,31]. This leads to chromosome mis-segregation as a result of kinetochore–microtubule disconnection during anaphase. This is not an inescapable outcome of *APC* mutations, however, as the karyotype of the DLD1 colorectal cancer cell line that carries an *APC* truncation is stable[6]. This implies the presence of a functional mitotic checkpoint to arrest premature advance to anaphase, as well as sufficiently stable kinetochore–microtubule connections to silence this checkpoint and initiate anaphase. Unstable attachments that produce a chronically-activated checkpoint can provoke cell death through a pathway that is not fully defined but that, in some cases, includes advance to interphase without cytokinesis and subsequent apoptosis[32,33].

Aneuploidy can also result from chromosome mis-segregation produced by defects that weaken mitotic checkpoint signalling sufficiently such that anaphase initiates before all chromosomes have established proper spindle attachments (FIG. 1d).

The mitotic checkpoint

Mitotic progression and sister-chromatid segregation are controlled by the ANAPHASE PROMOTING COMPLEX/CYCLOSOME (APC/C), a multi-subunit E3 ubiquitin ligase. APC/C activity requires a specificity factor, CDC20 (cell-division-cycle 20 homologue), in order to recognize and interact with mitotic substrates. Proteins that are targeted for degradation by APC/C include the 'master regulator' of mitosis, cyclin B1, as well as securin[34]. Degradation of securin leads to the activation of separase, which cleaves the cohesin links that hold together the sister chromatids. Degradation of cyclin B1, however, causes the inactivation of CDK1 (cyclin-dependent kinase 1) and initiates mitotic exit.

These proteolytic events are controlled by the mitotic checkpoint, the primary cell-cycle control mechanism in mitosis. The checkpoint prevents premature advance to anaphase and, in vertebrates, is activated every cell cycle immediately upon entry into mitosis or meiosis. The signal generators of this checkpoint are unattached kinetochores (FIG. 2). These recruit mitotic checkpoint components and catalytically convert and release some of these components in a form (or forms) that inhibits the CDC20-dependent recognition of cyclin B and securin by APC/C, thereby preventing advance to anaphase[35]. Classic experiments by Rieder and colleagues showed that, in one cell type, a single unattached kinetochore produced a signal that delayed anaphase for at least 3 hours (REF. 36). This is assumed to be true for all somatic cells and has led to a model in which a single unattached kinetochore generates a saturating 'wait anaphase' signal that can delay anaphase for as long as multiple unattached kinetochores can delay anaphase. However, this has not been experimentally established.

The first components of the checkpoint signal were identified by genetic screens in budding yeast and were dubbed Bub (budding uninhibited by benzimidazole) 1–3, Mad (mitotic arrest deficient) 1–3 and Mps1 (monopolar spindle 1)[37–39]. Vertebrate orthologues of Mad1, Mad2, Bub3 and the kinases Bub1 and Mps1 have all been implicated in mitotic checkpoint control[32,40–43] (TABLE 1). In addition, the vertebrate mitotic checkpoint requires the kinase BUBR1 (a hybrid of yeast Mad3 and Bub1, which is encoded by the *BUB1B* gene), the ZW10–ROD–zwilch protein complex, the microtubule motor protein centromere protein E (CENPE) (TABLE 1) and mitogen-activated protein kinase (MAPK)[44–48].

Some of the checkpoint components (including MAD2, BUBR1, BUB3 and MPS1) are rapidly bound by and released from the unattached kinetochores[49,50] that catalytically produce and release the anaphase inhibitor. The exact composition of the kinetochore-derived inhibitor (or inhibitors) has not been established. Some evidence indicates that it might be an alternative conformation of MAD2 (REFS 51–53) or BUBR1 (REFS 54,55), both of which directly bind to CDC20. The current model for the vertebrate mitotic checkpoint is shown in FIG. 2. Following nuclear envelope breakdown, the checkpoint proteins are recruited to the outer kinetochore surface of all unattached chromosomes. Direct binding of the kinetochore-bound microtubule motor protein CENPE to its binding partner BUBR1 activates the BUBR1 kinase activity[47] (FIG. 2a). BUBR1 kinase activity is required for the recruitment of a stable MAD1–MAD2 heterodimer, which, in combination with the other essential checkpoint components (see below), recruits and modifies MAD2 into an active conformation[45,47,49]. Activated MAD2 and/or BUBR1, possibly in a complex with BUB3, tightly associate with CDC20, preventing it from activating the APC/C and thereby inhibiting ubiquitylation of securin and cyclin B1 (REFS 51,56–58) (FIG. 2a).

Many more components are required to produce the inhibitory signal. Removing the kinase activity of either BUB1 or MPS1 weakens or abolishes mitotic checkpoint signalling and diminishes or prevents kinetochore recruitment of other checkpoint proteins[40,59]. There are additional contributions, probably through indirect roles, from CCT chaperonin, MAPK,

NUDE MICE
Strains of athymic mice bearing the recessive allele *nu*/*nu*, which are largely hairless and lack all, or most, of the T-cell population. Nude mice can accept either allografts or xenografts. *nu*/*nu* alleles on some backgrounds have near-normal numbers of T-cells.

KINETOCHORE
A multiprotein structure, positioned at the central constriction of each chromosome (centromere), which is responsible for chromosome attachment to the mitotic spindle, chromosome segregation during anaphase and mitotic checkpoint activity.

ANAPHASE PROMOTING COMPLEX/CYCLOSOME
Multisubunit E3 ubiquitin ligase required for mitotic progression by targeting key mitotic regulators such as cyclin B1 and securin for destruction through direct poly-ubiquitylation. Note that the nomenclature can be confusing here: the APC/C is completely distinct from the APC associated with β-catenin signalling and colorectal cancer.

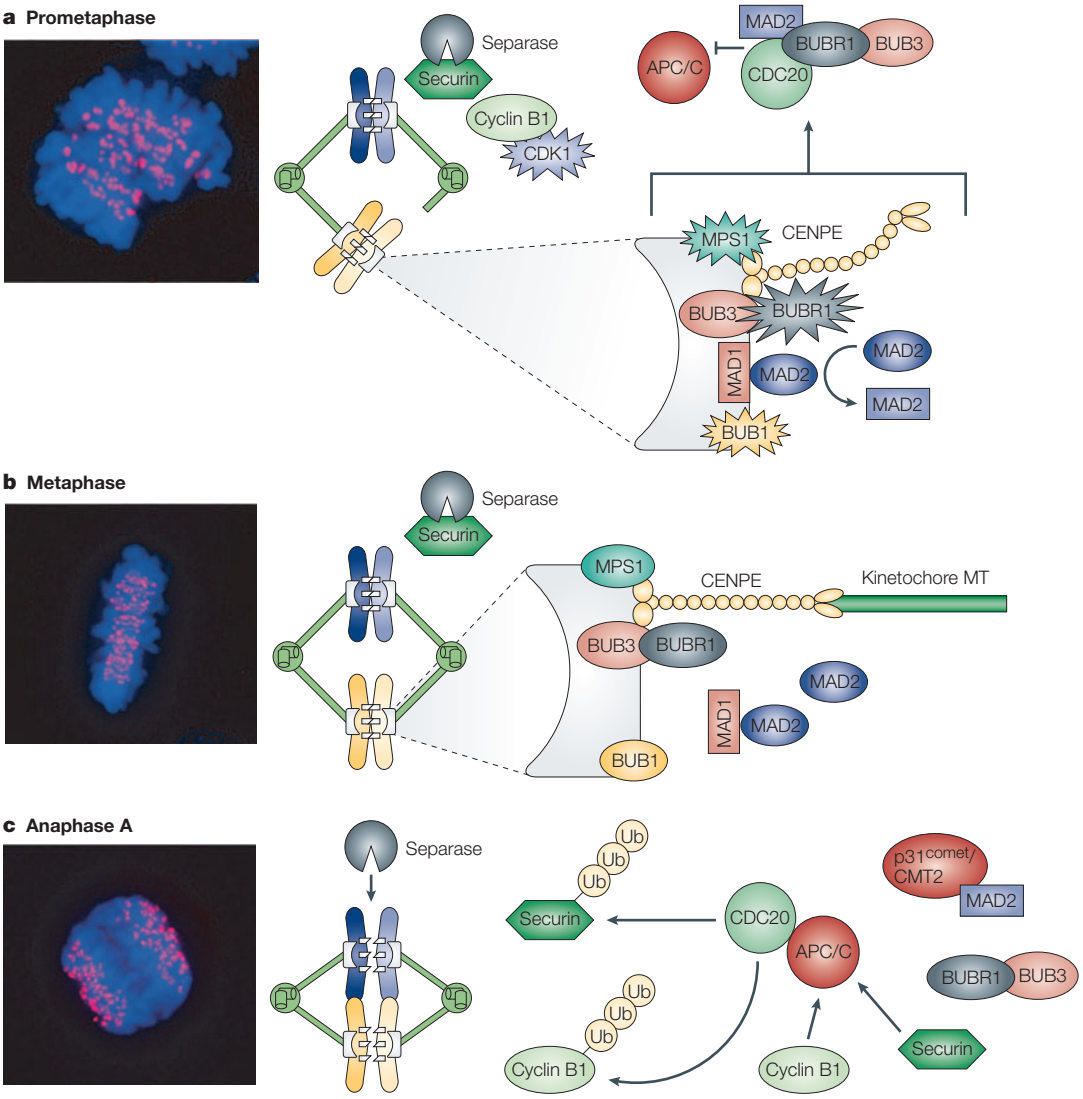


Figure 2 | **The mammalian mitotic checkpoint — signalling and silencing. a** | Kinetochore assembly in prophase/early prometaphase recruits MAD1 (mitotic arrest deficient homologue 1), MAD2, MPS1 (monopolar spindle 1), BUB1, BUB3, BUBR1 and CENPE (centromere protein E) to unattached kinetochores (grey sections, expanded diagram indicated by dashed lines). The kinase activity of BUBR1 is essential for checkpoint signalling and is activated by binding to CENPE. The actions of all of these components are required for rapid generation by, and release from, unattached kinetochores of a diffusible inhibitory 'wait anaphase' complex that inhibits or sequesters the anaphase-promoting complex/cyclosome (APC/C) activator CDC20 (cell-division-cycle 20). Separase, the protease that cleaves the cohesins that hold sister chromatids together, is inhibited by binding to securin. The photograph represents prometaphase in HeLa cells. DNA (stained with DAPI (4', 6-diamidino-2-phenylindole)) is in blue, kinetochores (immunostained with anticentromere antiserum (ACA)) are in red. **b** | As each pair of sister kinetochores attaches to kinetochore microtubules (MT, green), and microtubule motors generate tension that stretches them, generation of the checkpoint inhibitor is silenced at those kinetochores. At least one of the signal-transducing linkers is CENPE, which directly captures kinetochore microtubules and, in so doing, silences BUBR1 kinase activity. The photograph represents metaphase in HeLa cells. **c** | Following silencing of the signalling at each kinetochore and turnover of the inhibitor that transmits the wait anaphase signal, APC/C-mediated ubiquitylation (Ub) of securin and cyclin B1 and subsequent degradation by the proteosome triggers anaphase entry. Turnover of the wait anaphase complex is aided by p31^{comet}/CMT2. The photograph represents anaphase A in HeLa cells.

MICROTUBULE CAPTURE
Process in prometaphase in which unattached kinetochores each interact with one or more microtubules that emanate from one spindle pole. The interaction is mediated by microtubule-binding proteins that stably associate with the kinetochore.

GLC7/PP1 phosphatase, and chromosomal 'passenger' proteins such as aurora kinase B and survivin[48,60–63]. The role of aurora kinase B is of particular interest because its function unifies opposing views about what the mitotic checkpoint senses — lack of attachment or lack of tension. Tension between sister centromeres is normally achieved by forces from bipolar attachments that pull sister kinetochores apart, which are counteracted by centromeric cohesion. Aurora kinase B activity severs improper microtubule attachments that fail to produce tension between sister centromeres[60,64–66]. This creates unattached kinetochores that can generate the mitotic checkpoint inhibitor[60]. MICROTUBULE CAPTURE — which is mediated, at least in part, by CENPE[67,68]

Table 1 | **Mitotic checkpoint proteins**

Protein	Characteristics	Binding partners	Function in checkpoint	References
BUB1	122 kDa; serine/threonine kinase	BUB3	Inhibits CDC20 by phosphorylation	128
BUBR1	120 kDa; serine/threonine kinase	CENPE, BUB3, CDC20	Part of APC/C inhibitory complex. Directly binds to CDC20 and inhibits APC/C activity	54–56
BUB3	37 kDa; structure determined: 7-bladed propeller of WD40 repeats	BUB1, BUBR1	Part of APC/C inhibitory complex. Localizes BUB1 and BUBR1 to kinetochores	43,56,125
MAD1	83 kDa; coiled coil	MAD2	Directly recruits MAD2 to unattached kinetochores	129
MAD2	23 kDa; structure determined	MAD1, CDC20, CMT2/p31^{comet}	Part of APC/C inhibitory complex. Directly binds to CDC20 and inhibits APC/C activity	51,56,127
CMT2/p31^{comet}	31 kDa; none identified	MAD2	Inhibits mitotic checkpoint signalling by antagonizing MAD2	69,70
MPS1	97 kDa; dual-specificity kinase	Unknown	Unknown	NA
CENPE	312 kDa; plus-end directed microtubule motor	BUBR1	Activates BUBR1 at the unattached kinetochore	47,68
ZW10	89 kDa; none identified	ROD, Zwilch	Part of complex that recruits the MAD1–MAD2 heterodimer to unattached kinetochores	126
ROD	251 kDa; none identified	ZW10, Zwilch	Part of complex that recruits the MAD1–MAD2 heterodimer to unattached kinetochores	126
Zwilch	67 kDa; none identified	ROD, ZW10	Part of complex that recruits the MAD1–MAD2 heterodimer to unattached kinetochores	126

APC/C, anaphase promoting complex/cyclosome; BUB, budding uninhibited by benzimidazole; BUBR1, BUB1-related protein; CDC20, cell-division-cycle 20; CENPE, centromere protein E; MAD, mitotic arrest deficient; MPS1, monopolar spindle 1.

— is the most important signal transduction event as it silences production of the inhibitory complex[47] (FIG. 2a). The intrinsic turnover of the inhibitory complex (probably aided by p31^{comet}/CMT2, which antagonizes MAD2 function by binding the CDC20-bound active MAD2 molecules[69,70]) yields recognition by CDC20–APC/C of securin and cyclin B1 (FIG. 2c).

Mitotic checkpoint defects in tumorigenesis

Normal cells have a robust mitotic checkpoint in which one or more unattached kinetochores can produce a signal that is strong enough to inhibit all cellular APC/C activity and thereby block progression to anaphase. This is not necessarily true when checkpoint components are mutated or their concentrations are reduced. There are several examples of cells in which a checkpoint signal that is sufficiently robust to prevent anaphase onset can be generated when all kinetochores are unattached (after inhibition of spindle–microtubule assembly), but individual unattached kinetochores are unable to prevent anaphase[68,71]. For example, in mouse cells with deficiencies in CENPE, kinetochores cannot attract normal levels of the checkpoint proteins MAD1, MAD2 and BUBR1 (REF. 68). As a result, one or a few unattached chromosomes cannot generate enough inhibitory signal to delay anaphase onset and are mis-segregated at high frequency, both in cultured cells and in regenerating hepatocytes *in vivo*[68].

Following the report of mutations in BUBR1 and BUB1 in a subset of colon cancer cell lines[72], a weakened checkpoint was proposed to cause CIN that contributed to the oncogenic process. However, this model has not been proven. Analysis of mice with reduced levels of *Mad2*, *Bub1b* or *Bub3* has shown that all these mice have significant increases in the number of aneuploid fibroblasts[73–76]. Although this does not result in a large increase in spontaneous tumour development, small increases in cancer susceptibility have been reported. For example, tumour incidence was increased (to 6%) in mice with severely reduced BUBR1 levels[76] (that is, mice with ~10% of normal BUBR1 levels after construction of hypomorphic alleles). Also, 28% of mice heterozygous for *Mad2* develop small, self-limiting, late onset (18–19 months) papillary lung adeno-carcinomas[75], and mice heterozygous for functional BUBR1 or BUB3 are more prone to the development of colorectal[73] or lung[73,74] tumours after treatment with azoxymethane (AOM) or 9,10-dimethyl-1,2-benzanthracene (DMBA), respectively. Furthermore, colonies of primary human fibroblasts that survived short-term short interfering RNA (siRNA)-mediated reduction of BUB1 developed aneuploidy and the ability to grow in soft agar, but could not form tumours when injected into nude mice[77].

The type and incidence of tumours that develop in mice with checkpoint defects vary, depending on which gene is disrupted. It is unclear why that is, but

the differences might be caused by variations in the level of checkpoint impairment or, more likely, be owing to additional functions for the various gene products. The degree to which errors in the mitotic checkpoint contribute to tumour formation is further complicated by the possibility that many components, including BUBR1, BUB1 and MAD2, have functional roles outside of mitosis. Mice with reduced BUBR1 prematurely age and die by the age of 6 months[76], possibly before most tumours would have a chance to develop. In addition, induction of apoptosis in the subsequent interphase after escape from a chronically activated checkpoint requires BUBR1 and, to a lesser extent, BUB1 (REF. 32). *Bub1b*$^{+/-}$ mice develop defects in MEGAKARYOPOIESIS[78], a phenotype not reported in mice heterozygous for *MAD2* or *BUB3*. Bub1, Bub3 and the BubR1 homologue Mad3 have been implicated in the accumulation of gross chromosomal rearrangements in yeast[79]. Also, MAD2 is located at the nuclear envelope in interphase cells and might have a role in nuclear–cytoplasmic trafficking[80,81].

Rather than driving tumorigenesis, evidence indicates that a weakened checkpoint might be a facilitator of tumorigenesis, especially in collaboration with a mutated tumour suppressor. For example, *Apc*$^{Min/+}$ mice (which carry one mutant *Apc* allele encoding a truncated protein that causes intestinal neoplasias) developed ~0.4 colonic tumours per mouse by 3 months of age. Reduction of BUBR1 levels in these mice (*Apc*$^{Min/+}$*BubR1*$^{+/-}$ mice) resulted in a 10-fold increase in the number of tumours that developed (~4.1) by the same age — these tumours were also of a higher grade[82]. The interpretation of the effect of BUBR1 on tumorigenesis is complicated by the recent observations that BUBR1 is also involved in establishing stable kinetochore-microtubule interactions[60,66]. In another example, mice homozygous for a truncated form of BRCA2 (a tumour-suppressor protein implicated in DNA repair) develop thymic lymphomas[83,84]. Paradoxically, this mutant BRCA2 causes a progressive proliferation arrest in other tissues that is reversed by the introduction of a DOMINANT-INTERFERING MUTANT form of BUB1, which impairs the mitotic checkpoint response[33,85]. Sequencing of the BUB1 and BUBR1 alleles in four samples of BRCA2 mutant thymic lymphomas showed that, in all cases, either BUB1 or BUBR1 had acquired mutations that are presumed to impair mitotic checkpoint signalling. These findings imply that the ability of BRCA2 mutations to cause tumours in mice is increased by defects in this checkpoint[85].

In cell culture and animal models, weakening the mitotic checkpoint by reducing the levels of checkpoint proteins or by introducing interfering mutants correlates with an increase in the number of aneuploid cells and, in most cases, some features of oncogenicity. Substantiation of a true facilitator role, however, depends on the outcome of more experiments, similar to the ones described previously[82], in which checkpoint-impaired mice are crossed with oncogene or tumour-suppressor mutant strains that have a well-defined disease course.

So, the studies in which the levels of checkpoint proteins were experimentally reduced have revealed three important lessons. First, each of the central components of the mitotic checkpoint are essential, even though they might have roles other than those involved in checkpoint maintenance. Second, a point that should be readily obvious but that has been frequently overlooked[86], is that the biochemical signal produced by each unattached kinetochore is not an 'all-or-none' event, but can in fact be weakened. That is, the generation of the kinetochore-derived inhibitor that sends the wait-anaphase signal can be quantitatively reduced by mutation or reduction in concentrations of signal-producing components. This creates a situation in which more than one unattached kinetochore (the number depends on the strength of the signal) is needed to produce enough signal to inhibit anaphase onset, and in which chromosome separation can occur with unaligned chromosomes whenever fewer than that number of kinetochores is unattached. Indeed, absence of any of the checkpoint proteins that have been tested in mice so far (including MAD2 (REF. 75), BUB3 (REF. 74) and BUBR1 (REFS 73,76)) yields early embryonic lethality, whereas mice with reduced concentrations of any of these components show increased levels of chromosome misdistribution. Third, aneuploidy *per se* might not drive tumorigenesis and therefore might not be sufficient to initiate it, but might still facilitate tumorigenesis.

Observations in human tumours

The details of the mitotic checkpoint are becoming clearer, but what is the status of the mitotic checkpoint in human tumour cells, and is there any correlation with karyotype? Many tumour cell lines that have been investigated are aneuploid and, judging from the comprehensive analysis of over 20,000 tumour samples[5], most tumour types are predicted to be the same. By measuring the mitotic index in response to reagents that disrupt microtubule function, which determines the ability of a cell population to sustain a chronic mitotic arrest, the status of the mitotic checkpoint has been investigated in various tumour cell lines and clinical samples of divergent origins (for examples, see REFS 72,87–89). It should be recognized that this is really an assay for sustained checkpoint activation, which is an imprecise assay for true checkpoint signalling. The checkpoint has evolved to prevent even a single chromosome from being lost, and therefore can only be assayed by real-time video microscopy of chromosome distributions during mitosis in living cells. Nevertheless, judging by the ability to arrest (or remain arrested) after interference with spindle microtubules, mitotic checkpoint signalling often seems impaired, but not absent, with cells able to respond with an initial cell-cycle arrest but unable (or less able) to maintain it (for examples, see REFS 72,87–92).

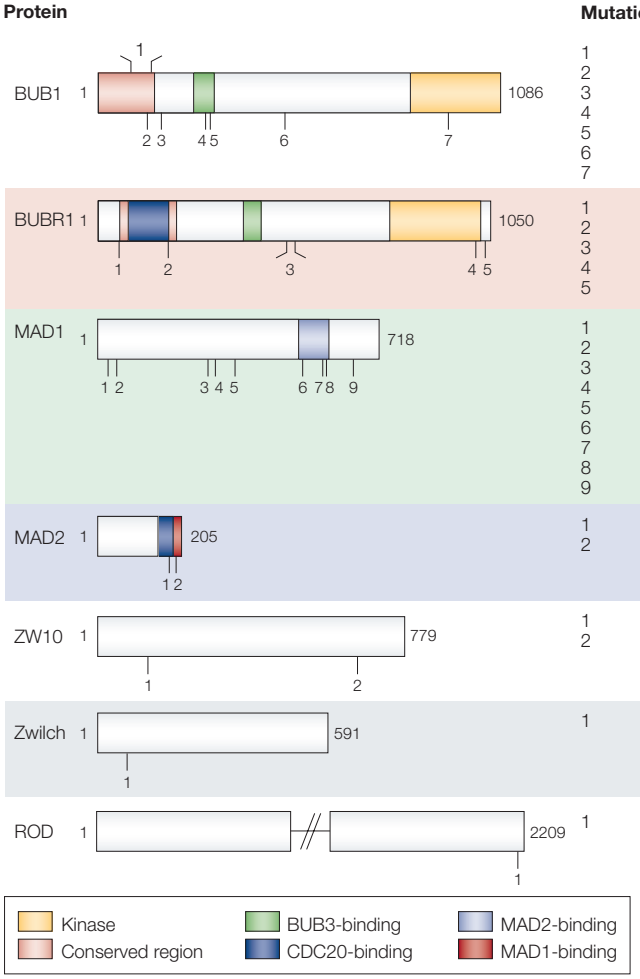
What causes the checkpoint to be weakened or unsustainable in those cells? Vogelstein and colleagues[72] reported that mutations in BUB1 or BUBR1 were present in 4 out of 19 colorectal cancer cell

MEGAKARYOPOIESIS
Process that leads to the production of megakaryocytes, the polyploid precursor to platelets. These precursors develop from haematopoietic stem cells by executing several cell-cycles in which cytokinesis is skipped (known as endoreduplication).

DOMINANT-INTERFERING MUTANT
Non-functional mutant protein that inhibits the function of the endogenous wild-type protein. These mutants often work by occupying subcellular binding sites required for the activation or correct subcellular positioning of the wild-type protein.

lines examined. Expression of two mutant forms of BUB1 (with amino-terminal mutations that were originally identified in cell lines with CIN) in tumour cell lines with stable chromosomes disrupted the sustained checkpoint arrest that is normally observed in response to microtubule depolymerization. These findings indicate a dominant, interfering action of these mutant forms of BUB1. Unfortunately, it is not known whether these mutants actually caused the CIN phenotypes in the cells that they were originally identified in, and a recent report has challenged the conclusion that these two cell lines were even subject to CIN[93]. It is important to note that the report by Vogelstein and colleagues did not determine whether the cell lines studied were completely devoid of mitotic-checkpoint signalling. Careful analysis of mitotic-checkpoint signalling in the CIN lines used, and in the chromosomally stable lines after expression of the mutant *BUB1* alleles, showed that these cells do have a checkpoint, albeit a weakened one[72,90].

Other studies have reported that human tumour cells contain mutations in mitotic checkpoint genes that encode BUB1, BUBR1, MAD1, MAD2 and all three members of the ZW10–ROD–zwilch



Protein	Mutation	Change in protein	Cancer	Reference
BUB1	1	Δ76–141+fs	Colon	72
	2	A130S	Colon	102
	3	H151D	Lung	94
	4	Y259C	Pancreatic	95
	5	H265N	Pancreatic	95
	6	S492Y	Colon	72
	7	S950G	Rectal	97
BUBR1	1	T40M	Colon	72
	2	E166D*	Lymphoid	98
	3	Δ523–538*	Lymphoid	98
	4	Fs 1023	Colon	72
	5	L1031Q*	Lymphoid	98
MAD1	1	S29L	Lymphoid	101
	2	R59C	Prostate	101
	3	T299A	Lung	99
	4	Δ318–end	Prostate	101
	5	R360Q#	Prostate	101
	6	E516K#	Breast	101
	7	R556C	Prostate	101
	8	E569K	Breast	101
	9	ΔY649–end	Lymphoid	101
MAD2	1	I190V	Bladder	96
	2	Δ192–end	Breast	100
ZW10	1	N123T	Colon	103
	2	S623G	Colon	103
Zwilch	1	ΔV67–end	Colon	103
ROD	1	E2199D	Colon	103

Figure 3 | **Mutations in mitotic checkpoint genes.** Somatic mutations in *BUB1*, *BUB1B* (which encodes BUBR1), *MAD1L1* (which encodes mitotic arrest deficient homologue 1, MAD1), *MAD2L1* (which encodes MAD2), *ZW10*, *FLJ10036* (which encodes zwilch) and *KNTC1* (which encodes ROD) have been found in various tumour samples. The locations of mutations in the genes are shown with respect to known functional domains in the proteins. Predicted amino-acid changes are shown for the gene product of the mutated alleles and the cancer type from which the alleles were isolated. Δ76–141+fs represents a deletion in BUB1 that is immediately followed by a frameshift. MAD1$^{\Delta Y649-end}$ was shown to be a dominant-negative form of MAD1 when overexpressed in osteosarcoma cells. It decreases the mitotic checkpoint response of these cells[101]. Expression of BUB1$^{\Delta 76-141+fs}$ and BUB1^{S492Y} decreased the response of colon carcinoma cells to nocodazole. These cancer cells normally express only wild-type BUB1 (REF. 72). Asterisk indicates that the listed codon numbers of the BUBR1 mutations found in lymphomas are different from the ones listed in the initial report by Ohshima *et al.*[98] Although Ohshima *et al.* used the correct consensus BUBR1 protein sequence (genbank accession AF046079), closer examination of the reported mutations revealed that the mutated codons were misnumbered in that report[98]. Ohshima *et al.* also report a BUB1 mutation (GGT→GAT, causing G250D), but no glycine is found at position 250 in human BUB1. As no information on the sequences surrounding the nucleotide mutation was provided, it is unclear what codon was found mutated in BUB1 and we have not included this mutation in the figure. Hash symbol (#): of eight mutations reported in MAD1 (REF. 101), two mutated codons were misnumbered.

complex[94–103] (FIG. 3). The effect of these mutations on mitotic checkpoint signalling has not been examined, with the exception of one mutant *MAD1* allele isolated from a lymphoma. This mutant was found to be dominant in overriding the mitotic-checkpoint signalling on spindle disruption[101].

Recently, the first germline mutations in a checkpoint gene associated with a human disease were reported in patients with a rare recessive condition called mosaic variegated aneuploidy (MVA). MVA is a complex disorder that is characterized by growth retardation, microcephaly and childhood cancer, and cells from individuals suffering from MVA display mosaicism for chromosomal gains and losses. In five families with MVA, a total of nine mutations were found in *BUB1B*, six of which are located in the kinase domain[104]. Although it has not been determined whether these mutant alleles affect checkpoint signalling, this finding supports a causal link between mitotic checkpoint defects, aneuploidy and tumour development. Nevertheless, although inactivation of mitotic checkpoint genes by somatic mutations does occur, it does not seem to be a common mechanism for aneuploidization (for example, see REFS 87,105–108).

Checkpoint regulation by tumour suppressors

Various oncogene products and tumour suppressors regulate mitosis. Microtubule instability or centrosome amplification are caused by the loss of tumour suppressors such as APC, p53, BRCA1 or BRCA2 and also by overexpression of oncogenes such as *MDM2*, *AURA* (aurora kinase A), *RAS*, *MYCN* and the E6 and E7 oncoproteins of the human papilloma virus[15–18]. In addition, mitotic entry or progression could be regulated by the putative tumour suppressors CHFR (checkpoint with forkhead and ring finger domains, an E3 ubiquitin ligase that is part of an ill-defined delay in entering mitosis, misnamed as an early mitotic stress checkpoint), WARTS (also known as large tumour suppressor homologue 1, LATS1) — a mitosis-specific serine/threonine kinase — and RASSF1A (a RAS-association-domain-containing protein that localizes to centrosomes)[109–111]. Even though RASSF1A, similar to the mitotic checkpoint complex, inhibits APC/C by binding CDC20, it is not essential for the mitotic checkpoint, as cells that are deficient in RASSF1A sustain mitotic arrest after NOCODAZOLE-induced microtubule disassembly[111]. It has not been tested whether loss of RASSF1A affects signalling from a single unattached kinetochore.

By contrast, cancer-associated defects in some tumour suppressors and oncogene products contribute to CIN by weakening the mitotic checkpoint signal from individual kinetochores as these proteins regulate the expression of checkpoint components. Altered expression of MAD1, MAD2, BUB1, BUB3, BUBR1 and MPS1 has been observed in various tumour samples and cell lines — most commonly in advanced stage tumours[32,106,112,113] — and has been correlated with reduced relapse-free survival time[101,112] and insensitivity to chemotherapeutics such as cisplatin[114].

NOCODAZOLE
Synthetic compound that binds tubulin dimers, thereby causing inibition of microtubule polymerization. Because of the highly dynamic nature of spindle microtubules in mitosis, this results in complete depolymerization of the cellular microtubule network.

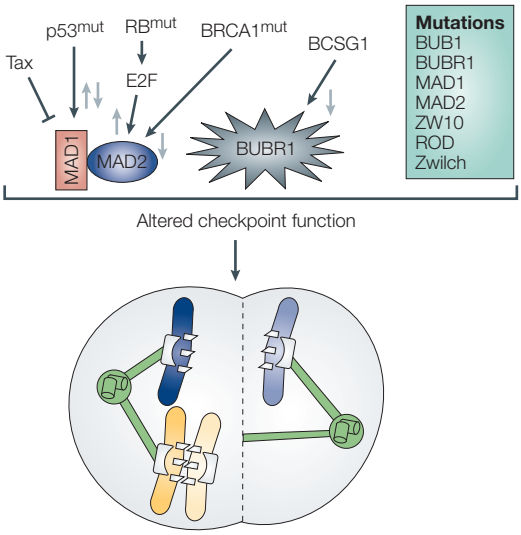


Figure 4 | **Mitotic checkpoint defects in cancer.** Mitotic checkpoint signalling is often impaired in tumour samples and cancer cell lines. Evidence has implicated tumour-suppressor proteins and oncogenes in transcriptional and post-transcriptional regulation of protein levels of the checkpoint components MAD1 (mitotic arrest deficient homologue 1), MAD2 and BUBR1. For example, mutations (mut) in *RB1* (which encodes the retinoblastoma protein, RB) or *BRCA1* (breast cancer 1) result in MAD2 dysregulation. (Black arrows indicate the effect on protein levels. The grey arrows indicate whether the effect is an increase or a decrease.) Wild-type p53 has also been proposed to regulate MAD1 transcription, so mutations in p53 can also disrupt MAD1 levels. The Tax oncoprotein from the human T-cell leukaemia virus type 1 directly binds MAD1 to inhibit its function. These types of alterations could contribute to chromosomal instability. However, except for MAD2 regulation by RB, these effects have not yet been tested *in vivo*. Additional somatic mutations have been identified in a number of mitotic checkpoint components (see FIG. 3).

How might oncogenes or tumour suppressors induce decreases in the levels of checkpoint components to produce a weakened mitotic checkpoint? BRCA1 regulates *MAD2* transcript levels directly by binding the *MAD2* promoter (FIG. 4), perhaps in concert with the transcription factor OCT1 (REF. 115). Mouse cells that express mutant forms of BRCA1 have decreased the expression of not only MAD2, but also BUB1, BUBR1 and ZW10. Re-expression of MAD2 partially reverts the mitotic checkpoint defects in these cells. Caution is advised, however, when interpreting the effects of MAD2 overexpression. Any defect in inhibition of the APC/C at, or upstream of, the mitotic checkpoint will probably be rescued by overexpression of MAD2, simply because of the fact that MAD2 directly binds CDC20, preventing APC/C recognition of securin and cyclin B1.

Although the mitotic checkpoint malfunction and CIN observed in cells that express mutant forms of BRCA1 can be attributed to a decrease in the levels of MAD2, paradoxically, overexpression of MAD2 has also been associated with clinical markers of tumour progression. For instance, the *MAD2L1* gene

CELL-AUTONOMOUS LETHAL
The situation in which a deleterious subcellular environment (such as a genetic lesion that inactivates the mitotic checkpoint) causes cell death from damage solely developed within that cell. Surrounding cells have no influence on this process.

is clustered with genes that have been correlated with disease outcome in patients with breast cancer[116], and increased MAD2 expression in neuroblastoma cells correlates with poor prognosis[113].

Many tumour-suppressor proteins control the expression levels of some of the mitotic checkpoint genes at the level of transcription (FIG. 3). The CIN phenotype of RB-negative tumour cells results from an E2F-dependent increase in MAD2 expression[113] (FIG. 4). E2F directly binds the MAD2 promoter[117], and partial reduction of MAD2 levels by siRNA reverses the increase in polyploidy observed in HCT116 cells that overexpress either E2F or E1A[113]. How can an increase in MAD2 cause CIN in the RB-deficient tumours? As the number of polyploid cells is increased tenfold in MAD2-overexpressing cells[113], one possibility is that part of the population can adapt to the hyperactive checkpoint that is induced by increased MAD2 levels and enter the next cell-division-cycle as polyploid cells. This could then cause chromosome misdistributions in subsequent mitoses (FIG. 1a).

Another tumour suppressor that transcriptionally regulates the mitotic checkpoint is p53 (FIG. 4). Wild-type p53 directly binds the *MAD1L1* promoter[118], but there are contradictory findings as to whether *MAD1L1* expression is increased or decreased following p53 activation[118,119]. In one report, *MAD1L1* expression was upregulated by gain-of-function mutations in p53, but a functional analysis of the mitotic checkpoint was not reported. Interestingly, even a less than two-fold decrease in MAD1 levels has been shown to decrease checkpoint signalling and cause CIN[71].

Finally, BCSG1 (breast-cancer-specific gene 1), which is expressed only by advanced-stage breast carcinoma cells, binds BUBR1 and causes its degradation[120]. Furthermore, the Tax oncoprotein from the human T-cell leukaemia virus type 1 (HTLV-1) binds MAD1 and prevents MAD2 activation[41] (FIG. 4). So, altering the protein levels of mitotic-checkpoint components to induce CIN seems to be one mechanism of action of both tumour suppressors and oncogenes.

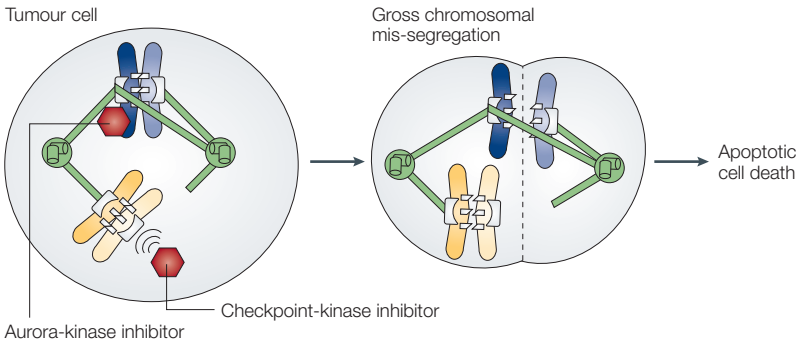


Figure 5 | **Induction of gross chromosome mis-segregations as an anticancer strategy.** Inhibition of mitotic checkpoint signalling (through checkpoint-kinase inhibitors) or inhibition of the attachment-error-correction mechanism (through aurora-kinase inhibitors), results in chromosome mis-segregations, leading to apoptotic cell death. So, it might be possible to design small molecule inhibitors that interfere with checkpoint signalling to disrupt tumour cell proliferation and kill tumour cells in patients.

Drug targets in the mitotic checkpoint

Traditional antimitotic chemotherapeutics, including the taxanes and the vinca alkaloids, are currently used to treat patients with breast and ovarian cancers. Both types of drugs produce unattached kinetochores in mitosis by altering microtubule dynamics and cause long-term mitotic arrest. An anti-mitotic drug that inhibits a mitosis-specific microtubule-dependent motor protein EG5 (also known as KSP), has also recently entered clinical trials. EG5 is required for spindle–pole separation and its inhibition generates monopolar spindles, unattached kinetochores and long-term mitotic arrest from activation of the mitotic checkpoint, similar to the mechansisms of taxanes and vinca alkaloids. Drug-mediated mitotic-checkpoint-dependent arrest is often followed by cell death[121]. Unfortunately, the link between prolonged mitotic checkpoint activation and cell death has not been well studied.

Paradoxically, the exact opposite effect of these antimitotic drugs might also be effective in killing tumour cells. Complete inhibition of the mitotic checkpoint is lethal to individual cells. Reducing MAD2 or BUBR1 to less than 10% of wild-type levels in various tumour cell lines caused complete inactivation of the mitotic checkpoint and resulted in massive chromosome misdistributions during mitosis, which, in turn, resulted in lethality within 2–6 cell divisions[7,8]. Inhibiting BUBR1 kinase activity to half its normal level was sufficient to induce checkpoint inhibition and subsequent cell death[7]. These observations are compatible with the embryonic-lethal phenotype of all mice that carry homozygous deletions of mitotic checkpoint alleles, and demonstrate that genetic mutations in tumour cells might weaken the fidelity of checkpoint signalling, but do not inactivate it. Additionally, aurora-kinase inhibitors diminish checkpoint signalling and cause mitoses with mis-segregation of chromosomes, failed cytokinesis and daughter cells with extra centrosomes[122]. Once the inhibitors have been removed, the cells undergo subsequent mitoses with multipolar spindles and produce largely inviable progeny, presumably because of rampant aneuploidy. In one study, inhibition of aurora kinases resulted in a 98% reduction in tumour volume in nude mice injected with human leukaemia cells[123].

So, whereas an initial weakening of the mitotic checkpoint might increase aspects of CIN-mediated tumorigenesis, further weakening (or silencing) of checkpoint signalling results in CELL-AUTONOMOUS LETHALITY. This raises the prospect of manipulating the mitotic checkpoint to inhibit tumour cell proliferation with drugs that target essential checkpoint functions, such as BUBR1 kinase activity, to treat certain cancers (FIG. 5). This offers a new way to use an old ally — the mitotic checkpoint — to kill tumour cell populations quickly and efficiently. Recently, an MPS1 inhibitor that causes chromosome mis-segregation and death in yeast cells was found by screening 140,000 small molecule drug compounds[124]. Although the MPS1 inhibitor was unable to inhibit the mitotic checkpoint response in human cells, this study provides the first indication of the feasibility of such an approach[124].

Box 2 | **Testing the 100-year-old hypothesis — does aneuploidy facilitate tumorigenesis?**

Mice with heterozygous disruptions in the genes encoding the mitotic checkpoint proteins MAD2 (mitotic-arrest deficient 2 homologue), BUB3 and BUBR1, display an increase in spontaneous (MAD2) or carcinogen-induced (BUB3 and BUBR1) tumour development. This provides an experimental test of how or whether aneuploidy contributes to tumour formation. Interpretation of the results is, however, complicated by the possibility that each of these proteins also has roles outside of mitosis. MAD2 is found at the nuclear envelope in interphase, where it might take part in nucleocytoplasmic trafficking[80,81] and BUBR1 has been implicated in the removal of cells that have exited an aberrant mitosis[32]. However, CENPE (centromere protein-E) accumulates just before mitosis and is degraded at mitotic exit like the mitotic cyclins. Mouse embryonic fibroblasts with reduced levels of CENPE mis-distribute chromosomes because of mitotic checkpoint impairment[68]. So, *Cenpe*$^{+/-}$ mice can be used to directly test the hypothesis that aneuploidy drives tumorigenesis. They can also be used to introduce chromosomal instability into mice with a defined disease course induced by a mutation in a tumour-suppressor gene or an oncogene. Analysing tissues of *Cenpe*$^{+/-}$/*Arf*$^{-/-}$ or *Cenpe*$^{+/-}$/*KrasG12D* mice, for instance, could teach us whether or not chromosomal instability accelerates tumour development, increases the aggressiveness or metastatic potential of tumours, or increases general tumour incidence. Perhaps finally, after more than a century, we will get a clear answer as to whether chromosomal instability is a participant in or a side-effect of the oncogenic process.

A strategy of inhibiting the mitotic checkpoint in cancer cells increases the risk of aneuploidy (and its contribution to tumorigenesis) in healthy cells when inhibition is incomplete. It is not known whether lethality by mitotic-checkpoint inhibition is tumour-cell specific, but this approach would not differ from the treatment of patients with drugs like paclitaxel or vincristine. These drugs do not differentiate between tumour cells or normal cells, but have been successfully used in the clinic for many years.

Conclusions

In the 100 years since Boveri's hypothesis on the origins of cancer, it has been established that genetic changes underlie tumorigenesis. Their effects on proliferation and survival of tumours have also been partly characterized. The discovery, 14 years ago, of components of the mitotic checkpoint, as well as the realization that many of the classic tumour suppressors and oncogene products regulate mitotic progression, has renewed interest in the role of CIN in tumorigenesis. With the generation of mice with weakened checkpoint signalling, the right toolbox to test the contribution of CIN to tumour development and perhaps drug resistance has now been acquired (BOX 2). Important challenges that remain include deciphering how the wait anaphase signal is produced at unattached kinetochores, identifying all the components of the anaphase inhibitory complex, and determining how capture by kinetochores of spindle-microtubules silences signalling. Most important for tumour treatment is deciphering the mechanism (or mechanisms) of escape from chronic checkpoint-mediated mitotic arrest, and identifying how this can trigger cell suicide. The rewards might be great; unravelling the molecular workings of this checkpoint and its defects in cancer cells might not only shed light on the origins of CIN, but could also provide new prospects for ways to interfere with tumour growth.

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Competing interests statement
The authors declare no competing financial interests.

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