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Centrosomal MPF triggers the mitotic and morphogenetic switches of fission yeast

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Abstract

Activation of mitosis promoting factor (MPF) drives mitotic commitment[1]. In human cells active MPF appears first on centrosomes[2]. We show that local activation of MPF on the equivalent organelle of fission yeast, the spindle pole body (SPB), promotes Polo kinase activity at the SPBs long before global MPF activation drives mitotic commitment. Artificially promoting MPF or Polo activity at various locations revealed that this local control of Plo1 activity on G2 phase SPBs dictates the timing of mitotic commitment. Cytokinesis of the rod shaped fission yeast cell generates a naïve “new” cell end. Growth is restricted to the experienced old end until a point in G2 phase called “New End Take Off” (NETO) when bipolar growth is triggered[3]. NETO coincided with MPF activation of Plo1 on G2 phase SPBs[4]. Both MPF and Polo activities were required for NETO and both induced NETO when ectopically activated at interphase SPBs. NETO promotion by MPF required polo. Thus, local MPF activation on G2 SPBs directs polo kinase to control at least two distinct and temporally separated, cell cycle transitions at remote locations.

Keywords

Polo kinase; Centrosome; *S. pombe*; NETO; MPF; Cdk1; Mitosis

Mutations in the SPB component Cut12 display a reciprocal relationship with mutations in the MPF activating phosphatase Cdc25. The loss of function mutation *cdc25.22* is suppressed by gain of function mutations in *cut12*, such as *cut12.s11*[5,6]. Conversely, increased Cdc25 levels suppress the loss of function mutant *cut12.1*[7]. The polo kinase Plo1 associates with the SPB towards the end of G2 phase. The timing of this recruitment is advanced in *cut12.s11* cells[4,8]. The antibody MPM2 recognizes SPBs in a Plo1 dependent manner[8] and so offers an ideal tool with which to assess the local activity of Plo1 on the SPB. Unlike bulk *in vitro* kinase assays, MPM2 assessment of Plo1 activity on SPBs is

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Contribution statement
IMH conceived the study. AK and VS generated *cdc2.as*. AP generated the conditional polo kinase alleles, including Plo1.RL. VAT and KYC did the imaging work in figure 1. AG performed the remainder of the experiments with occasional assistance from AP and KYC for more complex experiments and cloning. IMH wrote the manuscript with input and discussions from all authors.

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independent of any changes in Plo1 activities at any other locations. MPM2 SPB staining indicates that the Plo1 that associates with SPBs during G2 phase of both wild type and *cut12.s11* cells is active[8]. Prompted by the fact that the enhancement of Plo1 activity that accompanies mitotic commitment is driven by MPF activation[9], we now address the function of this SPB associated Plo1 in mitotic control.

MPM2 or Plo1 immunofluorescence staining reveals single (late G2 phase), or paired foci (mitotic) of SPB staining. The ratio of one:two foci (G2:Mitotic) in cell populations gives an indication of the point in G2 at which Plo1 is recruited to (Plo1 staining), or activated at (MPM2), SPBs. Staining patterns in an established *plo1.GFP* strain[10] revealed that the fusion of GFP sequences to the carboxyl terminus of Plo1 did not alter the timing or activation of Plo1 at SPBs (Figure 1a). Time lapse imaging revealed that Plo1 was initially recruited around 30 minutes before mitosis (Figure 1a-c). This initial recruitment was advanced by a further 30 minutes in *cut12.s11* cells (Figure 1a-c; Supplementary movie 1). Fluorescence recovery after photobleaching (FRAP) revealed a rapid turnover of Plo1.GFP (half-life 21-22 sec) at the SPBs of both *cut12*$^{+}$ and *cut12.s11* cells (Figure 1d; Supplementary movie 2).

To assess the impact of MPF activity on Plo1 SPB recruitment we exploited the *cdc2.as* mutation in the catalytic subunit of MPF that renders it sensitive to inhibition by ATP analogues[11]. *plo1.GFP cdc2*$^{+}$ cells were labeled by transient immersion in fluorescent lectin[12] and mixed with *plo1.GFP cdc2.as* cells for live cell imaging. Addition of the ATP analogue 1NA-PP1 prompted the immediate loss of Plo1.GFP from G2 SPBs of *cdc2.as* cells while it persisted on the G2 SPBs of neighbouring *cdc2*$^{+}$ cells (Figure 1e, f; Supplementary Movie 3, 4). Restoration of MPF activity by removal of the analogue promoted a rapid return (Figure 1f; Supplementary Movie 4), indicating that MPF activity is continuously required for Plo1 association with the late G2 SPB.

We wanted to extend the analogue sensitive approach to assess the contribution of Plo1 activity to mitotic control in unperturbed cell cycles. In strains harbouring the *plo1.as1, plo1.as2, plo1.as3* or *plo1.as4* mutations that are predicted to confer sensitivity[13] addition of analogue had little impact upon the most sensitive read out of Plo1 function, spindle formation[14]. However, septation was compromised in the first division after the addition of 40 μM 3MB-PP1 to *plo1.as3* cells (data not shown), indicating a modest degree of sensitization[14]. We therefore studied the structure of AMP-PNP bound human Plk1[15] to seek notable differences between the structure of this mammalian kinase that can be sensitized to ATP analogue inhibition and its refractory fission yeast counterpart, Plo1. Plk1 harbours a phenylalanine at position 170 at the bottom of the ATP binding pocket that is replaced by methionine in Plo1. Mutation of this methionine to phenylalanine in a *plo1.as3* backbone created the *plo1.as8* allele that responded to 3MB-PP1 or 3-BrB-PP1 addition by arresting mitotic progression with the monopolar spindle phenotype that is a characteristic of severely compromised Plo1 function[14] (Supplementary Figure 1a,b). Addition of 20 μM 3-BrB-PP1 to G2 *plo1.as8* cells delayed the phosphorylation of histone H3 serine 10 that accompanies mitotic commitment by 40 minutes (Figure 1g, h).

Plo1 comprises a catalytic domain and two polo boxes that direct the kinase to target molecules that are either direct substrates or platforms from which neighbouring substrates can be phosphorylated[16-18] (Supplementary Figure 1c). We reasoned that Plo1 could be directed to a site of choice by replacement of the polo boxes with a surrogate targeting sequence. We replaced the polo box domain of *plo1.as8* with sequences encoding the GFP binding protein, GBP[19-21], in a transgene that was expressed from an ectopic location. We incorporated mutations conferring constitutive activation[8] into the catalytic domain of this hybrid molecule in order to over-ride the controls that normally co-ordinate Plo1 activity

with cell cycle progression (Supplementary Figure 1c). Ectopic expression of this Plo1.RL chimera in the presence of inhibitory ATP analogue targets a restrained kinase to any location that hosts GFP (Supplementary Figure 2a). Subsequent analogue removal releases sustained Plo1 kinase activity at the target site. This approach enabled us to uncouple kinase activation from its normal cell cycle context and ask which events are triggered by forced Plo1 activation at a particular location?

Plo1.RL activation at SPBs via recruitment to Cut12.NEGFP promoted mitotic commitment (Figure 2a). A lower level of mitotic induction arose from recruitment to the SPB components Pcp1.GFP and Sid4.GFP while none followed targeting to any other location (Figure 2a, Supplementary Figure 2b). Bleaching the fluorescence signal derived from either the host molecule Cut12.NEGFP or the associated Plo1.RLTom chimeric kinase revealed no turnover of the Cut12.NEGFP/Plo1.RLTom complex at SPBs (Figure 2b). We therefore asked whether the anchored Plo1.RL chimeric kinase relied upon the endogenous, mobile, Plo1 to promote mitotic commitment. *plo1.ts41* cells (Supplementary Figure 1e,f) harbouring Cut12.NEGFP anchored Plo1.RL were shifted from 25°C to 36°C to inhibit Plo1 function as the inhibitory analogue was removed. Different induction times produced different levels of Plo1.RL at this point of release. Levels of Plo1.RL (18 or 20 h induction) that were competent to induce mitosis when the endogenous Plo1 kinase was a functional wild type molecule were unable to do so when it was the incapacitated Plo1.ts41 (Figure 2c).

As MPF associates with G2 SPBs[22,23] and controls SPB recruitment of Plo1 (Figure 1e-f), we asked whether it was also responsible for generating the pro-mitotic Plo1 signal at SPBs. We tested this possibility with a Cdc2/GBP fusion protein, Cdc2.RL (*cdc2. F84GY15F* Supplementary Figure 1c) that is insensitive to either Wee1 or Mik1 inhibition[24] and sensitive to ATP analogues[11]. Activation of Cdc2.RL by analogue removal from strains in which the chimeric kinase had been recruited to a variety of locations (Supplementary Figure 3a) mirrored the Plo1.RL data: SPB recruitment via Cut12.NEGFP gave a strong induction of mitosis, weaker levels followed SPB recruitment with either Pcp1.GFP or Sid4.GFP and no induction was seen following recruitment to any other location (Figure 2d, Supplementary Figure 3b). Bleaching Cut12.NEGFP anchored Cdc2.RLTom revealed a static association with the SPBs (Supplementary Figure 3c). MPM2 reactivity of SPBs showed that the wave of mitosis that arose from activation of Cut12.NEGFP anchored Cdc2.RL was preceded by Plo1 activation (Supplementary Figure 3d). Consistently, this ability of Cut12.NEGFP anchored Cdc2.RL to induce mitosis relied upon Plo1 activity (Figure 2e).

Three further Cut12.NEGFP/Cdc2.RL experiments were informative. 1) When the *cdc2* encoding sequence retained tyrosine 15 (Cdc2.RLY15, Supplementary Figure 1c) mitosis was not induced (Figure 2f), indicating that the balance of Wee1 and Cdc25 activities normally determines the timing at which SPB bound MPF activity is enhanced to trigger mitosis. 2) Mitotic induction by SPB tethered Cdc2-RL was blocked in a *cdc2.33* background in which the bulk population of MPF within the nucleus[22,23] was inactivated (Figure 2g, h), even though Plo1 activity was promoted on the SPBs of these interphase cells (Figure 2i). Thus, division is not driven from the SPB, rather MPF activation at the SPB serves as trigger event that promotes conversion of the bulk population MPF throughout the cell[22,23] to the active state. 3) The second wave of mitosis after analogue removal was abolished by the inclusion of hydroxyurea in the replacement, analogue free, medium (Figure 2f), indicating that the checkpoint remains intact.

The point in G2 phase at which Plo1 was initially recruited to G2 SPBs is strongly influenced by cell density (Figure 1a). Consistently, nutrient provision and TOR signaling is closely tied to SPB recruitment of Plo1 in mitotic control[25-28]. This context dependency is

highly reminiscent of the way by which the timing of the G2 event NETO changes in response to changes in nutrient supply[3]. We therefore examined the relationship between Plo1 recruitment to the G2 SPB and NETO. Strikingly, the two events were coincident: in counts of 100 cells, every post-NETO G2 cell displayed a Plo1.GFP signal at its SPB while neighbouring pre-NETO cells had none (Figure 3a). The correlation between NETO and Plo1 recruitment was even maintained when Plo1 SPB recruitment was advanced by 30 minutes by *cut12.s11* (Figure 3b).

To ask whether there was a direct causal relationship between Plo1 activity and NETO, NETO was monitored when small G2 *plo1.ts41* cells (Supplementary Figure 1e,f) were shifted from 25°C to 36°C. NETO execution was severely compromised by Plo1 inactivation (Figure 3c, Supplementary Figure 1g). To determine whether it was the SPB associated pool of Plo1 that triggered NETO, we targeted Plo1.RL to various locations in *cdc10.v50* cells. At 36°C, *cdc10.v50* arrests cell cycle progression in G1 phase before NETO[3]. NETO was triggered when Plo1.RL was activated at the SPBs of *cdc10.v50* cells but nowhere else, including recruitment to the cell end itself through either Tea1.v5GFP or For3.GFP fusion proteins (Figure 3d; Supplementary Figure 2a).

The observation that G2 arrested *cdc2.33* cells undergo NETO[3], yet NETO can be triggered by Plo1 activation on SPBs (an MPF dependent event (Figure 1e-f)), prompted us to re-examine the relationship between NETO and Cdc2 with the *cdc2.as* allele. Addition of 1 μM BrB-PP1 to G2 *cdc2.as* cells mimicked temperature mediated inactivation of *cdc2.33* in having no impact on NETO yet arresting cell cycle progression in G2. However, a stronger level of inhibition (20 μM BrB-PP1) did block NETO (Figure 4a, Supplementary Figure 4), indicating that the threshold of MPF activity required for NETO was lower than that for mitotic commitment.

The contribution of SPB associated MPF to the promotion of NETO cannot be assessed by the Cdc2.RL approach in wild type cultures because the immediate induction of mitotic commitment (Figure 2d) obscures any impact upon NETO. We therefore blocked the propagation of the mitotic commitment signal with the *cdc2.33* mutation (Figure 2h) before assessing NETO induction. Hydroxyurea treatment enriched the pool of S phase arrested (pre-NETO[3]) cells prior to temperature shift from 25°C to 36°C for 1 hour to inactivate Cdc2.33 before analogue removal activated the SPB associated Cdc2.RL (Figure 4b). NETO was triggered by Cdc2.RL activation on the SPB, but at no other location. Recruitment to Cut12 had a greater impact than to Pcp1 or Sid4 (Figure 4c, Supplementary Figure 3e). Critically, this ability of Cut12.NEGFP anchored Cdc2.RL to induce NETO was reliant on Plo1 activity (Figure 4d). It also required the polarity factors Tea1, Bud6 and Sla2 (Figure 4e).

We propose that Plo1 recruitment to SPBs in G2 phase both triggers the cell cycle dependent morphogenetic switch to bipolar growth and constitutes a priming event that licenses the cell to regulate the timing of mitotic commitment. The MPF dependency and activity of the Plo1 recruited to these G2 SPBs suggests a local activation of the feedback loops that will later be employed at remote locations to drive the global cell cycle during mitotic commitment (Figure 5a). Because NETO happens as soon as the feedback loop is running on the SPB the target/s for Plo1 in NETO control probably differ from those in G2/M commitment control. The fact that it is only constitutively active forms of MPF that can override the normal controls to induce mitosis and NETO when targeted to the SPB (Figure 2f, Supplementary Figures 2d, e, 3f) indicates that Wee1 activity blocks the propagation of the mitotic commitment signal from the SPB. Once the restraining activity of Wee1 is removed/ overcome cells can execute mitosis. Given the relative location of the different signaling components, it would seem likely that the anti-mitotic activity of the equatorial belt of

inhibitory Cdr1/Cdr2/Wee1 nodes that encircle the nucleus blocks the propagation of the signals emanating from the SPB (Figure 5b)[29,30].

The well documented ability of TOR signaling to influence the timing of mitotic commitment in fission yeast relies upon its ability to promote Plo1 recruitment to the SPB[25-28], indicating that, in some situations, enhancing the level of Plo1 signaling from the SPB is sufficient to advance the timing of mitotic commitment. Perhaps mitotic commitment is also finally triggered when Plo1 activity exceeds a critical threshold in unperturbed cell cycles.

Because NETO ensures that each daughter cell will receive an experienced “old” cell tip that will be optimal for growth in the subsequent cell cycle, Plo1 recruitment to G2 SPBs could be seen as the first phase of mitotic commitment. However, the question remains as to how a signal that emanates from the cell equator triggers an event at the cell extremity (NETO). A recent study may hold the clue[31]. The Cdc42 activation that drives tip growth is not a persistent signal at one end. Rather it alternates between the two cell tips throughout interphase. Thus, Cdc42 is activated at new ends every 3 minutes throughout early G2 up to NETO. However, NETO is only triggered once global Cdc42 activity exceeds a critical threshold level of global Cdc42 activation. A general signal emanating from the SPB could set the level of this general threshold by targeting Cdc42 or its regulators globally.

Given the impact of TOR and stress pathways upon SPB recruitment of Plo1[26,27,32] and the co-ordination of cytokinetic ring formation and function by a second cell cycle regulatory network on the same SPBs (the Septum Initiation Network[33]), we propose that the SPB (and by implication the centrosome) acts as a platform at which signals from diverse signaling pathways are integrated to generate, coordinated and coherent signals to control cell cycle progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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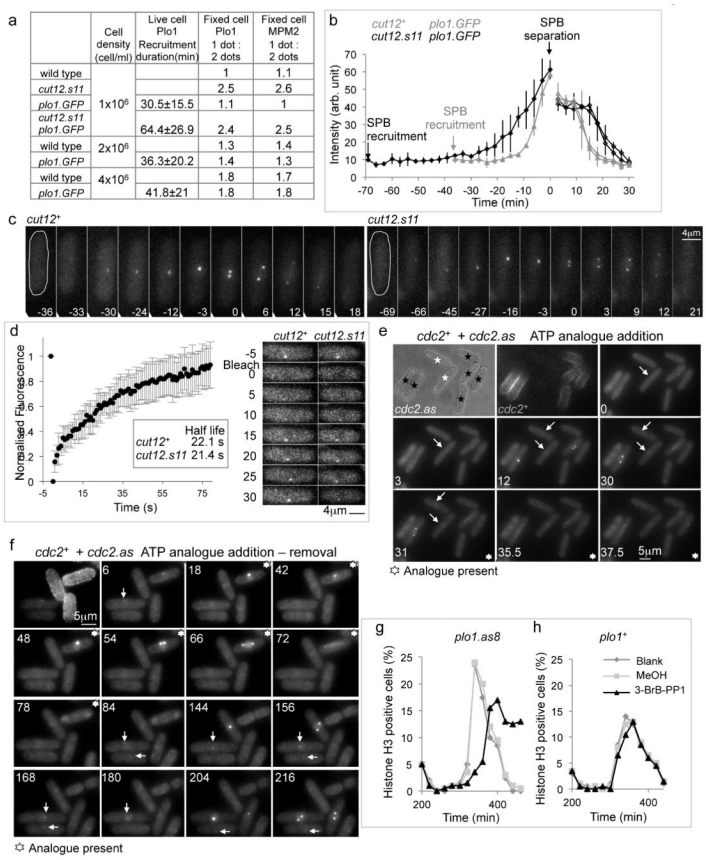

Figure 1. MPF activity controls Plo1.GFP recruitment to the SPB in late G2 phase

a) Timing of Plo1.GFP recruitment before SPB separation (living cells) and the ratios of 1 dot (G2, early mitotic SPB) to two dots (mitotic SPB) MPM2 staining. b) Plots of the intensity of the Plo1.GFP signals averaged for three cells. In each case the two plots of different shading after SPBs split indicate the signals arising from two individual SPBs that emerge from this single focus of paired SPB signals in G2. intensity of the two (see also Supplementary movie 1). (c) Time lapse images of *plo1.GFP* and *cut12.s11 plo1.GFP* cells. (d) FRAP of the signal from a late G2 Plo1.GFP SPB in wild type cells (left). The FRAP profile of a *cut12.s11 plo1.GFP* strain was indistinguishable from this wild type plot (right; see also Supplementary movie 2). e, f) Wild type (TRITC-lectin pre-treated) and analogue sensitive (*cdc2.as*) cells (no coating) recorded side by side in the same field of view at 25°C. The timing the addition of 25μM 1NA-PP1 is indicated in the panel. Control *cdc2*$^+$ cells advanced through mitosis irrespective of the presence of analogue with Plo1.GFP on their SPBs while Plo1.GFP signal left the SPBs of neighbouring *cdc2.as* mutant cells following analogue addition. White arrows indicate Plo1.GFP signals on SPBs. See also Supplementary movies 3, 4. g, h) Size selected cultures of either *plo1.as8* (g) or *plo1*$^+$ (h) cells were split into three following completion of the first synchronous division after size selection and treated as indicated in the legend to the graphs before processing for immunofluorescence with phospho-histone H3 serine 10 antibodies. The analogue 3-BrB-PP1 was added to a final concentration of 20 μM.

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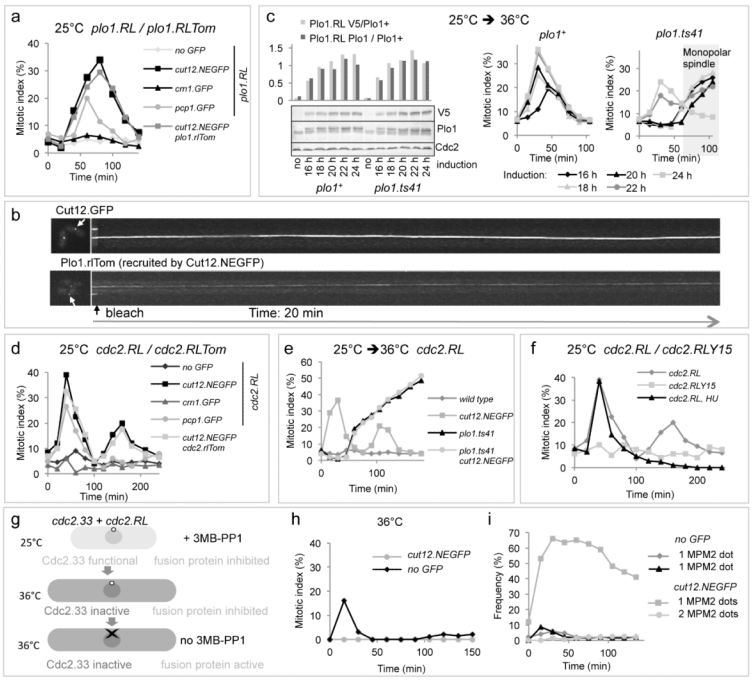

Figure 2. Ectopic activation of either Plo1 or MPF on G2 SPBs promotes mitotic commitment

a) 3-MB-PP1 removal from strains hosting a de-repressed *plo1.RL* allele (see also Supplementary Figure 2b). *crn1*$^{+}$ encodes the actin binding protein coronin, *pcp1*$^{+}$, pericentrin. b) Photobleaching of Cut12.NEGFP and Cut12.NEGFP anchored Plo1.RLTom. c) Expression of *plo1.RL* was induced in *cut12.NEGFP plo1*$^{+}$ or *cut12.NEGFP plo1.ts41* backgrounds by removal of thiamine at t=0. Levels of the endogenous and chimeric Plo1 molecules were assessed by Plo1 polyclonal antibodies while V5 monoclonal antibodies detected Plo1.RL alone. Cells were shifted from 25°C to 36°C at t=0 immediately after filtration into analogue free medium. Tubulin immunofluorescence revealed the mitotic index. d) 3-MB-PP1 removal from strains hosting a de-repressed *cdc2.RL* allele (for recruitment to other locations see Supplementary Figure 3b). e) 3-MB-PP1 removal from *cdc2.RL* expressing strains indicated that MPF activation relied upon Plo1 function to drive mitotic commitment. The accumulation of mitotic *plo1.ts41* cells from 60 mins reflected the block to mitotic progression that arose from Plo1 inactivation (Supplementary Figure 1e, f). f) 3-MB-PP1 removal from a *cut12.NEGFP* strains hosting either a de-repressed *cdc2.RL* or *cdc2.RL Y15* chimera. For *cdc2.RL* HU the analogue free medium that was substituted at t=0 to release the kinase activity contained 10 mM hydroxyurea to block DNA synthesis. g) A cartoon depicting the scheme used to generate the mitotic index (h) and MPM2 staining data in i. h) MPF activation via *cdc2.RL* expression and analogue release promoted a modest level of mitotic commitment in *cdc2.33* cells at 36°C when it was free to diffuse throughout the cell (*cut12*$^{+}$), but not when restricted to the SPB (*cut12.NEGFP*). i) MPM2 staining demonstrating that MPF activation on *cdc2.33* SPBs at 36°C triggered Plo1 activation on SPBs even though cells did not enter mitosis.

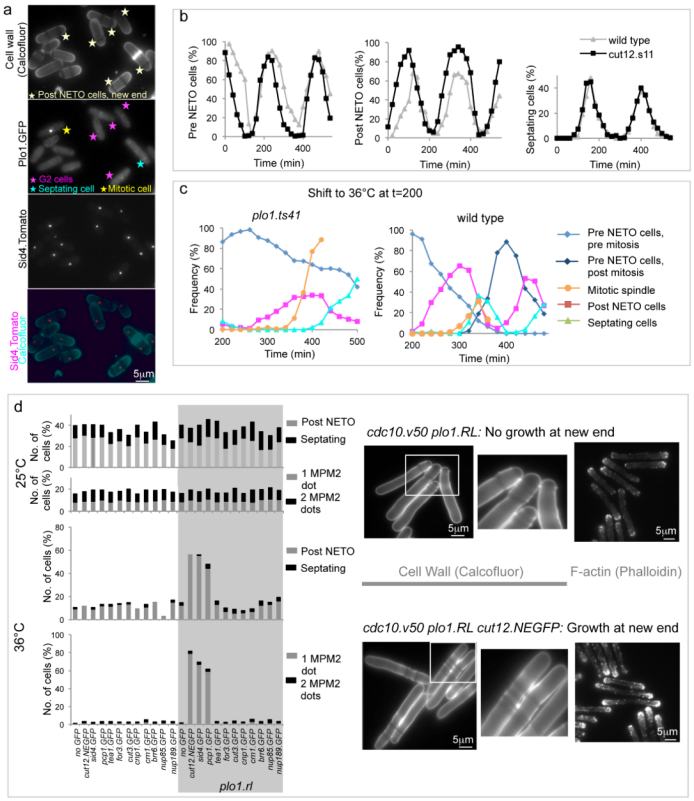

Figure 3. NETO is triggered by Plo1 activation/recruitment on the G2 SPB

a) Fluorescence imaging of cell wall signals (calcofluor, top), Plo1.GFP and Sid4.Tom of living cells of the indicated strains. Arrows identify cells with Plo1.GFP signals on SPBs. b) Size selected small G2 cells were fixed and scored for NETO status and septation index as they transited the cell division cycle. c) Cultures in which cell cycle progression had been synchronized by the selection of small cells at t=0 were allowed to transit one cell cycle (Supplementary Figure 1g) before the temperature was shifted to 36°C and the frequency of NETO and mitotic spindle index scored in fixed cells. As indicated in Figure 1g, inactivation of Plo1 delayed mitotic commitment in the *plo1.ts41* panel. Despite this delay, over 60% of cells failed to undergo NETO by the time the mitotic index rose sharply as cells accumulate with monopolar spindles (Supplementary Figure 1f). d) Mutants harbouring *cdc10.v50* and the indicated GFP fusion genes were grown to early log phase at 25°C before the *plo1.RL* allele was induced by removal of thiamine in the presence of 40 μM 3MB-PP1. After dilution and a further 24 hours at 25°C the early log phase cells were filtered into analogue and thiamine free medium and split into two, one half being kept at 25°C, the other being shifted to 36°C (lower). 5 hours later cells were fixed and the frequency of NETO scored (left). The panels on the right show examples of the wild type control (upper) and *cut12.NEGFP* (lower) cdc10.v50 strains after incubation at 36°C stained with Calcofluor to highlight cell wall material or TRITC phalloidin to stain F-actin.

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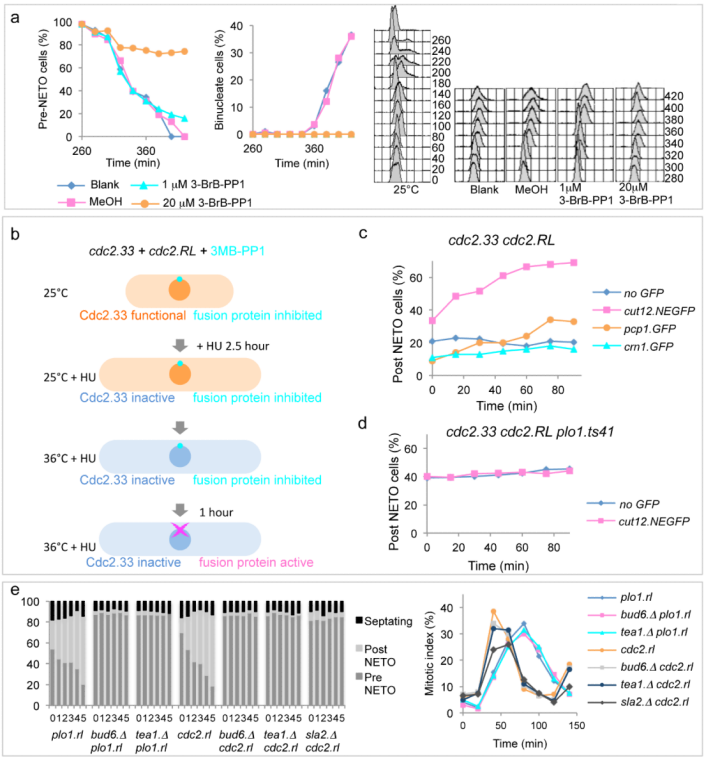

Figure 4. MPF activation on the SPB triggers NETO in a Plo1 dependent manner

a) Cell cycle progression was synchronized by the selection of small, G2, *cdc2.as* cells at 25°C. After transit of one cell cycle at 25°C (Supplementary Figure 4a) the culture was split into four equal aliquots which were all shifted to 32°C. Either 1 or 20 μM 3-BrB-PP1, solvent alone or nothing were added before NETO was scored in fixed samples at the indicated intervals. Samples were also processed for FACS analysis to monitor DNA content (right). For a repeat of this FACs analysis see Supplementary Figure 4c. b) The scheme used to assess whether analogue removal from strains harbouring *cdc2.RL* and the indicated GFP targeting proteins was able to induce NETO when the bulk population of MPF in the cell was inactivated by incubation of *cdc2.33* cells at 36°C. c, d) While NETO was induced when Cdc2.RL was targeted to the SPB by Cut12.NEGFP (c), no induction occurred when Plo1 kinase was inhibited (d). e) Left *cut12.NEGFP* cells containing the indicated additional mutations were treated as for Figure 3d except that cells were maintained at 25°C throughout. The time interval is in hours. Activation of Plo1 or Cdc2 on the SPB did not induce NETO in the polarity mutants *bud6.Δ, tea1.Δ* or *sla2.Δ* despite the fact that processing of the *cut12.NEGFP* mutant strains as described for Figure 2a, revealed that none of them compromised the ability of activation of either kinase on SPBs to induce mitosis (right).

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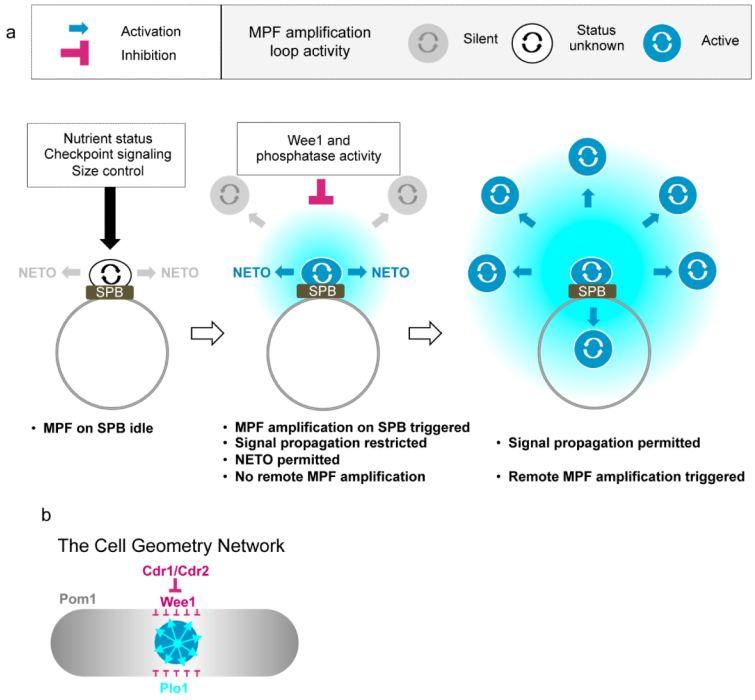

Figure 5. Model of Feedback Loop activation on the SPB and its subsequent propogation throughout the cell to promote mitotic commitment

a) The absence of a readout for MPF activity on the SPB in early G2 means that it is not possible to propose activity at this site before activation/enhancement promotes Plo1 turnover in late G2 (left). MPF feedback loop enhancement/activation on the G2 SPB subsequently triggers the pathway that ultimately leads to NETO, yet this level of G2 activation is insufficient to trigger mitotic commitment (centre). Mitosis is triggered later when there has either been a reduction in the level of inhibition by Wee1 or phosphatases such as PP2A and the Cdc14 homologue Flp1/Clp1 or loop activity is enhanced to exceed an inhibitory threshold, or a combination of the two (right). It is likely that the different pathways that impact upon the timing of mitotic commitment will target different parts of this switch. (b) The spatial organization of Plo1 activation on the SPB relative to the Pom1/Cdr1/Cdr2 pathway that couples tip extension to mitotic commitment[29,30]. Note that the close relationship between the position of the Cdr1/Cdr2/Wee1 nodes and the position of the nucleus that is established by the cycles of nuclear import/export of Mid1 places this inhibitory network directly over the SPB, putting it in the optimal location to dampen/neuter the pro-mitotic signal emerging from the SPB.

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