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ORIGINAL ARTICLE

# Impact of $G_2$ checkpoint defect on centromeric instability

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**Centromeric instability is characterized by dynamic formation of centromeric breaks, deletions, isochromosomes and translocations, which are commonly observed in cancer. So far, however, the mechanisms of centromeric instability in cancer cells are still poorly understood. In this study, we tested the hypothesis that $G_2$ checkpoint defect promotes centromeric instability. Our observations from multiple approaches consistently support this hypothesis. We found that overexpression of cyclin B1, one of the pivotal genes driving $G_2$ to M phase transition, impaired $G_2$ checkpoint and promoted the formation of centromeric aberrations in telomerase-immortalized cell lines. Conversely, centromeric instability in cancer cells was ameliorated through reinforcement of $G_2$ checkpoint by cyclin B1 knockdown. Remarkably, treatment with KU55933 for only 2.5 h, which abrogated $G_2$ checkpoint, was sufficient to produce centromeric aberrations. Moreover, centromeric aberrations constituted the major form of structural abnormalities in $G_2$ checkpoint-defective ataxia telangiectasia cells. Statistical analysis showed that the frequencies of centromeric aberrations in $G_2$ checkpoint-defective cells were always significantly over-represented compared with random assumption. As there are multiple pathways leading to $G_2$ checkpoint defect, our finding offers a broad explanation for the common occurrence of centromeric aberrations in cancer cells.**
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**Keywords:** $G_2$ checkpoint; defect; centromere; instability

## Introduction

Centromeres are integral chromosomal elements where sister chromatids are constricted and microtubules are attached for chromosome segregation during cell division. Investigations on chromosomal structural dynamics indicate that centromeres, being hotspots for rearrangements during species evolution, are intrinsically predisposed to instability (Eichler and Sankoff, 2003). Cytogenetic studies have shown that centromeric or pericentromeric aberrations, such as whole-arm translocations, deletions and isochromosomes, are common in human cancer cell lines and primary solid tumors of various origins (Jin *et al*., 1995; Johansson *et al*., 1995; Zhu *et al*., 1995; Beheshti *et al*., 2000; Wong *et al*., 2000; Padilla-Nash *et al*., 2001). The frequent occurrence of centromeric aberrations in tumor cells suggests that centromeric instability may contribute to tumor development. However, the mechanisms of centromeric instability in carcinogenesis remain poorly understood. Elevated levels of centromeric instability are well characterized in ICF (immunodeficiency, centromeric region instability, facial anomaly) patients, and are ascribed to hypomethylation of centromeric DNA, leading to centromeric aberrations specifically on chromosomes 1, 16 and sometimes 9 (Ehrlich, 2002). Yet, centromeric aberrations in most human tumors are not limited to the three chromosomes. Therefore, other mechanisms are probably involved in the genome-wide centromeric instability in tumor cells.

Human centromeres consist largely of repeated short sequences known as α-satellite DNA sequences, which are tightly packed into centromeric heterochromatin. It has been proposed that the condensed structure of heterochromatin presents barriers to DNA replication, such that replication fork stalling occurs; and unresolved stalled replication forks may generate DNA double-strand breaks (Leach *et al*., 2000). In normal cells, the $G_2$ checkpoint exerts its protective function by delaying cell cycle progression from $G_2$ to M phase to provide time for correction of postreplication errors and DNA damage repair. We, therefore, hypothesize that centromeric DNA may be preferentially subjected to erroneous replication that fails to be corrected in cells with defective $G_2$ checkpoint, leading to centromeric instability. Cyclin B1 is one of the specific and pivotal genes driving $G_2$ to M phase transition. The overexpression of cyclin B1 is expected to induce $G_2$ checkpoint defect. In this study, for the first time, we obtained the evidence that defective $G_2$ checkpoint, induced by manipulation of cyclin B1 overexpression and inhibition of its upstream regulator ATM (ataxia telangiectasia mutated), indeed promotes centromeric instability in the context of spontaneous DNA damage preferentially occurring at or near centromeres.

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## Results

*Cyclin B1 overexpression promotes $G_2$ checkpoint defect and centromeric instability*

To study the causative role of $G_2$ checkpoint defect in centromeric instability, we stably overexpressed cyclin B1 in two human telomerase-immortalized cell lines derived from normal esophageal epithelial cells (NE2-hTERT) (Cheung *et al*., 2010) and nasopharyngeal epithelial cells (NP460-hTERT) (Li *et al*., 2006), which were chosen because they had low background levels of centromeric instability. The cells were infected with retroviral plasmids, expressing cyclin B1 or empty vector, and selected with puromycin for 6 days. Western blotting analysis demonstrated the successful overexpression of cyclin B1 (Figure 1a, lanes 1–4). This was also accompanied by the increased expression of active form of phospho-cdc2, p-cdc2(Thr161), which is known to form complex with cyclin B1 to promote $G_2$ to M phase transition, whereas there was no remarkable change in the total levels of cdc2. Because intact $G_2$ checkpoint enforces $G_2$ arrest after DNA damage, the function of $G_2$ checkpoint was readily monitored by the percentage of mitotic cells 2 h after 1 Gy γ-ray irradiation relative to that of unirradiated control cells (that is, relative mitotic index) (Xu *et al*., 2002; Terzoudi *et al*., 2005; Deckbar *et al*., 2007). We confirmed that the cyclin B-overexpressing cells had impaired $G_2$ checkpoint function, as evidenced by the higher relative mitotic indices (Figure 1b, lanes 1–4 and Supplementary Table S1) compared with empty-vector-infected cells after γ-ray irradiation, indicating inefficient $G_2$ arrest after cyclin B1 overexpression.

Unirradiated cells were analyzed for spontaneous chromosome aberrations using 24-color spectral karyotyping (SKY) and pan-centromere fluorescence *in situ* hybridization (FISH) at the 6th population doubling after puromycin selection. The most remarkable finding was a ~20-fold increase in the frequencies of non-clonal centromeric aberrations in cyclin B1-overexpressing cells compared with empty-vector-infected cells (Figure 2, lanes 1–4). The new aberrations (Supplementary Table S2)

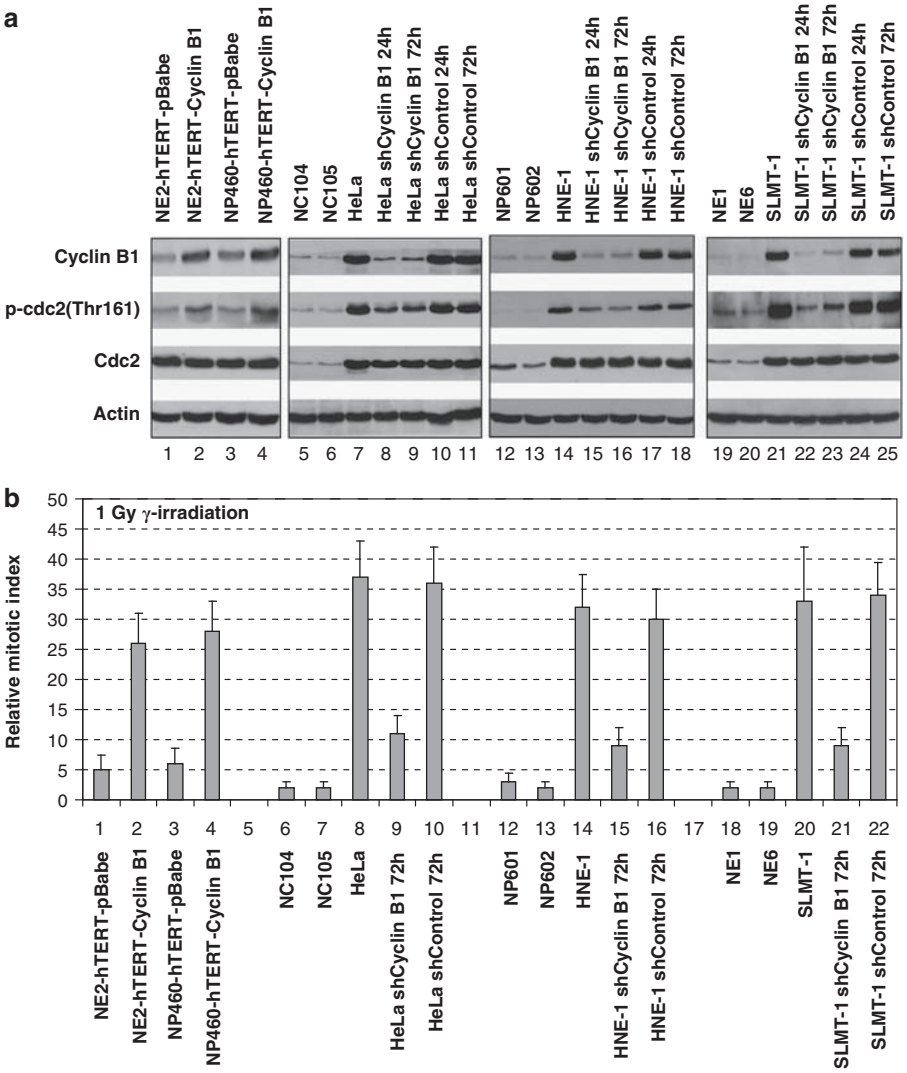


**Figure 1** Cyclin B1 overexpression and $G_2$ checkpoint function. (**a**) Western blot analysis and (**b**) relative mitotic indices expressed as percentages of mitotic cells 2 h after irradiation relative to unirradiated cells.

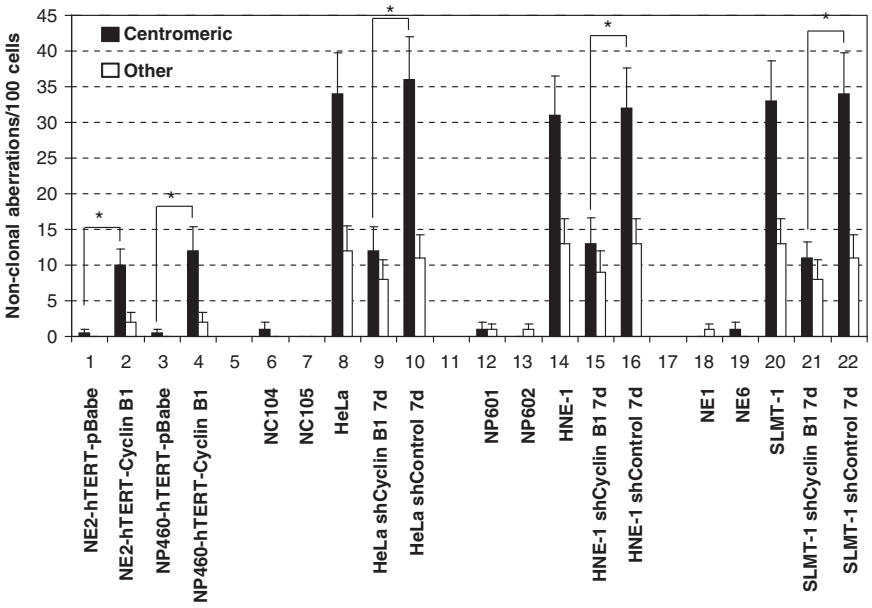


**Figure 2** Frequencies of non-clonal chromosome aberrations per 100 metaphases analyzed using SKY and centromere FISH. $^*P<0.05$.

included centromeric chromatid breaks, centromeric chromosomal deletions, centromeric translocations and isochromosomes, as exemplified in Figure 3a. The centromeric aberrations were confirmed by the presence of centromere FISH signals at the broken ends or chromosome rejoining points (Figure 3a, right). These results represent the first direct evidence that $G_2$ checkpoint defect promotes centromeric instability.

*Cyclin B1 knockdown reinforces $G_2$ checkpoint function and reduces centromeric instability in cancer cells*

We next tested the impact of $G_2$ checkpoint defect on centromeric instability in cancer cells. Three cancer cell lines of different cell types: HeLa (cervical cancer), SLMT-1 (esophageal cancer) (Tang *et al*., 2001) and HNE-1 (nasopharyngeal cancer) (Glaser *et al*., 1989) were examined. Although cancer cells are known to retain some degree of $G_2$ checkpoint function, we anticipated that the $G_2$ checkpoint in cancer cells may not be as stringent as in normal cells. To obtain normal control cells, we cultured primary epithelial cells from normal tissues donated by six independent individuals (NC104 and NC105 for cervical epithelial cells, NP601 and NP602 for nasopharyngeal epithelial cells, NE1 and NE6 for esophageal epithelial cells). SKY analysis confirmed that an average of 98% of these primary epithelial cells had a normal karyotype. Western blotting analysis showed that the control cells had significantly lower protein expression of cyclin B1, active form of phospho-cdc2 and total cdc2 than the cancer cells (Figure 1a, lanes 5–7, 12–14 and 19–21). The relative mitotic indices of normal and cancer cells after γ-ray irradiation decreased to an average of 2 and 34%, respectively (Figure 1b, lanes 6–8, 12–14 and 18–20), demonstrating that the cancer cells had defective $G_2$ checkpoint. Detailed mitotic indices are given in

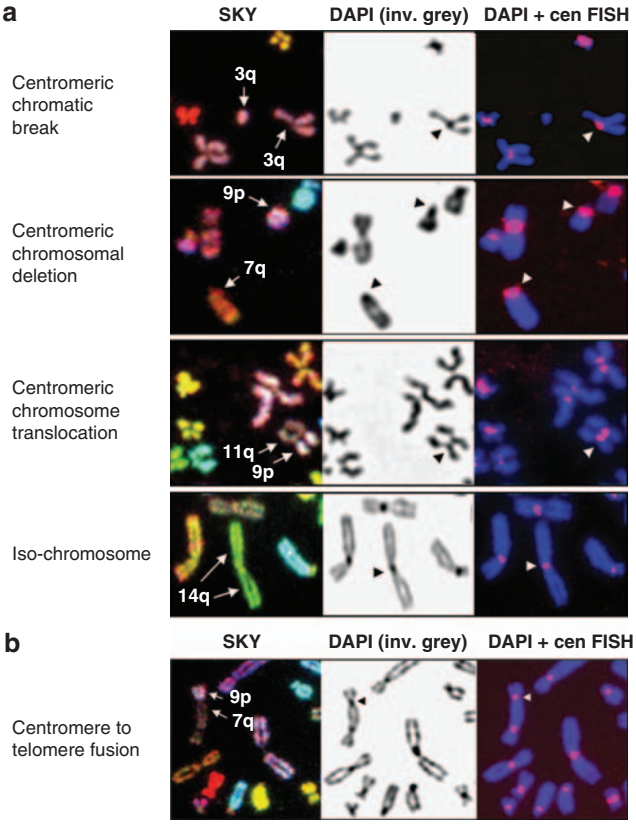


**Figure 3** Cytogenetic analysis of chromosome aberrations. Left, middle and right images show SKY, inverse DAPI and centromere FISH signals of the same metaphase, respectively. Arrows indicate aberrant chromosome arms. (**a**) Examples of centromeric aberrations in telomerase-immortalized cells overexpressing cyclin B1. Arrowheads indicate centromeres at the broken ends or chromosome rejoining points. (**b**) Example of fusion between a centromeric end and a telomeric end in AG02496 cells. Arrowhead indicates the fusion point between a centromere and a telomeric end of another chromosome.

Supplementary Table S3). Karyotype analysis revealed that each cancer cell line (unirradiated and pooled culture) had specific clonal structural aberrations that were present in all analyzed metaphases. However, the cells from each cancer cell line also had high frequencies of non-clonal structural aberrations, which predominantly involved centromeric regions (Figure 2, lanes 8, 14, 20 and Supplementary Table S4), indicating severe centromeric instability. Strikingly, the majority of clonal structural aberrations in the cancer cell lines were also centromeric aberrations. Examples of karyotypes of the three cell lines are shown in Supplementary Figure S1.

To examine whether defective $G_2$ checkpoint truly contributes to centromeric instability in the cancer cell lines, we reinforced $G_2$ checkpoint function by cyclin B1 knockdown to see if centromeric instability could be reduced. RNA interference directed against cyclin B1 was performed using plasmids containing a human cyclin B1 sequence that, when expressed, forms a short-hairpin RNA (shRNA), which gets processed into a cyclin B1-specific short interfering RNA. Figure 1a (lanes 8, 9, 15, 16, 22 and 23) shows the effective knockdown of cyclin B1 protein expression in the three cancer cell lines measured at 24 and 72 h after the plasmid transfection. Interestingly, the active form of phospho-cdc2 protein expression also showed some degree of decrease, but not the total levels of cdc2. The cyclin B1 shRNA-transfected cells had significantly lower mitotic indices ($P<0.05$, Supplementary Table S3) and were more sensitive to γ-ray irradiation compared with parental and control plasmid-transfected cells (Figure 1b and Supplementary Table S3), suggesting the improvement of $G_2$ checkpoint function after cyclin B1 knockdown. To achieve sustained cyclin B1 knockdown, we repeated cyclin B1 shRNA plasmid transfections twice with an interval of 48 h, and the cells were harvested 72 h after the third transfection. By the time of harvest, the cells had been transfected with cyclin B1 shRNA or control plasmids for 7 days. We then analyzed metaphases for spontaneous chromosome abnormalities and found that total non-clonal centromeric aberrations in the cells with cyclin B1 knockdown decreased significantly ($P<0.05$) to about 40% of those in the parental and control plasmid-transfected cell lines (Figure 2, lanes 9, 10, 15, 16, 21, 22 and Supplementary Table S4), indicating the amelioration of centromeric instability. The decreased centromeric aberrations included chromatid-type (chromatid breaks) and chromosome-type (chromosomal deletions, isochromosomes, centromeric translocations, centromeric-to-telomeric fusions). Although the frequencies of chromatid-type aberrations were expected to decrease with the checkpoint improvement within a single $G_2$ phase, new chromosome-type aberrations could be generated by rearrangements of chromatid-type aberrations after DNA replication in the next cell cycle. Therefore, the decrease in frequencies of both centromeric chromatid-type and chromosome-type aberrations was observed in cells harvested on day 7 (which allowed cell proliferation for multiple cell cycles) of cyclin B1 knockdown. The frequencies of other non-clonal, non-centromeric aberrations also showed a trend of decrease but to a lesser extent than centromeric aberrations (Figure 2, lanes 9, 15 and 21). Taken together, the above data enabled us to conclude that $G_2$ checkpoint defect induced by cyclin B1 overexpression plays an important role in the manifestation of centromeric instability in cancer cells.

We also studied the growth kinetics of cancer cells under cyclin B1 knockdown. By day 7, the numbers of cells transfected with cyclin B1 shRNA were about 50% that of cells transfected with control plasmids (Supplementary Figure S2), indicating that cyclin B1 knockdown decreased cell proliferation rate by about one cell population doubling within 7 days of experiments. The slower population doubling of cancer cells after $G_2$ checkpoint improvement with cyclin B1 knockdown might offer a trivial explanation for the reduction of centromeric and non-centromeric, non-clonal aberrations.

### *$G_2$ checkpoint defect induced by ATM inhibitor promotes centromeric instability*

We then examined whether the upstream regulator of cyclin B1 also affects centromere instability. It is well established that ATM is essential in maintaining $G_2$ checkpoint function (Terzoudi *et al*., 2005; Deckbar *et al*., 2007) through inhibition of cyclin B1/cdc2 (Abraham, 2001). We, therefore, examined the effect of a specific and potent ATM inhibitor, KU55933 (Rainey *et al*., 2008), which is known as a 'molecular switch' because of its rapid and reversible inactivation of ATM (White *et al*., 2008), on centromeric instability. Being aware that ATM also has $G_1$/S checkpoint functions (Abraham, 2001), we particularly designed experiments to examine the effect of KU55933 treatment without the confounding factor of $G_1$/S checkpoint inactivation. The NE2-hTERT and NP460-hTERT cells were treated with 10 μM KU55933 or dimethyl sulfoxide (DMSO) for 2.5 h, with the addition of colcemid 0.5 h after KU55933 or DMSO treatment to enable the collection of metaphases accumulated from $G_2$ cells. The data in Figure 4a confirmed the $G_2$ checkpoint inactivation by KU55933. An average of 11 non-clonal centromeric aberrations (mainly centromeric chromatid breaks) per 100 metaphases was detected after KU55933 treatment (Figure 4b and detailed data in Supplementary Table S5). This frequency was 21-fold higher than that in control (DMSO-treated) cells (0.5 non-clonal centromeric aberrations per 100 metaphases). Other intra-arm aberrations were also induced by the inhibitor treatment but the frequencies were lower than centromeric aberrations (Figure 4b and Supplementary Table S5). Because the total duration of the inhibitor treatment was only 2.5 h, and the duration of $G_2$ phase of a typical human cell cycle lasts about 4 h even under the condition of ATM inhibition (Pincheira and Lopez-Saez, 1991), it is unlikely that the new aberrations in the metaphases after the transient inhibitor treatment stemmed from $G_1$ or S phase. We, therefore, conclude that the centromeric aberrations can be induced by the ATM inhibition through the inactivation of $G_2$ checkpoint function.

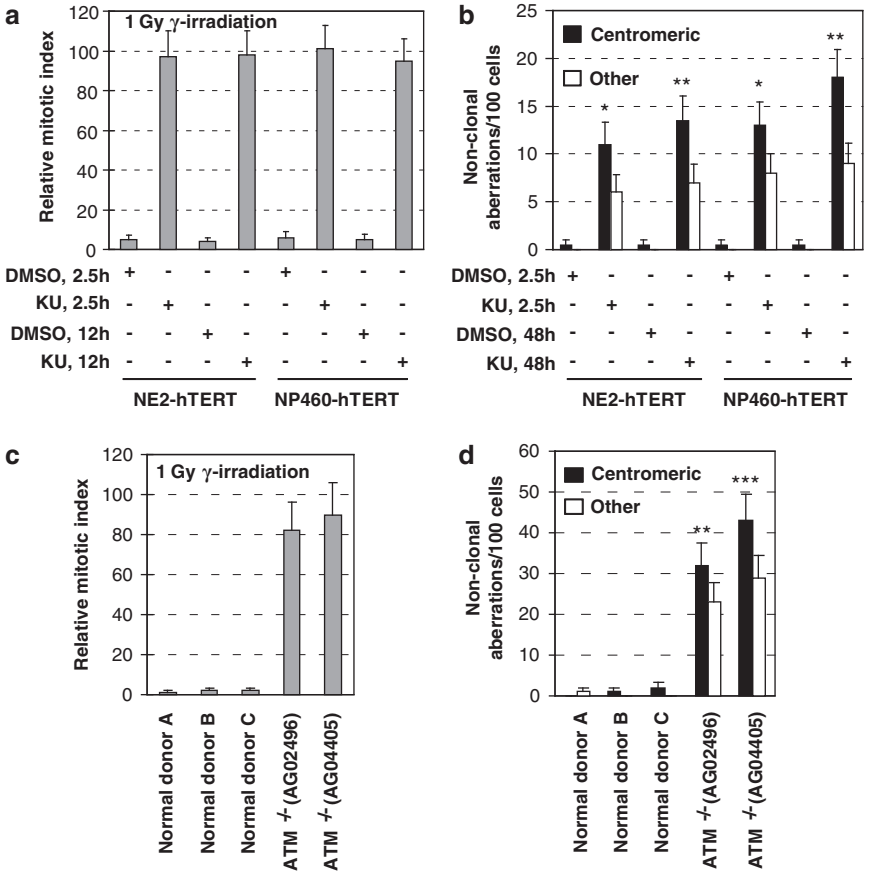


**Figure 4** Effect of KU55933 (KU) treatment and ATM mutation on $G_2$ checkpoint and chromosome instability. (**a**) Relative mitotic indices (percentages of mitotic cells 2 h after irrdiation relative to unirradiated cells). (**b**) Frequencies of non-clonal chromosome aberrations per 100 metaphases after DMSO or KU55933 treatment. A total of 200 metaphases were analyzed for DMSO- or KU55933-treated cells. (**c**) Comparison between fibroblasts from A-T patients and normal donors for relative mitotic indices after irradiation. (**d**) The frequencies of spontaneous non-clonal chromosome aberrations in 100 fibroblasts from A-T patients and normal donors. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ for ratio of centromeric aberrations to non-centromeric aberrations compared with 0.27, which is the expected value based on random assumption.

It is of interest to examine if the functions of ATM in $G_1$ and S phases also play a role in regulating centromeric instability. We cultured the NE2-hTERT and NP460-hTERT cells in KU55933- or DMSO-containing medium for 48 h (with medium change every 12 h). Colcemid was added into the culture medium 18 h before cell harvest to allow metaphase accumulation from $G_2$, S and $G_1$ phases. Chromosome aberration analysis showed $\sim$30% increases in the frequency of non-clonal centromeric aberrations in both cell lines compared with 2.5 h treatment with KU55933 (Figure 4b and Supplementary Table S5), indicating that $G_1$/S checkpoint inactivation by ATM inhibition also induced centromeric instability but to a lesser extent than $G_2$ checkpoint inactivation in the cell lines.

*Human primary fibroblasts from A-T patients exhibit elevated centromeric instability*

To further confirm the role of $G_2$ checkpoint defect in centromeric instability, we used primary fibroblasts (without any ectopic gene expression) from patients with ataxia telangiectasia (A-T) syndrome, a cancer-prone disorder, to investigate whether these cells also show centromeric instability. The A-T cells were used as additional cell models because they are well known to have defective $G_2$ checkpoint due to the mutations in ATM and are frequently used in $G_2$ checkpoint functional studies (Xu *et al*., 2002; Terzoudi *et al*., 2005; Deckbar *et al*., 2007). Analyses of relative mitotic indices after γ-radiation showed that the primary A-T cells from two patients (AG02496 and AG04405) had severe $G_2$ checkpoint defect (Figure 4c). We found 55 and 72 spontaneous structural chromosome aberrations in 100 AG02496 and AG04405 metaphases, respectively, whereas $\leqslant$2 aberrations were detected in 100 primary fibroblasts derived from normal individuals. Strikingly, chromosome breakpoint analysis using SKY and centromere FISH showed that the majority of the aberrations in the unirradiated A-T cells occurred in centromeric regions (Figure 4d), producing centromeric chromatid breaks, whole-arm translocations, centromeric chromosomal deletions, isochromosomes and another unexpected form described below.

Primary A-T cells are known to have telomeric instability (Pandita, 2002). Our analysis showed that

**Table 1** Statistical analysis of ratios of centromeric to non-centromeric aberrations in KU55933-treated and A-T cells

| | *Metaphases analyzed* | R[a] *(ratio of centromeric to non-centromeric aberrations)* ± *s.d.* | P-*value (compared with* R *based on random assumption)* |
|---|---|---|---|
| NE2-hTERT (KU55933, 2.5 h) | 200 | 1.83 ± 0.62 | <0.02 |
| NE2-hTERT (KU55955, 48 h) | 200 | 1.93 ± 0.64 | <0.01 |
| NP460-hTERT (KU55933, 2.5 h) | 200 | 1.60 ± 0.52 | <0.05 |
| NP460-hTERT (KU55933, 48 h) | 200 | 2.00 ± 0.64 | <0.01 |
| AG02496 | 100 | 1.39 ± 0.38 | <0.005 |
| AG04405 | 100 | 1.48 ± 0.36 | <0.001 |

[a]Calculated according to the following formula:

$$R = A \div B$$

$$(\sigma R/R)^2 = (\sigma A/A)^2 + (\sigma B/B)^2$$

where $\sigma R/R$, $\sigma A/A$ and $\sigma B/B$ are relative s.d. of $R$, $A$ and $B$, respectively. $A$ (frequency of centromeric aberrations), $\sigma A$ (s.d. of $A$), $B$ (frequency of non-centromeric aberrations) and $\sigma B$ (s.d. of $B$) were from data in Figures 4b and d. The value of $R$ under random assumption is 0.27.

these A-T cells not only had telomeric end-to-end fusions but also dicentrics formed by fusion between centromeric and telomeric ends (Figure 3b). The centromeric aberrations that were involved in fusions with telomeric ends accounted for about one fifth of the total centromeric aberrations in the A-T cells (Supplementary Table S6). These results demonstrate that centromeric instability not only occurs independently but also cooperates with telomeric instability to generate complex genetic changes in $G_2$ checkpoint-defective A-T cells. Although centromeric instability was not previously identified as a particular form of instability in A-T lymphocytes probably due to the high background of random genomic instability, previous cytogenetic analysis of A-T fibroblasts did show that centromeric or pericentromeric regions are hot spots of breakage (Kojis *et al*., 1989), consistent with our results.

*Statistical validation of significant overrepresentation of centromeric aberrations in $G_2$ checkpoint-defective cells*

Statistical analysis of the chromosome aberration data in Figure 2 showed that the frequencies of non-clonal centromeric aberrations were always significantly higher ($P<0.05$) than those of non-centromeric aberrations in cyclin B1-overexpressing immortalized cells and $G_2$ checkpoint-defective cancer cells (Figure 2, lanes 2, 4, 8, 10, 14, 16, 20 and 22). The frequencies of non-clonal centromeric aberrations in other $G_2$ checkpoint-defective cells (KU55933-treated and A-T cells) were also higher than non-centromeric aberrations (Figures 4b and d), although the differences were not statistically significant ($P>0.05$). However, it is important to emphasize that the band ratio of centromeric (p11–q11) to non-centromeric bands is only about 0.27 in the male haploid genome (Stewenius *et al*., 2005). If the chromosome aberrations were randomly distributed along chromosomes, the expected ratio of centromeric to non-centromeric aberrations would be 0.27. Yet, our experimental ratios of centromeric to non-centromeric aberrations in KU55933-treated and A-T cells (from male donors) ranged from $1.39 \pm 0.38$ to $2.00 \pm 0.64$ (Table 1), which were significantly ($P<0.05$) higher than the expected value based on random assumption. These results together suggested that centromeric aberrations were significantly overrepresented in $G_2$ checkpoint-defective cells.

## Discussion

In this study, we uncovered a previously uncharacterized role of $G_2$ checkpoint defect in chromosome instability. We have shown, for the first time, that defective $G_2$ checkpoint preferentially promotes the manifestation of centromeric instability. Cyclin B1 is one of the central and specific effector proteins driving $G_2$ to M phase transition. We found that cyclin B1 overexpression in telomerase-immortalized cell lines compromised $G_2$ checkpoint and increased the frequencies of non-clonal centromeric aberrations. We also showed that centromeric instability in cancer cells was associated with $G_2$ checkpoint defect. Conversely, centromeric instability in cancer cells was reduced by $G_2$ checkpoint improvement, using cyclin B1 knockdown by RNA interference. We further demonstrated that inhibition of ATM, the upstream regulator of cyclin B1/cdc2 and the well-recognized potent regulator of $G_2$ checkpoint, induced *de novo* centromeric aberrations. It is important to note that although ATM also has $G_1$ and S phase checkpoint functions, our experiments showed that transient (2.5 h) treatment with the specific ATM inhibitor, KU55933, was sufficient to induce centromeric aberrations. Because the treatment duration was shorter than $G_2$ phase duration (usually lasts about 4 h), the confounding factor of $G_1$/S phase checkpoint inhibition was avoided. Moreover, we analyzed detailed chromosome aberrations in primary fibroblasts derived from A-T patients. We particularly chose to test primary A-T cells because they are close to the *in vivo* situation and are frequently used in $G_2$ checkpoint functional studies. We found that centromeric or pericentromeric aberrations were the most prominent form of spontaneous chromosome structural abnormalities in primary A-T fibroblasts. KU55933 treatments and ATM mutations also promoted non-centromeric chromosome instability, but to lesser extents than centromeric instability. Collectively, the above data lead us to conclude that $G_2$ checkpoint

defect plays a critical role in promoting centromeric instability.

It is envisaged that centromeric regions intrinsically present replication barriers due to the condensed structure of heterochromatin, and unresolved replication barriers and/or asynchronous replication may result in DNA damage such as DNA double-strand breaks (Leach *et al*., 2000). Overstimulation of cell proliferation pathways has been shown to generate replication stress and DNA double-strand breaks at regions difficult to replicate, due to the conflict between unscheduled DNA synthesis and uncoordinated prereplicative complex assembly (Bartkova *et al*., 2005; Gorgoulis *et al*., 2005). Indeed, $p16^{INK4a}$ deletion, which promotes cell proliferation, is detected in both of our telomerase-immortalized cell lines (Li *et al*., 2006; Cheung *et al*., 2010).

Based on the above information, we suggest the following model to explain centromeric instability. In cells overstimulated to proliferate, centromeric regions are predisposed to spontaneous DNA damage; defective $G_2$ phase may impair the correct repair of the damage, which then manifests as chromosomal breaks or rearrangements. The spontaneous DNA damage and response at or near centromeric regions in $G_2$ checkpoint-defective cells is currently under active investigation in our laboratory.

Extensive centromeric instability is believed to have oncogenic potential in at least two ways. First, most centromeric aberrations result in whole-arm losses or gains, which lead to large-scale alterations of gene dosage. Ample amount of data from comparative genomic hybridization showed that whole-arm imbalances are common in tumors (Struski *et al*., 2002). Second, centromeric heterochromatin encompasses multiple forms of inactive chromatin structure that can lead to gene silencing, so that translocations at centromeric or pericentromeric regions may result in gene deregulation (Dillon and Festenstein, 2002; Perrod and Gasser, 2003). We thus propose that centromeric instability represents one of the basic forms of genomic instability and may play a functional role in cancer development.

The role of $G_2$ checkpoint defect in the manifestation of centromeric instability has important implications for genomic instability in cancer. In the context that low levels of DNA damage can escape normal $G_2$ checkpoint (Deckbar *et al*., 2007; Lobrich and Jeggo, 2007), it has been shown that $G_2$ checkpoint defect further reduces the efficacy of DNA damage repair (Terzoudi *et al*., 2005). Our data demonstrate that the $G_2$ checkpoint in cancer cells is not as stringent as in normal cells. One of the direct causes of $G_2$ checkpoint defect is the overexpression of cyclin B1. In fact, cyclin B1 overexpression has been frequently detected in numerous types of cancer (Ito *et al*., 2000; Takeno *et al*., 2002; Yoshida *et al*., 2004; Nakamura *et al*., 2005; Suzuki *et al*., 2007). Multiple pathways are able to upregulate cyclin B1. One of the well-studied classical pathways is through mutation or inactivation of ATM (Abraham, 2001). Another classical pathway is through inactivation of p53, which can regulate $G_2$ checkpoint through inhibition of cyclin B1 (Innocente *et al*., 1999), and p53 pathway inactivation has been detected in most cancers (Hanahan and Weinberg, 2000). Furthermore, oncogenes, such as H-Ras (Santana *et al*., 2002), c-Myc (Yin *et al*., 2001) and the viral oncogene human papillomavirus type 16 E6 (Kaufmann *et al*., 1997), can also activate cyclin B1. Therefore, the existence of a plethora of pathways leading to the upregulation of cyclin B1, thus $G_2$ checkpoint defect, offers a novel and broad explanation for the common occurrence of centromeric aberrations in cancer cells. Further studies on the upstream mechanisms underlying the preferential centromeric DNA damage and the role of centromeric instability in early process of cancer development are warranted.

## Materials and methods

*Cell culture, chemicals and irradiation*

Immortalized and primary normal epithelial cells were cultured as reported (Li *et al*., 2006; Deng *et al*., 2008; Cheung *et al*., 2010). Fibroblasts from A-T patients (obtained from Coriell Cell Repositories) and cancer cells were cultured in Dulbecco′s modified Eagle′s medium supplemented with 10% fetal bovine serum. Informed consents for normal tissue donation were obtained from the patients before surgery. KU55933 (Calbiochem, San Diego, CA, USA) was dissolved in DMSO. $^{137}Cs$ γ-ray irradiation was carried out in a GammaCell 220 irradiator (MDS Nordion, Ottawa, ON, Canada) at a dose rate of 1 Gy/min.

*Retroviral infection*

The NE2-hTERT and NP460-hTERT cells were infected with retroviral vector pApuro-CyclinB1 or control vector pBabe-puro using 4 μg/ml polybrene (Sigma-Aldrich, St Louis, MO, USA). The cyclin B1 expression vector was a kind gift from Dr Prochownik, Pittsburgh, PA (Yin *et al*., 2001). The pApuro vector was modified from pBabe-puro vector (Takata *et al*., 1994). Two days after retroviral infection, the cells were selected with 0.5 μg/ml puromycin for 6 days. The resistant cells were pooled for experiments.

*RNA interference*

shRNA plasmid against cyclin B1 (pKD-Cyclin B1-v4) and negative control plasmid (pKD-NegCon-v1) were purchased from Millipore (Billerica, MA, USA). Plasmid transfections were carried out according to the recommended protocols of the company.

*Chromosome spreads preparation, SKY and centromere FISH*

The cells in the absence of γ-ray irradiation were analyzed for chromosome aberrations. To accumulate metaphases, cells were treated with colcemid (Sigma-Aldrich, 0.03 μg/ml) for 2 h unless otherwise specified. Chromosome spreads were prepared as described (Deng *et al*., 2003). SKY and centromere FISH were done sequentially as reported (Deng *et al*., 2007). The rhodamine-labeled pan-centromere DNA probes (Cambio Ltd., Cambridge, UK) were used for centromere FISH. A total of 1–200 metaphases from multiple experiments were analyzed for detailed chromosome aberrations using SKY and centromere FISH. Only non-clonal aberrations were used to quantify chromosome instability.

*$G_2$ checkpoint function analysis*

The function of $G_2$ checkpoint was monitored by the decrease in the percentage of mitotic spreads 2 h after 1 Gy γ-ray irradiation relative to unirradiated control cells (relative

mitotic index) (Terzoudi *et al*., 2005; Deckbar *et al*., 2007). For each experiment point, at least 5000 cells were counted. Mitotic cells were identified after chromosome spreading (without colcemid treatment).

*Western blotting*
A total of 10 μg protein was separated by SDS–polyacrylamide gel electrophoresis and blots were prepared on a polyvinylidene fluoride membrane (Amersham, Piscataway, NJ, USA). Primary antibodies against cyclin B1 and actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phosph-cdc2(Thr161) and total cdc2 were from Cell Signaling Technology (Beverly, MA, USA). The membrane was probed with secondary antibody against peroxidase-conjugated mouse, rabbit or goat immunoglobulin G, and the blots were visualized by the enhanced chemiluminescence western blotting system (Amersham).

*Statistical analysis*
The two-tailed *t*-test was used to examine the statistical differences. *P*-values $<0.05$ were deemed significant. In all bar graphs, error bars represent standard deviations.

## Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)