Published in final edited form as:

Nat Cell Biol. 2014 June ; 16(6): 538–549. doi:10.1038/ncb2952.

Cyclin B2 and p53 control proper timing of centrosome separation

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Abstract

Cyclins B1 and B2 are frequently elevated in human cancers and are associated with tumour aggressiveness and poor clinical outcome; however, whether and how B-type cyclins drive tumorigenesis is unknown. Here we show that cyclin B1 and B2 transgenic mice are highly prone to tumours, including tumour types where B-type cyclins serve as prognosticators. Cyclins B1 and B2 both induce aneuploidy when overexpressed but through distinct mechanisms, with cyclin B1 inhibiting separase activation, leading to anaphase bridges, and cyclin B2 triggering aurora-A-mediated Plk1 hyperactivation, resulting in accelerated centrosome separation and lagging chromosomes. Complementary experiments revealed that cyclin B2 and p53 act antagonistically to control aurora-A-mediated centrosome splitting and accurate chromosome segregation in normal cells. These data demonstrate a causative link between B-type cyclin overexpression and tumour pathophysiology, and uncover previously unknown functions of cyclin B2 and p53 in centrosome separation that may be perturbed in many human cancers.

Genomic instability provides the genetic diversity through which preneoplastic cells acquire the capabilities to become tumorigenic and eventually malignant. Loss of genomic integrity in human tumours involves both nucleotide changes and chromosomal instability (CIN; refs 1–3). CIN increases the rate of numerical or structural karyotypic change and promotes formation of cells with an abnormal chromosome content, a state defined as aneuploidy. As many as 500 human genes may cause CIN when defective, but which of these so-called CIN genes are deregulated in human tumours and responsible for the chromosomal heterogeneity associated with cancer lethality remains unknown^{4–6}. To identify such CIN gene alterations we focused on two related genes, *Ccnbl* and *Ccnb2*, whose overexpression is a characteristic

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AUTHOR CONTRIBUTIONS

H-J.N. and J.M.v.D. conceived the research and designed experiments. H-J.N. carried out experiments and analysed results. H-J.N. and J.M.v.D. discussed results and wrote the paper.

Note: Supplementary Information is available in the online version of the paper

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

of multiple molecular signatures that predict tumour aggressiveness and poor prognosis in a broad spectrum of cancers⁷⁻¹⁰.

Human cyclins B1 and B2 have unique amino-terminal segments but are highly homologous over the last 300 residues¹¹⁻¹³. Both proteins activate cyclin-dependent kinase 1 (Cdk1) and are expressed in most cell lines, tissues and organs with a high mitotic index^{14,15}. B-type cyclin levels are low in G1, gradually increase during S and G2, and peak during mitosis^{16,17}. Cdk1–cyclin B1 activation at the G2/M transition initiates the phosphorylation of various proteins that control key early mitotic events, including chromosome condensation, nuclear envelope breakdown and spindle assembly^{18,19}. Furthermore, Cdk1–cyclin B1 coordinates anaphase onset by controlling the activity of separase, a protease that cleaves cohesion complexes that hold sister chromatids together²⁰. By phosphorylating separase, Cdk1–cyclin B1 prevents cohesin cleavage until proper attachment of the last kinetochore to spindle microtubules in metaphase silences the mitotic checkpoint and activates APC/C^{CdG20}, an E3 ubiquitin ligase that targets cyclin B1 for proteasomal degradation. Little is known about cyclin B2 other than that it is localized to centriolar satellites in somatic cells and implicated in spindle formation in *Xenopus* oocytes²¹⁻²⁴. Here, we show that both B-type cyclins cause chromosome missegregation and tumour formation when overexpressed in mice. Furthermore, we show that endogenously expressed cyclin B2 and the p53 tumour-suppressor protein act antagonistically on aurora A to control proper timing of centrosome separation and correct chromosome segregation.

RESULTS

Cyclin B1 and B2 transgenic mice become aneuploid through distinct segregation errors

To explore whether and how overexpressed B-type cyclins promote tumorigenesis, we generated doxycycline (dox) inducible cyclin B1 and B2 transgenic mice using site-directed integration in KH2 embryonic stem (ES) cells^{25,26} (Fig. 1a,b). Two cyclin B1 (*Ccnb1*^{T7} and *Ccnb1*^{T11}) and two cyclin B2 (*Ccnb2*^{T15} and *Ccnb2*^{T16}) transgenic lines obtained were further characterized. Western blot analysis of mouse embryonic fibroblast (MEF) lysates confirmed that dox tightly controlled transgene expression (Fig. 1c), with dox-treated *Ccnb1*^{T7} and *Ccnb2*^{T16} MEFs showing ~10-fold cyclin B1 and ~20-fold cyclin B2 overexpression, respectively (Fig. 1d,e). Dox-treated animals expressed considerably more transgenic than endogenous protein in a broad spectrum of tissues, although the fold overexpression varied slightly among tissues and transgenic lines (Fig. 1f,g and Supplementary Fig. 1a–c).

Late in G2, cyclin B1 raises Cdk1 kinase activity above a threshold level required for mitotic entry²⁰. At mid-mitosis, this burst of Cdk1 activity is rapidly eliminated through proteasomal degradation of cyclin B1 to initiate sister chromatid separation and anaphase onset. Consistent with this, endogenous cyclin B1 levels of *Ccnb1*^{T7} MEFs cultured without dox were low in G1, sharply increased from G2 until prometaphase, and then progressively declined in meta phase and anaphase (Supplementary Fig. 2a,b). This pattern of expression was maintained on transgene induction, although absolute cyclin B1 levels were now substantially increased at each mitotic stage. Cdk activity, as measured using an antibody against phosphorylated Cdk substrates, changed in accordance with cyclin B1 levels and was

thus elevated in dox-treated *Ccnb1*^{T7} MEFs during mitosis (Supplementary Fig. 2c,d). The temporal expression pattern of endogenous cyclin B2 was similar to that of cyclin B1 (Supplementary Fig. 3a,b). However, cyclin B2 levels and Cdk activity in dox-treated *Ccnb2*^{T16} cells were elevated not only in mitosis, but also in G2 (Supplementary Fig. 3a-d).

To determine whether cyclin B1 and cyclin B2 overexpression might affect karyotype stability, we analysed metaphase spreads of MEFs cultured with or without dox for 48 h. Without dox, aneuploidy rates of *Ccnb1*^{T17}, *Ccnb1*^{T11}, *Ccnb2*^{T15} and *Ccnb2*^{T16} MEFs were 12, 15, 15 and 16%, respectively (Table 1a). In contrast, on transgene induction, these rates markedly increased to 33, 31, 25 and 36%, respectively. Both near-diploid and near-tetraploid aneuploidies were elevated in cyclin-B 1-overexpressing MEFs. To measure in vivo-acquired aneuploidy, we counted chromosomes of splenocytes from 5-month-old *Ccnb1*^{T7}, *Ccnblm*, *Ccnb2*^{T15} and *Ccnb2*^{T16} mice maintained on dox after weaning. Splenocytes from dox-treated transactivator (TA) mice served as controls and contained 6% aneuploidy (Table 1b). Aneuploidy substantially increased on transgene induction, with *Ccnb1*^{T7}, *Ccnb1*^{T11}, *Ccnb2*^{T15} and *Ccnb2*^{T16} splenocytes with 20, 12, 18 and 18% aneuploidy, respectively (Table 1b).

In live-cell imaging experiments, dox-treated *Ccnb1*^{T7} and *Ccnb2*^{T16} MEFs both showed markedly increased chromosome missegregation rates when compared with untreated counterparts (Fig. 2a,b). However, the main mitotic errors resulting from each transgene showed no substantial overlap, with cyclin B1 overexpression inducing chromatin bridges or anaphase failure, and cyclin B2 overexpression causing lagging chromosomes (Fig. 2a,b). Thus, although the extents to which cyclin B1 and B2 perturb the fidelity of chromosome segregation are very similar, the fundamental basis for the inaccuracies is unique in each case.

Cyclin B1 overexpression perturbs separase activation

The anaphase failure and chromatin bridging phenotypes of cyclin B1 transgenic cells were reminiscent of the mitotic defects of cells lacking theseparase^{27,28}. This led to the idea that cyclin B1-Cdk1, which inhibits separase through phosphorylation at Ser 1121 (ref. 29) and is hyperactive when cyclin B1 is overexpressed (Supplementary Fig. 2c,d), might interfere with the timely activation of separase on chromosome bi-orientation. To test this hypothesis, we monitored separase activity in live cells using a biosensor consisting of two separase cleavage sites flanked by mCherry-histone H2B and enhanced green fluorescent protein (eGFP) at the amino- and carboxy-terminal ends, respectively (Fig. 2c). In non-induced *Ccnb1*^{T7} MEFs, this biosensor resided at chromatin during interphase and the first part of mitosis, producing a yellow colour in merged images of eGFP and mCherry fluorescence (Fig. 2c). Cleavage of the biosensor on separase activation in late metaphase released eGFP from chromosomes, thus converting yellow to red. Quantitation of eGFP fluorescence at chromosomes revealed that activation of separase starts 6-8 min before onset of anaphase (Fig. 2d). A similar pattern of Seel cleavage was observed in dox-induced *Ccnb1*^{T7} MEFs undergoing accurate chromosome separation (Fig. 2c,d). In contrast, chromosomes of cells with anaphase bridges, which we find have higher levels of cyclin B1

overexpression than their counterparts without bridges (Supplementary Fig. 4a), released only relatively small amounts of eGFP, remaining yellow throughout anaphase and telophase. Cells experiencing anaphase failure showed even less separase activity than those with bridges (Fig. 2d), indicating that the severity of mitotic phenotypes inversely correlates with separase activity. Biosensor cleavage was normal in dox-treated *Ccnb2*^{T16} MEFs (Supplementary Fig. 4b,c).

Incomplete decatenation of centromeric DNA and replication fork stalling are alternative causes of chromatin bridge formation^{30,31}. Top2a, the decatenase that disentangles centromeric DNA, was normally expressed and properly targeted to inner centromeres of mitotic MEFs with elevated cyclin B1 (Supplementary Fig. 4d,e). The number of stalled replication forks, as determined by immunostaining for the replication protein RPA2 (ref 30), was not increased in cyclin B1 overexpressing MEFs (Supplementary Fig. 4f). These MEFs also showed no evidence for increased double strand DNA breaks (Supplementary Fig. 4g). Thus, DNA replication and decatenation problems seemed to play a limited or no role in the chromatin bridging phenotype. Timing of S/G2, prophase, prometaphase and metaphase also seemed normal in cyclin B1 overexpressing MEFs (Supplementary Fig. 4h,i). Classical growth rate analysis revealed a slight, yet significant, reduction in cell number at high cyclin B1 levels (Supplementary Fig. 4j), which is consistent with the modest rate of anaphase failure observed in these cells (Fig. 2b).

Cyclin B2 overexpression hyperactivates Plk1 through aurora A to deregulate mitosis

Cyclin B2 overexpression primarily causes lagging chromosomes. Although this type of segregation error has been associated with mitotic checkpoint failure³², no such defect was observed (Supplementary Fig. 5a). Lagging chromosomes are due to merotelic attachments, a type of kinetochore-microtubule malattachment that can be caused by defects in attachment error correction, microtubule dynamics or spindle geometry^{33,36}. However, monastrol washout assays indicated that correction of merotelic attachments was unperturbed in cyclin B2 overexpressing cells (Supplementary Fig. 5b). Microtubule dynamics also seemed normal in these cells, as measured using photo-activatable GFP-cHubulin (Supplementary Fig. 5c). To evaluate spindle geometry, *Ccnb2*^{T16} MEFs were stained for γ - and cHubulin. At high cyclin B2 levels, there were noticeably more metaphases in which the spindle poles were not lying perpendicular to the metaphase plate (Fig. 3a). We quantitated this defect by determining the percentage of cells that had an acute angle between the spindle pole axis and the metaphase plate of less than 85° (Fig. 3a). According to these criteria, 44% of dox-induced *Ccnb2*^{T16} metaphases were abnormal, compared with 23% of control metaphases (Fig. 3b). Cyclin B1 overexpression was not associated with spindle geometry defects (Fig. 3b).

Abnormal spindle geometry has been linked to defects in centrosome separation³⁷. As a first step to probe for such defects, we assessed whether cyclin B2 is centrosome associated. Indeed, cyclin B2 co-localized with centrin 2, a well-established centrosomal marker protein, both before and after mitosis onset (Supplementary Fig. 5d,e). Next, we stained dox-treated and untreated *Ccnb2*^{T16} MEFs for γ -tubulin to determine the timing of centrosome separation in G2 phase, and found that overexpression of cyclin B2 significantly

accelerated centrosome separation (Fig. 3c,d and Supplementary Fig. 5f). To decipher the underlying mechanism, we focused on Plk1, a mitotic kinase that plays a central role in disengagement and full separation of centrosomes³⁸. Plk1 activity typically increases in late G2, resulting in phosphorylation and activation of Mst2, which in turn phosphorylates and activates Nek2A^{67,68}. Activated Nek2A then phosphorylates C-Nap1 and rootletin, two proteins that hold duplicated centrosomes, resulting in centrosome separation^{69,70}. In cooperation with Cdk1, Plk1 also regulates recruitment of the kinesin Eg5 to centrosomes, driving the full separation of centrosomes in prophase⁴⁰. To evaluate whether cyclin B2 overexpression alters Plk1 activity at centrosomes, we stained *Ccnb2*^{T16} MEFs with an antibody specific for Plk1 containing the activating phosphorylation on Thr 210 (ref. 41) (Fig. 3e). Indeed, centrosomal p-Plk1T210 staining was nearly twice as high in dox-treated MEFs as in their untreated counterparts (Fig. 3f,g).

To determine whether the observed Plk1 hyperactivity might drive the mitotic defects in cyclin B2 overexpressing cells, we used low amounts of a small-molecule inhibitor of Plk1, BI2536, to normalize Plk1 activity in dox-treated *Ccnb2*^{T16} MEFs. As shown in Supplementary Fig. 5g, 10nM BI2536 corrected the accelerated centrosome separation phenotype of cells overexpressing cyclin B2 and had no impact in cells in which the transgene was not induced. Furthermore, spindle geometry defects in dox-treated *Ccnb2*^{T16} MEFs declined from 48 to 15% (Fig. 3h), and chromosome missegregation rates decreased from 36 to 14% (Table 2a). Consistent with improved chromosome segregation, aneuploidy rates dropped from 39 to 21% (Table 2b). Importantly, 10 nM BI2536 had no impact on spindle geometry, chromosome segregation or aneuploidy rates of *Ccnb2*^{T16} MEFs not expressing the transgene (Fig. 3h and Table 2a,b). These findings indicated that the mitotic phenotypes of cyclin B2 overexpressing cells were due to Plk1 hyperactivity.

Aurora A kinase activates centromeric Plk1 through phosphorylation of Thr 210 (refs 42-44), which prompted us to test whether aurora A hyperactivity is responsible for hyperactivation of Plk1 in cyclin B2 overexpressing cells. When stained with an antibody specific for phosphorylated aurora proteins, which visualizes activated aurora A at centrosomes, cyclin B2 overexpressing MEFs showed markedly increased fluorescence in prophase (Fig. 3i,j). No such increase was observed in cyclin B1 transgenic MEFs (Supplementary Fig. 5h). Low amounts (10nM) of the aurora A inhibitor MLN80S4 normalized p-Plk1^{T210} levels and corrected the spindle geometry, centrosome separation and chromosome segregation defects in cells overexpressing cyclin B2 (Fig. 3k,l, Table 2c and Supplementary Fig. 5i,j). Collectively, these data indicate that cyclin B2 overexpression promotes Plk1 hyperactivity through hyperactivation of aurora A. Notably, Plk1 has been implicated in several other mitotic processes, including mitotic spindle positioning⁴⁵ and attachment error correction⁴⁶, but these functions seemed unperturbed by cyclin B2 overexpression (Supplementary Fig. 5k-m).

Endogenous cyclin B2 and p53 antagonistically regulate centrosome separation

To explore whether endogenous cyclin B2 regulates centrosome separation in normal cells, we depleted cyclin B2 from wild-type MEFs using two independent short hairpin RNAs (shRNAs). Low cyclin B2 markedly increased the percentage of G2 cells with unseparated

centrosomes (Fig. 4a–c). Importantly, cyclin B2 depletion significantly reduced centrosomal levels of phosphorylated Plk1 and aurora A, and increased rates of asymmetrical mitotic spindles and chromosome lagging (Fig. 4d–h). Centrosome nondisjunction on cyclin B2 depletion was also observed in primary epithelial cell lines established from murine lung and mammary gland tissue (Fig. 4i). The same holds true for human primary fetal lung fibroblasts (IMR-90) and adult human primary skin fibroblasts (Fig. 4j,k), indicating that cyclin B2 functions in centrosome separation across mammalian species. Cyclin B2 loss, however, did not impair centrosome separation in HeLa cells (Fig. 4k).

To investigate whether this might be due to cell transformation, we immortalized wild-type MEFs by expressing SV40 large T antigen, which perturbs p53 and RB function⁴⁷. Indeed, cyclin B2 depletion failed to inhibit centrosome separation in immortalized MEFs (Fig. 5a). Subsequent studies revealed that cyclin B2 depletion does not impair centrosome disjunction in *p53*^{−/−} MEFs (Fig. 5a). Furthermore, we noticed that the percentage of unseparated centrosomes was significantly lower in *p53*^{−/−} MEFs than in wild-type MEFs (Fig. 5a). A recent report documented that p53 negatively regulates aurora A transcript and protein abundance⁴⁸, which we confirmed by quantitative PCR with reverse transcription (qRT–PCR) and western blot analysis of wild-type and *p53*^{−/−} MEFs (Supplementary Fig. 6a,b). This led us to hypothesize that elevated aurora A expression due to p53 loss increases levels of phosphorylated aurora A. This would lead to increased amounts of p-Plk1^{T210}, accelerated centrosome splitting, abnormal spindle geometry and chromosome missegregation. Consistent with this notion, *p53*^{−/−} MEFs had significantly higher amounts of p-aurora A and p-Plk1^{T210} at centrosomes, as well as significantly increased rates of spindle geometry defects and lagging chromosomes (Fig. 5b–f,i). The observation that errors in centrosome separation, spindle geometry and chromosome segregation were largely corrected by culturing *p53*^{−/−} MEFs in low amounts of aurora A inhibitor further supported our hypothesis (Fig. 5g–i). Centrosomes of cyclin B2-depleted *p53*^{−/−} MEFs had elevated p-aurora A and p-Plk1^{T210} levels, indicating that p53 loss leads to cyclin B2- independent aurora A activation (Supplementary Fig. 6c,d). Collectively, these data uncover previously unknown functions for cyclin B2 and p53 in proper timing of centrosome separation to ensure correct spindle pole positioning and chromosome segregation (Fig. 5j).

Cyclins B1 and B2 both drive tumorigenesis when overexpressed

To determine whether cyclin B1 or cyclin B2 overexpression drives tumorigenesis, we established cohorts of *Ccnb1*, *Ccnb2* and TA control mice that were on dox-containing water from weaning on. These mice were killed and screened for tumours at 14 months of age. Tumours were collected and analysed by histopathology. Both B- type cyclin transgenic strains showed marked increases in tumour incidence (Fig. 6a–c). Fluorescence *in situ* hybridization (FISH) analysis confirmed that the tumours had increased numbers of aneuploid cells (Supplementary Table 1). Lung tumours were the most prevalent tumour type in both models (Fig. 6b,c), which is interesting because several studies have documented elevated cyclin B1 expression in human lung cancer^{10,49–51}. To corroborate these studies and to determine the frequency of cyclin B2 overexpression in human lung cancers, we measured *CCNB1* and *CCNB2* transcript levels of primary tumour samples of lung cancer patients and compared these with normal lung tissue (Supplementary Fig. 7a,b).

CCNB1 transcript levels were more than 10-fold elevated in 43% of adenocarcinomas and 37% of squamous cell carcinomas, whereas *CCNB2* transcript levels were more than 10-fold increased in 20% of adenocarcinomas and 23% of squamous cell carcinomas, indicating that subsets of human lung cancers overexpress *CCNB1* and *CCNB2*. Measurements of *BUB1* and *Ki67* transcript levels confirmed that the observed increases in *CCNB1* and *CCNB2* expression were not simply a consequence of increased numbers of cycling cells (Supplementary Fig. 7c,d).

Next we focused on colon cancer, a human tumour type characterized by elevated B-type cyclin levels^{52–54}. *Ccnb1*^{T7}, *Ccnb2*^{T16} and TA control mice were bred onto a *APC*^{+/^{Min} genetic background, which predisposes mice to intestinal tract polyps⁵⁵, placed on dox water at weaning age and screened for intestinal tumours at day 90. Colon tumour incidence and multiplicity were significantly increased in *Ccnb1*^{T7};*APC*^{+/^{Min} and *Ccnb2*^{T16};*APC*^{+/^{Min} mice when compared with TA;*APC*^{+/^{Min} control mice (Fig. 6d–f). Furthermore, colon tumours from *Ccnb1*^{T7};*APC*^{+/^{Min} and *Ccnb2*^{T16};*APC*^{+/^{Min} mice were significantly larger than those of TA;*APC*^{+/^{Min} mice, and showed marked intramucosal invasion (Fig. 6d–g), indicating that B-type cyclin overexpression promotes a more aggressive tumour phenotype. There were no significant differences in small intestinal tumour multiplicity between the various cohorts (Supplementary Fig. 8a). When we repeated the above experiments but stopped dox treatment 30 days before tumour analysis, colon tumour incidence, multiplicity and size did not significantly decline when compared with counterparts that were continuously treated with dox (Fig. 6e–g). Tumour histopathology was similar regardless of dox treatment regimen (Supplementary Fig. 8b). These data indicate that cyclin B1 and B2 overexpression is important for initial tumour formation but dispensable for continued cancer cell viability and tumour growth in this model.}}}}}}}

The carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) predisposes wild-type mice to lung and skin tumours when it is applied to the dorsal skin at low dose³¹. We found that the occurrence of DMBA-induced skin tumours was significantly higher in cyclin B1 and B2 transgenic mice than in TA control mice (Fig. 6h). However, DMBA-induced lung tumours were only elevated in cyclin B2 transgenic mice. The observation that both lung and skin tumour multiplicity were elevated in cyclin B2 transgenic mice underscored that cyclin B2 is the more potent proto-oncogene in this tumour model (Fig. 6i).

DISCUSSION

Cyclins B1 and B2 are frequently elevated in human cancers and are associated with tumour aggressiveness and poor clinical outcome. Here, we demonstrate that both these cyclins robustly promote tumour development when overexpressed in mice. Analysis of MEFs from these mice revealed that each cyclin perturbs chromosome segregation through a distinct mechanism, with cyclin B1 inhibiting separase activation and cyclin B2 accelerating aurora-A-mediated Plk1 activation and centrosome separation. Our data strongly indicate that cyclin B1 overexpression induces chromatin bridging through continued inhibition of separase after chromosome biorientation and mitotic checkpoint silencing have occurred. However, contributions of other mechanisms cannot be fully excluded, given the complex

nature of the sister chromatid separation process and the multitude of mitotic substrates that cyclin B1–Cdk1 is thought to phosphorylate.

The role of endogenous cyclin B2 has remained elusive for decades, but the collective results from the cyclin B2 knockdown and overexpression studies presented here now indicate that cyclin B2–Cdk1 acts to initiate centrosome separation in late G2 by triggering the phosphorylation of aurora A. Overexpression of cyclin B2 accelerates this process, resulting in early centrosome disjunction and formation of aberrant spindles that tend to separate chromosomes inaccurately. Although activated aurora A is readily detectable at centromeres in prophase, we were unable to demonstrate this during G2. One interpretation of this result is that aurora-A-mediated Plk1 activation in G2 does not require the activator to be stably centrosome bound. The same could hold true for cyclin B2-mediated activation of aurora A, although our finding that cyclin B2 localizes to centrosomes in G2 leaves open the possibility that the centrosome-associated pool of cyclin B2 establishes a local increase in soluble aurora A activity.

Three observations reported here indicate that p53 mediates proper timing of centrosome disjunction by negatively regulating aurora A kinase activity. First, cells lacking p53 are prone to rapid centrosome separation in G2 phase. Similarly to cyclin B2 overexpression, p53 loss results in formation of asymmetrical spindles that promote chromosome lagging. Second, p53 null cells have increased amounts of phosphorylated aurora A and Plk1 at centrosomes. Third, normalization of aurora A activity in p53 null cells with a low amount of aurora A inhibitor restores timely centrosome separation, spindle geometry and accurate chromosome segregation. The implication of these findings is that tumours with p53 defects, which represent a relatively large fraction of human tumours, may acquire chromosomal plasticity that promotes tumour formation through a mechanism that involves aberrant centrosome segregation. Collectively, our data best fit a model whereby p53 and cyclin B2–Cdk1 act antagonistically to control aurora-A-mediated centrosome disjunction, with p53 keeping aurora A protein levels in check through transcriptional and post-translational inhibitory mechanisms and cyclin B2–Cdk1 activating the residual aurora A pool in a timely manner (Fig. 5j). Our data further indicate that p53 inactivation bypasses cyclin B2-dependent initiation of centrosome disjunction, whereas cyclin B2 overexpression overrides p53-mediated inhibition of aurora A kinase activity.

METHODS

Methods and any associated references are available in the online version of the paper.

METHODS

Mouse strains

All mice were housed in a pathogen-free barrier environment. Mouse protocols were reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee. All animals were mixed in a 129/Sv x C57BL/6 genetic background. *Ccnb1*–HA and *Ccnb2*–HA transgenic mice were generated using KH2 ES cells (Origene Technologies) according to a previously described method²⁶. Briefly, wild-type *Ccnb1* and *Ccnb2*

complementary DNA sequences with an HA tag at the 3' end were PCR-amplified from established murine cDNA clones¹¹ and cloned into the EcoRI site of Flp-in vector pBS31. The resulting plasmids were electroporated into KH2 ES cells with pCAGGS-FLPe-puro to target integration of *Ccnb1-HA* and *Ccnb2-HA* to an Flp recognition target site downstream of *Col1a1*. KH2 ES clones properly expressing *Ccnb1-HA* or *Ccnb2-HA* were injected into blastocysts, and chimaeras from two independent *Ccnb1-HA* (*Ccnb1*^{T7} and *Ccnb1*^{T11}) and *Ccnb2-HA* (*Ccnb2*^{T15} and *Ccnb2*^{T16}) clones achieved germline transmission. These transgenes were maintained on an M2-rtTA (TA) hemizygous background (*ROSA26*). Both sexes were used for experimentation. After weaning, transgenic mice were continuously administered 2 mg ml⁻¹ dox in drinking water containing 5% sucrose. Mice in spontaneous tumour susceptibility studies were monitored daily. At 14 months, mice were killed and major organs were screened for overt tumours. Tumours were processed for histopathology by standard procedures. Mice on an *APC*^{+/-Min} genetic background⁵⁶ received dox either from day 21 to 90 or from day 21 to 60. All mice were killed at 90 days to screen for tumours. All tumour histology is representative for at least three independent animals. For carcinogen-induced tumorigenesis, mice were given a single application of 50 µl 0.5% DMBA to the dorsal skin on postnatal day 3–5 and killed 4 months later.

Generation and culture of MEFs

MEFs were generated at embryonic day 13.5 and cultured as previously described⁵⁷. Primary MEFs were frozen at passage 2 (P2) or P3 and used for experimentation between P4 and P6. At least three independently generated MEF lines per genotype were used. In experiments in which the impact of cyclin B1 or cyclin B2 overexpression was examined, 1 µg ml⁻¹ dox was added to the culture medium 72 h before analysis, unless otherwise stated.

Statistical analyses

GraphPad Prism software was used for all statistical analyses. A two-tailed unpaired *t*-test was used for comparisons in the following figures: Figs 2b,d, 3b,d,f,h,j-l, 4c,g,i,k, 5a,c,d,f-i, 6f,g; Supplementary Figs 2b,d, 3b,d, 4a,c,f-j, 5a-c, g-j,m, 6b-d, 8a; Tables 1 and 2b,c. A one-sample *t*-test against a theoretical mean of unity was used for comparisons in Fig. 4e,f. A one-tailed unpaired *t*-test was used for Fig. 4h and Table 2a. A two-tailed Mann-Whitney test was used for pairwise significance analysis in Fig. 6i and Fisher's exact tests were used in Fig. 6a,h. A one-tailed chi-squared test was used in Fig. 6b,e. Graphs are indicated with the significance score of **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. We note that no power calculations were used. Sample sizes were based on previously published experiments where differences were observed. No samples were excluded. The experiments were not randomized and the investigator not blinded to allocation during experiments and outcome assessment.

Western blot analysis

Western blot analysis was carried out as previously described⁵⁸. Tissue lysates were prepared by snap-freezing tissues and grinding into powder. Ten milligrams of the powder was suspended in 100 µl PBS and boiled for 10 min after the addition of 100 µl 2× Laemmli lysis buffer. Primary antibodies used for western blotting were mouse anti-cyclin B1

(1:1,000, SC-245/clone CNS-1, Santa Cruz), rabbit anti-cyclin B2 (1:2,000, SC-22776, Santa Cruz), mouse anti-tubulin (1:10,000, T9026/clone DM1A, Sigma), mouse anti-actin (1:10,000, A5441/clone AC-15, Sigma-Aldrich) and mouse anti-HA (1:1,000, 12013819001/clone 3F10, Roche). Ponceau S staining served as a loading control for tissue blots. All western data are representative for two or three independent experiments.

Immunostaining and confocal microscopy

Indirect immunofluorescence was carried out as described previously⁵⁹. Primary MEFs were cultured in the presence or absence of dox for 72 h. For cyclin B1, cyclin B2, p-CDK substrate and p-Plk1^{T210} staining, cells were fixed in PBS/3% paraformaldehyde for 12 min at room temperature (RT), permeabilized in PBS/0.2% Triton X-100 for 10 min and blocked in PBS/1% BSA for 30 min at RT. For γ -tubulin, cyclin B2 and p-Plk1^{T210} staining at centrosomes, cells were first permeabilized in PHEM buffer (25 mM HEPES, 10 mM EGTA, 60 mM PIPES and 2 mM MgCl₂ at pH 6.9) containing 0.5% Triton X-100 for 5 min at RT and then fixed in ice cold methanol for 10 min at RT. For p-aurora and centrosome staining, cells were fixed with PBS/1% paraformaldehyde for 5 min and then incubated in ice-cold methanol for 10 min at RT. A laser-scanning microscope (LSM 510 or LSM 780; Carl Zeiss) with an inverted microscope (Axiovert 100M; Zeiss) was used to analyse immunostained cells and capture images. Quantification of p-Cdk substrates, cyclin B1, cyclin B2, p-Plk1^{T210} and p-aurora signals was carried out using ImageJ software (NIH). Confocal microscopy images were converted to eight-bit grey scale, cell edges were traced and the mean pixel intensity density (arbitrary units) within the marked area was calculated. G2 cells were identified using rabbit anti-pH3^{S10}, which selectively stains heterochromatin foci in G2 phase. In triple stainings for p-Plk1^{T210}, γ -tubulin and pH3^{S10}, primary antibodies for γ -tubulin and pH3^{S10} used were both raised in rabbits. We visualized both these rabbit antibodies with the same fluorophore-conjugated secondary antibody. This did not create a detection conflict because γ -tubulin and pH3^{S10} are confined to the cytoplasm and nucleus, respectively. G2 cells in which the centrosomes were localized in the cytoplasmic area flanking the nucleus were selected for p-Plk1^{T210} quantitation. All confocal microscopy images are representative of at least three independent experiments. Primary antibodies for immunostaining were as follows: mouse anti-cyclin B1 (1:100, 554178/clone GNS-11, BD); rabbit anti-cyclin B2 (1:200, SC-22776, Santa Cruz); rabbit anti-p-Cdk substrates (1:1,000, 9447, Cell Signaling Technology); mouse anti-centrin 2 (1:1,000)⁶⁰; mouse or rabbit anti- γ -tubulin (1:300, T6557/clone GTU-88 or T5192, Sigma); mouse anti- α -tubulin (1:1,000, T9026/clone DM1A, Sigma); rabbit anti-HA (1:100, 3724, Cell Signaling Technology); mouse anti-p-Plk1^{T210} (1:300, ab39068/clone 2A3, Abcam); rabbit anti-p-aurora (1:100, 2914, Cell Signaling Technology); rabbit anti-Top2a (1:300, TG2011-1, Topogen); human anti-centromeric antibody (1:100, 15-234-0001, Antibodies, Inc.); and rabbit anti-pH3^{S10} (1:10,000, 06-570, Millipore).

Aneuploidy analyses

Chromosome counts were carried out on metaphase spreads from colcemid-treated transgenic MEFs grown in the presence or absence of dox for 48 h or on splenocytes of 5-month-old transgenic mice treated with dox since weaning⁵⁷. Interphase FISH analysis with probes for chromosomes 4 and 7 was carried out on single cell suspensions of various

tissues and tumours as previously described⁶¹. To evaluate aneuploidy rates after correction of aurora A or Plk1 hyperactivity, MEFs were cultured in the presence or absence of dox for 24 h. 10 nM BI2536 or 10 nM MLN8054 was then added to the culture medium and metaphase spreads prepared 48 h later.

Live-cell imaging

For chromosome missegregation analysis, chromosome movements of monomeric red fluorescent protein (mRFP)–H2B-positive primary MEFs progressing through an unchallenged mitosis were followed at interframe intervals of 3 min as previously described^{32,62}. MEFs were seeded onto 35 mm glass bottom dishes (MatTek). All experiments were carried out using a microscope system (Axio Observer; Zeiss) with CO₂ Module S, TempModule S, Heating Unit XL S, a Plan Apo ×63 NA 1.4 oil differential interference contrast III objective (Zeiss), camera (AxioCam MRm; Zeiss) and AxioVision 4.6 software (Zeiss). Imaging medium was kept at 37 °C. BI2536 or MLN8054 was then added to a final concentration of 10 nM and MEFs immediately analysed for chromosome segregation errors by live-cell imaging. All chromosome missegregation error images are representative of three independent experiments. Nocodazole and taxol challenge assays were carried out as previously described⁵⁸. To generate the biosensor to measure separase activity, a 390-base pair (bp) BglII–SacI H2B cDNA fragment was ligated into pEGFP-N1 (Clontech). In two consecutive rounds of cloning, a 720-bp HindIII–SalI mCherry and a 982 bp KpnI–ApaI human SCC1 fragment were cloned into the resulting plasmid. Finally, the H2B–mCherry–hSCC1–eGFP fragment from this vector was excised with BglII–NotI, blunted, and ligated into a blunted XhoI site in pTSIN. Two days after transduction with biosensor containing pTSIN lentivirus, cells were followed through an unchallenged mitosis by live-cell imaging 24 h later. Time-lapse images of mCherry and eGFP fluorescent signals were captured every 3 min and quantified using ImageJ. Specifically, the DNA area was outlined in mCherry images using the freehand tool. The integrated fluorescence density within this area was measured after background subtraction. The same area was similarly analysed for eGFP (ref. 63). The ratio (eGFP—background)/(mCherry—background) was calculated for all images aligned on the time axis according to anaphase onset, as determined by sister chromatid separation. The value corresponding to the –12 min time point before anaphase onset was considered as 100% and subsequent time-lapse values were normalized against it. The plots represent the average data for the indicated number of cells for each genotype. All separase biosensor fluorescence images are representative of five independent experiments. Error bars represent s.e.m. For analysis of cell cycle duration, *Ccnb1*^{T7} MEFs were co-transduced with mKO2–hCdt1(30/120)/pCSII–EF and mAG–hGeminin(1/110)/pCSII–EF lentiviruses⁶⁴ grown in the presence or absence of dox for 48 h, and then followed by live-cell imaging for 36 h. Time-lapse images of differential interference contrast (DIC), mCherry and Venus fluorescent signals were captured every 5 min using a Plan Apochromat ×20/0.8 M27 D = 0.55 objective. Fluorescence intensity was quantified semi-automatically for each mCherry and Venus individual image using the mean region of interest (ROI) tool within the ZEN software. Specifically, a constant circular ROI was selected to encompass the cell of interest and the same ROI area size was used for background subtraction. The G1/S time was defined as the time with the lowest Venus intensity. The S/G2 time was defined as the time for which mCherry ends the high intensity

plateau and begins a descending slope. Nuclear envelope breakdown (mitosis entry) was identified by DIC and by the diffusion of nuclear Venus into mitotic cytosol.

Measurement of centrosome distance, spindle geometry and spindle position

For centrosome distance measurements, serial optical sections (z-series) were collected from pH3^{S10} / γ -tubulin stained MEFs using a laser-scanning microscope (LSM 780 with an Axiovert 100M inverted microscope, Zeiss). Following maximum intensity projection, the distance between centrosomes (γ -tubulin signals) was measured using ZEN software (Zeiss). For spindle geometry analysis, serial optical sections were collected from γ -tubulin/ α -tubulin stained MEFs using a laser-scanning microscope. Following maximum intensity projection, the angle between the spindle and the metaphase plate was measured using ZEN software (Zeiss). G2 cells with centrosome distances $\geq 3 \mu\text{m}$ were classified as accelerated. Abnormal spindle geometry experiments were conducted in the presence of 10 nM BI2536 or 10 nM MLN8054 (Selleck Chemicals), which was added 6 h before immunostaining. *p53*^{-/-} MEFs with supernumerary centrosomes were excluded from spindle geometry and centrosome distance analysis. For analysis of spindle position, cells were stained for γ -tubulin antibodies to mark spindle poles and with Hoechst to mark chromosomes. Images were acquired using a laser-scanning microscope (LSM 780; Zeiss) under a $\times 100$ 1.4 NA oil-immersion objective, and spindle pole-to-cell cortex distance was measured using ZEN software. Spindle pole-to-cell cortex distance was calculated with ZEN software by marking the centrosome positions, drawing a line between the centrosomes and then marking the points of intersection with the cell edges, as determined under the DIC image. The corresponding two distances between the spindle pole and the point of intersection with the cell edge were measured for 20 cells of each group, and the ratio (smaller/larger value) was averaged and plotted in Excel.

GFP- α -tubulin photoactivation and analysis

MEFs transduced with photoactivatable GFP (PA-GFP)- α tubulin lentivirus were grown in the presence or absence of dox and photoactivated as previously described^{36,65}. Briefly, mitotic cells were identified by DIC microscopy using a $\times 100$ 1.4 NA oil-immersion objective on a Zeiss LSM780 microscope. PA-GFP- α tubulin in a thin area near chromosomes (500 nm wide and as long as the metaphase plate) was photoactivated with ten pulses from a 405 nm laser at 100% power. $3 \times 1 \mu\text{m}$ stacks of fluorescent images were acquired less than 1 s before and after photoactivation. Subsequent images were acquired every 30 s up to 4 min and saved as maximum projection time-lapse images. To calculate fluorescence dissipation after photoactivation, we used ImageJ to measure pixel intensities within the photoactivation area. Background pixel intensity was subtracted using an equal ROI from the non-activated opposite spindle. The fluorescence values were corrected for photobleaching by determining the percentage of fluorescence loss during 4 min in activated cells treated with 10 μM taxol. For each cell, fluorescence values were normalized to the first time after photoactivation. Normalized fluorescence was then averaged for multiple cells at each time and plotted against time using Excel 2011.

Ccnb2 knockdown using lentiviral shRNA

Non-targeting shRNA TRC2 negative control vector (SCH202), *Ccnb2* shRNA TRC2 clones NM_007630.2-1098s21c1 (referred to as *Ccnb2* shRNA 1), NM_007630.2-1332s21c1 (*Ccnb2* shRNA 2), NM_007630.2-454s21c1 (*CCNB2* shRNA 1) and NM_004701.2-1190s21c1 (*CCNB2* shRNA 2) were purchased from Sigma and lentiviruses prepared according to the manufacturer's manual. Cells were infected with cyclin B2 shRNA or non-targeting shRNA as a control for 48 h (every 24 h supernatants were refreshed), selected with puromycin (2 µg ml⁻¹) for 24 h and analysed over the next 48 h.

Quantitative real-time PCR

Total RNA was extracted from cryosections of normal human lung tissue ($n = 17$), human adenocarcinomas ($n = 35$) and human lung squamous cell carcinomas ($n = 35$), or from cultured wild-type and *p53*^{-/-} MEFs. cDNA synthesis was carried out with random hexamers and SuperScript III reverse transcription (Invitrogen). PCR reactions on human cDNA samples were carried out in duplicate with Taqman probes using the ABI PRISM Sequence Detection System 7900 Biosystems (Applied Biosystems). For *b.b.Ct* calculations, TBP was used for normalization. The relative fold change was determined after normalizing to normal lung samples. TaqMan primer IDs were as follows: Hs01030097 and Hs00270424 for *CCNB1* and *CCNB2*, respectively, 4332659 for TBP, Hs01032443 for Ki-67 and Hs00177821 for *BUB1*. PCR reactions on MEF cDNA samples used SYBR green PCR Master Mix (Applied Biosystems) to a final volume of 12 µl, with each cDNA sample measured in triplicate in the ABI PRISM 7,900 Sequence Detection System (Applied Biosystems) according to the protocol of the manufacturer. The expression of genes was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primer sequences were forward 5' -CTTA CTGCTTGGCTCAAACG-3' and reverse 5' -TCAATCATCTCTGGGGGC-3' for *Aurka* set 1, forward 5' -GAGAGTTGAAGATTGCAGACTTC-3' and reverse 5' -AA GTCTCCTGGTACGTGTGTGCC-3' for *Aurka* set 2, forward 5' -CCAAGCACAT CAACCCAGTG-3' and reverse 5' -TGAGGCAGGTAATAGGGAGACG-3' for *Plk1* set 1 and forward 5' -CCATTGAGTGCCACCTTAGTGA-3' and reverse 5' -TGTCCT GAATAGTCCACCCACT-3' for *Plk1* set 2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank W. Zhou and M. Li of Mayo Clinic's Gene Knockout Mouse Core Facility for ES cell microinjection and chimaera breeding, and L. Malureanu, K. Jeganathan and D. Baker for help with live-cell imaging, karyotyping and tumour analyses, respectively. We are grateful to P. Galarzy, R. Ricke, R. Naylor, D. Baker and L. Malureanu for discussions and critical evaluation of this manuscript, J. Sali5bury for sharing antibody, J-M. Peters for sharing the *Scc1* construct, D. Compton and L. Kabeche for sharing photoactivable GFP- α -tubulin construct and help with measurements of microtubule dynamics, V. Shridhar for human lung (tumour) samples and the Mayo Clinic Cytogenetics shared resource for FISH analysis. This work was supported by the National Institutes of Health (CA126828 to J.M.v.D.).

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Figure 1.

Ccnbl and *Ccnb2* transgene production and expression analyses. (a) Generation of wild-type *Ccnbl* and *Ccnb2* transgenic mice: schematic representation of the transgenic strategy. Using recombination at Flp recognition target (FRT) sites, DNA sequences encoding mouse wild-type cyclin B1 or cyclin B2 were inserted downstream of the 3' untranslated region of the *Col1a1* locus in KH2 ES cells. SA-polyA, splice acceptor-polyA; TetO, tetracycline operator; *PGKp*, phosphoglycerate kinase promoter; ATG, start codon. (b) Western blots of lysates from hygromycin B-resistant KH2 ES clones with targeted insertion of *HA-Ccnbl* or *HA-Ccnb2* probed for haemagglutinin (HA). (c) Western blots of extracts from *HA-Ccnb1* and *HA-Ccnb2* MEFs grown in the presence or absence of dox for 72 h. (d, e) Quantitation of western blots for cyclin B1 or cyclin B2 overexpression (fold change) in MEFs derived from *HA-Ccnb1*^{T7} or *HA-Ccnb2*^{T16} transgenic strains. (f, g) Western blot analysis of tissue extracts from six-week-old dox-treated *HA-Ccnb1* (strains T7 and T11), *HA-Ccnb2* (strains T15 and T16) and control (TA) transgenic mice. Ponceau S staining of blotted proteins served as a loading control. Blots in c are representative for three independent experiments, and blots in b and d-g for two independent experiments. Uncropped images of blots are shown in Supplementary Fig. 9.

Figure 2. B-type cyclins induce distinct chromosome missegregation when overexpressed. **(a)** Representative images of cells with indicated chromosome segregation errors. Scale bar, 10μm. **(b)** Live-eel I imaging analysis of chromosome segregation errors in *Ccnb1*^{r7} and *Ccnb2*ⁿ⁶ transgenic primary MEFs with and without transgene induction for 72 h. Anaphase failure refers to complete failure of sister chromatid separation producing one daughter with 4N DNA and one with out DNA. The analysis was carried out on three independent MEF I ines per genotype (>20 cells per line were analysed). Statistical significance was determined by a two-tailed, unpaired t-test. Statistics source data are provided in Supplementary Table 2. **P* < 0.05; ***P* < 0.01. **(c)** Schematic representation of the separase activity biosensor (top) and time-lapse images of biosensor-positive *Ccnb1*^{r7} MEFs progressing through mitosis in the absence or presence of transgenic eye I in B1. hSCC1, human sister chromatid cohesion protein 1. Sea le bars, 5μm. **(d)** Quantification of biosensor fluorescence at chromosomes as cells progress through mitosis. Data represent mean ± s.e.m. from five independent experiments. We note that the level of overexpression varied among dox-treated *Ccnbz*^{r7} MEFs, with those showing anaphase bridges expressing significantly higher levels of HA-cyclin B1 than those without bridges.

Figure 3.

Cyclin B2 overexpression causes aurora-A-mediated hyperactivation of Plk1. (a) Maximum intensity projection images of *Ccnb2n⁶* metaphases immunostained for α -tubulin (red) and γ -tubulin (green). (b) Incidence of spindle geometry abnormalities. (c) Images of MEFs in G2 immunostained for γ -tubulin (visualizing centrosomes) and pH3⁵¹⁰ (phosphorylated histone H3, used as a marker for G2). (d) Quantitation of accelerated centrosome separation. Cells with centrosome distances $\geq 3 \mu\text{m}$ were classified as accelerated. (e) Images of cells immunostained for p-Plk1¹²¹⁰, γ -tubulin and pH3⁵¹⁰. Primary antibodies for γ -tubulin and pH3⁵¹⁰ were both raised in rabbits and were visualized with the same fluorophore-conjugated secondary antibodies. This did not create a detection conflict because γ -tubulin and pH3⁵¹⁰ are confined to the cytoplasm and nucleus, respectively. (f) Quantitation of p-Plk1¹²¹⁰ signal at centrosomes in *Ccnb2n⁶* MEFs. (g) Western blots of mitotic extracts from *Ccnb2n⁶* MEFs grown in the presence or absence of dox for 72 h. Taxol and okadaic acid were added to the culture medium 5 h before cell harvest. Blots are representative for two independent experiments. Uncropped images of blots are shown in Supplementary Fig. 9. (h) Quantitation of metaphases with spindle geometry abnormalities in the presence or absence of 10nM 812536. (i) Images of prophase immunostained for p-aurora and γ -tubulin. P-aurora is a pan-aurora antibody: signal at spindle poles represents p-aurora A and signal at inner centromeres is p-aurora B. (j) Quantification of p-aurora signal at centrosomes in *Ccnb2n⁶* MEFs at the indicated stages of mitosis. (k) The same as h except with 10nM MLN8054. (l) The same as d except with 10 nM MLN8054. DNA in a,c,e,i was visualized with Hoechst. Arrows mark centrosomes. Scale bars, 5 μm in a and 10 μm in c,e,i. Quantifications in b,d,f,h and j-l were done on three independent MEF cell lines (per line, 20 cells in b,h,k and l, 10 cells in f and j, and >20 cells in d, were analysed). Data represent mean $\pm$ s.e.m. Statistical significance was determined by a two-tailed, unpaired t-test. Statistics source data are provided in Supplementary Table 2. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4.

Endogenous eye Iin B2 regulates aurora-A-mediated centrosome separation. **(a-h)** Wild-type (WT) MEFs transduced with cyclin B2 shRNA or non-targeting shRNA negative control lentiviruses and analysed 72 h later. **(a)** Western blot analysis for cyclins B1 and B2. **(b)** Immunostaining for cyclin B2 and γ -tubulin. **(c)** Quantification of cells with unseparated centrosomes ($<1 \mu\text{m}$ in G2). **(d)** G2 cells immunostained for p-Pik1¹²¹⁰, γ -tubulin and p-H3⁵¹⁰. We note that p-Pik1¹²¹⁰ levels at centrosomes of cyclin B2 overexpressing cells were increased irrespective of inter-centrosome distance. **(e)** Quantification of p-Pik1¹²¹⁰ signal at centrosomes. **(f)** Quantification of centrosomal p-aurora A signal in prophase. p-aurora A levels at centrosomes of cyclin B2 overexpressing cells were increased irrespective of intercentrosome distance. **(g)** Analysis of spindle geometry. **(h)** Analysis of chromosome segregation errors by live-cell imaging. The analysis was carried out on three independent MEF lines (15-22 cells were analysed per line). Statistical significance was determined by a one-tailed, unpaired *t*-test. **(i)** The same as c for mouse epithelial cells. **(j,k)** Different human cell lines transduced with cyclin B2 shRNA or non-targeting shRNA negative control lentiviruses and analysed 72 h later (HF, human skin fibroblasts). **(j)** Western blot analysis of human skin fibroblast lysates for cyclins B1 and B2. **(k)** Quantification of cells with unseparated centrosomes in G2 phase. Colour codes in e-g and i are as in c. DNA in b and d was visualized with Hoechst. Scale bars in b and d, 10 μm . Quantifications in c,e-g and k were done on three independent cell lines (per line, 20 cells in c,g and k and 10 cells in e and f were analysed). Quantifications in i were done on the same line in triplicate (20 cells for each analysis). Data represent mean $\pm$ s.e.m. Statistical significance was determined by a two-tailed, unpaired *t*-test in c,g,i and k and by a one sam

pie I-test against a theoretical mean of unity in e and f. $*P < 0.05$; $**P < 0.01$. Statistics source data are provided in Supplementary Table 2. Blots in **a** and **j** are representative for three independent experiments. Uncropped images of blots are shown in Supplementary Fig. 9.

Figure 5.

Cyclin B2 and p53 antagonistically control aurora-A-mediated centrosome separation. **(a)** WT, WT SV40 large T antigen-transformed and p53^{-/-} MEFs transduced with cyclin B2 sh RNA or non-targeting shRNA negative control lentiviruses and analysed for G2 cells with unseparated centrosomes (< 1μm). **(b)** Images of prophase cells immunostained for p-aurora and γ-tubulin. **(c)** Quantification of p-aurora signal at centrosomes in prophase. **(d)** Incidence of spindle geometry abnormalities. **(e)** Images of G2 cells immunostained for p-Plk1^{T210}, γ-tubulin and pH3^{S10}. **(f)** Quantification of p-Plk1^{T210} signal at centrosomes in G2 cells. **(g)** Quantitation of cells with unseparated centrosomes in G2 in the presence or absence of 10nM MLN8054. **(h)** Incidence of spindle geometry abnormalities in the presence or absence of 10nM MLN8054. **(i)** Analysis of chromosome segregation errors by live-cell imaging. The analysis was carried out on three independent MEF lines (14-26 cells were analysed per line). **(j)** Model for antagonistic control of the centrosome splitting signalling cascade by p53 and cyclin B2-Cdk1. In this model, p53 functions to limit the amount of activated (phosphorylated) aurora A by inhibiting AURKA gene transcription and promoting proteasomal degradation of aurora A protein. Cyclin B2-Cdk1, on the other hand, triggers the phosphorylation of the residual aurora A protein pool in a timely manner to induce phosphorylation of centrosome-associated Plk1 and permit centrosome disjunction^{38,67-70}. Whether cyclin B2-Cdk1 triggers aurora A activation directly or indirectly remains to be determined. Colour codes in **d** and **f** are as in **c**. Colour code in **h** is as in **g**. DNA in **b** and **e** was visualized with Hoechst. Scale bars represent 10 μm. Arrows mark centrosomes. p53^{-/-} cells with supernumerary centrosomes were excluded from the analyses in b-h. Quantifications in **a,c,d** and **f-h** were done on three independent MEF lines

(per line, 20 cells in **a,d,g** and **h** and 10 cells in **c** and **f** were analysed). Data represent mean $\pm$ s.e.m. Statistical significance was determined by a two-tailed, unpaired *t*-test. Statistics source data are provided in Supplementary Table 2. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

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Figure 6.

Cyclins B1 and B2 drive tumorigenesis when overexpressed. **(a)** Spontaneous tumour incidence of 14-month-old mice. **(b)** Spectrum of spontaneous tumour types in 14-month-old mice. **(c)** Representative images and histological analysis of representative spontaneous tumours from transgenic mice. Scale bar, 100µm. **(d-g)** Overexpressed B-type cyclins synergize with *APC*^{+/-Min} to develop aggressive colonic tumours. Colour codes and cohort sizes in **f** and **g** are as indicated in **e**. **(d)** Haematoxylin-eosin stained sections of colonic polyps in 90-day-old mice continuously treated with dox. Enlargements (right) of the *Ccnb1*; *APC*^{+/-Min} and *Ccnb2*; *APC*^{+/-Min} tumours show areas where intramucosal invasion has occurred to illustrate that these tumours have a more aggressive tumour phenotype. The TA; *APC*^{+/-Min} tumour is less advanced as it lacks invasive properties. Black and red scale bars are 1mm and 100 µm respectively. **(e)** Colon tumour incidence of mice that were continuously on dox (3 months (mo) +dox) or taken off dox for the last 30 days of the experiment (2 months +dox and 1 month -dox). **(f)** Colon tumour burden. **(g)** Average colon tumour size. **(h)** Tumour incidence of DMBA-treated mice. **(i)** Lung and skin tumour burden of DMBA-treated mice. *n* in **a**, **e** and **h** represents number of mice per genotype. Data represent mean ± s.e.m. in **f**, **g** and **i**. Statistical significance was determined by Fisher's exact test in **a** and **h**, a one-tailed chi-squared test in **b** and **e**, a two-tailed unpaired *t*-test in **f** and **g** and a two-tailed Mann-Whitney test in **i**. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Table 1

B-type cyclin overexpression induces aneuploidy.

(a) Overexpression of B-type cyclins in MEFs leads to increased aneuploidy

MEF genotype (n)	Mitotic cells inspected	% aneuploid figures (s.d.)	% (near) tetraploid figures (s.d.)	Karyotypes with the indicated chromosome number												
				36	37	38	39	40	41	42	43	71-79	80	>80		
<i>Ccnb1</i> ^{T7} -Dox (4)	200	12(3)	4(4)		1	4	9	174	3	1		6		2		
<i>Ccnb1</i> ^{T7} +Dox (4)	200	33(7)**	11(3)*		4	10	21	131	8	3	1	13		3		
<i>Ccnb1</i> ^{T11} -Dox (3)	150	15(3)	6(5)		4	3	7	125	1	1		4		2		
<i>Ccnb1</i> ^{T11} +Dox(3)	150	31(4)**	17(5)		3	6	10	96	5	4		15		8		3
<i>Ccnb2</i> ^{T15} -Dox (3)	150	15(3)	5(3)			5	6	123	4	3	2	2		4		1
<i>Ccnb2</i> ^{T15} +Dox (3)	150	25(1)**	8(2)		1	4	10	108	12	2	1	4		5		3
<i>Ccnb2</i> ^{T16} -Dox (3)	150	16(3)	7(1)		4	1	2	3	122	3	3	1	7	4		
<i>Ccnb2</i> ^{T16} -Dox (3)	150	36(8)*	10(2)		3	2	8	19	91	11	11	8		5		2

(b) Splenocytes of transgenic mice with high levels of cyclin B1 or B2 are prone to aneuploidization

Mouse genotype (n)	Mitotic cells inspected	% aneuploid figures (s.d.)	% (near) tetraploid figures (s.d.)	Karyotypes with the indicated chromosome number												
				36	37	38	39	40	41	42	43	44	79	80		
TA control (3)	150	6(2)	0				3	5	141	1						
<i>Ccnb1</i> ^{T7} (3)	150	20(3)**	1(1)		4	2	4	9	119	8	2		1	1		
<i>Ccnb1</i> ^{T11} (3)	150	12(0)**	0		5	3	4	2	132	1	2	1				
<i>Ccnb2</i> ^{T15} (3)	150	18(4)**	0		3	5	4	7	123	5	3					
<i>Ccnb2</i> ^{T16} (3)	150	18(3)**	0		2	7	9	123	5	3		1				

Asterisks in a mark values statistically different from -dox counterparts. Asterisks in b mark values statistically different from dox-treated transactivator (TA) control values.

* $P<0.05$;

** $P<0.01$.

Table 2
Chromosome missegregation in cyclin-B2-overexpressing MEFs is due to aurora A and Plk1 hyperactivity.

(a) Normalization of Plk1 activity in cyclin-B2-overexpressing MEFs restores accurate chromosome segregation												
MEF genotype (n)	Mitotic cells inspected	% cells with segregation defects (s.e.m.)	% metaphases with misaligned chromosomes (s.e.m.)	% anaphases with chrom. bridges (s.e.m.)	% anaphases with lagging chromosomes (s.e.m.)	% anaphase failure (s.e.m.)	% cytokinesis defects (s.e.m.)					
<i>Ccnb2</i> ^{T16} -Dox (3)	65	13(1)	1(1)	5(3)	4(2)	2(2)	0(0)					
<i>Ccnb2</i> ^{T16} +Dox (3)	65	36(10)*	7(4)	9(3)	15(4)	3(3)	2(2)					
<i>Ccnb2</i> ^{T16} -Dox (3) + BI	69	19(3)	0(0)	10(3)	7(1)	2(2)	0(0)					
<i>Ccnb2</i> ^{T16} +Dox (3) + BI	77	14(1)	3(1)	6(2)	3(3)	3(1)	0(0)					

(b) Cyclin-B2-overexpressing MEFs in which Plk1 activity is normalized are not prone to aneuploidy												
MEF genotype (n)	% aneuploid figures (s.e.m.)	% (near) tetraploid figures (s.e.m.)	Karyotypes with the indicated chromosome number									
			37	38	39	40	41	42	43	44	45	46
<i>Ccnb2</i> ^{T16} -Dox (3)	18(1)	7(2)	2	5	5	118	6	3	1	2	2	5
<i>Ccnb2</i> ^{T16} +Dox (3)	39(1)***	7(1)	3	2	22	89	13	6	4	1	2	5
<i>Ccnb2</i> ^{T16} -Dox(3)+BI	21(1)	8(0)	3	5	9	114	7		4	3	1	4
<i>Ccnb2</i> ^{T16} +Dox(3)+BI	21(2)	7(1)	2	6	11	112	7	1	1	1	1	6

(c) Normalization of aurora A activity in cyclin-B2-overexpressing MEFs prevents aneuploidy												
MEF genotype (n)	% aneuploid figures (s.e.m.)	% (near) tetraploid figures (s.e.m.)	Karyotypes with the indicated chromosome number									
			37	38	39	40	41	42	43	44	45	46
<i>Ccnb2</i> ^{T16} -Dox (3)	17(1)	8(1)	1	2	8	119	5	1	1	1	2	6
<i>Ccnb2</i> ^{T16} +Dox (3)	34(3)**	11(1)	5	5	14	96	12	2	3	2	3	4
<i>Ccnb2</i> ^{T16} -Dox(3)+MLN	23(1)	8(1)	5	4	13	109	6	1	1		2	6
<i>Ccnb2</i> ^{T16} +Dox(3)+MLN	22(2)	8(3)	4	5	11	112	5	1	1	1	1	2

Asterisks mark values statistically different from -dox counterparts.

* $P<0.05$;

.1001

 $P < 0.001$

;101
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