Clks 1, 2 and 4 prevent chromatin breakage by regulating the Aurora B-dependent abscission checkpoint

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When chromatin is trapped at the intercellular bridge, cells delay completion of cytokinesis (abscission) to prevent chromosome breakage. Here we show that inhibition of Cdc-like kinases (Clks) 1, 2 or 4 accelerates midbody resolution in normally segregating cells and correlates with premature abscission, chromatin breakage and generation of DNA damage in cytokinesis with trapped chromatin. Clk1, Clk2 and Clk4 localize to the midbody in an interdependent manner, associate with Aurora B kinase and are required for Aurora B-serine 331 (S331) phosphorylation and complete Aurora B activation in late cytokinesis. Phosphorylated Aurora B–S331 localizes to the midbody centre and is required for phosphorylation and optimal localization of the abscission protein Chmp4c. In addition, expression of phosphomimetic mutants Aurora B–S331E or Chmp4c-S210D delays midbody disassembly and prevents chromatin breakage in Clk-deficient cells. We propose that Clks 1, 2 and 4 impose the abscission checkpoint by phosphorylating Aurora B–S331 at the midbody.
Chromatin bridges represent incompletely segregated chromosomal DNA connecting the anaphase poles or daughter nuclei and have been linked to chromosomal instability in human tumours and tumourigenesis in mice1,2. In response to chromatin bridges or to lagging chromosomes that are trapped in the intercellular bridge in late cytokinesis, eukaryotic cells delay abscission, the final cut of the narrow cytoplasmic canal that connects the daughter cells, to prevent chromosome breakage or tetraploidization by regression of the cleavage furrow1-6. In mammals, this abscission delay is called the abscission checkpoint and is dependent on Aurora B kinase3. Aurora B localizes to the midbody and imposes the abscission checkpoint by phosphorylating the endosomal sorting complex required for transport-III (ESCRT-III) subunit charged multivesicular body protein 4C (Chmp4c) on serines 210, 214 and 215 in human cells6,7. This phosphorylation has been proposed to target Chmp4c to the midbody centre, to prevent downstream endosomal sorting complex required for transport components including the ATPase Vps4 from relocating to the abscission site and deliver the final cut5-9. In addition, in normally segregating cells, that is, in the absence of trapped chromatin at the intercellular bridge, inhibition of Aurora B accelerates abscission, suggesting that the abscission checkpoint may function more generally as an abscission timer5,6. However, the mechanism of Aurora B activation in the abscission checkpoint is a matter of active investigation.

Complete Aurora B kinase activity requires phosphorylation at S331 (ref. 10). The DNA damage kinases Chk1 and Chk2 phosphorylate Aurora B–S331 in mitosis: Chk2 phosphorylates Aurora B–S331 in early prometaphase, while Chk1 phosphorylates S331 in late prometaphase and metaphase11-13. However, the kinase that activates Aurora B in the late stages of cytokinesis has not been previously reported.

The Cdc-like kinases (Clk1–4 in human cells) are an evolutionary conserved family of dual specificity protein kinases, which can autophosphorylate at tyrosine residues and phosphorylate their substrates on serine/threonine residues14,15. Clks localize in the cytoplasm and in the nucleus where they regulate alternative splicing through phosphorylation of serine/arginine-rich domains on splicing factors16-18. Clks recognize the minimum consensus sequence R-x-x-S/T also shared by Chk1 and Chk2; however, our current understanding of Clk biological targets and function is relatively limited15,19,20.

In the present study, we show that depletion of Clk1, Clk2 or Clk4 by small interfering RNA (siRNA) or pharmacological inhibition of Clk catalytic activity accelerates midbody resolution in normally segregating human cells. Furthermore, Clk-deficient cells exhibit premature abscission, chromatin breakage and generation of DNA damage in cytokinesis with chromatin bridges. Clks 1, 2 and 4 phosphorylate Aurora B–S331 in vitro and are required for optimal Aurora B–phosphorylation and complete Aurora B activation in late cytokinesis. In addition, Clk1, Clk2 and Clk4 localize to the midbody in an interdependent manner and associate with Aurora B in cell extracts after enrichment of cells in cytokinesis. Using cells transiently expressing siRNA-resistant forms of Aurora B–S331 in BE cells (Clk4:GFP) proteins localized on midbody arms and inside the midbody, we investigated localization of Clks in midbody resolution, human colon carcinoma BE cells transiently expressing α-tubulin fused to mCherry (mCherry-tubulin) were monitored by time-lapse microscopy and the kinetics of tubulin disassembly at the midbody determined (Fig. 1a)11. In control cells, the midbody remained visible for a median time of 35 ± 5 min after formation (n = 9; Supplementary Movies 1 and 2). In contrast, treatment of cells with 1 μM TG003, an inhibitor of Clk1, Clk2 and Clk4 catalytic activity at this concentration12, accelerated midbody disassembly (t = 18 ± 3 min, n = 8) compared with controls (P < 0.001; Supplementary Movies 3 and 4). This correlated with reduced frequency of cells at midbody stage after treatment with TG003 or depletion of Clk1, Clk2 or Clk4 by two independent siRNAs, but not with an increase in binucleate or multinucleate cells compared with controls (Fig. 1b–d and Supplementary Figs 1a–f and 2b). Furthermore, Clk-deficient and control cells exhibited similar frequency of cells in prometaphase (Supplementary Fig. 2a,b), suggesting that Clk inhibition does not prevent mitotic entry and that Clk-deficient cells can progress through abscission and disassemble their midbodies more rapidly than controls. We propose that Clks 1, 2 and 4 regulate proper timing of midbody disassembly in normally segregating cells.

**Results**

**Clk inhibition accelerates midbody disassembly.** To investigate a role for Cdc-like kinases in midbody resolution, human colon carcinoma BE cells transiently expressing α-tubulin fused to mCherry (mCherry-tubulin) were monitored by time-lapse microscopy and the kinetics of tubulin disassembly at the midbody determined (Fig. 1a)11. In control cells, the midbody remained visible for a median time of 35 ± 5 min after formation (n = 9; Supplementary Movies 1 and 2). In contrast, treatment of cells with 1 μM TG003, an inhibitor of Clk1, Clk2 and Clk4 catalytic activity at this concentration12, accelerated midbody disassembly (t = 18 ± 3 min, n = 8) compared with controls (P < 0.001; Supplementary Movies 3 and 4). This correlated with reduced frequency of cells at midbody stage after treatment with TG003 or depletion of Clk1, Clk2 or Clk4 by two independent siRNAs, but not with an increase in binucleate or multinucleate cells compared with controls (Fig. 1b–d and Supplementary Figs 1a–f and 2b). Furthermore, Clk-deficient and control cells exhibited similar frequency of cells in prometaphase (Supplementary Fig. 2a,b), suggesting that Clk inhibition does not prevent mitotic entry and that Clk-deficient cells can progress through abscission and disassemble their midbodies more rapidly than controls. We propose that Clks 1, 2 and 4 regulate proper timing of midbody disassembly in normally segregating cells.

Clks localize to the midbody. We then investigated localization of Clk1, Clk2 and Clk4 by immunofluorescence using CENP-A as a midbody marker (Supplementary Fig. 2c–e)13,14. Complete Aurora B kinase activity requires phosphorylation at B–S331 with chromatin bridges. Clks 1, 2 and 4 phosphorylate Aurora B in cell extracts after enrichment of cells in cytokinesis. In control cells, Clk1 fused to green fluorescent protein (GFP) (Clk1:GFP) and Clk2 or Clk4 fused to GFP (Clk2:GFP and Clk4:GFP) proteins localized on midbody arms and inside the midbody. As shown in Supplementary Fig. 2c–e, Clk1 and Clk2 were localized to the midbody in normally segregating cells (Fig. 1e).

During cytokinesis, the microtubule bundles at the midbody progressively get narrower21,22 and microscopic examination showed that midbody thickness ranges from ~400 to 1,400 nm in BE cells (n = 100). To further investigate localization of Clks in cytokinesis, ‘early’ or ‘late’ midbodies exhibiting midbody thickness of 800–1,400 or 400–700 nm, respectively, were examined. In control cells, Clk1 fused to green fluorescent protein (GFP) (Clk1:GFP) and Clk2 or Clk4 fused to GFP (Clk2:GFP and Clk4:GFP) proteins localized on midbody arms and inside the midbody, that is, the narrow region in the midbody centre where tubulin staining by immunofluorescence is blocked21,22, in early or late midbodies, and they partially co-localized with Aurora B (Fig. 1f and Supplementary Fig. 2f). Depletion of Clk1 or Clk4 diminished localization of Clk2 to early or late midbodies compared with controls (Fig. 1f and Supplementary Fig. 2f). These results show that Clks 1, 2 and 4 localize to the midbody in normally segregating cells, and that Clk1 and Clk4 are required for the localization of Clk2 in the structure.

Clks phosphorylate Aurora B–S331 at the midbody. Recruitment of active Aurora B at the midbody delays abscission23. We therefore investigated Aurora B–S331 phosphorylation, a maker of Aurora B activation, at the midbody10. In early midbodies, phosphorylated Aurora B–S331 localized on midbody arms in control cells where it partially overlapped with midbody microtubules (Fig. 2a). Inhibition of Clk catalytic activity by TG003 diminished Aurora B–S331 phosphorylation (Fig. 2a,c); however, it did not prevent localization of total Aurora B on midbody arms compared with control cells (Supplementary Fig. 2g). Surprisingly, in late midbodies, phosphorylated Aurora B–S331 localized inside the Fleming body in control cells.
(Fig. 2b). Treatment of cells with TG003 or depletion of Clk1, Clk2, Clk4 or Aurora B by siRNA reduced phospho-Aurora B–S331 staining in the Flemming body compared with controls (Fig. 2b,c and Supplementary Fig. 3a,b). The majority of total Aurora B localized on midbody arms in late midbodies; however, a relatively small proportion of total Aurora B was also detectable in the midbody centre in Clk-deficient or control cells (Fig. 2d and Supplementary Fig. 3c). These results show that Clks 1, 2 and 4 are required for Aurora B–S331 phosphorylation at the midbody in normally segregating cells. In contrast, inhibition of Chk1 using the Chk1-selective inhibitor UCN-01 or depletion of Chk2 by siRNA did not reduce Aurora B–S331 phosphorylation.
phosphorylation at the midbody compared with control cells (Supplementary Fig. 3d,e).

Recombinant proteins Clk1, Clk2 or Clk4 phosphorylated Aurora B in an in vitro kinase assay and substrate phosphorylation was remarkably reduced after mutation of S331 to alanine (S331A) or inhibition of Clks by TG003 compared with WT or kinase-dead (KD) Aurora B (Fig. 2e and Supplementary Fig. 4a–c). Transiently expressed Clk1:GFP, Clk2:GFP or Clk4:GFP proteins associated with glutathione S-transferase (GST)–Aurora B, but not with GST, in pull-down assays after enrichment of cells in cytokinesis (Fig. 2f and Supplementary Fig. 4d). Furthermore, Aurora B was precipitated from cell extracts using antibodies against endogenous Clk1, Clk2 or Clk4 after enrichment of cells in cytokinesis but not from asynchronous cells (Fig. 2g and Supplementary Fig. 4e), suggesting that Clks 1, 2 and 4 interact with Aurora B in cytokinesis, but not in interphase. In addition, Clk4 was precipitated from cell extracts from cytokinesis-enriched cells using antibodies against Clk1 or Clk2, and Clk2 was precipitated by antibodies against Clk1 or Clk4, which is consistent with Clks 1, 2 and 4 participating in the same protein complexes (Supplementary Fig. 4f,g). Taken together, we propose that Clk1, Clk2 and Clk4 associate with Aurora B and phosphorylate Aurora B–S331 at the midbody in normally segregating cells.

Clks regulate localization of Chmp4c to late midbodies. Aurora B phosphorylates Chmp4c to delay abscission6,7. To investigate Chmp4c phosphorylation at the midbody, an antibody against phosphorylated S210, S214 and S215 of human Chmp4c (pChmp4c) was used7. In control cells, phosphorylated Chmp4c localized on midbody arms in early midbodies and inside the Flemming body in late midbodies (Fig. 3a and Supplementary Fig. 5a). Inhibition of Clk catalytic activity by TG003 or depletion of Chmp4c by siRNA (siChmp4c) diminished Chmp4c phosphorylation in late midbodies compared with control cells (Fig. 3a,b). However, Chmp4c or Aurora B protein levels per se were not affected by TG003-treatment (Supplementary Fig. 5b). In addition, phosphorylation of Chmp4c in early midbodies after Clk inhibition was similar to control cells (Supplementary Fig. 5a,b). As Clk inhibition reduced Aurora B–S331 phosphorylation in early midbodies as described in the previous section, one possibility is that there is redundancy in phosphorylation of Chmp4c at this stage, and that an unidentified kinase can phosphorylate Chmp4c at midbody arms when Aurora B activity is diminished. In late midbodies, total Chmp4c localized inside the Flemming body in control cells but mislocalized on midbody arms in TG003-treated cells (Fig. 3c). Importantly, this antibody recognized Chmp4c but not Chmp4a or Chmp4b proteins (Supplementary Fig. 5c). These results show that Cdc-like kinase activity is required for optimal phosphorylation and localization of Chmp4c to late midbodies in normally segregating cells.

Aurora B–S331 phosphorylation delays midbody resolution. To investigate the significance of Aurora B–S331 phosphorylation, BE cells transiently expressing Myc-tagged, siRNA-resistant versions of WT (WT8) or a phosphomimetic S331 to glutamic acid (S331E) mutant Aurora B were analysed after depletion of the endogenous Aurora B by siRNA (Supplementary Fig. 5d). Expression of S331E, but not WT8, Myc-Aurora B restored the frequency of cells at midbody stage after Clk inhibition by TG003 compared with controls, whereas mitotic entry, judged by the frequency of cells in prometaphase, was similar in all treatments (Fig. 3d and Supplementary Fig. 5e). These results suggest that phosphorylated Aurora B–S331 delays midbody disassembly in Clk-deficient cells.

To investigate the importance of Chmp4c phosphorylation, WT Chmp4c fused to GFP (WT Chmp4c:GFP), S210D Chmp4c:GFP harbouring a phosphomimetic mutation of S210 to aspartic acid or S210A Chmp4c:GFP containing a non-phosphorylatable mutation of S210 to alanine were transiently expressed at approximate levels tenfold higher than the endogenous protein, to disrupt endogenous Chmp4c functions (Supplementary Fig. 5f). Correct localization of Chmp4c:GFP proteins at the midbody was maintained as will be shown in Supplementary Fig. 9c. Expression of S210D, but not WT, Chmp4c:GFP rescued the frequency of cells at midbody stage after treatment with TG003 compared with controls (Fig. 3e and Supplementary Fig. 5g), suggesting that Chmp4c-S210 phosphorylation prevents faster midbody resolution in Clk-deficient cells compared with control cells. In contrast, expression of S210A Chmp4c:GFP accelerated midbody disassembly in the absence or presence of TG003 as judged by the reduced frequency of cells at midbody stage compared with controls expressing WT Chmp4c:GFP (Fig. 3e and Supplementary Fig. 5g). We propose that proper timing of midbody resolution in normally segregating cells requires Clk-mediated Aurora B–S331 phosphorylation and subsequent Chmp4c-S210 phosphorylation by Aurora B at the midbody.

Clks prevent chromatin breakage in cytokinesis. To investigate the role for Clks in the abscission checkpoint in the presence of chromatin bridges, HeLa cells stably expressing the inner nuclear envelope marker LAP2b fused to red fluorescent protein (RFP) (LAP2b-RFP) were monitored in cytokinesis by time-lapse microscopy. Gradual thinning of chromosome bridges during mitotic exit limits their detection by time-lapse imaging of chromatin markers; however, LAP2b-RFP localizes around chromatin from late anaphase and always correlates with chromatin bridges in high-resolution still images in which DNA-binding stains are used5. Control cells exhibiting

Figure 1 | Clk inhibition accelerates midbody disassembly in normally segregating cells. (a) Midbody disassembly in the absence (control) or in the presence of TG003. Cells expressing mCherry:tubulin were analysed by time-lapse microscopy. Phase-contrast (right panels) and fluorescence images (left panels) are shown. Time from midbody formation to midbody disassembly is indicated. Midbodies are shown by solid arrows and midbody disassembly is indicated by broken arrows. (b) Frequency of bi-/multinucleate or midbody stage cells. Cells were transfected with negative siRNA (control), Clk1 siRNA (siClk1), Clk2 siRNA (siClk2), Clk4 siRNA (siClk4) or treated with TG003 for 5 h. Error bars show the s.d. from the mean from three independent experiments. A minimum of 300 cells were analysed per experiment. ***P<0.001 compared with control. The Student’s t-test was used. (c) Examples of midbody stage cells (shown in brackets). Green, α-tubulin; blue, DNA. (d) Example of a binucleate cell after TG003 treatment. Green, actin; blue, DNA. (e) Localization of Clk1, Clk2 or Clk4 in cytokinesis. Green, Clk1, Clk2 or Clk4; red, CENP-A; blue, DNA. Twenty cells from two independent experiments were examined. (f) Localization of Clk1 in cells transfected as in b. Green, Clk1; red, α-tubulin; blue, DNA. Tubulin values indicate midbody thickness. Insets show magnified midbodies. (g) Mean midbody intensity of Clk2 in cells treated as in f. Data are from n cells from three independent experiments. Error bars show s.d. Values in control were set to 1. ***P<0.001 compared with control. The Mann–Whitney U-test was used. (h) Localization of Clk1:GFP, Clk2 or Clk4:GFP in purified midbodies. Green, GFP or Clk2; red, Aurora B. Scale bars, 5 μm.
LAP2b:RFP intercellular bridges sustained those bridges for at least 60 min (n = 9; Fig. 3f and Supplementary Movie 5). In contrast, in 11/12 cells treated with the Clk inhibitor TG003, LAP2b:RFP bridges were rapidly ruptured (average time to LAP2b bridge breakage 11 ± 7 min, n = 11; Fig. 3f, Supplementary Fig. 6a and Supplementary Movies 6 and 7).

Confocal microscopy analysis of fixed HeLa or BE cells in telophase showed that 30–36% cells treated with TG003 or depleted of Clk1, Clk2 or Clk4 by two independent siRNAs exhibited broken DNA bridges compared with 2–3% controls (Fig. 4a,b and Supplementary Fig. 6b–e). In addition, all broken DNA bridges exhibited broken LAP2b intercellular bridges and vice versa (Supplementary Fig. 6b,c). Furthermore, 35/35 BE control cells with stable chromatin bridges exhibited intact intercellular bridges as evidenced by staining with α-tubulin and actin, and 35/35 TG003-treated cells with broken DNA bridges exhibited broken intercellular bridges, indicating that these cells had completed abscission (Fig. 3g).

Figure 2 | Aurora B associates with Clks in cell lysates. (a,b) Aurora B-S331 phosphorylation (pS331) in the absence (control) or presence of TG003 for 5 h. Green, pS331; red, α-tubulin; blue, DNA. (c) Mean midbody intensity of pS331 in cells treated as in a,b. Data are from n cells from three independent experiments. Values in control were set to 1. Error bars show s.d. ***P < 0.001 compared with control. The Mann–Whitney U-test was used. (d) Localization of total Aurora B in cells treated as in b. Green, Aurora B; red, α-tubulin; blue, DNA. Thirty cells from three independent experiments were examined per treatment. Tubulin values indicate midbody thickness. Insets show magnified midbodies. Scale bars, 5 μm. (e) Clk2 in vitro kinase assay. Autoradiography analysis of Aurora B substrates (top) and Ponceau staining (bottom). (f) GST pull-down assay from cytokinesis-enriched cells. Lysates expressing Clk1:GFP, Clk2:GFP or Clk4:GFP proteins were incubated with 10 μg glutathione–agarose-bound WT GST–tagged Aurora B (GST-Aur BWT) or GST. Associated GFP (top) or total GFP and actin (bottom) were detected by western blotting and GST proteins (middle) by Ponceau staining. (g) Immunoprecipitation assay from cytokinesis-enriched cells. Immunoprecipitated (IP) Aurora B (top) and total Aurora B and actin (bottom) were detected by western blotting.
These results suggest that Clk activity is required to prevent abscission and chromatin breakage in cytokinesis with chromatin bridges.

To further investigate whether broken chromatin bridges were caused by abscission, cells expressing mutant Vps4 protein fused to GFP in which Vps4-lysine 173 was changed to glutamine...
Figure 4 | Clk inhibition correlates with chromatin breakage and generation of DNA damage. (a) Cells were transfected with negative siRNA (control), Clk1 siRNA (siClk1), Clk2 siRNA (siClk2), Clk4 siRNA (siClk4) or treated with TG003 for 5 h. Intact DNA bridges are indicated by solid and broken bridges by open arrowheads. (b) Broken bridges index analysis in cells treated as in a. Error bars show the s.d. from the mean from three independent experiments. A minimum of 50 cells with chromatin bridges were analysed per experiment. (c) Cells expressing GFP or GFP:Vps4-K173Q were treated as in a. Broken bridges index shows the percentage of green cells with broken chromatin bridges/total green cells with chromatin bridges. Error bars show the s.d. from the mean from three independent experiments. A minimum of 300 cells were analysed per experiment. (d) The abscission checkpoint can also be activated by partial depletion of Clk1, Clk2 or Clk4 increased the frequency of cells exhibiting micronuclei compared with controls (Supplementary Fig. 6f), suggesting that chromatin breakage in Clk-deficient cells was caused by abscission.

Fragmented chromatin bridges can lead to the formation of micronuclei and accumulation of DNA damage1. Treatment with TG003 compared with cells expressing GFP (Fig. 4c and Supplementary Fig. 6f), suggesting that chromatin breakage in Clk-deficient cells was caused by abscission. B-negative micronuclei in Clk-deficient cells was not due to an overall increase in the frequency of cells with chromatin bridges (both intact and broken) compared with controls (Supplementary Fig. 7a). Furthermore, in Clk-deficient cells, micronuclei were positive for phospho-Ser139 histone H2A.X (γ-H2AX) staining, which is a marker for double-strand DNA breaks (Fig. 4e,f)28. These results show that Clks 1, 2 and 4 are required to prevent formation of micronuclei and generation of DNA damage in cytokinesis with chromatin bridges.

The abscission checkpoint can also be activated by partial depletion of nucleoporin 153 (Nup153) and is evidenced by an accumulation of cells at the midbody stage that are unable to
complete abscission. Importantly, accumulation of cells at midbody stage after Nup153 depletion by Nup153 siRNA (siNup153) was prevented by co-transfection of cells with Clk1, Clk2 or Clk4 siRNA (Fig. 4g,h), whereas the frequency of cells in prometaphase was similar in all the above treatments (Supplementary Fig. 7b). Taken together, we propose that Clk1, Clk2 and Clk4 are required for the abscission checkpoint in response to chromatin bridges or partial depletion of Nup153.

**Clks localize to the midbody in an interdependent manner.** In control cells with chromatin bridges, endogenous Clk1, Clk2 or Clk4, or transiently expressed Clk1:GFP, Clk2:GFP or Clk4:GFP localized to the midbody where they co-localized with endogenous Aurora B (Fig. 5a,b and Supplementary Fig. 7c,d). Depletion of one Clk1, Clk2 or Clk4 protein by siRNA, but not inhibition of Clks by TG003, reduced localization of the other two Clks to the midbody by 86–95% compared with controls (Fig. 5b–d and Supplementary Fig. 8a–c), suggesting that all three Clk proteins stabilize each other’s presence in the midbody to impose the abscission checkpoint in response to chromatin bridges.

**Clks phosphorylate S331 in cytokinesis with DNA bridges.** Control cells exhibited phosphorylated Aurora B–S331 at the midbody in late cytokinesis with DNA bridges (Fig. 5e). Inhibition of Clks by TG003 or depletion of Clk1, Clk2 or Clk4 by siRNA diminished Aurora B–S331 phosphorylation at the midbody compared with controls (Fig. 5e,f and Supplementary Fig. 9b–d).
This was not due to impaired localization of total Aurora B at the midbody in Clk-deficient cells (Fig. 6a,b and Supplementary Fig. 9a).

We also examined localization of Mklp1 as a surrogate marker of Aurora B catalytic activity in late cytokinesis. It was proposed that phosphorylation by Aurora B prevents premature entry of Mklp1 to the nucleus. In control cells with chromatin bridges, Mklp1 did not localize to daughter nuclei, indicating active Aurora B (Supplementary Fig. 9b). In contrast, cells treated with TG003 or depleted of Aurora B by siRNA (as a positive control) exhibited Mklp1 inside the daughter nuclei and this was not due to premature abscission, because an intact intercellular bridge

Figure 6 | Clk inhibition reduces Chmp4c phosphorylation at the midbody in cells with chromatin bridges. (a) Localization of total Aurora B. Cells were transfected with negative siRNA (control) or treated with TG003 for 5 h. Green, Aurora B; red, CENP-A; blue, DNA. (b) Mean midbody intensity of Aurora B in cells treated as in a or transfected with Clk1 siRNA (siClk1), Clk2 siRNA (siClk2) or Clk4 siRNA (siClk4). (c) Localization of phosphorylated Chmp4c-S210, S214 and S215 (pChmp4c) in the absence (control) or presence of TG003 for 5 h. Green, pChmp4c; red, CENP-A; blue, DNA. (d) Mean midbody intensity of pChmp4c. Data are from n cells from three independent experiments. Error bars show s.d. Values in control were set to 1. ***P<0.001 compared with control. The Mann–Whitney U-test was used. (e) Localization of total Chmp4c in cells treated as in c. Green, Chmp4c; red, CENP-A; blue, DNA. Insets show magnified midbodies. Scale bars, 5 μm. (f) Broken bridges index analysis. Cells expressing sRNA-resistant forms of WT or S331E Myc-tagged Aurora B (Myc-Aurora B) were depleted of endogenous Aurora B by siRNA and treated as in c. Error bars show the s.d. from the mean from three independent experiments. A minimum of 50 cells in cytokinesis with chromatin bridges were analysed per experiment. ***P<0.001 compared with control. Student’s t-test was used.
It has been proposed that local auto-activation through Aurora B clustering onto chromatin bridges can prevent DNA breakage by abscission in yeast10,26. Our model suggests a novel mechanism for activating the abscission checkpoint in human cells, through Clk-mediated Aurora B phosphorylation. This is in agreement with previous findings that Aurora B activity at intercellular canals does not exclusively depend on its auto-activation8. Our model also proposes a common pathway for activating an abscission delay in the absence or presence of trapped chromatin, consistent with a role for Aurora B in delaying abscission in normally segregating cells9. One possibility is that the abscission checkpoint is activated spontaneously, perhaps by monitoring midbody formation5, and that chromatin bridges sustain checkpoint signalling until chromosome segregation is completed, for example, through counteracting dephosphorylation of Aurora B by inhibitory phosphatases. In conclusion, our study describes the identification of a potential novel component of the abscission checkpoint acting upstream of Aurora B.

Methods

Antibodies and plasmids. Monoclonal antibody against Myc (9E10), rabbit polyclonal antibodies against GFP (FL), Mdklp1 (N-19) and Nup153 (H-161), and a goat polyclonal antibody against Clk1 (N-17) were from Santa Cruz Biotechnology. Antibodies against normal rabbit (sc-2027) or goat IgG (sc-2028) used in immunoprecipitations were also from Santa Cruz Biotechnology. Rabbit polyclonal anti-Aurora B (ab2254; used in immunofluorescence and in Fig. 2g) antibodies were from Abcam. Mouse monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, anti-CENP-A (1-39) was from GeneTex, anti-AIM1 (Aurora B; used in western blotting and in Fig. 1b) was from Cell Signaling, and anti-Aurora B (ab2254; used in immunofluorescence and in Fig. 2g) antibodies were from Abcam. Mouse monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, anti-CENP-A (1-39) was from GeneTex, anti-AIM1 (Aurora B; used in western blotting and in Fig. 1b) was from Cell Signaling, and anti-Aurora B (ab2254; used in immunofluorescence and in Fig. 2g) antibodies were from Abcam. Mouse monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, anti-CENP-A (1-39) was from GeneTex, anti-AIM1 (Aurora B; used in western blotting and in Fig. 1b) was from Cell Signaling, and anti-Aurora B (ab2254; used in immunofluorescence and in Fig. 2g) antibodies were from Abcam. Mouse monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, anti-CENP-A (1-39) was from GeneTex, anti-AIM1 (Aurora B; used in western blotting and in Fig. 1b) was from Cell Signaling, and anti-Aurora B (ab2254; used in immunofluorescence and in Fig. 2g) antibodies were from Abcam. Mouse monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, anti-CENP-A (1-39) was from GeneTex, anti-AIM1 (Aurora B; used in western blotting and in Fig. 1b) was from Cell Signaling, and anti-Aurora B (ab2254; used in immunofluorescence and in Fig. 2g) antibodies were from Abcam. Mouse monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, anti-CENP-A (1-39) was from GeneTex, anti-AIM1 (Aurora B; used in western blotting and in Fig. 1b) was from Cell Signaling, and anti-Aurora B (ab2254; used in immunofluorescence and in Fig. 2g) antibodies were from Abcam. Mouse monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, anti-CENP-A (1-39) was from GeneTex, anti-AIM1 (Aurora B; used in western blotting and in Fig. 1b) was from Cell Signaling, and anti-Aurora B (ab2254; used in immunofluorescence and in Fig. 2g) antibodies were from Abcam. Mouse monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, anti-CENP-A (1-39) was from GeneTex, anti-AIM1 (Aurora B; used in western blotting and in Fig. 1b) was from Cell Signaling, and anti-Aurora B (ab2254; used in immunofluorescence and in Fig. 2g) antibodies were from Abcam. Mouse monoclonal antibodies against α-tubulin (DM1A) and actin (AC-40) were from Sigma, anti-CENP-A (1-39) was from GeneTex, anti-AIM1 (Aurora B; used in western blotting and in Fig. 1b) was from Cell Signaling, and anti-Aurora B (ab2254; used in immunofluorescence and in Fig. 2g) antibodies were from Abcam.
(DE3) cells (Agilent Technologies). Proteins were purified using the 6 × His purification kit (B-PER; Thermo Fisher Scientific) and used as substrates in kinase reactions. Human GST-tagged Aurora B protein was expressed in BL21 (DE3) cells and purified using glutathione-agarose beads (Santa Cruz Biotechnology).

**Mutagenesis and cloning.** Point mutations were generated using the QuikChange site-directed mutagenesis kit (Agilent Technologies). To generate siRNA-resistant forms of Myc-Aurora B, the Myc/Aurora B plasmid coding 6 × Myc-tagged human Aurora B in pcDNA5/FRT/TO vector (Invitrogen) was used to introduce C919T.
G921C and C922T point mutations giving resistance to the Aurora B siRNA. This gift from Daniel Gerlich, Institute of Molecular Biotechnology, Vienna, Austria)5

To visualize mCherry:tubulin, BE cells were transfected with the mCh-expressing LAP2b:RFP were seeded onto Petri dishes with a 30-mm glass base pre-warmed (37°C). Actin was visualized with Fluorescein–Phalloidin (Life Technologies) or antibodies (Jackson ImmunoResearch Laboratories, Inc.) were used as appropriate. For CLM, RNase, and DNA staining, cells were extracted in a solution containing 1 mM PIPES pH 7, 1 mM EGTA, 1% NP40, 5 μg ml−1 taxol, 5 μg ml−1 leupeptin, 50 μg ml−1 PMSF and 5 μg ml−1 aprotonin, and vortexed vigorously. After addition of 0.3 volumes of cold 50 mM 2-(N-morpholino) ethane sulfonic acid (MES) pH 6.3, cells were incubated on ice for 20 min and then centrifuged at 250 g for 10 min. The supernatant was then transferred to a new tube and centrifuged at 740 g for 20 min, to pellet midbodies. The pellet was resuspended in 50 mM MES pH 6.3, layered over a cushion of 40% glycerol in 50 mM MES pH 6.3 and centrifuged at 2,800 g for 45 min. Midbodies were resuspended in MES, plated on poly-lysine-coated coverslips and processed for immunofluorescence microscopy.

**In vitro kinase assays.** For Clk1, Clk2 or Clk4 in vitro kinase assays, 0.5 μg recombinant Clk1, Clk2 or Clk4 was incubated with 0.5–1 μg protein substrate in 20 μl kinase buffer (40 mM MOPS-NaOH pH 7.0, 1 mM EDTA, 1 μM ATP and 2 μg μl−1 ATP) for 30 min at 30°C before analysis by SDS-PAGE and autoradiography.

**GST pulldowns and immunoprecipitations.** Cells were sonicated for 3–4 μg cell lysate was incubated with 0.5 μg anti-Clk or anti-Ima antibody for 16 h followed by addition of 20 μl protein A/G PLUS–agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. Samples were spun down, washed four times with immunoprecipitation buffer and analysed by SDS-PAGE.

**Western blotting.** Cells were lysed in ice-cold whole-cell extract buffer (20 mM HEPES, 5 mM EDTA, 10 mM EGTA, 0.4 M KCl, 0.4% Triton X-100, 10% glycerol, 5 mM NaF, 1 mM dithiothreitol, 5 μg ml−1 leupeptin and 10 μg ml−1 aprotonin). For GST pull-down assays, 1 mg cell lysate was incubated with 10 μg agarose-bound GST proteins for 6 h at 4°C. For immunoprecipitations, 3–4 μg cell lysate was incubated with 0.5 μg anti-Clk or anti-Ima antibody for 16 h followed by addition of 20 μl protein A/G PLUS–agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. Samples were spun down, washed four times with immunoprecipitation buffer and analysed by SDS-PAGE.

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**Author contributions**

E.P. performed the experiments and analysed the results. G.Z. designed the study and wrote the paper.

**Additional information**

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

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