Chromosome Res (2011) 19:291–306
DOI 10.1007/s10577-010-9178-z

Mitosis in vertebrates: the G2/M and M/A transitions and their associated checkpoints

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Published online: 31 December 2010

Abstract In this review, I stress the importance of direct data and accurate terminology when formulating and communicating conclusions on how the G2/M and metaphase/anaphase transitions are regulated. I argue that entry into mitosis (i.e., the G2/M transition) is guarded by several checkpoint control pathways that lose their ability to delay or stop further cell cycle progression once the cell becomes committed to divide, which in vertebrates occurs in the late stages of chromosome condensation. After this commitment, progress through mitosis is then mediated by a single Mad/Bub-based checkpoint that delays chromatid separation, and exit from mitosis (i.e., completion of the cell cycle) in the presence of unattached kinetochores. When cells cannot satisfy the mitotic checkpoint, e.g., when in concentrations of spindle poisons that prohibit the stable attachment of all kinetochores, they are delayed in mitosis for many hours. In normal cells, the duration of this delay depends on the organism and ranges from ~4 h in rodents to ~22 h in humans. Recent live cell studies reveal that under this condition, many cancer cells (including HeLa and U2OS) die in mitosis by apoptosis within ~24 h, which implies that biochemical studies on cancer cell populations harvested in mitosis after a prolonged mitotic arrest are contaminated with dead or dying cells.

Keywords cell cycle · mitosis · mitotic checkpoint · G2/M · FACS

Abbreviations

A	Anaphase
ATM	Ataxia telangiectasia mutated kinase
ATR	Ataxia telangiectasia and Rad-3-related kinase
B/CDK1	Cyclin B/CDK1 kinase
BRCA2	Breast cancer 2 susceptibility protein
BubR1	Budding uninhibited by benzimidazole 1 homologue beta (yeast)
CDC	Cell division control
CENP	Centromere protein
CHFR	Checkpoint with Forkhead-associated and Ring (a ubiquitin ligase)
CHK	C-terminal Src kinase-homologous kinase
ERK1/2	Extracellular signal-regulated kinase 1/2

Responsible Editors: James Wakefield and Herbert Macgregor

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FACS	Fluorescence-activated cell sorting
ICFR	bis(2,6-Dioxopiperazine) derivative 193 (topoisomerase II inhibitor)
M	Metaphase
Mad	Mitosis arrest deficient
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MPM2	Mitosis phosphoprotein monoclonal antibody 2
MT	Microtubule
NEB	Nuclear envelope breakdown
P53/p21	Protein 53/protein 21
RPE1	Human retinal pigment epithelia cells

Introduction

During mitosis, the replicated chromosomes become equally segregated (via karyokinesis) into two identical twin sister nuclei, each of which then becomes enclosed (via cytokinesis) in its own separate cytoplasmic compartment. The series of events that prepare the cell for mitosis are collectively termed the cell cycle. Under appropriate environmental conditions, unicellular organisms like yeast exist perpetually in the cell (or "life") cycle, constantly growing and dividing without interruption. Although the same is true for some cancer cells, most cells in multicellular organisms are not cycling. Many, including those that have differentiated, are no longer (normally) capable of dividing. Others have exited the cycle, but can reenter it in the presence of proper cues.

In the early 1950s, it became evident that the "mitotic cycle" of plant (Howard and Pelc 1953) and animal (Lajtha et al. 1954) cells could be divided into four distinct phases "by means of the fact that S, the period of DNA synthesis (which can be recognized by radio-autographic determination of incorporated H3-thymidine) is separated from mitosis by an interval of several hours, which was called G2. Similarly, the period between the end of mitosis and the beginning of S was labeled G1" (Puck and Steffen 1963). At the time, progression through the mitotic cycle, now simply termed the *cell cycle*, was referred to as "mitotic progression." Although the terms *mitotic cycle* and *mitotic progression* remain in use, the latter has become ambiguous because mitosis investigators also use it to describe progression through the various phases of mitosis (not the entire cell cycle). As a result, a PubMed search using the key phrase *mitotic progression* (without the italics) produces 3,864 articles encompassing two distinctly different types of topics that are not always easy to differentiate from one another (all the PubMed searches cited in this review are for the italicized word or phrase without the italics). The question of whether a particular agent inhibits mitotic progression because it blocks cells in interphase or mitosis is significant: A failure to draw a clear distinction between the two possibilities leads to claims that are true under one definition and not the other.

Work in the 1960s and 1970s revealed that normal non-transformed cells stop dividing under crowded (confluent) conditions, whereas under the same conditions, those from many tumors continue to divide. Importantly, normal non-proliferating cells in post-confluent cultures could be stimulated to divide simply by adding serum (via a media change). If labeled thymidine was added to confluent cultures at the same time as the serum, mitotic cells appeared at later time points and were heavily labeled, meaning that serum stimulates cells from the G1 phase and before DNA synthesis (Temin 1968). This suggests that in the absence of serum, such non-proliferating cells reside in a "no cell cycle" (Lajtha et al. 1962). These observations, as well as conclusions from regenerating liver that cells do have a true resting phase, led Lajtha to opine in a roundtable discussion chaired by Hans Ris (Ris et al. 1963) that "… following the 'gap' nomenclature. This true resting stage would then be G_0 and in this G_0 period the cells would just sit, minding their own biochemical business. But they don't have a G1-S-G2 cell cycle in respect to growth. From this stage, lasting perhaps **7**years, the cells can be triggered into a cell cycle as and when required." Many intervening years of study have shown that this reversible block in G_0 is mediated by the Rb-E2F and *myc* proto-oncogene pathways (reviewed in Seville et al. 2005). [All cells in a transformed population like HeLa, U2OS, etc., have lost the ability to arrest in G_0 and are constantly cycling. As a result, they can be synchronized in mitosis by first transiently blocking them in S-phase with thymidine followed by release into a microtubule

(MT) poison like nocodazole. This protocol is, however, of limited value for non-transformed cells, many of which in a given population are, due to contact inhibition, no longer cycling].

Cells can also exit the cycle during G2, after DNA synthesis, and before mitosis (reviewed in Gelfant 1977), although this is rarely mentioned in books or reviews. As during G_0, these cells can remain in $G2_0$ for prolonged periods before being stimulated by various environmental or hormonal cues to reenter the cycle. $G2_0$ cells are commonly found in epithelia, sometimes in high numbers (Kaneko et al. 1984), which at times must rapidly increase in number, e.g., as in the gut of hibernating animals as they wake (e.g., Kruman et al. 1988) or during wound healing (Gordon and Lane 1980). The mechanism(s) responsible for G2 cells exiting into $G2_0$, and their return to G2, remain to be defined and are an important area for future work. Cells can also be transiently induced to delay in G2 within an organism by stress, e.g., as in the esophagus and duodenum of starved chicks (Cameron and Cleffmann 1964), which may involve the p38 MAPK pathway (see below).

An important conceptual breakthrough in cell cycle research came in the late 1980s when it became evident that progress through the cycle is regulated by a series of surveillance or control mechanisms, termed checkpoints (Weinert and Hartwell 1988; Hartwell and Weinert 1989). These control pathways "enforce dependency in the cell cycle" by delaying progress in response to specific problems so that they can be corrected or resolved prior to division. [The term "checkpoint" is starting to be used to also describe other surveillance mechanisms not involved in cell cycle regulation including, e.g., one that prevents the expression of aberrant RNA transcripts (see references in Kazerouninia et al. 2010; Eberle et al. 2010)]. Cell cycle checkpoints evolved to minimize the production and propagation of genetic mistakes. They consist of at least three components (Murray 1992), including a sensor that detects a mistake or problem, a signal generated by the sensor by a signal transduction pathway, and a response element in the cell cycle machinery (often, but not always, a cyclin-dependent kinase) that the signal targets to block progression. The term checkpoint was coined because such control mechanisms "have a role of checking to see that prerequisites…have been properly satisfied" (Weinert and Hartwell 1988)…before the cycle is allowed to continue. The popular notion that checkpoints are activated or triggered by a problem is a fallacy: Checkpoints are constitutive control pathways, external to the event being monitored, that are always checking for problems. Although the checkpoint may trigger, activate, or lead to a cell cycle block or delay, the control mechanism itself is always working, i.e., the checkpoint is not the block but rather the pathway that leads to the block. The block occurs because the checkpoint is not satisfied, not because it is activated or triggered.

Checkpoint controls guard key cell cycle transitions including start (the G1/S), entry into mitosis (G2/M), and exit from mitosis (metaphase/anaphase, M/A). This brief review will focus on some popular but fictitious idea surrounding the G2/M and M/A transitions in vertebrates as well as some of the misleading terminologies currently used to characterize events during the final phases of the cell cycle. The opinions expressed are this author's alone and, although supported by (mostly) direct data, some are likely not shared by my colleagues.

Entry into mitosis (the G2/M transition)

Traditionally, mitosis is defined as beginning when chromosome condensation first becomes evident within the "prophase" nucleus. For several reasons, this is no longer a useful definition. Chromosome condensation is a gradual process that is initiated shortly after S-phase as the aurora kinases begin to phosphorylate histones (Hendzel et al. 1997; Crosio et al. 2002) and condensation can be detected, with modern live cell imaging methods (Hiraoka et al. 1989; Sarkar et al. 2002), in early to mid-G2 well before it is obvious that the cell is in prophase. Furthermore, up to a point, the process of prophase chromosome condensation is *reversible*. Once this point is passed, however, the cell is committed to divide and only death can prevent it from undergoing nuclear envelope breakdown (NEB). The period at the end of G2, just before the cell becomes independent of environmental conditions and thus committed to mitosis, has been termed "antephase" (Bullough and Johnson 1951). Important to my argument is that cells from vertebrates (salamanders to humans) do not become committed to mitosis until late prophase, ~10 min before NEB, near the time when active

cyclin B/CDK1 begins to accumulate in the nucleus (see Pines and Rieder 2001). Before this, various insults (e.g., Fig. 1) arrest chromosome condensation, or induce chromosome decondensation, and delay the cell in G2 (reviewed in Rieder and Cole 2000; Mikhailov et al. 2005). Thus, although Bullough and Johnson (1951) posit that antephase occurs "just before the prophase becomes visible," modern imaging reveals that it actually extends well into the period of chromosome condensation. This being the case, antephase really represents the terminal stage of G2, and in normal vertebrate cells, G2 ends and mitosis begins in late prophase (at the end of antephase) when cells become committed to undergo NEB (see Mazia 1961; Pines and Rieder 2001). The physiological differences between an early to mid-prophase cell, and one committed to divide, can therefore be expected to be considerable.

The G2/M transition, i.e., progression through antephase, is guarded by at least two checkpoint control pathways that work throughout G2, including one, mediated by the ataxia telangiectasia mutated kinase (ATM)/ataxia telangiectasia and Rad-3-related kinase (ATR) kinase, which responds to DNA breaks. In G2 cells, this pathway works through the CHK1 kinase and Cdc25 phosphatase to rapidly prevent entry into mitosis by depressing the activity of cyclin A- and B-dependent kinases. The other pathway is based on the p38 kinase and CHFR, a RING finger-containing protein with ubiquitin ligase activity (Matsusaka and Pines 2004). CHFR activity has been implicated in various G2 events, including, e.g., the aurora A kinase-mediated phosphorylation of CENP-A (Kunitoku et al. 2003; Yu et al. 2005). Like the DNA damage pathway, the p38 pathway must also depress A and B cyclin-dependent kinase activity (Mikhailov et al. 2005). The p38-mediated checkpoint senses a sudden onset of stress caused by insults ranging from drug treatments that disassemble MTs (Matsusaka and Pines 2004), inhibit topoisomerase II and histone deacetylase (Mikhaiov et al. 2004), or induce ribotoxic stress (Mikhailov et al. 2007) to excessive illumination during microscopy (Rieder and Cole 2000) and media changes (osmotic shocks; e.g., Mikhailov et al. 2005; Fig. 1). [The rapid transient cessation of the antephase/mitosis transition reported in 1950 in response to shocking mice with tourniquets or ATP injections (Green And Bullough 1950) was likely mediated by p38].

Both the DNA damage and stress-activated checkpoints are rapid-response systems that do not require

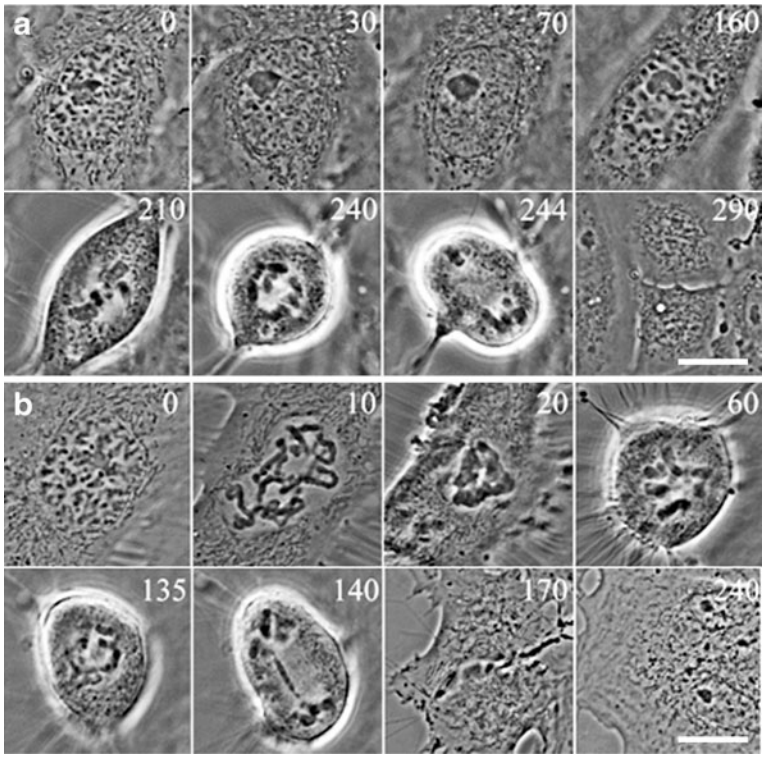

Fig. 1 Rat kangaroo (PtK_1) cells after treatment during prophase with hypotonic medium before (**a**) or just after (**b**) the commitment to mitosis. Perfusions before the commitment transiently arrest (and reverse) chromosome condensation via the p38 checkpoint pathway. Note that with time, the cell "adapts" to the stress, recondenses its chromosomes, and completes a normal mitosis. Perfusions after the commitment cannot delay entry into mitosis and lead to sticky chromosomes, which in this example prevent the completion of cytokinesis. Time is in minutes from the treatment (courtesy of A. Mikhailov, Northwestern University, Chicago, IL). *Scale*, 10 μm

transcription or translation to quickly and transiently block the cell cycle in G2, although both can ultimately produce a sustained block via the transcription-dependent p53/p21 pathway. Both controls also share common component(s) during G2, including Cdc25 (reviewed in Karlsson-Rosenthal and Millar 2006), so that, e.g., in response to IR or UV irradiation during late G2, either pathway can induce a cell cycle block (reviewed in Bulavin et al. 2002; Thornton and Rincon 2009). Reports that the G2/M transition is also regulated by an ATM and p38-independent DNA "decatenation checkpoint" (e.g., Demming et al. 2001) were based principally on ICRF inhibitors of topoisomerase II that were subsequently shown to both induce DNA damage and activate p38 (Mikhaiov et al. 2004; Park and Avraham 2006). The idea that this putative checkpoint pathway is really independent of the ATM kinase has also been questioned (Bower et al. 2010).

Antephase is unique: The sudden cessation and/or reversal of chromosome condensation (Fig. 1a) are clear visible manifestations that cell cycle progression has been delayed. This has fostered the idea that progress through the terminal stage of G2 is guarded by an "antephase checkpoint" that works only during this period (Chin and Yeong 2010). This is a fallacy: In mammals, the p38 stress-activated checkpoint pathway functions throughout the cell cycle and can induce a delay or an arrest during G1 (e.g., Escote et al. 2004; Lafarga et al. 2009) or S (e.g., Jirmanova et al. 2005), as well as anytime during G2. At the same time, there is no reason to suspect that the CHFR component of this pathway, which in normal cells is expressed at constant levels throughout the cell cycle (Burgess et al. 2008), works only during antephase and not throughout all of G2 (or even G1) where one of its functions is to modify transcription activity through histone deacetylase 1 (Oh et al. 2009). The same has been known to be true for the DNA damage checkpoint which also functions throughout G2, including antephase (Rieder and Cole 1998).

By far, the most popular method for studying the G2/M transition is by fluorescence-activated cell sorting (*FACS*=7,791 articles). This technique is used to differentiate cells in a population that contains 2N and 4N nuclei, and intermediates, often for subsequent biochemical analyses. The 2N cells are considered to be in G1; those between 2N and 4N in S, while all 4N cells are relegated to G2/M. G2 cells can then be differentiated from M cells by conducting a secondary fluorescence assay using an antibody that recognizes an epitope found only during mitosis. Unfortunately, few investigators bother to do this, and those that do usually use histone phosphorylation as a marker for mitosis. Yet, as noted above, histone phosphorylation is not specific for mitosis because it starts in early G2, shortly after S-phase ends. [A more appropriate antibody is the MPM2, which recognizes >40 phosphoproteins unique to mitotic cells; see Westendorf et al. 1994]. The failure to clearly distinguish G2 cells from those in mitosis leads to vague and confusing conclusions on "*G2/M cells*." In fact, *G2/M cells* (*G2/M cell*=6,281 articles; *G2-M cell*=8,138 articles) are now considered by many to exist in a distinct phase of the cell cycle (*G2/M phase*=4,483 articles; *G2-M phase*=5,797 articles) and, in response to various treatments, undergo a *G2/M arrest* (2,747 articles; *G2-M arrest*=3,211 articles). This is in spite of the fact that neither a *G2/M cell* nor a *phase* nor an arrest exists: G2/M is a transitional state (*G2/M transition*=only 830 articles; *G2-M transition* = 1,042 articles) and at any given time a cell is either in G2 or mitosis, and these are biochemically very different states. When a treatment is said to arrest cell cycle progression at G2/M, and many treatments do (see above), one wonders whether this is due to checkpoint controls that exist during interphase (G2) or mitosis, or even in the next G1. The latter is because when cells exit mitosis without dividing, as happens in high concentrations of spindle poisons, they enter the next G1 as 4N cells (discussed below). In many FACs studies, these 4N cells are interpreted as G2/M cells.

Although the shortcomings of FACS are evident, they are often ignored both by those who work with this technique and also by those who review the results. The failure to conduct reliable supporting live cell studies and to interpret FACS data using proper controls has resulted in the publication of some spectacular and persistent fallacies in highly respected journals. These errors include, e.g., that p53 (Cross et al. 1995; Beltrami et al. 2004), ATM (Takagi et al. 1998; Bayart et al. 2004), and p38 (Takenaka et al. 1998; Tang et al. 2008) are all required for a functional mitotic checkpoint. This is in spite of the fact that knocking bona fide mitotic checkpoint proteins (see below) out of mice, like Mad2 (Dobles et al. 2000) or BubR1 (Wang et al. 2004), is lethal at the embryonic level, while p53 (Donehower et al. 1992) and p38 (Adams et al. 2000) are dispensable

for embryonic development, and humans with null mutations in ATM (ataxia telanglectasia) are viable for many (20–40) years. Once in the literature, these erroneous claims require considerable effort, time, and expense to correct. One lesson from these mistakes is that it is essential to determine at the very start of a study whether mice (or humans) lacking the protein are viable before claiming that it is a critical component of the mitotic checkpoint. It is hard to take seriously reports that the breast cancer gene BRCA2 is involved in the mitotic checkpoint (Futamura et al. 2000) and is required for the completion of cytokinesis in MEF cells (Daniels et al. 2004), when it was evident well beforehand that homozygous mice mutant for BRCA2 can survive to adulthood (Connor et al. 1997).

Simply because inhibiting or knocking down a particular protein delays entry into mitosis, or leads to problems in spindle assembly, does not mean that it is required during the G2/M transition or even in mitosis. Timely entry into and progression through mitosis are both critically dependent on the action of many proteins, events, and pathways during earlier points in the cell cycle. This is especially true of constitutive kinases and transcription factors that turn on transcription programs required for entry into mitosis and normal spindle assembly (e.g., FoxM1; Laoukili et al. 2005). Thus, although inhibiting the ERK1/2 kinase delays the G2/M transition, this is not, as originally concluded, because its activity is required during this transition for timely entry into mitosis. Rather, it is because ERK1/2 activity is required during very early G2 for timely progression through G2 (Shinohara et al. 2006).

The M/A transition

Once it commits to mitosis, a cell undergoes NEB (Fig. 1b), a non-reversible event that allows the dynamic MT arrays generated by the two separating centrosomes to interact with the chromosomes to form a bipolar mitotic spindle. During spindle assembly, the two (sister) kinetochores on each replicated chromosome must become attached to the opposing spindle poles so that each is attached to just one pole and the sisters are attached to opposing poles. This "amphitelic" attachment is the only orientation which guarantees that the two chromatids of a chromosome will be incorporated into different daughter nuclei during the ensuing anaphase.

In vertebrates, chromosomes attach to the forming spindle as their kinetochores capture MTs growing from the centrosomes, each of which normally defines a spindle pole. During this process, sister kinetochores seldom attach simultaneously to opposing centrosomes. Rather, the kinetochore closest to and facing a centrosome at NEB usually attaches first while its sister, located on the opposite side of the centromere, remains unattached (reviewed in Rieder 1990). This "monotelic" attachment results in motion of the chromosome toward, and positions it adjacent to, the centrosome it has just attached to (Fig. 2). Monotelic or pole-associated chromosomes are *a regular transient feature of astral spindle assembly.* Their presence in fixed cells is not, as interpreted by some, a manifestation of abnormal spindle assembly.

As the spindle forms, sister kinetochores can become erroneously attached. In some cases, both acquire a MT attachment to the same pole. Although such "syntelic" chromosomes are commonly seen during normal spindle assembly, they are more prevalent on monopolar spindles (Kapoor et al. 2000; Lampson et al. 2004) and on the multipolar spindles that form in taxol-treated cells (Yang et al. 2009). Alternatively, a single kinetochore may also become attached by MTs to both poles at the same time. Live cell studies reveal that this "merotelic" condition is much more common than previously thought (reviewed in Salmon et al. 2005). Fortunately, both types of attachment errors are *rapidly* corrected by a highly efficient Aurora-B kinase-based mechanism located in the centromere of each chromosome, between the two kinetochores (reviewed in Cimini 2007). Although the correction of a merotelic attachment does not generate an unattached kinetochore, the correction of a syntelic attachment does (Lampson et al. 2004; Pinsky et al. 2006), and this is important for how certain drugs work to block cells in, or promote exit from, mitosis (see below).

To minimize the production of aneuploid progeny during mitosis, cells have evolved a checkpoint that delays chromatid separation (anaphase onset) in the presence of kinetochores that are not attached to microtubules (Rieder et al. 1994, 1995; Li and Nicklas 1995). Genetic studies reveal that this "mitotic checkpoint" works by preventing large E3 ubiquitin ligase assemblies, known as cyclosomes (or

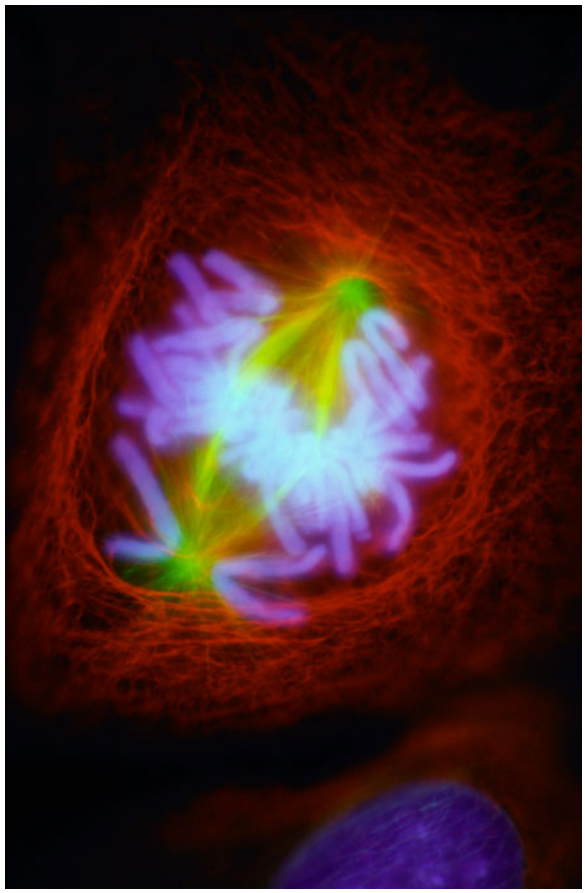

Fig. 2 This newt (salamander) lung cell was fixed in prometaphase of mitosis after which it was stained for the fluorescence localization of chromosomes (*blue*), spindle microtubules (*yellow*), and keratin filaments (*red*). As the spindle assembles during prometaphase, many chromosomes first form a monotelic attachment and move into a single spindle pole (centrosome) before acquiring an amphitelic attachment and congressing to the spindle equator. The presence of monotelic chromosomes is not evidence of a problem in spindle assembly. Rather, they are a common transient intermediate in the formation of normal bipolar spindles

anaphase-promoting complexes), from targeting for proteolytic destruction securin and the cyclin B regulatory subunit of CDK1 (reviewed in Manchado et al. 2010). The degradation of securin results in sister chromatid disjunction, while the degradation of cyclin B induces exit from mitosis (telophase and cytokinesis). In brief, during spindle assembly, unattached kinetochores catalyze the production of an inhibitory complex via a pathway involving Mad1, Mad2, BubR1, and other proteins, some of which are associated with unattached kinetochores (reviewed in Kops 2008). In turn, by sequestering Cdc20, this complex prevents the cyclosomes from recognizing securin and cyclin B. Then, when the last unattached kinetochore becomes stably attached to microtubules, production of the inhibitory complex ceases, allowing the cyclosomes to do their job. The discovery of the mitotic checkpoint provided a molecular explanation for the early observation that during prometaphase, the spindle can be destroyed and then allowed to reform multiple times without ultimately compromising the fidelity of mitosis (Mazia 1961). This is true as long as the spindle is destroyed before a point of no return, which occurs several minutes after the checkpoint has been satisfied. After this point, the cell is committed to enter anaphase.

In mammals, the mitotic checkpoint is so sensitive that a single unattached kinetochore can delay anaphase for at least several hours (Rieder et al. 1994). This implies that the "wait-anaphase" signal generated by an unattached kinetochore is amplified to inhibit all cyclosomes within the cell (by sequestering all Cdc20). Unfortunately, it is not possible to test this idea experimentally by, e.g., generating cells in mitosis that contain progressively decreasing numbers of persistently unattached kinetochores. At the moment, the best we can do is compare the duration of mitosis in drug-treated cells containing 100% unattached kinetochores, as in nocodazole, with that of cells containing ~15% unattached kinetochores, as on the monopolar spindles generated in Eg5 inhibitors (Kapoor et al. 2000). Under both conditions, non-transformed human RPE-1 cells remain in mitosis for ~20 h at 37°C before slipping into the next G1 as tetraploid (4N) cells (Brito et al. 2008). Thus, the duration of the block when ~15% of the kinetochores are unattached at any one time is about the same as when 100% remain unattached, which is consistent with an amplification step. However, exactly how long anaphase onset is delayed in a *normal* human cell that contains just one unattached and 95 attached kinetochores is unknown. In some very large cells, like newt epithelia containing 22 chromosomes, a single unattached kinetochore can delay anaphase onset for many hours, but not indefinitely (Rieder et al. 1986; Fig. 3). On the other hand, once NEB occurs, 99.9% of normal human cells satisfy the mitotic checkpoint and enter anaphase in under 15 min (Meraldi et al. 2004). This makes it highly unlikely that an individual cell within a population of normal

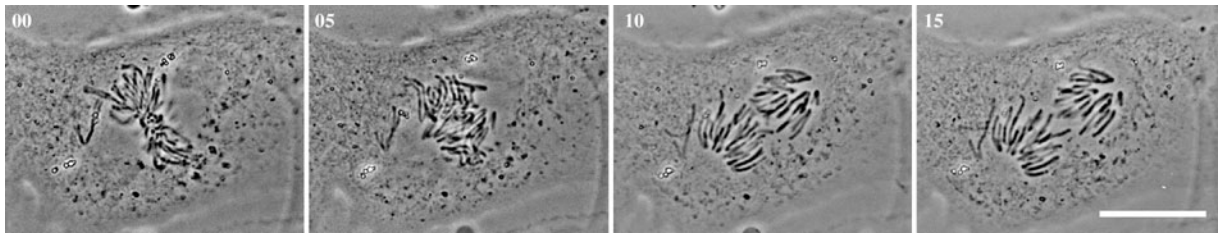

Fig. 3 Series of photomicrographs of a newt lung epithelial cell entering anaphase in the presence of a single monotelic chromosome. Normally, this event, which leads to the production of aneuploid daughter cells, is prevented by the mitotic checkpoint. However, newt cells are extremely large and at room temperature can take many (≥8) hours to complete spindle assembly when highly flattened on an artificial substrate. As evident here, occasionally, a cell will enter anaphase before attaching all of its kinetochores. Time is in minutes from first frame. *Scale*, 25 μm

cells will encounter a problem in which the checkpoint needs to remain functional for many hours. Yet within an organism, conditions *may suddenly occur in situ* in which a prolonged checkpoint-induced delay in mitosis may be beneficial, as when highly proliferating gut epithelia are suddenly and transiently exposed via ingestion to toxic agents that perturb spindle assembly.

A number of studies report that G2 checkpoint controls, including those based on the ATM/ATR and p38 kinases as well as p53, also function to regulate progression through mitosis. For the most part, these are based on FACS and other indirect analyses (see above) and are not supported by direct observations. Inactivating the Mad/Bub-based mitotic checkpoint by antibody microinjection or RNAi depletion of Mad2 induces mitotic cells to rapidly exit mitosis under every experimental condition that I am aware of, and claims to the contrary are spurious. The most recent report that p38 signaling is required for mitotic checkpoint function (Yen and Yang 2010) is simply not consistent with the fact that inhibiting or knocking down p38 in living non-transformed human cells does not override the mitotic block induced by nocodazole (Lee et al. 2010a); nor is it consistent with the decade-old report that when the placental defect is rescued, mice lacking p38 grow to adulthood and appear normal (Adams et al. 2000).

Evidence is accumulating, however, that the sensor and signal transduction pathways behind G2 checkpoints do continue to function during mitosis. For example, when chromosomes are damaged during mitosis, the ATM kinase still phosphorylates histone H2AX (Rogakou et al. 1999; Mikhailov et al. 2002) and initiates the DNA damage response (Giunta et al. 2010), while in response to stress in mitosis, p38 still activates its major downstream MK2 target (Tang et al. 2008). However, during mitosis, these pathways do not have target(s) that can delay anaphase onset independent of the Mad/Bub-based mitotic checkpoint. Thus, although p38 activity increases as cells enter mitosis, live cell studies reveal that inhibiting this kinase at the time of NEB has no effect on the fidelity of the division process and in fact slightly delays satisfaction of the Mad/Bub-based mitotic checkpoint (Lee et al. 2010a). Similarly, when chromosomes are damaged during mitosis by UV or gamma rays, the division process may be considerably prolonged. Again, however, live cell studies reveal that this prolongation depends on the mitotic checkpoint (Mikhailov et al. 2002; Dotiwala et al. 2010), and it does not occur if the damage is external to the centromere regions (Rieder et al. 1995).

In spite of the evidence that DNA damage and stress-activated checkpoint controls cannot delay progression through mitosis, data are emerging that their activity during this time plays a critical role in determining the subsequent fate of the cell(s) produced from the division. For example, stress during mitosis, whether induced by disrupting centrosome assembly (Uetake et al. 2007) or prolonging mitosis with low concentrations of spindle poisons (Vogel et al. 2004; Uetake and Sluder 2010), produces a subsequent prolonged p38-dependent, p53-mediated G1 arrest in the daughter cells.

Many continue to express the opinion that the mitotic checkpoint detects a lack of tension between an unattached kinetochore and its underlying centromere rather than the absence of a MT attachment. This position is no longer defensible. It is clear from recent live cell studies that the checkpoint can be satisfied under conditions in which there is *no tension*

between kinetochores and their underlying centromere. Examples of this include cells undergoing mitosis with unreplicated genomes (O'Connell et al. 2008) or in the presence of high concentrations of taxol (Yang et al. 2009). In fact, in one of his last studies before retiring, R.B. Nicklas, the original proponent of tension (Li and Nicklas 1995), concluded from laborious same-cell correlative LM/EM studies that "microtubule attachment determines both Mad2 binding and phosphorylation….[while]…tension elevates the number of kinetochore microtubules to the level necessary for the complete loss of Mad2 and dephosphorylation from all kinetochores. This gives a reliable "all clear" signal to the checkpoint, allowing the cell to progress into anaphase" (Nicklas et al. 2001). Clearly, there is no room in this scheme for a tension-sensing kinetochore/centromere-based checkpoint element. [The recent report that after a long search this element has finally been discovered (Baumann et al. 2007) was erroneous (Hubner et al. 2010)]. Instead, the checkpoint detects unattached kinetochores, and the checkpoint signal is shut down at a kinetochore as MTs progressively accumulate on its surface (Rieder et al. 1994; Waters et al. 1998); this is a quick process (McEwen et al. 1997) promoted by tension which slows the turnover rate of kinetochore-associated MTs (King and Nicklas 2000). Recent incarnations of the tension hypothesis envision that it is tension *within* each kinetochore (intra), and no longer between the kinetochore and centromere (inter), that shuts down the checkpoint signal (reviewed in Maresca and Salmon 2010). However, it is highly likely that the "tension" seen by these workers simply represents a structural rearrangement of the many proteins that define a kinetochore as it binds MTs and sheds its checkpoint proteins (McEwen and Dong 2009).

In the introduction to a talk or manuscript on the mitotic checkpoint, it often happens that investigators state that anaphase is delayed in the presence of lagging chromosomes or until chromosomes are properly attached to and/or positioned/aligned on the spindle. This is not true. The presence of a lagging chromosome can only be determined *after a cell enters anaphase*, when such chromosomes "lag" behind others in their motion toward a spindle pole (Fig. 4). This condition frequently arises when cells enter anaphase in the presence of an uncorrected merotelic attachment, and it is no doubt a major source of aneuploidy (Cimini et al. 2004). Furthermore, the checkpoint is not sensitive to the position of a chromosome on the spindle (Nicklas and Arana 1992), nor do erroneous kinetochore attachments signal a problem: The checkpoint can be satisfied in the presence of multiple merotelic (Cimini et al. 2004) or (stabilized) syntelic attachments (Loncarek et al. 2007; Yang et al. 2009). The fact that cells rarely enter anaphase in the presence of these errors can be attributed to the speed and efficiency of the error correction mechanism (see above), not to the prolongation of mitosis by the error (see Khodjakov and Rieder 2009). It is the constant production of unattached kinetochores from syntelic attachments by the error correction mechanism that delays checkpoint satisfaction in taxol-treated cells (Yang et al. 2009) and prohibits satisfaction on monopolar spindles (Lampson et al. 2004; Brito et al. 2008). Thus, although the mitotic checkpoint forestalls anaphase in the presence of a problem, this problem does not include mis-positioned or erroneously attached chromosomes. Statements to the contrary ascribe attributes to the checkpoint that do not exist and do a disservice to students and those entering the field, confusing this complicated lexicon more than necessary.

In some cases, inhibiting, knocking down, or mutating a particular protein allows otherwise untreated cells to prematurely enter anaphase before all chromosomes have "congressed" to the spindle equator, even after treatment with some drugs (e.g., Eg5 inhibitors, taxol) that normally delay checkpoint satisfaction by disrupting spindle assembly. This does not necessarily mean, however, that the protein under study is a component of the mitotic checkpoint, as is often interpreted. A good example here is the Aurora B kinase. When Aurora B is inhibited during mitosis, cells rapidly enter anaphase in the presence of "misaligned" chromosomes, and it also induces rapid anaphase onset in cells containing monopolar spindles (e.g., Hauf et al. 2003) or spindles formed in the MT-stabilizing drug taxol (Yang et al. 2009). However, in each case, this is due to the fact that inhibiting Aurora B inhibits the error correction mechanism which leads to the stabilization of syntelic and merotelic kinetochore attachments. This, in turn, promotes checkpoint satisfaction in cells containing MTs by preventing the production of free kinetochores, the presence of which is required to sustain the block. In fact, if

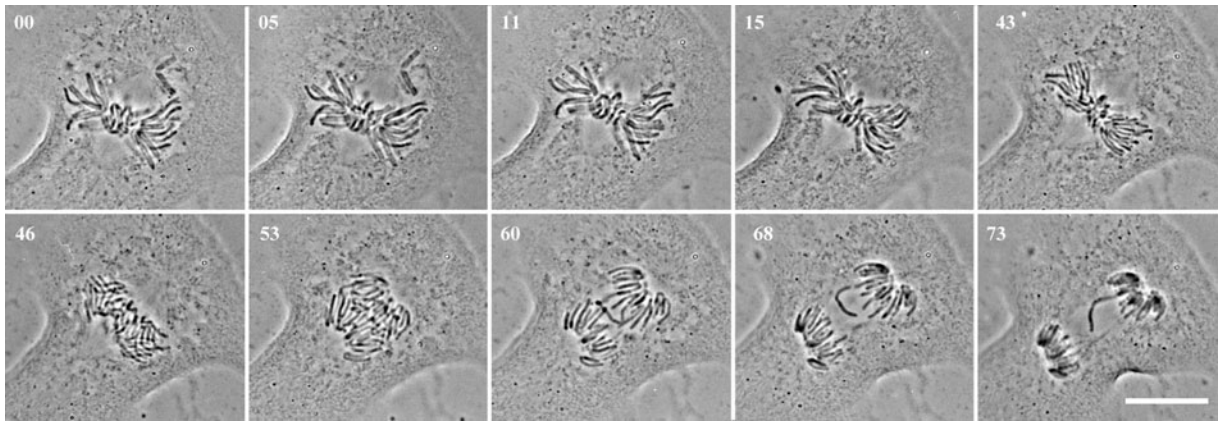

Fig. 4 The mitotic checkpoint delays anaphase onset until the last monotelic chromosome acquires an amphitelic connection, which results in its congression to the spindle equator. Only after anaphase began did this cell exhibit a single lagging chromosome. Time is in minutes from first frame. *Scale*, 25 μm

Aurora B is inhibited in nocodazole-treated cells lacking MTs, which therefore cannot satisfy the checkpoint, they remain arrested in mitosis for a period similar to controls not treated with Aurora B inhibitors (Yang et al. 2009). The conclusion that Chk1, which is reported to influence the activity of Aurora B, is a component of the mitotic checkpoint (e.g., Zachos et al. 2007; Carrassa et al. 2009) is similarly questionable until it is shown by direct methods that inhibiting or knocking it out of cells lacking MTs abrogates the checkpoint.

Finally, it is worth reiterating that a protein is part of the mitotic checkpoint only if inhibiting, depleting, or mutating it allows cells to rapidly exit mitosis *under conditions in which the checkpoint cannot be satisfied*. [Contrary to conventional wisdom and as discussed below, many conditions that are commonly assumed to inhibit checkpoint satisfaction, including taxol treatment, do not!] This criterion is known as "relief of dependence" (Hartwell and Weinert 1989), and it is a hallmark of a checkpoint control. It means that proteins or complexes that block cells in mitosis after being inhibited, mutated, or knocked down, including, e.g., CENP-E (Abrieu et al. 2000), proteasomes (Ehrhardt and Sluder 2005), and cyclosomes, are not part of the checkpoint.

Spindle poisons and mitotic slippage

The term *antimitotic agent* (64,881 articles) or its synonym *mitotic inhibitor* (67,177 articles) are routinely applied to drugs that "prevent or interfere with mitosis," "inhibit or disrupt cell division," or "block cell growth by stopping mitosis." Both of these terms have multiple meanings and are therefore ambiguous: Inhibiting or preventing mitosis is not the same as disrupting or interfering with mitosis. Does a particular antimitotic agent or mitotic inhibitor prevent cells from entering mitosis by arresting them in interphase, as implied by each term, or does it prevent cells from escaping mitosis by disrupting spindle assembly, as is more commonly understood? A more appropriate term for drugs that prevent or delay cells from exiting mitosis is *spindle poison* (395 articles), which leaves no doubt about where the drug works in the cell cycle. All drugs that perturb MT dynamics or prevent centrosome separation "impair" spindle assembly, which is one of Webster's definitions of a poison.

In 1905, Walter Dixon reported in his "Manual of Pharmacology" (freely available online) that colchicine "excites karyokinesis." That this increased mitotic index was due to an accumulation of arrested mitoses, rather than from the stimulation of mitosis, was not evident until cell culture methods became widespread in the 1930s. We now know that like other spindle poisons, colchicine blocks cells in mitosis by delaying or preventing satisfaction of the mitotic checkpoint. As discussed below, the duration of this delay depends on a number of factors. However, like all checkpoints, satisfying the mitotic checkpoint is not necessarily a prerequisite for exiting mitosis. Instead, many non-transformed cells, and to a lesser degree cancer cells, ultimately slip out of mitosis when they cannot satisfy

the checkpoint and enter the next G1 as a single 4N cell. Under this condition, non-transformed cells are arrested in G1via the p53 pathway (references in Uetake and Sluder 2004), while transformed cells lacking functional p53 can continue to cycle in the presence of the drug, ultimately producing 8N to 16N cells before finally dying (see Gascoigne and Taylor 2008). This route for increasing the ploidy level is increasingly referred to as endocycling or endoreduplication. This, however, is a misuse of terminology and therefore confusing. In an "endocycle, also referred to as the endoreplicative cycle, cells undergo successive rounds of DNA replication *without an intervening mitosis*" (Lilly and Duronio 2005). The ploidy level of cells that complete multiple cell cycles in the presence of spindle poisons increases not because the cells fail to enter mitosis, as during an endocycle, but because chromosome segregation and cytokinesis are prevented during the successive mitoses.

Slippage in non-transformed cells occurs in the continuous presence of kinetochore-associated checkpoint proteins like Mad2 and BubR1, and it correlates with a slow progressive cyclosome-mediated destruction of cyclin B (Brito and Rieder 2006; Lee et al. 2010b). It occurs because like all biochemical pathways, the mitotic checkpoint is not 100% efficient at blocking the ubiquitination and proteolysis of cyclin B. As a result, over time, the level of cyclin B slowly drops until it falls below the threshold needed to maintain the mitotic condition. Unlike in non-transformed human RPE-1 cells, slippage in HeLa is reported to correlate with a caspase-mediated cleavage of BubR1 during the block (Shin et al. 2003; Baek et al. 2005; Kim et al. 2005). However, since HeLa tend to die in mitosis in response to spindle poisons (see below), it is unknown from these indirect studies whether the destruction of BubR1 occurs gradually during the block as part of the slippage process or suddenly as part of the death process. When RPE-1 cells are treated with nocodazole or concentrations of Eg5 inhibitors that prohibit satisfaction of the checkpoint, it takes ~20 h for slippage to occur; this approximates the duration of a normal cell cycle, and 70–85% of the cells that enter mitosis, respectively, survive mitosis to slip (Brito et al. 2008).

Figure 5a depicts the percentage of HeLa cells in mitosis after a 20-h treatment with varying nocodazole concentrations (other mitotic poisons produce very similar plots). The common interpretation of this graph is that the higher the drug concentration, the more cells are blocked in mitosis until a point when most or all cells are blocked. Although true, this interpretation fails to consider that since HeLa are transformed, every cell in the population cycles into mitosis during the 20-h treatment period (see Puck and Steffen 1963). As a result, the more informative meaning of Fig. 5a is *that the higher the drug concentration, the longer the cells are delayed in mitosis* until a point (~100 nM for HeLa) when they are delayed for a maximum period, after which they either die in mitosis or slip into G1. In other words, the duration a HeLa cell spends in mitosis increases with increasing nocodazole concentration, until ~100 nm after which it spends ≥20 h.

This same figure (Fig. 5a) also reveals that the mass of spindle MTs formed in HeLa progressively decreases with increasing nocodazole concentration until none are formed at ~5 μM. [It takes ~3 μM nocodazole to completely inhibit spindle MT formation during prometaphase in non-transformed RPE1 cells and 4.0 μM in transformed U2OS (Brito and Rieder 2009)]. This response, when combined with the timing data in the same figure, reveals an inverse correlation between the length of time a cell spends in mitosis and the number of spindle MTs it can form. This relationship, as well as other indirect data, has been interpreted to mean that the rate cyclin B is destroyed during mitotic slippage is accelerated by the presence of MTs (Andreassen and Margolis 1994). This interpretation, however, hinges on the assumption that all concentrations of spindle poisons prohibit satisfaction of the mitotic checkpoint, which turns out to be incorrect (Brito et al. 2008). Instead, increases in nocodazole concentration progressively delay, but do not prevent, checkpoint satisfaction until a concentration is reached, after which the checkpoint can no longer be satisfied. From Fig. 5a, it is evident that HeLa cannot satisfy the checkpoint at or above ~100 nM nocodazole and, as a result, are blocked in mitosis for ≥20 h. In contrast, in 50 nM nocodazole, the majority of cells satisfy the checkpoint within ~4–5 h, which is why only 20% are in mitosis after 20 h.

A similar situation exists for taxol and its derivatives which stabilize and promote spindle MT assembly. However, unlike for drugs like nocodazole that prevent MT assembly, the duration of mitosis in taxol increases with drug concentration until a point is

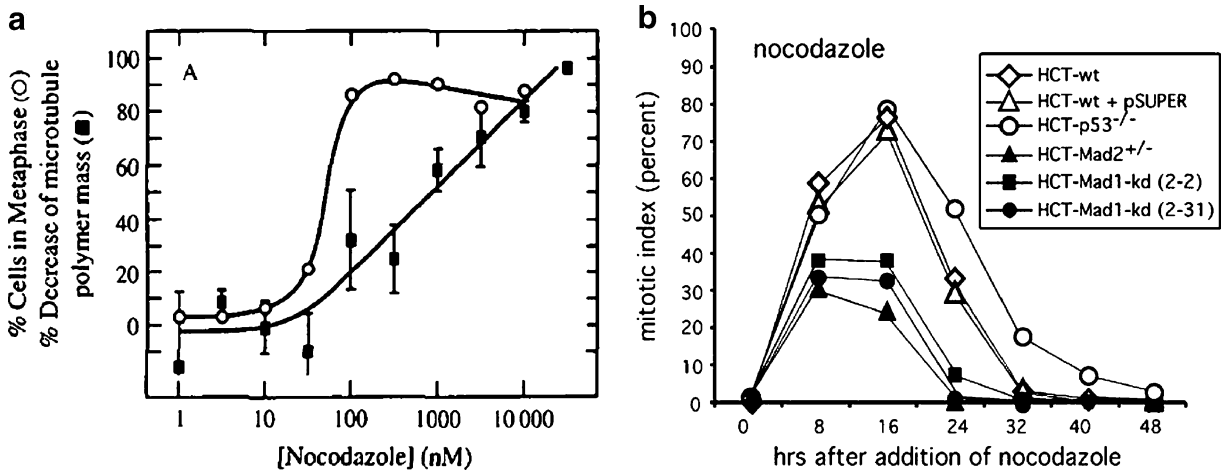

Fig. 5 **a** Plot depicting the percentage of HeLa cells in mitosis and the percentage decrease of spindle microtubule polymer mass after 20 h in various concentrations of nocodazole (from Jordan et al. 1992). **b** Plot showing the relationship between the mitotic index (percentage of cells in mitosis) of various strains of HCT cells fixed various times after treatment with 150 nM nocodazole. Note that those strains in which the expression of Mad2 or Mad1 was reduced (*solid symbols*) are blocked in mitosis for ~50% of the duration exhibited by controls (from Kienitz et al. 2005). See text for details

reached (about 500 nm), after which *it radically decreases*. This is because cells ultimately satisfy the checkpoint in all concentrations of taxol, but do so more rapidly at very low and high concentrations. As a result, in 5 nM taxol, the duration of mitosis in non-transformed RPE-1 cells is similar to that in 10 μM taxol (about 2–3 h; Yang et al. 2009). Importantly, not only do cells satisfy the checkpoint in low concentrations of spindle poisons, but within a range, they also successfully divide into two or more aneuploid daughters (Ikui et al. 2005; Brito and Rieder 2009). This raises the possibility that clinically therapeutic doses of taxol kill cancer cells *by promoting their division into non-viable progeny.*

Within a given line, the duration of a mitotic arrest induced by spindle poisons varies considerably between neighboring cells (Gascoigne and Taylor 2008; Orth et al. 2008; Brito and Rieder 2009). This likely partly reflects their genotypic diversity, especially those derived from cancers. The duration of the arrest also varies widely between different cell lines, and when the checkpoint cannot be satisfied, the average delay in cancer cell lines can be significantly greater than (e.g., HeLa) or less than (e.g., U2OS) that of non-transformed control lines (Brito and Rieder 2009). As a rule, cancer cell lines that possess a less than fully functional mitotic checkpoint (e.g., Kasai et al. 2002; Saekl et al. 2002), or lines constructed to have reduced expression of checkpoint proteins (e.g., Kienitz et al. 2005), slip through mitosis significantly faster than non-transformed controls. [Although such studies often interpret faster slippage in the presence of spindle poisons as evidence that checkpoint is lost, inactivated, or otherwise non-functional, this is a gross exaggeration. Usually, the cells delay in mitosis for 4–8 h (Fig. 5b), whereas when the mitotic checkpoint is truly rendered non-functional, the duration of mitosis is <20 min (Meraldi et al. 2004)]. Depending on the line, the rate a cell slips through mitosis may also depend on the type of spindle poison used: While RPE-1 cells average ~20 h in division before slipping in nocodazole or Eg5 inhibitors, in vinblastine, they average ~30 h (Brito et al. 2008). Based on how slippage occurs, vinblastine, in addition to its effect on MTs, may also depress cyclosome or proteosome function. In nocodazole-treated RPE-1 cells, the duration of mitosis is also significantly longer in cells treated with p38 kinase inhibitors (Lee et al. 2010a), but it is significantly shorter when caspases 3 and 9 are inhibited or knocked out (K. Lee and C.L. Rieder, unpublished observation). The reasons for this are currently under investigation. Finally, when the checkpoint cannot be satisfied, the duration of mitosis is also species-specific: In concentrations of nocodazole that inhibit MT assembly, normal human cells spend ~20 h in mitosis, while mouse, hamster, and rat (and rat-kangaroo), cells spend only 4–5 h in mitosis before slipping into the next G1 (Lanni and Jacks 1998; see

also Haller et al. 2006)—a fact that is rarely considered when conducting checkpoint studies on such cells.

Compared to non-transformed cells, a much higher percentage of cancer cells fail to undergo slippage and instead die in mitosis (see Gascoigne and Taylor 2008). Based on live cell analyses, Mitchison and colleagues (Shi et al. 2008) reported that for every HeLa cell which survives to slip through mitosis in a spindle poison, 30 die in mitosis. In a similar study that focused on timing, we found that 50% of the HeLa cells that entered mitosis in nocodazole died in mitosis within ~20 h (Brito and Rieder 2009; see also Orth et al. 2008). This was also true for 500 nM taxol. These findings have potentially important negative consequences for population studies that base their conclusions on HeLa cells harvested in mitosis 24–48 h after releasing them into spindle poisons from an S-phase block (*HeLa* + *mitosis* = 3,421 articles). Since G2 in HeLa is ~4 h (Puck and Steffen 1963), ~100% of these cells will have been in mitosis for ≥20 h. Based on the in vivo data on how HeLa cells respond to a prolonged exposure to spindle poisons during mitosis, it is reasonable to conclude that many of these biochemical studies were conducted on a high percentage of dead or dying cells.

Similarly, indirect analyses of cancer cell populations that have been synchronized in mitosis by nocodazole treatment usually fail to consider the possibility that subsequent experimental manipulation may potentiate either mitotic slippage or death in mitosis. These are important considerations because a treatment that promotes slippage leads over time to a decrease both in the mitotic index of fixed cell populations and in the number of rounded cells—which can give the erroneous impression that the treatment abrogated the mitotic checkpoint (e.g., caspase 3 inhibition; Hsu et al. 2006). Likewise, treatments that promote cell death during mitosis (e.g., tumor necrosis factor-related apoptosis-inducing ligand; Kim et al. 2008) will be reflected in FACS analyses as a decrease in the number of G2/M cells which, in lieu of corresponding direct data, can also be interpreted as "inactivation" of the mitotic checkpoint. The lesson here is that one should always determine directly how individual living cells respond to a protocol before scaling it up for population studies—information that can be easily obtained by simply following live cell populations under low-magnification phase-contrast light microscopy (Gascoigne and Taylor 2008; Brito and Rieder 2009).

Acknowledgments Work in my lab has been supported by a grant (40198) from the National Institutes of Health, National Institute of General Medical Sciences. Many thanks to members of Dr. William Earnshaw's lab for discussions related to the material presented here and also to Susan Nowogrodzki for editorial assistance.

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