The spindle assembly checkpoint (SAC) is essential in mammalian mitosis to ensure the equal segregation of sister chromatids. The SAC generates a mitotic checkpoint complex (MCC) to prevent the anaphase-promoting complex/cyclosome (APC/C) from targeting key mitotic regulators for destruction until all of the chromosomes have attached to the mitotic apparatus. A single unattached kinetochore can delay anaphase for several hours, but how it is able to block the APC/C throughout the cell is not understood. Present concepts of the SAC posit that either it exhibits an all-or-nothing response or there is a minimum threshold sufficient to block the APC/C. Here, we have used gene targeting to measure SAC activity, and find that it does not have an all-or-nothing response. Instead, the strength of the SAC depends on the amount of MAD2 recruited to kinetochores and on the amount of MCC formed. Furthermore, we show that different drugs activate the SAC to different extents, which may be relevant to their efficacy in chemotherapy.

The SAC is activated by unattached kinetochores that seem to generate an APC/C inhibitor. The exact nature of the inhibitor, or inhibitors, is not yet clear but genetic, biochemical, and structural data indicate the MCC, composed of MAD2, BUBR1 and BUB3, prevents CDC20 from activating the APC/C. This evidence is consistent with unattached kinetochores catalysing a conformational change in MAD2 as a necessary prerequisite to binding CDC20. How a single unattached kinetochore is able to generate sufficient inhibitor to block the APC/C throughout the cell, however, is not understood: in particular, whether the inhibitory signal is amplified to generate an all-or-nothing response, or whether it generates a minimum SAC threshold. The true nature of the SAC is important both for our understanding of the mechanism behind it, and because it has been postulated that the SAC can be weakened in cancer cells.

Our understanding of the SAC has been hampered by the lack of an assay to measure its activity. We recently showed that cyclin A2 could be degraded when the SAC was active because it bound to CDC20 in competition with the MCC. We thought that this should provide an assay for SAC activity, that is, the rate of MCC production, because the rate of cyclin A2 degradation should be determined by competition for CDC20 with the MCC. To test this, we needed to measure precisely the prometaphase destruction of cyclin A2. Therefore, we used recombinant adenovirus-associated virus (rAAV)-mediated gene targeting to introduce the open reading frame (ORF) of yellow fluorescent protein (Venus) into the last exon of one allele of the CCNA2 (cyclin A2) gene in hTert-RPE1 cells (RPE1; retinal pigment epithelial; Fig. 1a and Supplementary Fig. S1a,b). (Note that this fusion generated a functional protein.) We chose RPE1 cells because they have a normal diploid karyotype, are not transformed and exhibit little cell death when arrested in mitosis; tagging the endogenous cyclin A2 protein in RPE1 cells avoided the complications of mosaic protein levels in the cell population produced by ectopic expression. Immunoblotting analysis showed that cells expressed the fusion protein to the same level as the untagged protein (Fig. 1b). Time-lapse microscopy showed that individual cells in the population had very similar kinetics of cyclin A2–Venus degradation and mitotic progression (Supplementary Fig. S1c and Video S1).

Our hypothesis predicted that the rate of cyclin A2 degradation should be accelerated when we eliminated the production of MCC, either by depleting MAD2, or by inhibiting MPS1, an essential SAC kinase. Conversely, maintaining the SAC, for example by, treating cells with the Eg5 spindle motor poison di-methyl anastron (DMA), should reduce the rate of cyclin A2 degradation. Both of these predictions were verified (Supplementary Fig. S1d), from which we concluded that the rate of degradation of cyclin A2–Venus could serve as a quantitative assay for SAC activity.

Taxanes and vinca alkaloids are effective anti-cancer agents, possibly through their ability to activate the SAC (refs 21,22), so understanding how such compounds affect the SAC could be therapeutically valuable. To investigate this we treated RPE1 CCNA2Venus/+ cells with Taxol, DMA or nocodazole and found that although all three drugs delayed...
cells in mitosis, the rate of cyclin-A2–Venus destruction was faster in Taxol than in nocodazole or DMA (Fig. 1c); therefore, different microtubule poisons generated different levels of SAC activity. Furthermore, when we inhibited Mps1, the rate of cyclin A2 degradation increased whereby a gradual increase in activity of a pro-apoptosis pathway is which cells delay in mitosis depends on two antagonistic pathways, (Supplementary Fig. S2d,e). Quantitative fluorescence imaging showed (Fig. S2f; control and Video S2) and was regulated by the SAC of ectopic cyclin B1 (refs 21,23), but as altering the level of cyclin-B1–CDK1 activity affected the fate of cells in mitosis21, we chose to assay the destruction of endogenous cyclin B1 by using rAAV-mediated gene targeting to fuse exon 9 of the CCNB1 locus (cyclin B1) locus by rAAV-mediated homologous recombination. The rAAV vector contains the coding sequence of Venus, which was inserted between two gene-specific homology arms to replace the STOP codon at the junction between non-coding (white square) and coding (black square) regions in exon 8 and two inverted terminal repeats (L-ITR, R-ITR; red rectangles). Integrants were selected by fluorescence-activated cell sorting (FACS). (b) Western blot analysis of parental RPE1 and a cyclin-A2–Venus clone. Cell lysates were probed with anti-cyclin A2 (left panel) and anti-GFP (right panel) antibodies. Note the two forms of cyclin A2 in the cyclin-A2–Venus clone (the upper one uniquely recognized by the anti-GFP antibody). Relative molecular mass markers are shown on the left. (c,d) Single-cell cyclin-A2–Venus destruction assays of asynchronously growing RPE1 cyclin-A2–Venus cells treated with nocodazole (n = 15 cells), Taxol (n = 12 cells) or DMA (n = 12 cells; c) or different concentrations of AZ3146 in the presence of 10 μM DMA (n = 15 cells for all conditions except 5 μM AZ3146, where n = 11 cells; d). Images were acquired at 3–5 min intervals and the total cell fluorescence was quantified. Fluorescence intensities were normalized to the level at NEBD. Error bars indicate s.d. and are representative of at least two independent experiments. The time to reach half-maximum fluorescence after NEBD was used for statistical tests.

Mitotic slippage had previously been measured by the degradation of ectopic cyclin B1 (refs 21,23), but as altering the level of cyclin-B1–CDK1 activity affected the fate of cells in mitosis21, we chose to assay the destruction of endogenous cyclin B1 by using rAAV-mediated gene targeting to fuse exon 9 of the CCNB1 (cyclin B1) locus to the ORF of Venus in RPE1 cells (Fig. 2a,b and Supplementary Fig. S1e), which produces a functional fusion protein25. Just as for cyclin-A2–Venus, the cyclin-B1–Venus protein accumulated and was destroyed with similar kinetics to the untagged endogenous protein (Supplementary Fig. S2d,e). Quantitative fluorescence imaging showed that cyclin-B1–Venus destruction began at metaphase (Supplementary Fig. S2f; control and Video S2) and was regulated by the SAC (Supplementary Fig. S2f; reversine and nocodazole).

We treated RPE1 CCNB1Venus+ cells with various microtubule poisons and assayed them by time-lapse fluorescence microscopy. We
**Figure 2** Different spindle poisons activate the SAC to different extents. 

(a) Venus ORF targeting into the human CCNB1 locus by rAAV-mediated homologous recombination. The rAAV vector contains both the coding sequence of Venus and a G418 resistance cassette (flanked by loxP sites, red triangles), which were inserted between two gene-specific homology arms to replace the STOP codon at the junction between non-coding (white square) and coding (black square) regions in exon 9 and two inverted terminal repeats (L-ITR, R-ITR; red rectangles). Integrants were selected by survival in G418-containing medium. 

(b) Western blot analysis of parental RPE1 and a cyclin-B1–Venus clone. Cell lysates were probed with anti-cyclin B1 and a cyclin-B1–Venus clone. Cell lysates were probed with anti-cyclin B1 (left panel) and anti-GFP (right panel) antibodies. Note the two forms of cyclin B1 in the cyclin-B1–Venus clone (the upper one uniquely recognized by the anti-GFP antibody). Relative molecular mass markers are shown on the left. 

(c) Scatter plot showing the duration of the mitotic arrest of RPE1 cyclin-B1–Venus cells treated with the same drugs as in Fig. 1c (n = 50 in each condition). The black line represents the mean. 

(d) Single-cell analysis of mitotic slippage in asynchronously growing RPE1 cyclin-B1–Venus cells treated with nocodazole (n = 5 cells), Taxol (n = 6 cells) or DMA (n = 5 cells). Images were acquired at 5 min intervals and quantified, plotted and analysed as in Fig. 1c. The inset shows the slopes of the linear regressions in the main panel. Error bars indicate s.d. and are representative of at least two independent experiments. 

(e) Single-cell analysis of asynchronously growing RPE1 cyclin-B1–Venus cells treated with siRNA against MAD2 in the presence of nocodazole (n = 6 cells), Taxol (n = 13 cells) or DMA (n = 16 cells). Note that only cells with mitosis durations of 9–12 min (minimum mitosis duration in RPE1 cells) were considered. Error bars indicate s.d. and are representative of at least two independent experiments. 

(f) Effect of the rate of cyclin-B1–Venus slippage in d on the duration of mitosis in different drug treatments in c. Error bars indicate s.d. and are representative of at least two independent experiments. 

(g) Single-cell analysis of mitotic slippage in asynchronously growing RPE1 cyclin-B1–Venus cells treated with nocodazole (n = 7 cells) and increasing AZ3146 concentrations (n = 5 cells for 0.31 μM; n = 6 cells for 0.62 μM; n = 6 cells for 1.25 μM; n = 9 cells for 2.5 μM and n = 7 cells for 5 μM). Images were acquired at 5 min intervals and total fluorescence was quantified. Fluorescence intensities were normalized to the level at NEBD. The insets show the slopes of the linear regressions in the respective main panel. Error bars indicate s.d. and are representative of at least two independent experiments.
reasoned that assaying cyclin-B1–Venus levels over time in cells blocked in mitosis should provide a measure of the effectiveness with which the SAC could repress APC/C activity because weaker SAC activity should result in a higher rate of cyclin B1 destruction. In agreement with other studies, we found that Taxol-treated cells spent less time in mitosis (555.9 ± 345.6 min) when compared with cells treated with DMA (1,101 ± 410.1 min) or nocodazole (1,481 ± 529 min; Fig. 2c), even though DMA produces similar kinetochore attachment defects to Taxol. The reason for this discrepancy was different rates of proteolysis: cyclin-B1–Venus levels fell faster in Taxol-treated cells when compared with DMA, and faster in DMA than in nocodazole (0.33 μM; Fig. 2d). This was not caused by a direct effect of the drugs on APC/C activity because when MAD2 was depleted by short interfering RNA (siRNA), cyclin-B1–Venus was degraded with the same kinetics in the different drugs (Fig. 2e). Instead, the kinetics of cyclin-B1–Venus destruction correlated with the strength of the checkpoint we had previously measured using the disappearance of cyclin-A2–Venus. The average slope of cyclin-B1–Venus destruction in different drug treatments correlated inversely ($r^2 = 0.9482$) with the time cells spent in mitosis (Fig. 2f), indicating that the length of mitotic arrest probably depended on how effectively the APC/C was inhibited by the SAC. To test this further, we used serial dilutions of the MPS1 inhibitor AZ3146 in cells treated with 0.33 μM nocodazole, and found that a progressive increase in AZ3146 concentration caused an increase in the rate of cyclin-B1–Venus destruction (Fig. 2g), and, as previously observed, a reduced mitotic delay (Supplementary Fig. S2g).

The differences in cyclin A2 and cyclin B1 degradation rates in response to different drugs indicated that the strength of the SAC was variable rather than all-or-nothing. To understand how the strength of the SAC could vary we needed a quantitative measure of the SAC. To test this, we assayed the effect on the SAC of depleting MAD2 to different extents by sub-optimal siRNA treatment in RPE1 cells (Fig. 2h). The molecular endpoint of the SAC is the incorporation of CDC20 into the MCC (refs. 4,10,11,32,33); therefore, we examined the abundance of MCC components (MAD2, BUBR1 and CDC20) bound to the APC/C and CDC20 in extracts of mitotic cells treated with different drugs (Fig. 5a). This showed that CDC20 bound to more MAD2 in cells treated with nocodazole when compared with DMA, and more in DMA when compared with Taxol (Fig. 5b,d), whereas the amount of BUBR1 bound to CDC20 remained unchanged (Fig. 5b,d). We interpreted this as a change in the ratio of MCC complexes with and without MAD2, because we, and others, had previously found that MAD2 was substoichiometric to BUBR1 and CDC20 in the MCC (refs. 10,12,13). To test this, we compared MCC that was free or bound to the APC/C by immunoprecipitation (Fig. 5a) and size exclusion chromatography (Supplementary Fig. S4a–c). This indicated that, at the molecular level, the strength of the SAC could be determined by the amount of MCC generated per unit time, and thus its concentration in the cell, and that binding to the APC/C was limiting. Whereas the levels...
Figure 3 The amount of MAD2-positive kinetochores correlates with the length of a mitotic block. (a) Venus ORF targeting into the human MAD2LI locus by rAAV-mediated homologous recombination. The rAAV vector contained the coding sequence of Venus, which was inserted between two gene-specific homology arms to replace the ATG at the junction between non-coding (white square) and coding (black square) regions of exon 1 and two inverted terminal repeats (L-ITR, R-ITR; red rectangles). Integrants were selected by FACS. (b) Western blot analysis of parental RPE1 and a Venus–MAD2 clone. Cell lysates were probed with anti-MAD2 (left panel) and anti-GFP (right panel) antibodies. The asterisk indicates a cross-reacting band. Note the two forms of MAD2 in the Venus–MAD2 clone (the upper one uniquely recognized by the anti-GFP antibody). Molecular mass markers are shown on the left. (c) Asynchronous RPE1 cells were treated for 12–16 h with nocodazole, Taxol or DMA, fixed with methanol and processed for immunofluorescence using anti-β-tubulin (green) and ACA antibodies to stain the kinetochores (red). DNA was stained using DAPI (blue). The signal intensity for HEC1, a stable kinetochore component, was used as a measure of kinetochore size. Kinetochore-associated MAD2 (molecules per cell) was used as a measure of kinetochore size. (d) Typical localization of Venus–MAD2 in living mitotic cells treated with different spindle poisons as in c. (e) Distribution of Venus–MAD2-positive kinetochores per cell following 12–16 h of treatment with different drugs (n = 42 cells for nocodazole; n = 38 cells for Taxol and n = 34 cells for DMA), representative of at least two independent experiments. (f) Scatter plots obtained by fluorescence correlation using the data shown in Supplementary Fig. S4 for the number of Venus–MAD2 molecules per kinetochore in cells treated with different drugs (n = 57 kinetochores from 8 cells for nocodazole; n = 38 kinetochores from 11 cells for Taxol; and n = 55 kinetochores from 8 cells for DMA), representative of at least two independent experiments. The black line represents the mean. (g) Asynchronous (top) or 3:1 (bottom) RPE1 Venus–MAD2 cells were fixed in methanol and processed for immunofluorescence against HEC1 (red), and DNA stained with DAPI (blue). The signal intensity for HEC1, a stable kinetochore component, was used as a measure of kinetochore size. Kinetochore-associated MAD2 signals (green in image) were quantified and plotted as a function of the corresponding HEC1 fluorescence (n = 56 kinetochores from 6 cells for asynchronous and n = 60 kinetochores from 3 cells for 3.33 μM nocodazole). Data are representative of two independent experiments. (h) Plot of the relationship between the duration of mitosis (from Fig. 2c) and the integrated molecule number of Venus–MAD2 at kinetochores in the different poisons (from f,e). Error bars indicate s.d. and are representative of at least two independent experiments. Scale bars, 10 μm.
of APC/C-bound MCC proteins remained approximately the same in all drug treatments, the levels bound to non-APC/C-associated CDC20 increased as the strength of the SAC increased. The strongest effect was seen in nocodazole-treated cells, where BUBR1 accumulated ~2-fold and MAD2 ~3-fold (Fig. 5b,c and Supplementary Fig. S4b,c).

To test this further, we arrested cells with nocodazole, DMA or Taxol, then inactivated the SAC with AZ3146 and monitored the kinetics of mitotic exit, predicting that cells with more MCC should take longer to exit mitosis because more time would be required for all of the MCC to disassemble. In agreement with this, the rate at which cells exited mitosis was inversely proportional to the amount of the MCC (Supplementary Fig. S5a). Note that each half-life value was corrected by subtracting the minimum half-life for mitotic exit in the presence of a CDK1 inhibitor RO3306 (Fig. 5i, inset). Although some of these differences might be attributed to the error-correction role of MPS1 (refs 29,34–36), because cells satisfy the checkpoint quicker in Taxol or DMA when compared with nocodazole, this would make only a minor contribution because AZ3146 reduced the mitotic delay by 95.4% for Taxol, 94.8% for DMA and 91.9% for nocodazole (Figs 2c and S1), a difference of only 3.5% between error-correction conditions and nocodazole.

Together, our data support a model in which the SAC does not behave as an all-or-nothing toggle switch. Instead, the SAC is like a rheostat: it can be activated to different levels, and thereby inhibit the APC/C to different extents, which dictates the length of a mitotic delay. The strength of the SAC depends on the number of signalling centres (kinetochores), and critically on the amount of MAD2 recruited to kinetochores, which probably determines the rate of MCC production (Supplementary Fig. S5a).

Our findings have implications for the control of mitosis. At the start of an open mitosis most kinetochores are unattached, which would produce a pulse of MCC to inhibit the APC/C. As chromosome attachments are established, the declining numbers of unattached kinetochores generate sufficient inhibitory complex to prevent premature anaphase, but because the MCC is constantly disassembled this will reduce the amount of MCC (Supplementary Fig. S5b). The progressive reduction in MCC level, allied with the role of protein phosphatases in chromosome attachment and SAC
Figure 5 CDC20 incorporation into the MCC correlates with the strength of the SAC. (a) Isolation of different complexes and western blot analysis of the different lysates (n = 5 extracts for nocodazole; n = 4 extracts for Taxol; and n = 3 extracts for DMA). The error indicates s.e.m. and is representative of at least three independent experiments. (b) Anti-APC4 or anti-CDC20 immunoprecipitates from extracts of RPE1 cells treated with nocodazole (n = 3 extracts) and isolated by mitotic shake-off 12–16 h later. (c–e) The amount of MCC proteins that co-purified with APC4 (c) or CDC20 (d,e) was analysed by quantitative immunoblotting, normalized to the level in Taxol-treated cells, and shown as bar graphs. Error bars indicate s.e.m. and are representative of at least three independent experiments. Note that the CDC20 immunoprecipitates in b (last three lanes to the left) and e were obtained from APC4-free extracts (in a). (f) Schematic of experiments in g,h.

silencing 38,39, would contribute to the efficient coupling between correct bipolar attachment of chromosomes and rapid anaphase onset (Supplementary Fig. S5c and unperturbed). In drug-induced arrests, MCC production will be continuous, at a rate depending on the ability of the drug to prevent correct kinetochore attachments (Supplementary Fig. S5c; Taxol, DMA and nocodazole). This may explain why depleting

\[ \text{Relative binding to APC4} \]

\[ \text{Relative binding to CDC20} \]

\[ \text{Mitotic cells (%)} \]

\[ \text{Time (min)} \]

\[ \text{MCC production will be continuous, at a rate depending on the ability of the drug to prevent correct kinetochore attachments (Supplementary Fig. S5c; Taxol, DMA and nocodazole). This may explain why depleting} \]
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AUTHOR CONTRIBUTIONS

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METHODS

Plasmids and cloning. Homology arms to human cyclin A2 (CCNA2), human cyclin B1 (CCNB1) and human MAD2 (MAD2L1) genes were amplified from h-TERT-RPE1 genomic DNA using High-Fidelity polymerase (Roche). Targeting construct was assembled in a NotI-digested pAAV vector by 4-piece DNA ligation with the coding sequence of a fluorescent protein fused or not with a neomycin-resistant cassette from the pXv vector23 and two homology arms. Clones were screened by colony PCR and restriction enzyme digestion. All constructs were sequenced to ensure their integrity.

Production of rAAV particles and cell infections. All rAAV preparations were made as previously published25. For FACS selection, h-TERT-RPE1 cells were seeded on a single T75 flask 24 h before infection. The next day, cells were washed twice in PBS+Mg+Ca (100 mM−1) and infected with 3 ml of viral supernatant complemented with 7 ml of DEEM/F12 medium (Sigma). After 48 h, cells were washed twice in PBS+Mg+Ca, trypsinized, seeded into two 140 mm tissue culture dishes and allowed to grow until 80% confluence was reached. Cells were split into 4 x 140 mm tissue culture dishes and treated the next morning with 5 mM thymidine for 24 h. Cells were then washed twice in PBS+Mg+Ca and released into fresh medium for 4-6 h to enrich for cells in G2 phase.

Culture conditions and treatments. Normal RPE1 and knock-in cell lines were cultured in F12/DMEM (Sigma) medium supplemented with GlutaMAX (Invitrogen), 10% FBS (Gibco), 0.348% sodium bicarbonate, penicillin (100 U ml−1), streptomycin (100 µg ml−1) and Fungizone (0.5 µg ml−1). Cells were maintained in a 37 °C incubator with 5% CO2, and cultured for a minimum of 48 h before experiments and or drug treatments. For experiments on populations of cells arrested in mitosis (biochemistry, kinetochore and molecule numbers counts), cells were treated for 12–16 h with Taxol (0.116 µM), DMA (10µM) and nocodazole (0.33 µM). For other imaging experiments, cells were seeded either on 96-well plates (µClear, Greiner) or 8-well chamber slides (µSlide, ibidi) and drugs were added directly into the imaging medium before acquisition unless otherwise stated. For RNA interference, RPE1 cells were transfected with Lipofectamine RNAiMAX (Invitrogen) and MAD2 siRNA oligonucleotide (5'-GGAAAGAGUCGGGACACACGUAU-3') at 30 nM for 48 h before analysis, except for partial siRNA experiments, where a 30 nM starting concentration and 2- and 4-fold dilution of the final mix were used for 24 h.

Flow cytometry. Infected or uninfected RPE1 cells were resuspended in conditioned F12/DMEM (Sigma) medium and filtered using a 30 µm mesh (PARTEC) to remove aggregates. A MoFlo high-speed cell sorter (Beckman Coulter) was used to isolate cells by fluorescence. Cells were analysed for forward and side scatter to eliminate debris, and doublet discrimination to ensure that only single cells were sorted. ToPro-3 (Molecular Probes, Invitrogen) (excitation at 633 nm, detection at 670 nm) was added to the cells to exclude dead cells. Venus fluorescence (excitation at 488 nm) was detected using a 530/30 nm filter and to distinguish between autofluorescent cells and Venus-positive cells, the 530/30 nm channel was selected against the 580/40 nm filter that showed autofluorescent cells at a 45 degree angle. Venus-positive cells formed a discrete population that was dimmer than the autofluorescent cells on the 580/40 axis. Negative cells were used to set the voltages for all channels and to set the positive gate. Venus-positive cells were individually sorted into single wells of a 96-well plate filled with conditioned medium.

Diagnostic PCR and western blot analysis. Neomycin-resistant clones and clones retained following microscopy screens were subjected to diagnostic PCR analysis using genomic DNA extracted from cells in 96- and 24-well plates, respectively. The following sequences of oligonucleotides were used for diagnostic PCR: 5’ cyclin A2: 5’-TCGTCACACAGTAACTACG-3’, 5’ cyclin B1: 5’-CACCACGAATTGCGAAG-3’, 5’ cyclin B1: 5’-TTACCGGTGTTGACAGGTAT-3’, 5’ cyclin B1: 5’-ACCACAGTTGATCTGTAATC-3’, 5’ Neo: 5’-AGGTTGCGTTCGGAATTCG-3’, 5’ Neo: 5’-GGTGTGCGGCACATGACGCGG-3’, 5’ MAD2(Venus): 5’-CTGTCGCTTCATTCCACAC-3’, 5’ MAD2(Venus); 5’-CCTGTTCTATTGCGCACAAT-3’, 5’ Venus: 5’-CTCCTGCGCCCTTGGCTCAAC-3’ and 5’ Venus: 5’-GAGTGCAACAGCTGCTACAA-3’.

For immunoblot analyses, cells were typically cultured in 6-well dishes to 70% confluence, washed in PBS and lysed in 100 µl sample buffer. Ten microtubes of the extracts were loaded and run on a 4–12% NuPAGE gel (Invitrogen) and transferred to PVDF membrane before immunoblotting. The following antibodies were used at the indicated dilution: CCNA2 (monoclonal antibody, clone GNS-1, 1:2,000, BD Pharmingen), MAD2 (polyclonal antibody, 1:1,000; Bethyl Laboratories) BUBR1 (polyclonal antibody A300-386A, 1:1,000, Bethyl Laboratories), AP3C (monoclonal antibody, clone 35/CDC27, 1:1,000, BD Transduction Laboratories), AP4C (monoclonal antibody against a carboxy-terminal peptide, 1:1,000), anti-GFP (monoclonal antibody, clone JL-8, 1:1,000; Clontech), TUBB (beta-tubulin) (polyclonal antibody, ab6046, 1:200, Abcam), HEC1 (monoclonal antibody, clone GC93, 1,500, Abcam). Antibodies were detected and analysed using the LiCOR Odyssey system according to the manufacturer’s instructions (LI-COR Biosciences).

Immunoprecipitations and size-exclusion chromatography. Cells were lysed for 30 min on ice in lysis buffer (PBS, 0.5% NP40, 0.2 µm microcristin and Roche complete protease inhibitor cocktail) and lysates were cleared by centrifugation (15 min, 16,100g). Immunoprecipitations were carried out with antibodies coincidentally coupled to protein G Dynabeads (Invitrogen) with 500 µg of protein extracts per condition. Beads were washed three times with lysis buffer and protein complexes were eluted using LDS-sample buffer (Invitrogen). Size-exclusion chromatography was carried out on a Superose 6 PC 3.2/30 column (GE Healthcare) as previously described27.

Microscopy. Primary screens were conducted 7–10 days after cell sorting. Each 96-well plate was scanned using an ImageXpress micro high-throughput microscope (Molecular Devices) for destruction curves or using a DeltaVision Core microscope (Applied Precision) equipped with a QuantEM512sc EMCCD camera (Photometrics) and an environmental chamber at 37 °C (Applied Precision) for Fig 4h. For higher resolution microscopy and molecule counts, cells were imaged using an Intelligent Imaging Innovations confocal spinning-disc microscope equipped with a CSU-X1 head (Yokogawa) and a QuantEM:512sc EMCCD camera (Photometrics). For molecule counts, 50 0.3 µm slices were acquired with a ×60 objective with a 100 ms exposure time (488 nm laser excitation). For correlative studies between Venus–MAD2 foci intensity and the strength of the checkpoint, cells were imaged every 10 min with 15 1 µm slices per acquisition on the same microscope, but using a ×40 objective. In all experiments 10% laser power (Neutral density) was used.

Quantification of fluorescence microscopy data. For cyclin-A2–Venus and cyclin-B1–Venus destruction analysis, fluorescence intensities were measured and analysed using ImageJ software as previously described28. To calculate Venus–MAD2 molecule numbers (Fig 3f), integrated fluorescence intensities were measured from the sum of intensity projections of z-sections and correlated to total molecule numbers in a cell obtained by antibody calibration with cell extracts and recombinant MAD2 protein25. The method described in ref. 46 was used to determine local fluorescence intensities at kinetochores and local molecule numbers were obtained by using the correlation above. Total fluorescence intensity (Ftot) at a kinetochore was calculated using the following equation: Ftot = Fst − (A1/A3 − A1) × (Fst − F3), where Fst is the total fluorescence in the inner (kinetochore) circle whose area is A1 and F3 is the total fluorescence in the outer (local cytoplasm) circle whose area is A3. All local fluorescence intensities were corrected for background and bleaching. Isolated kinetochores from maximum intensity projections were carefully selected after analysing z–planes. Only kinetochores around the middle z–section were quantified to minimize differences in fluorescence absorption by the cell.

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To evaluate the number of Venus–MAD2-positive kinetochores per cell in different drug treatments, each z-section of individual cells was visually screened for kinetochore-like fluorescence patterns using CENPA–Cerulean as an internal control. The fluorescence of a single kinetochore typically spanned 2–3 z-sections (one z-section brighter than the others). In the case of kinetochore clusters, the number of individual kinetochores was estimated by surface area.

For the quantification of Venus–MAD2 fluorescence in the partial siRNA experiments (Fig. 4c–e), confocal sections were projected (maximum intensity projection) and total fluorescence intensities were measured when cells finished rounding up (10–20 min after NEBD). Integrated kinetochore intensities of Venus–MAD2 were obtained by thresholding raw images (~10 min after NEBD) until cytoplasmic signals were completely excluded and measuring the entire area containing the extracted kinetochore signals.

For mitotic profiling in Figs 5g and h, RPE1 CCNB1/Venus+/− knock-in cells were automatically detected in each image using a modified count nuclei journal in MetaXpress and plotted against time.

**Statistical analysis.** Graphics and statistical analyses for all figures were carried out using GraphPad Prism 6 software. Data in this study are presented as the mean ± s.d. (except Fig. 5a,c–e and Supplementary Fig. S5c, which show mean ± s.e.m.). Statistical significance between population distributions was determined by a two-tailed unpaired Mann–Whitney test. Statistical significance definitions are summarized as follows: NS (not significant), $P$ value <0.05; * $P$ value 0.01 to 0.05; ** $P$ value 0.001 to 0.01 and *** $P$ value <0.001.

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Figure S1 Identification and characterization of Cyclin A2-Venus RPE1 clones. (a) Genomic PCR screen for correctly targeted Cyclin A2-Venus integrants following flow cytometry selection. Primer sets consisted of an oligonucleotide annealing outside the targeting construct (either 5’ (#1) or 3’ (#4)) and oligonucleotides annealing to the ORF of Venus (2 or 3). (b) PCR analysis with different primer combinations of a positive clone compared with parental RPE1 cells. (c) Single-cell Cyclin A2-Venus destruction assays of unsynchronised RPE1 Cyclin A2-Venus cells (n=8 cells). Error bars indicate s.d. and are representative of two independent experiments. (d) Single-cell Cyclin A2 destruction assays of asynchronously growing RPE1 Cyclin A2-Venus cells treated with DMA (10 µM) and MPS1 inhibitor reversine (0.5 µM) or Mad2 siRNA (20 µM) for 48 hours (n=6 cells for each condition). Images in c) and d) were acquired at 3 minutes intervals and the total cell fluorescence was quantified. Fluorescence intensities were normalized to the level at nuclear envelope breakdown (NEBD) and aligned at metaphase and NEBD respectively. Error bars indicate s.d. and are representative of two independent experiments. (e) Scatter plot of the time between nuclear envelope breakdown (NEBD) and exit from mitosis as judged by the disappearance of the Cyclin B1-Venus signal in Cyclin B1-Venus cells were treated simultaneously with DMA (10 µM) and the indicated concentration of AZ3146. The black line in each condition represents the mean, n= 50 cells per condition. Representative of three independent experiments. Scale bar = 10 µm.
Figure S2 Identification and characterization of Cyclin B1-Venus RPE1 clones. (a) Genomic PCR screen for correctly targeted Cyclin B1-Venus-neoR integrants following antibiotic selection. Primer sets consisted of an oligonucleotide annealing in the neomycin resistance cassette (1) and another outside the targeting construct (either 5’ (#5) or 3’ (#4)), and oligonucleotides annealing to the ORF of Venus (2 or 3). (b) PCR analysis of neomycin resistant pools compared with parental RPE1 cells. (c) PCR analysis of a Cyclin B1-Venus RPE1 clone following Cre-recombination compared to RPE1 control cells. (d) Serum-starved RPE1 Cyclin B1-Venus cells were released from quiescence by adding FBS and the levels of Cyclin B1 and Cyclin B1-Venus were followed by immunoblotting. Note the similar kinetics of appearance of the untagged and tagged proteins. Molecular mass markers on the left. (e) Proteolysis of Cyclin B1 and Cyclin B1-Venus after release from a DMA-induced mitotic block. Note that both Cyclin B1 and Cyclin B1-Venus accumulate during the block. Molecular mass markers on the left. (f) Analysis of Cyclin B1-Venus levels by time-lapse fluorescence imaging during mitosis under conditions that either activate or eliminate the SAC. Representative of 15 cells in each condition and from at least two independent experiments. (g) Scatter plot of the time between nuclear envelope breakdown (NEBD) and exit from mitosis as judged by the disappearance of the Cyclin B1-Venus signal in Cyclin B1-Venus cells were treated simultaneously with nocodazole and the indicated concentration of AZ3146 (n=46 cells for nocodazole control; n=21 cells for 0.31 µM; n=27 cells for 0.62 µM; n=19 cells for 1.25 µM; n=36 cells for 2.5 µM and n=55 cells for 5 µM). The black line in each condition represents the mean. Representative of at least two independent experiments.
Figure S3 Characterization of Venus-Mad2 RPE1 clones and estimation of the number of molecules of Venus-Mad2 in a cell and at kinetochores. (a) Genomic PCR screen for correctly targeted Venus-Mad2 integrants following flow cytometry selection. Primer sets consisted of an oligonucleotide annealing outside the targeting construct (either 5' (#1) or 3' (#4)) and an oligonucleotide annealing to the ORF of Venus (2 or 3). (b) PCR analysis with different primer combinations of a positive clone compared with parental RPE1 cells. Note that in lane 1+4 the upper band corresponds to the targeted allele whereas the bottom band corresponds to the wild-type allele. The asterisk shows a hybrid product caused by the annealing of DNA from the two other bands. (c) Localisation of Venus-Mad2 in prometaphase and metaphase. Candidate Venus-Mad2 RPE1 cells expressing ectopic CENP A-Cerulean were imaged by spinning-disk confocal microscopy. Note the co-localisation of the two signals in prometaphase that disappears in metaphase once all the kinetochores are attached. (d) Asynchronous RPE1 cells were treated similarly to Fig.3c, fixed with methanol and processed for immunofluorescence. Mad2 foci that colocalised with ACA staining were counted (n=20 cells for nocodazole; n=7 cells for Taxol and n=10 cells for DMA) and compared to the data obtained by live-cell imaging (Fig. 3d,e). Error bars indicate s.d. and are representative of at least two independent experiments. (e) Quantification of Venus-Mad2 molecules in protein extracts by immunoblotting using an anti-Mad2 antibody and recombinant His6-Mad2. Mitotic Venus-Mad2 RPE1 cells were collected by mitotic shake-off after nocodazole treatment, counted and processed for immunoblotting. Protein extracts were prepared from known numbers of cells. Molecular mass markers on the left. (f) Calibration curve for the Mad2 antibody obtained by quantifying band intensities in (e). The dotted line represents the linear regression. The inset graph shows the frequency distribution of Venus-Mad2 fluorescence in cells treated with nocodazole and analyzed by confocal microscopy (n=100 cells). Note that by knowing the mean number and mean fluorescence of Venus-Mad2 molecules per cell we can calculate the mean fluorescence per molecule of Venus-Mad2. (g) A maximum intensity projection of 50 z-sections taken at 0.5 µm intervals was used to identify individual kinetochores. (h) An inner circle (red) was drawn around the single kinetochore. An outer circle (blue) just around the inner circle was used to measure the local background around the kinetochore. (i) Since kinetochore signals span more than one 0.5 µm z-section, sum intensity projections were used to measure the integrated fluorescence intensity. (j) Equation used to obtain the total fluorescence intensity (Ftot) of a single kinetochore. This takes into account the local background and the respective areas of measurements. Note that all integrated fluorescence intensities were initially corrected for camera noise by subtracting fluorescence intensities of regions adjacent to cells. Scale bar = 10 µm.
**Figure S4** Size-exclusion chromatography of RPE1 cells treated with Taxol and nocodazole. Extracts from (a) Taxol- and (b) nocodazole-treated RPE1 cells were fractionated by size-exclusion chromatography and analysed by quantitative immunoblotting with the indicated antibodies. Signal intensities (right panels) are relative to the highest value for each protein. Representative of three independent experiments. Molecular mass markers on the left. (c) Absolute amount of checkpoint proteins per Cdc20 in the MCC fractions of both Taxol- and nocodazole-treated RPE1 cells (n=3). Taxol values were set to 1. Error bars indicate s.e.m. and are representative of three experiments.
Figure S5 Model. (a) Each unattached kinetochore has an influence on the rate of MCC formation in a cell. (b) We hypothesise that Cdc20 can be inhibited by the SAC in two ways: by sequestration and inactivation by the MCC, or by binding of the MCC away from the normal co-activator site on the APC/C. To recognize its metaphase substrates the APC/C needs first to bind to a free Cdc20 molecule to generate the bi-partite destruction box receptor. Assuming constant MCC turn over, dependent on p31Comet and APC15 pathways, the higher the concentration of MCC is in a cell, the higher the probability (p) that it will bind APC/C complexes. Consequently, this will decrease the probability that APC/C complexes can bind to free Cdc20.

(c) In unperturbed cells, the stability of metaphase APC/C substrates is largely due to the initial strong “pulse” of MCC formation at NEBD. In drug-treated cells, the continuous presence of unattached kinetochores brings MCC concentration to “supra-physiological” levels (assuming constant MCC turn over), keeping the APC/C inhibited. Drug-treated cells will slip out of mitosis at a rate that is inversely related to the number of improperly attached kinetochores that recruit Mad2. In nocodazole there are very few MTs, therefore Mad2 is recruited to most kinetochores and maintains strong inhibition of the APC/C. In DMA, MTs attach to kinetochores but are efficiently detached by the error-correction machinery. In Taxol, most kinetochores attach to MTs and are only inefficiently detached by the error correction machinery, generating a smaller number of MCC per unit time than in nocodazole or DMA-treated cells, which allows a higher level of APC/C activity and faster slippage.
Figure S6 Complete scans of all Western blot analyses presented in Figs 1-5 and Supplementary Figs 1-5. Cropped regions are indicated by dashed boxes. 700 nm or 800 nm channels indicate scans of the same blot using secondary antibodies coupled to fluorophores that are excited by 680 nm or 800 nm light, respectively.
Supplementary Video legends:

Supplementary Video 1. Movie of the RPE1 Cyclin A2-Venus knock-in cell line going through mitosis.
A Cyclin A2-Venus RPE1 cell imaged on a single plane and at 3 minute intervals. 40X objective. Representative of >50 cells from more than three experiments.

Supplementary Video 2. Movie of the RPE1 Cyclin B1-Venus knock-in cell line going through mitosis.
A Cyclin B1-Venus RPE1 cell imaged on a single plane and at 15 seconds intervals. 60X objective. Representative of >50 cells from more than three experiments.

Supplementary Video 3. Movie of the RPE1 Venus-Mad2 knock-in cell line going through mitosis.
A Venus-Mad2 RPE1 cell imaged on a single plane and at 15 seconds intervals. 40X objective. Representative of >50 cells from more than three experiments.