Aurora promotes cell division during recovery from TOR-mediated cell cycle arrest by driving spindle pole body recruitment of Polo

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Summary

The coordination of cell division and growth in response to changes in nutrient supply is mediated by TOR signalling. In fission yeast, increased nutrient provision transiently delays mitotic onset without affecting growth. The result is an increase in cell size at division. We find that this block to cell division relies upon TOR and MAPK signalling and that mitotic entry during recovery from this block is regulated by the Aurora kinase Ark1. We show that Ark1 phosphorylation of polo kinase Plo1 within the linker region between the kinase domain and polo boxes drives Plo1 onto the spindle poles where it promotes mitosis. Interestingly, the use of Ark1 to phosphorylate Plo1 and promote mitotic entry is dependent on the environment.

Key words: Aurora kinase, Mitotic entry, Nutrient, Polo, TOR kinase

Introduction

Commitment to mitosis and cell division is driven by a protein kinase complex known as mitosis promoting factor (MPF: Cdc2–cyclin B). Interphase MPF activity is restrained as a consequence of phosphorylation by Wee1-related kinases. The satisfaction of a range of criteria for cell division drives the reversal of this phosphorylation by Cdc25 phosphatase to promote division (Nurse, 1990). In this way, the balance of Wee1 and Cdc25 activities determines the timing of mitotic commitment. Following MPF activation, additional mitotic kinases assist in executing mitotic commitment. Two such kinases, Aurora A and Polo, have essential roles in the timing of mitotic entry. In fission yeast, the single Aurora kinase, Ark1, is a homologue of both its higher eukaryotic counterparts, Aurora A and Aurora B. Importantly, it carries out the functions of both kinases. Its similarity to Aurora A, in driving mitotic entry (Carmena et al., 2009; Glover et al., 1995), is manifested by its requirement for spindle formation and its ability to influence the timing of mitotic commitment. Complete loss of Ark1 delays mitotic entry (Petersen et al., 2001), whereas overexpression advances mitotic commitment to promote cell division at a reduced size (Petersen and Hagan, 2003).

The role of Polo kinases in the timing of mitotic entry and cell division is generally conserved (Archambault and Glover, 2009). The control of Polo recruitment to its target molecules and substrates appears to be a major mechanism by which Polo kinase signalling is regulated. A number of these controls are intrinsic to Polo itself. Polo kinases have a characteristic docking domain for phosphorylated serine and/or threonine residues, called the ‘Polo-box’ domain (PBD) in their C-termini (Elia et al., 2003). Additional phosphorylation-independent substrate interaction motifs exist alongside the phosphorylation-dependent docking motifs within this PBD (Miller et al., 2009). Outside the PBD, N-terminal catalytic and linker (between the kinase domain and the PBD) regions have also been shown to influence Polo localisation (García-Alvarez et al., 2007; Petersen and Hagan, 2005). In fission yeast, it is the phosphorylation of serine 402 (S402) in the linker region that increases the affinity of the Polo kinase, Plo1, for the spindle pole bodies (SPBs) to control mitotic commitment (Petersen and Hagan, 2005).

Control of cell proliferation is highly integrated with the cellular environment. When cells commit to mitosis and cell division, cell growth is blocked (Mitchison, 2003). Thus, cell size homeostasis is achieved by adjusting MPF activation through the balance of Wee1 and Cdc25 activities, to suit particular growth conditions. A key environmental factor influencing this balance is the level of nutrient available (Dolznig et al., 2004; Fantes and Nurse, 1977; Jorgensen and Tyers, 2004; Kim et al., 2002; Mitchison, 2003). Target of rapamycin (TOR) kinases are key nodes in nutrient sensing in many eukaryotes, such that inhibition of TOR activity reduces cells size at division (Wullschleger et al., 2006). In fission yeast, changing the nitrogen source from glutamate to proline (good to poor), termed ‘nutrient stress’, inhibits TOR signalling. The decline in TOR signalling during this nutrient stress boosts mitogen-activated protein (MAP) kinase (Sty1) signalling. Increased Sty1 signalling drives the phosphorylation of S402 in the linker region of Plo1, which increases the affinity of Plo1 for the SPBs and promotes MPF activation and cell division (Hartmuth and Petersen, 2009; Petersen and Hagan, 2005; Petersen and Nurse, 2007). Similarly, cell number increases following mild rapamycin treatment of Drosophila cell cultures and wings (Wu et al., 2007), and rapamycin or nutrient-induced inhibition of TOR reduces cell size at division in proliferating mammalian cells (Fingar et al., 2002; Kim et al., 2002).
Many environmental signals arrest cell division, but only temporarily. A key feature of the cell cycle following the recovery from environmentally induced arrest is that it is a modified type of mitotic commitment, in that it needs to be actively promoted. Activation of the human Polo-like kinase, PLK1, drives cell division following repair of DNA damage (Macurek et al., 2008). The phosphorylation of the activating T-loop residue by Aurora A kinase plays an important role in the recovery. However, because the functional phosphorylation-mimicking Plk1-T210D mutation only partially complements an Aurora A depletion, this modification is unlikely to be the sole means by which PLK1 is regulated to promote mitosis following DNA damage.

In this study, we characterise the impact of nutrient-induced arrest of mitosis and cell division in fission yeast, Schizosaccharomyces pombe. We find that a TOR and MAP kinase-dependent transient delay to cell division is followed by recovery and division at an increased cell size. We find that Ark1 phosphorylates S402 in the non-catalytic linker region of Plo1 to promote its localisation to SPBs and thereby controls mitotic commitment in the recovery phase. The presence of similar Aurora kinase consensus phosphorylation sites at similar locations within the linker regions of Xenopus and human Polo-like kinases suggests that this control of Polo by Aurora might be generally conserved.

**Results**

**Nutrient enrichment transiently blocks mitotic entry to increase cell size at division**

Nutrient stress alters the timing of mitotic commitment in fission yeast such that a reduction in nutrient quality accelerates mitotic commitment, leading to a concomitant reduction in cell size at division (Fantes and Nurse, 1977). To further examine the control of mitotic commitment and cell division, we investigated the converse of nutrient stress and looked at the impact of enriching the nutrient source upon the timing of cell division. We call this switch ‘nutrient enrichment’.

Wild-type cells were subjected to nutrient enrichment by shifting them from a poor nitrogen source (proline) to a good nitrogen source (glutamate). In cultures synchronised by size selection, commitment to mitosis and cell division was delayed by nutrient enrichment (Fig. 1A). In asynchronous cultures, this delay in mitotic entry and cell division was seen immediately as a transient drop in the frequency with which dividing cells could be found in the population (Fig. 1B) (Fantes and Nurse, 1977). Fission yeast cells only turn off tip growth upon commitment to mitosis and cell division (Fantes and Nurse, 1977). To further examine the control of mitotic commitment and cell division, we investigated the converse of nutrient stress and looked at the impact of enriching the nutrient source upon the timing of cell division. We call this switch ‘nutrient enrichment’.

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We recently demonstrated that a change in TOR signalling controls mitotic entry in fission yeast and that Tor1 kinase activity is lower in medium containing proline, a poor nitrogen source, than in medium containing glutamate, a better nitrogen source (Petersen and Nurse, 2007). Thus, the nutrient stress drives a reduction in TOR signalling to advance the timing of mitotic commitment. To examine the role of TOR signalling during nutrient enrichment, we inhibited TOR kinase activity with the specific inhibitor rapamycin. Rapamycin abolished the mitotic delay that normally accompanies nutrient enrichment, and cell division continued at the smaller size at which cells divided in proline before nutrient enrichment (Fig. 1D). Thus, the
glutamate-induced cell cycle arrest and delay in cell division, which increased cell size, appears to be dependent on an increase in TOR signalling (Fig. 1D).

MAP kinase signalling pathways are key signalling networks by which cells adjust their growth and division with changes in their environment. In fission yeast, the timing of mitotic commitment is acutely sensitive to alterations in the activity of the stress MAP kinase, Sty1 (Hartmuth and Petersen, 2009; Shiozaki and Russell, 1995). Because TOR signalling modulates Sty1 activity to control mitotic onset in response to nutrient stress (Petersen and Nurse, 2007), we investigated whether Sty1 signalling was required for the TOR-mediated cell cycle arrest following nutrient enrichment. Subsequent to nutrient enrichment, in contrast to wild-type cells, sty1Δ mutant cells failed to fully delay the timing of mitotic onset and induce a transient block to cell division (Fig. 1E), indicating that Sty1 signalling is indeed required for the nutrient-induced mitotic arrest.

**Ark1 activity promotes mitotic entry following nutrient induced cell cycle arrest**

Because Aurora kinases play an important role in promoting mitosis, we next assessed the impact of inhibiting fission yeast Ark1 activity upon recovery from nutrient-induced cell cycle arrest. We generated an analogue-sensitive *ark1* allele (*ark1.as3*) in which the kinase domain had been modified (Zhang et al., 2005) to allow inhibition with the ATP analogue 1NM-PP1 (a cell permeable inhibitor of kinases that have been mutated to be ‘analogue sensitive’).

Synchronous wild-type and *ark1.as3* cultures were subjected to the nutrient enrichment, and cell cycle progression was followed in both cultures. The timing of mitosis and cell division was identical in the two cultures (Fig. 2A), indicating that the mutations within the *ark1.as3* allele have no impact upon mitotic commitment in the absence of the ATP analogue.

Next we split an *ark1.as3* culture into four aliquots, and each was treated in one of the following ways: solvent alone or 1NM-PP1 was added, either 20 minutes before the nutrient enrichment or at the time of nutrient shift. Although inhibiting Ark1 had no effect on the initial block, strikingly the recovery from the cell cycle arrest was delayed by 70 minutes (Fig. 2B). As a control for the impact of 1NM-PP1 upon cell division under these circumstances, 1NM-PP1 was also added to nutrient-enriched wild-type cultures. Surprisingly, cell division was delayed by approximately 30 minutes in unmodified wild-type *ark1+* cultures (Fig. 2C); this cell cycle delay was also observed in a synchronous wild-type culture to which 1NM-PP1 was added (Fig. 2D). Together these results indicated that in wild-type cells 1NM-PP1 reduced the activity of a kinase required at the G2–M transition in which the kinase domain had been modified (Zhang et al., 2005) to allow inhibition with the ATP analogue 1NM-PP1 (a cell permeable inhibitor of kinases that have been mutated to be ‘analogue sensitive’).

Fig. 2. Inhibition of Ark1 kinase activity results in delayed recovery from a nutrient-induced cell cycle arrest. (A) Cultures of JP350 (wt) and JP612 (*ark1.as3*) cells were synchronised by centrifugal elutriation in EMM-P to isolate early G2 phase cells that were then washed and resuspended in pre-warmed EMM-G or EMM-P for the control. Samples were taken every 20 minutes to score the frequency of dividing cells. (B) Early exponential cultures of JP612 (*ark1.as3*) cells were shifted from EMM-P to EMM-G, and 1NM-PP1 was added either at the time of the shift (0’) or 20 minutes before (–20’). For controls, media-shifted cells were treated with the same volume of solvent (added at 0 or 20 minutes before the media shift). Samples were taken at the indicated time points to score the frequency of dividing cells. (C) Early exponential cultures of JP350 (wt) cells were shifted from EMM-P to EMM-G, and 1NM-PP1 or an identical volume of solvent was added at the time of the shift and samples taken at the indicated time points to score the frequency of dividing cells. (D) JP350 (wt) cells were grown in EMM-G, and G2 phase cells were isolated by centrifugation and filtration before resuspension in EMM-G. 1NM-PP1 or an equal volume of solvent alone was added at 220 minutes after harvesting. Samples were taken every 20 minutes to score the frequency of dividing cells. (E–H) Extracts were prepared from IH2117 (wt) or JP792 (*ark1.as3*) cultures that had been blocked in G2 phase by incubation at 37°C for 4 hours 30 minutes (G2 sample) and from the same cultures 70 minutes after release to 25°C to induce mitosis (mitotic sample). Ark1.PK protein was immunoprecipitated and used in combination with histone H3 as a substrate for kinase assays. Antibodies against histone H3 S10-P were used to detect Ark1 activity. 1NM-PP1 or the same volume of solvent alone was added to the kinase assays as indicated and the reaction performed for 20 minutes (E–G) or 40 minutes (H).
transition. Wild-type Ark1 kinase activity is elevated when cells commit to mitosis (Fig. 2E) (Petersen and Hagan, 2003). We therefore assessed whether 1NM-PP1 could inhibitArk1 purified from mitotic wild-type cells. Interestingly, the kinase activity of wild-type Ark1 was indeed reduced, but crucially not fully inhibited by 1NM-PP1 (Fig. 2G,H). Importantly, compared with the wild-type Ark1 kinase, the activity of mitotic Ark1.as3 was significantly further reduced (Fig. 2H). Therefore, the 30-minute delay in cell division seen in unmodified cultures following addition of 1NM-PP1 could be due to a partial reduction in wild-type Ark1 kinase activity. However, because it is unclear whether 1NM-PP1 affects other mitotic kinases possibly involved in the 30-minute cell cycle delay, this highlighted the need for 1NM-PP1 treatment of control cultures in all subsequent experiments.

Therefore, both wild-type ark1 + and ark1.as3 were simultaneously exposed to nutrient enrichment, and 1NM-PP1 was added to both cultures. When Ark1 was inhibited, the delay in the re-initiation of cell division was far greater than that seen in the analogue-treated wild-type control. This extended delay in cell cycle re-entry was observed in both asynchronous and synchronous cultures (Fig. 3A,B). Consistently, cell size at division following nutrient enrichment was increased to a much greater extent in ark1.as3 cells than in wild-type controls (Fig. 3A2).

Given that Ark1 also regulates events during mitotic progression (Petersen et al., 2001; Petersen and Hagan, 2003; Hauf et al., 2007; Vanoosthuyse and Hardwick, 2009; Tsukahara et al., 2010; Nakazawa et al., 2011), it was possible that the increase in cell length at division was a consequence of later mitotic defects rather than a delay in mitotic entry. To address this, we followed the timing of spindle assembly as a more direct readout of mitotic entry (Fig. 3C). When Ark1 was inhibited, mitotic spindle assembly was delayed in the ark1.as3 culture to a much greater extent than in the analogue-treated wild-type control. We therefore conclude that Ark1 activity controls the timing of mitotic entry during recovery from a nutrient-induced cell cycle arrest.

We next asked whether Ark1 activity also was required to promote cell division in unstressed cultures. Wild-type and ark1.as3 cultures were synchronised by centrifugal elutriation and 1NM-PP1 was added to both cultures after the first cell division. Interestingly, Ark1 inhibition has no additional effect on the timing of mitosis and cell division in an unperturbed cell cycle (Fig. 3D).

Ark1 phosphorylates Plo1 S402

In addition to Aurora A, Plo1 plays an important role in controlling mitotic entry. Our previous work on the phosphorylation of S402 in the linker region of fission yeast Plo1, established that this modification promotes mitotic commitment by driving the recruitment of Plo1 to the SPB. Importantly, the sequence around S402 in Plo1 is virtually

Fig. 3. Ark1 activity controls mitotic entry and cell length at division following nutrient enrichment. (A) Early exponential cultures of JP350 (wt) and JP612 (ark1.as3) cells were shifted from EMM-P to EMM-G and 1NM-PP1 was added at the time of the shift. Samples were taken at the indicated time points to score the proportion of dividing cells (panel 1), and cell length at division was measured at 0 and 150 minutes after the media shift (panel 2). Error bars show ± s.e.m. of 100 measurements. (B) Cultures of JP350 (wt) and JP612 (ark1.as3) cells were synchronised by centrifugal elutriation in EMM-P as in Fig. 2A, shifted into EMM-G, and 1NM-PP1 was added at the time of the shift. Samples were taken every 20 minutes to score the frequency of dividing cells. (C) Early exponential cultures of JP350 (wt) and JP612 (ark1.as3) cells were treated as in A, fixed, and mitotic cells frequency was counted as a percentage of cells with spindles (visualised by tubulin immunofluorescence; panel 1). Panel 2 shows tubulin immunofluorescence and DAPI staining of the cells of the two strains 130 minutes after media shift. Wild-type cells are in the early stages of mitosis as can be seen by the presence of short mitotic spindles and initiation of chromosome segregation (yellow arrows), while in the ark1.as3 cells the persistence of interphase microtubules arrays and absence of spindles indicates a persistent G2 arrest. (D) JP350 (wt) and JP612 (ark1.as3) cultures were synchronised by centrifugal elutriation in EMM-G, and small G2 cells were resuspended in EMM-G. 1NM-PP1 was added 220 minutes after the harvest. Samples were taken every 20 minutes to score the proportion of dividing cells.
identical to that surrounding histone H3 serine 10 (H3S10; Fig. 4A) (Petersen and Hagan, 2005). Given that Ark1 phosphorylates H3S10 and that H3S10 phosphorylation in fission yeast cells depends on Ark1 activity (Petersen and Hagan, 2003), we investigated whether Ark1 directly targets Plo1 S402 phosphorylation to promote mitotic entry in the nutrient-controlled mitotic commitment we were studying.

Ark1 kinase activity is elevated when cells commit to mitosis (Fig. 2E) (Petersen and Hagan, 2003). We therefore assessed whether Ark1 purified from mitotic fission yeast cells could phosphorylate recombinant Plo1 on S402. S402-P-specific antibodies were able to detect Ark1-dependent phosphorylation of wild-type Plo1 but not Plo1 S402A in which the target serine had been mutated to alanine (Fig. 4B), indicating that Ark1 is indeed able to phosphorylate S402 in vitro. To extend this analysis to the in vivo setting, total protein extracts were prepared from inhibited ark1Δ and ark1.as3 cells over the course of the nutrient-enrichment protocol. A decline in Plo1 S402 phosphorylation was observed in both cultures during the transient block to mitosis and cell division (Fig. 4C,D). However, whereas S402 phosphorylation then increased in the wild-type cells as they re-entered mitosis, no increase was seen in the ark1.as3 cells (Fig. 4C,D). This raised the possibility that Ark1 might control Plo1 S402 phosphorylation to promote mitotic entry during recovery from nutrient-induced cell cycle arrest.

Ark1 controls mitotic entry through Plo1 S402 phosphorylation

To directly test the hypothesis that Ark1 phosphorylation of Plo1 S402 regulates the timing of mitotic commitment during recovery from nutrient induced cell cycle arrest, fission yeast ark1.as3 plo1.S402X double mutants were subjected to nutrient enrichment. If Ark1 controls mitotic onset through Plo1 S402 phosphorylation, inhibition of Ark1 in a plo1.S402E double mutant would not delay the recovery from the cell cycle arrest, because Plo1 with a glutamic acid at residue 402 mimics phosphorylation (Petersen and Hagan, 2005). Ark1 inhibition in the ark1.as3 plo1.S402E double mutant had no impact upon the timing of mitotic commitment during the recovery phase (Fig. 5A2). Indeed, ark1.as3 plo1.S402E cells recover at the same rates as wild-type cells, indicating that this phosphomimetic mutation eliminates the requirement for Ark1 activity. Inhibition of Ark1 in the ark1.as3 plo1.S402A mutant had no additive effects (Fig. 5A3). In this mutant, the delay in cell cycle re-entry was identical to that arising from only inhibiting Ark1, indicating that Plo1 S402 is the main target of Ark1.

Plo1 localisation to SPBs is the first indication of commitment to mitosis in fission yeast (Mulvihill et al., 1999). As mentioned above, we have previously shown that Plo1 S402 phosphorylation influences mitotic commitment because it alters the affinity of Plo1 for the SPBs; specifically Plo1.S402E has an increased affinity whereas Plo1.S402A has a reduced affinity for the SPBs (Petersen and Hagan, 2005). Therefore, we next monitored the affinity of Plo1 for the SPBs following nutrient enrichment. During the recovery from the nutrient-induced arrest of mitosis, when mitotic entry needs to be actively promoted, Plo1 was recruited to SPBs (Fig. 5B). Inhibition of Ark1 reduced the affinity of Plo1 for the SPBs. Importantly, incorporation of the plo1.S402E mutation overcame this deficiency.

We conclude that Ark1 controls mitotic entry during recovery from cell cycle arrest imposed by nutrient enrichment by phosphorylating S402 of Plo1 to drive its recruitment to the SPBs, thereby promoting mitotic entry and cell division.

Reliance upon Ark1 for phosphorylation of Plo1.S402 to promote mitotic entry is context specific

When cells are exposed to an increase in temperature i.e. heat stress, mitotic onset and cell division is also transiently blocked (Nurse, 1975). We previously demonstrated that Plo1 S402 phosphorylation is required for timely recovery from this heat-stress-induced cell cycle arrest (Petersen and Hagan, 2005). We, therefore, asked whether Ark1 is responsible for S402 phosphorylation during recovery from heat stress.

Wild-type and ark1.as3 cultures were shifted from 28 to 37°C. Inhibition of Ark1 delayed the recovery and commitment to mitosis after heat stress, suggesting that it could be the S402 kinase that mediates recovery in this context as well (Fig. 6A1).
To test this possibility directly, *ark1.as3* *plo1.S402E* double mutant cells were subjected to heat stress. However, in contrast to nutrient enrichment, the phosphorylation-mimicking *plo1.S402E* mutation was unable to complement the delay in mitosis and cell division (Fig. 6A2). Consistently, inhibition of Ark1 following heat stress did not reduce Plo1 S402 phosphorylation in total protein extracts (Fig. 6B). Therefore the Plo1 S402-dependent recovery from heat-stress-induced cell cycle arrest is Ark1 independent. At this stage, the nature of Ark1-dependent control of mitotic entry following heat stress is unclear, but will be an interesting topic for future studies.

**Discussion**

We have shown here that nutrient enrichment transiently delays commitment to mitosis and cell division, thereby increasing cell size at division. We recently demonstrated that a change in TOR signalling controls mitotic entry in fission yeast (Petersen and Nurse, 2007). More specifically, we showed that inhibition of TOR signalling with rapamycin advanced mitosis to reduce cell size at division. Consistently, the nutrient enrichment described here triggered a cell cycle arrest to increase cell size, which is dependent on increased TOR signalling (Fig. 1D). MAP kinase signalling pathways are key signalling networks by which cells adjust their growth and division with changes in their environment. We recently demonstrated that the change in TOR signalling to promote mitotic entry is dependent on the MAP kinase Sty1 (Petersen and Nurse, 2007). Here we have shown that subsequent to nutrient enrichment, *sty1.1* mutant cells failed to fully delay the timing of onset of mitosis and induce a transient block to cell division (Fig. 1E), indicating that Sty1 signalling is also required for a nutrient-induced mitotic arrest.

Our previous work on the phosphorylation of S402 in the linker region of Polo established that this modification promotes mitotic commitment by driving the recruitment of Plo1 to the SPBs (Petersen and Hagan, 2003). Here we show that Ark1 controls mitotic entry during recovery from nutrient-stimulated cell cycle arrest by phosphorylating S402 of Plo1 to promote its SPB recruitment. Interestingly, Ark1 inhibition had no additional effect on the timing of mitosis and cell division in an unperturbed cell cycle when 1NM-PP1 was also added to both wild-type and *ark1.as3* cultures (Fig. 3D). However, we did find that cell division was delayed by approximately 30 minutes when 1NM-PP1 was added to wild-type cultures compared with solvent treated controls (Fig. 2C,D). In addition, we found that wild-type Ark1 kinase activity was reduced, but not fully inhibited (Fig. 2G,H). Nevertheless, whether the 30-minute delay in the cell cycle in unperturbed cultures is due to partial Ark1 inhibition or combined inhibition of additional mitotic kinases remains to be elucidated. Together, our results indicate that the threshold for Ark1 activity needed to promote mitosis is lower, or it is not required, in an unperturbed cell cycle. Thus, a high level of Ark1 activity is only required when mitosis has to be actively promoted after an environmentally induced cell cycle arrest. We conclude that Ark1 function in fission yeast resembles that of its higher eukaryotic counterpart, Aurora A, in driving mitotic entry during recovery from an environmentally induced delay of the cell cycle (Macurek et al., 2008). Importantly, the different type of stress imposed on the cells in this study suggests that Aurora kinases are generally used to ‘kick start’ mitosis after diverse environmentally induced cell cycle arrests.

**Context-specific reliance upon Ark1 to promote mitotic entry**

We have shown that cells recover from a nutrient- or heat-stress-induced mitotic arrest through phosphorylation of Plo1 S402 (Petersen and Hagan, 2005; Petersen and Nurse, 2007). The
independence of Ark1 in the recovery from heat stress was initially surprising (Fig. 6). However, on reflection there is clear evidence that kinases other than Ark1 must be able to phosphorylate S402 of Plo1. We have previously shown that phosphorylation of S402 increases before mitotic commitment in unperturbed cell cycles and that mitotic commitment is delayed by Plo1.S402A and accelerated by Plo1.S402E (Hartmuth and Petersen, 2009; Petersen and Hagan, 2005; Petersen and Nurse, 2007). However, perturbation of Ark1 had no impact upon the timing of mitotic entry in unperturbed cell cycles (Fig. 3D), therefore it cannot be the kinase acting on S402 in this context. Thus, Ark1 appears to only control Plo1 S402 phosphorylation to promote mitotic entry upon recovery from nutrient enrichment. Therefore, another kinase(s) must target the same site to coordinate different aspects of growth control with cell cycle progression. Importantly, this scenario is very similar to histone H3 S10 phosphorylation, a sequence almost identical to Plo1 S402 (Fig. 4A), as it has been shown that H3 S10 is phosphorylated by several cell cycle (stress) kinases in mammalian cells (Nowak and Corces, 2004; Soloaga et al., 2003).

Is linker region phosphorylation of Polo kinase by Aurora kinase conserved?

Previous studies in Xenopus and human cells have shown that a key rate-limiting step in the promotion of Polo-like kinase activity in driving mitotic commitment is Aurora-A-dependent phosphorylation of the activating T-loop residue (Macurek et al., 2008; Seki et al., 2008). However, whether or not Ark1 similarly targets the analogous T-loop residue of Plo1, phosphorylation on this residue cannot be rate limiting for Plo1 activation during recovery from nutrient-enhanced cell cycle delay. This is because a single mutation outside the catalytic domain (plo1.S402E) complements the delay to mitotic entry arising from inhibition of Ark1 activity (Fig. 5A). Therefore, Aurora kinases can target Polo kinases by at least two distinct mechanisms to promote mitotic commitment during recovery from environmentally induced cell cycle arrest. It might therefore be highly relevant that the T-loop phosphomimetic mutations in Plk1 were only able to partially substitute for Aurora A during recovery from DNA damage in the human system (Macurek et al., 2008). In addition, Aurora A still phosphorylates Xenopus Polo-like kinase Plx1.T201D (T201 is the T-loop) (Seki et al., 2008). Together, these data suggest that Aurora A enhances Polo activity by additional means. Sequence alignments of Plo1 with Plx1 and PLK1 of Xenopus and human cells are provocative in this respect because they reveal potential Aurora kinase phosphorylation sites at a similar location in the linker region of the two Polo-like kinases (Fig. 7). Thus, it is tempting to speculate that Aurora-A-mediated control of localised Polo activity is a generally conserved mechanism that assists in promoting mitotic entry.
**NUTRIENT CONTROL OF THE G2/M TRANSITION:**

**Cell size homeostasis.**

- **Nutrient enrichment environment**
  - G2
  - M

**Block of mitotic onset to increase cell size threshold.**

**Fig. 8. Ark1 control of recovery from a nutrient-induced cell cycle arrest.**
A model of the proposed signalling pathway that controls cell size homeostasis. Nutrient enrichment blocks mitotic onset to establish a new (increased) cell size threshold at division. This G2 arrest is dependent on TOR and Sty1 activity. Once the new size threshold is attained, the return to mitotic division is promoted by Ark1 phosphorylation of S402 that lies within the linker region of Plo1. S402 phosphorylation (P-S402) promotes Plo1 localisation to the spindle pole bodies where it regulates mitotic onset. (Arrows and lines indicate positive and negative signals, respectively.)

**Conclusions**
Fission yeast cells transiently block mitotic commitment and arrest in G2 phase when challenged with an enhanced nutrient environment. Once cells have attained the increased size appropriate for the new conditions, a cell cycle recovery pathway promotes mitotic entry. We show that nutrient-induced arrest of division relies upon signalling from both the TOR and MAP kinase networks. The return to cell division in the recovery phase is promoted by Ark1-dependent phosphorylation of Plo1 at S402 in the non-catalytic linker region of the kinase. This phosphorylation event promotes Plo1 recruitment to the SPB (Fig. 5B) (Petersen and Hagan, 2005), and thus mitosis (Fig. 8). The identification of sequence motifs similar to those surrounding S402 of fission yeast Plo1 in Polo kinases of higher eukaryotes prompt speculation that phosphorylation of Polo kinases within the linker region by Aurora kinases might be a conserved module in mitotic control.

**Materials and Methods**

**Cell cultures and strains**

Strains used are listed in supplementary material Table S1. Standard fission yeast techniques were employed throughout. Cells were cultured at 28°C in yeast extract (YES) or in Edinburgh minimal medium (EMM) with either 20 mM glutamic acid (EMM-G) or 20 mM proline (EMM-P) as a nitrogen source (Fantes and Nurse, 1977). Nutritional shift or rapamycin experiments were performed as previously described (Petersen and Nurse, 2007). Non-starved starter cultures in EMM-G were washed and grown to exponential phase in EMM-P for 40 hours. At a density of 1.8×10⁶ cells/ml (OD600=0.15), cells were washed and shifted into EMM-G. For inhibition of Ark1, as3 kinase activity, cultures were treated with 15 μM INM-PPI (Toronto Research Chemicals) immediately after changing media unless otherwise stated, re-incubated at 28°C and sampled as indicated for fluorescence microscopy or protein extraction. INM-PPI was added from a 15 mM stock solution dissolved in methanol. calc25.22 mutants were grown at 25°C under permissive conditions and at 37°C under non-permissive conditions.

**Molecular manipulations**
Two point mutations were introduced by site-directed mutagenesis to generate the ATP-analogue-sensitive ark1 allele as3 (Bishop et al., 2001). Leucine (L) 166 and S229 were both mutated to alanine (ark1.L166A-S229A). The entire mutant ark1 open reading frame was sequenced and cloned into the integration vector pINTA (Petersen et al., 2001) under the control of the nmt81 promoter (pJP134). This plasmid was digested with NotI and integrated into the leu1 locus of fission yeast. This drug-sensitive allele was combined with the ark1:LEU2 (Petersen et al., 2001) to generate strain Jp612.

Plasmids pET-41a.plo1.S402 and pET-41a.plo1.S402A containing the plo1.S402 or plo1.S402A sequences fused with the glutathione S-transferase (GST) tag sequence were constructed as follows: plo1.S402 or plo1.S402A fragments were cloned by PCR from pREP41PK-N plasmid harbouring the plo1 or plo1.S402A genes (Petersen and Hagan, 2005) as a template and subcloned into the pET-41a plasmid (Novagen) using BamHI and SalI restriction sites included in the primers 5’-GCCGGAATTCCTTTGCTTCTGCCAACTAAAATCA-3’ and 5’-GGCGGTCCGACTTATGCTTCCAATGCGATCCGATTTAAAAGCC-3’; restriction sites are indicated in bold.

**Purification of plo1.S402 and plo1.S402A peptides fused with the GST tag protein**
*Escherichia coli* strain BL21 pLysS was transformed using pET-41a.plo1.S402 or pET-41a.plo1.S402A containing the plo1.S402 or plo1.S402A peptides fused with the GST tag sequence. Expression of the fusion peptides in the *E. coli* transformants was induced by addition of 2 mM isopropylthiogalactoside (IPTG) and subsequent purification of GST-tagged fusion peptides was performed according to standard protocols. Purified peptides were used as substrates for kinase assays.

**Microscopy**
Immunofluorescence was as described previously (Hagan and Asycough, 2000). For anti-tubulin immunofluorescence with TAT1 antibodies (Woods et al., 1989), standard solvent fixation was used. TAT1 antibodies were used at a dilution of 1:500 and appropriate FITC-conjugated secondary antiserum (Sigma) at a dilution of 1:100. For all other stainings, cells were fixed in culture with 0.37% formaldehyde. Plo1 antibodies (BN184) were used at a dilution of 1:20. Calcofluor staining of septa was described previously (Moreno et al., 1991). At least 100 cells were counted for each time point. Images and cell length measurements were obtained using a CoolSNAP HQ2 CCD camera and processed with ImageJ. More than 100 dividing cells per strain were measured.

**Western blotting and kinase assay**
Total protein extracts were prepared by trichloroacetic acid (TCA) precipitation (Caspari et al., 2000), run on SDS-PAGE gels and blotted using alkaline phosphatase-coupled secondary antibodies. Protein kinase (PK)-dependent immunoprecipitation of Ark1 was performed using protein A Dynal beads (Invitrogen) with covalently coupled γ-PK antibodies, and the kinase assay was performed as described previously (Petersen and Hagan, 2005). Phosphorylation-specific antibodies at a dilution of 1:500 were used to detect phosphorylation of Plo1 S402 or Plo1 fusion peptides (Petersen and Hagan, 2005). Ark1.PK was detected with mAb336 anti-Pk (SV5) epitope antibodies at a dilution of 1:200. S10 phosphorylation of histone H3 was detected with H3SP antibodies (Upstate Biologicals) at a dilution of 1:200. Tubulin was detected using anti-α-Tat1 antibodies (Woods et al., 1989) at a dilution of 1:1500.

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