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Fission yeast Pcp1 links polo kinase-mediated mitotic entry to γ-tubulin-dependent spindle formation

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The centrosomal pericentrin-related proteins play pivotal roles in various aspects of cell division; however their underlying mechanisms remain largely elusive. Here we show that fission-yeast pericentrin-like Pcp1 regulates multiple functions of the spindle pole body (SPB) through recruiting two critical factors, the γ-tubulin complex (γ-TuC) and polo kinase (Plo1). We isolated two *pcp1* mutants (*pcp1-15* and *pcp1-18*) that display similar abnormal spindles, but with remarkably different molecular defects. Both mutants exhibit defective monopolar spindle microtubules that emanate from the mother SPB. However, while *pcp1-15* fails to localise the γ-TuC to the mitotic SPB, *pcp1-18* is specifically defective in recruiting Plo1. Consistently Pcp1 forms a complex with both γ-TuC and Plo1 in the cell. *pcp1-18* is further defective in the mitotic-specific reorganisation of the nuclear envelope (NE), leading to impairment of SPB insertion into the NE. Moreover *pcp1-18*, but not *pcp1-15*, is rescued by overproducing nuclear pore components or advancing mitotic onset. The central role for Pcp1 in orchestrating these processes provides mechanistic insight into how the centrosome regulates multiple cellular pathways.

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Introduction

The centrosome was classically defined as the primary microtubule-organising centre (MTOC) of the cell. However, recent advances of our understanding about this organelle have further linked it to many other processes, including cell-cycle transition, DNA-damage checkpoint, asymmetric cell division, and ageing (Cowan and Hyman, 2004; Hachet *et al*, 2007; Cheng *et al*, 2008). Despite the recognition of its ever-growing importance, the molecular mechanisms by which the centrosome executes its diverse roles have remained unsolved. In yeast, the centrosome equivalent spindle pole body (SPB) is also known to regulate multiple pathways. For example, in fission yeast this structure is important for spindle microtubule formation (Hagan and Yanagida, 1995; Vardy and Toda, 2000), mitotic entry (Bridge *et al*, 1998; Petersen and Hagan, 2005), SIN (Septation Initiation Network) signalling (Krapp and Simanis, 2008), and cell morphogenesis and polarity (Kanai *et al*, 2005). Despite this, we are still largely ignorant at the molecular levels as to how the SPB is involved in each distinct pathway.

Fission-yeast Pcp1 is an evolutionarily conserved pericentrin-related protein that localises to the inner plaque of the SPB throughout the cell cycle (Flory *et al*, 2002). Its orthologues, pericentrin (human beings), Spc110 (budding yeast), and centrosomin (*Drosophila*), are all linked to a role of spindle microtubule nucleation and assembly via the γ-tubulin complex (γ-TuC) during mitosis (Knop and Schiebel, 1997, 1998; Zimmerman *et al*, 2004; Muller *et al*, 2005; Zhang and Megraw, 2007). Spc110 was shown to interact with the γ-TuC (Knop and Schiebel, 1997, 1998) and its mutation leads to dissociation of the Spc110-containing complex from the main SPB structure towards the nucleoplasm (Yoder *et al*, 2005). In human and fly cells, it is shown that disrupting the function of pericentrin and centrosomin, respectively, caused γ-TuC delocalisation from the centrosome (Zimmerman *et al*, 2004; Zhang and Megraw, 2007). Mto1 is another fission-yeast SPB protein structurally similar to Pcp1. Unlike Pcp1, however, Mto1 localises to distinct cytoplasmic foci, including the outer side of the SPB, the nuclear periphery (interphase MTOC), and the medial region (equatorial MTOC), whereby it is responsible for the assembly of cytoplasmic microtubules nucleating from these sites during interphase (Sawin and Tran, 2006). In contrast to these precise analyses of Mto1, the roles for Pcp1 in cell division and microtubule formation have not been pursued except to identify that it is essential for cell viability (Flory *et al*, 2002), and it is proposed that this protein is involved in sensing proper spindle alignment (Rajagopalan *et al*, 2004).

Polo kinase plays vital roles during mitotic progression, DNA-damage checkpoint, and cytokinesis, and acts both upstream and downstream of CDKs (Nigg, 2007; Takaki *et al*, 2008; Archambault and Glover, 2009). Unlike other mitotic kinases such as CDK1 and aurora kinases, polo kinase does not have regulatory subunits that act as molecules

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responsible for targeting kinase catalytic subunits to specific cellular locations. Instead this kinase has been shown to localise by associating with proteins already recruited to a distinct cellular position (Elia *et al*, 2003). In fission yeast, as in other organisms, polo kinase (Plo1) executes myriad functions, including cell-cycle control, SIN signalling, and stress-responsive MAP kinase pathways (Ohkura *et al*, 1995; Bähler *et al*, 1998a; Cullen *et al*, 2000; Tanaka *et al*, 2001; Anderson *et al*, 2002; Petersen and Hagan, 2005). Plo1 localises to multiple sites in the cell in a cell cycle-dependent manner. For instance, Plo1 localises to the SPB, spindle microtubules, and the medial ring specifically during the M phase (Bähler *et al*, 1998a; Mulvihill *et al*, 1999). Despite this distinct cell-cycle-dependent Plo1 localisation, its spatial regulation remains largely elusive except that the involvement of the SPB component Cut12 and NIMA-related kinase Fin1 has been reported (Grallert and Hagan, 2002; MacIver *et al*, 2003).

In this study we characterise the molecular roles of Pcp1 during the cell cycle. We have found that like other members of pericentrin-related molecules, Pcp1 is required for γ-TuC recruitment to the SPB. In addition our analysis unveils unexpected roles of Pcp1 in mitotic entry and structural reorganisation of the nuclear envelope (NE) during mitosis. We further uncover that Pcp1 executes these roles by recruiting polo kinase to the mitotic SPB. We discuss the significance of our findings in view of conserved mechanisms underlying centrosome/SPB-mediated mitotic control.

Results

pcp1 mutants are defective in bipolar spindle assembly and chromosome segregation

To analyse the essential functions of Pcp1 in cell division, we isolated temperature-sensitive (ts) mutants of $pcp1^+$ using an error-prone PCR method (Figure 1A; see Supplementary data and Supplementary Figure S1). As Pcp1 was expected to perform multiple roles at the SPB, we wanted to isolate mutant alleles that were specifically defective in distinct aspects of Pcp1 functions, rather than a complete loss-of-function mutant. To this end, ts mutants that exhibited Pcp1 delocalisation from the SPB at the restrictive temperature were first excluded, as these mutants were expected to mimic Pcp1 deletions. Among six mutants screened in this manner, we focused our analysis on two representative alleles, *pcp1-15* and *pcp1-18* (Supplementary Figure S2).

In order to define the cell-cycle stage for which Pcp1 function is essential, we performed synchronous culture analyses with centrifugal elutriation. Cultures with early-G_2 cells were harvested by elutriation at 27°C, shifted up to 36°C, and cell viability was monitored as cells proceeded through the cell cycle. Both *pcp1-15* and *pcp1-18* mutants exhibited a sharp decline in viability after 100 min at 36°C, which coincided with mitotic entry (light red columns in Figure 1B). This viability drop paralleled the emergence of massive chromosomal mis-segregation (blue lines in Figure 1B and post-mitotic cells with mis-segregated chromosomes are shown in Figure 1C). This result demonstrates that Pcp1 plays an essential role in cell proliferation during mitosis.

Overexpression of Pcp1 results in spindle abnormalities (Flory *et al*, 2002). These precedent results suggest a role for Pcp1 in spindle assembly like its orthologues in other species. We then examined spindle behaviour in the *pcp1* mutants by observing GFP–Atb2 (α2-tubulin) and Sid4–mRFP (an SPB component) in live cells incubated at 36°C. Wild-type cells formed bipolar spindles with two SPBs flanking the ends of the spindle as they progressed through mitosis (top 3 rows in Figure 1D and Supplementary Movie S1). By contrast, in ~80% of *pcp1-15* and *pcp1-18* cells spindle microtubules that emanated from only one SPB (we refer to monopolar spindles hereafter) were formed (bottom 6 rows, emphasised by arrowheads at 8 and 24 min; Supplementary Movies S2 and S3). Duplication of the SPB is unlikely to be affected in these *pcp1* mutants, as we detected two separate Sid4–mRFP signals (e.g., time point 12 min; Figure 1D). Thus the results indicate that Pcp1 is required for efficient nucleation/assembly of spindle microtubules from the SPB. In addition, live-image analysis showed that mitotic progression was delayed in both *pcp1* mutants (~15 min in the wild type versus >30 min in *pcp1* mutants; Figure 1D).

Monopolar spindles in *pcp1* mutants emanate from the mother SPB

As noted earlier, the majority of *pcp1-15* and *pcp1-18* cells managed to nucleate spindles from one of the two SPBs, instead of displaying a complete lack of spindle formation (see Figure 1D). Fission-yeast SPB duplicates in a conservative manner (Grallert *et al*, 2004). We posited that mutant Pcp1 proteins would be less heat-sensitive once they are incorporated into the mother SPB prior to its duplication and separation, thereby retaining the capacity of spindle formation in an asymmetrical manner. To evaluate this possibility, we exploited asymmetric SPB localisation of Cdc7, one of the SIN kinases (Sohrmann *et al*, 1998). Cdc7 localises to both SPBs in early mitosis, but remains only on the daughter SPB in late anaphase (Figure 2A, top, wild type). Indeed, in the *pcp1* mutants, Cdc7–GFP localised to the SPBs devoid of spindles (the second and third rows), confirming that the mother SPB is the origin of monopolar spindles in these cells. In addition, compared with wild-type spindles, these mutant spindles exhibited significantly lower GFP–Atb2 signal intensities (Figure 2B). This result shows that both *pcp1* mutants exhibited compromised nucleating activities of spindle microtubules, in which only the mother SPB manages to retain nucleation capability to some extent.

γ-TuC is not recruited to the SPB in the absence of Pcp1 function

Human pericentrin and *Drosophila* centrosomin were shown to be required for γ-TuC recruitment to the mitotic centrosome (Zimmerman *et al*, 2004; Zhang and Megraw, 2007). We therefore asked whether the *pcp1* mutants exhibit γ-TuC delocalisation from the SPB at the restrictive temperature. In wild-type cells, Alp4–GFP (orthologue of the γ-TuC component GCP2/Spc97) (Vardy and Toda, 2000) localised normally to the SPB in almost all the cells (Figure 3A and B). On the contrary, ~80% of *pcp1-15* cells lost Alp4–GFP from the SPB after 4-h incubation at 36°C (Figure 3A and B). Similar results were obtained when localisation of another γ-TuC component, Alp6 (orthologue of GCP3/Spc98) (Vardy and Toda, 2000), was analysed (Supplementary Figure S3). This shows that Pcp1 is required for γ-TuC recruitment to the SPB.

Intriguingly γ-TuC localisation was largely unaffected in *pcp1-18* cells, suggesting that *pcp1-15* and *pcp1-18* mutants are defective in distinct functions, which are genetically

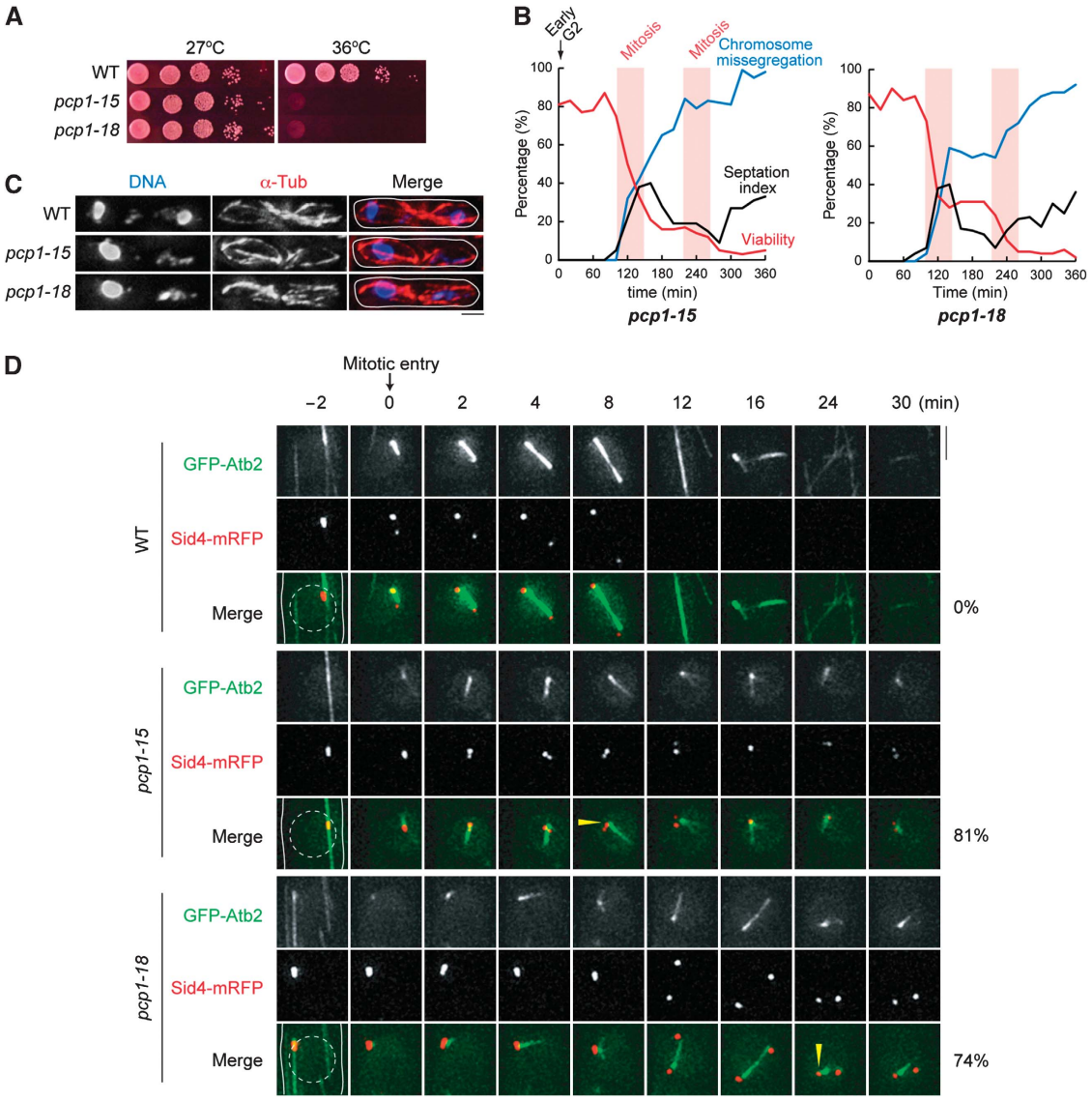

Figure 1 Two *pcp1* mutants exhibit similar mitotic defects in spindle formation and chromosome segregation. (**A**) Ten-fold serial dilution assays on rich YES media. A total of 5×10^4 cells were applied in the first spot and incubated at 27 or 36°C for 3 days. (**B**) Viability of cells in synchronous culture analyses. Small G_2 cells grown at 27°C were collected with centrifugal elutriation and shifted to 36°C at time 0. The percentage of septated cells was used to monitor cell-cycle progression. Light red columns mark periods in mitosis ($n > 100$). (**C**) Chromosome segregation defects. Immunofluorescence microscopy was performed with anti-α-tubulin antibody (TAT-1, red) and DAPI (blue) at the 120-min time point in synchronous culture experiments at 36°C as described in panel **B**. Cells completed the first mitosis, as indicated by the presence of post-anaphase arrays with two unequally segregated chromosomes. (**D**) Time-lapse fluorescence montages of spindle microtubules (GFP–Atb2, green) and SPB (Sid4–mRFP, red) structures during the first mitosis. A representative cell of each strain is shown. Note that during wild-type anaphase-B (the second row, 8 min and thereafter) SPBs became out of frame due to spindle elongation. Dashed circles outline the nuclei. Arrowheads (8 and 24 min): SPBs nucleating spindle microtubules ($n > 20$). The percentage indicates the proportion of cells displaying monopolar spindle phenotype. Live images are shown in Supplementary Movies S1–3. Scale bars, 2 μm.

separable (see below). It is of interest to point out that as shown earlier (see Figures 1D and 2A), mitotic spindles nucleate from the mother SPB despite an apparent loss of γ-TuC from the SPB. We envisage that under this condition a residual low amount of the γ-TuC at the mother SPB would manage to support the nucleation of spindle microtubules. Similar phenotypes were observed previously in temperature-sensitive *alp4* and *alp6* mutants (Vardy and Toda, 2000).

To substantiate our finding that Pcp1 is required for γ-TuC recruitment, we tested whether restoring γ-TuC localisation to the SPB could suppress the temperature sensitivity of *pcp1-15* cells. Mild overexpression of Alp4 (note that strong over-expression of Alp4 is lethal) (Vardy and Toda, 2000) restored Alp6–GFP localisation to the SPB (Figure 3C and D) and moreover indeed ameliorated the growth of *pcp1-15* cells at the semi-restrictive temperature (34°C; Figure 3E). The same condition did not confer growth advantage to *pcp1-18* cells (Figure 3E), consistent with the fact that γ-TuC localisation is not the underlying defect in this mutant. Collectively, our data demonstrate that one of the essential functions of Pcp1 lies in γ-TuC recruitment to the SPB.

Pcp1 is required for γ-TuC recruitment to the SPB during mitosis but not interphase

Previous work showed that although Pcp1 and the γ-TuC appear to colocalise to the SPB, there is a profound difference

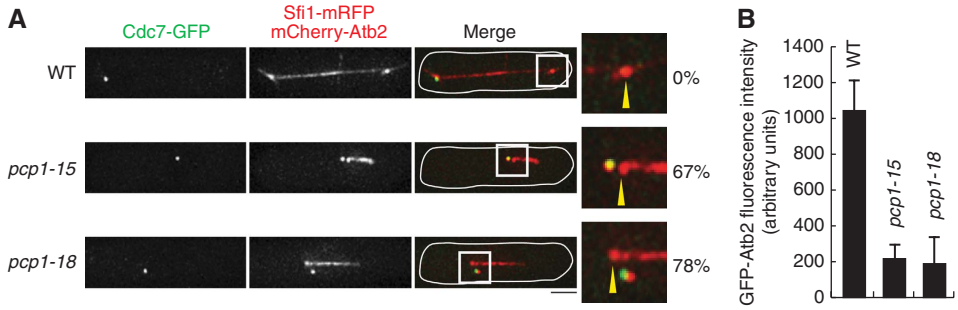

Figure 2 Only the mother SPB nucleates spindle microtubules in *pcp1* mutants. (**A**) Monopolar spindles emanating from the mother SPB. Cdc7–GFP (green) is a marker for the daughter SPB (Sohrmann *et al*, 1998). Arrowheads: Mother SPBs (negative for Cdc7–GFP). Sfi1–mRFP and mCherry–Atb2 (red) mark the SPB and the spindle, respectively. Merged images of emanating spindles from the mother SPB to which Cdc7–GFP does not localise (white squares) are enlarged on the right ($n>15$). The percentage indicates the proportion of cells displaying monopolar spindles emanating from the mother SPB. (**B**) Quantification of GFP–Atb2 fluorescence signals ($n>50$); the error bars represent standard error. Scale bars, 2 μm.

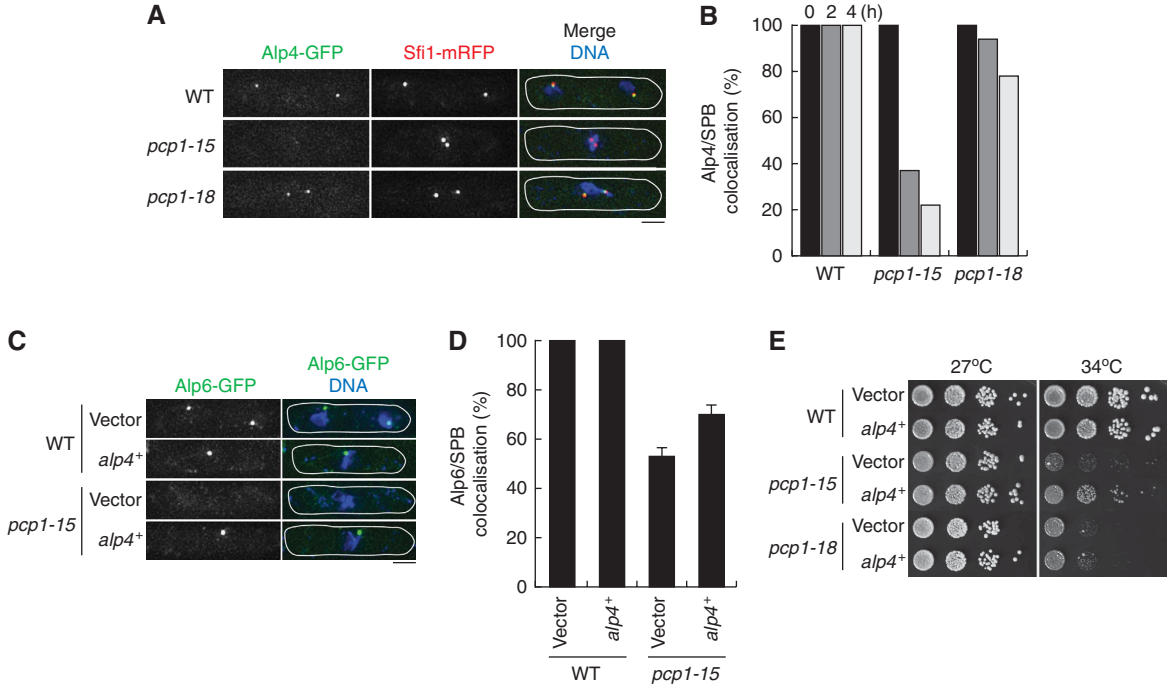

Figure 3 Pcp1 plays an essential role in γ-TuC recruitment to the SPB. (**A**) Alp4–GFP localisation after 2-h incubation at 36°C. Sfi1–mRFP is an SPB marker. (**B**) Percentage of cells with Alp4–GFP at the SPB in the experiment described in panel A ($n>100$). (**C**) Alp6–GFP localisation at the SPB with or without mild overexpression of Alp4. Alp4 was mildly overexpressed through repression of the *nmt1* promoter (pREP1–Alp4) on rich liquid YES media incubated for 4 h at 34°C. (**D**) Percentage of cells with Alp6–GFP at the SPB in the experiment described in panel **C** ($n>100$); the error bars represent standard deviation from three independent experiments. (**E**) Ten-fold serial dilution assays on YES media. Scale bars, 2 μm.

in their geometry between interphase and mitosis (Flory *et al*, 2002). While the γ-TuC localises predominantly inside the nucleus throughout the cell cycle (Ding *et al*., 1997), Pcp1 stays in the cytoplasm during interphase and enters the nucleus only upon mitotic entry. It is, therefore, conceivable that Pcp1 is only required for γ-TuC recruitment during mitosis. To clarify the temporal involvement of Pcp1 in γ-TuC recruitment to the SPB, centrifugal elutriation was performed again in the *pcp1-15* mutant and Alp4–GFP signals were followed over time at 36°C (Figure 4A, culture 1). We found that Alp4–GFP is retained at the SPB during interphase, but started to delocalise from the SPB coincidentally as *pcp1-15* cells entered mitosis (Figure 4B, 60–80 min).

To exclude the possibility that the timing of Alp4–GFP delocalisation reflects the minimum duration required for heat inactivation of the Pcp1-15 mutant protein, half of the initial culture was incubated at 27°C for a further 40 min during G_2 phase, followed by a shift up to 36°C (Figure 4A, culture 2). Similar to the previous observation, Alp4–GFP in this parallel experiment delocalised from the SPB as cells entered mitosis (Figure 4B, 60–80 min). Note that the same result was obtained in the deletion background of another pericentrin-related Mto1, which plays a critical role in the organisation of cytoplasmic microtubules (Sawin and Tran, 2006, data not shown). Taken together, our findings not only establish that Pcp1 is required for γ-TuC recruitment to the

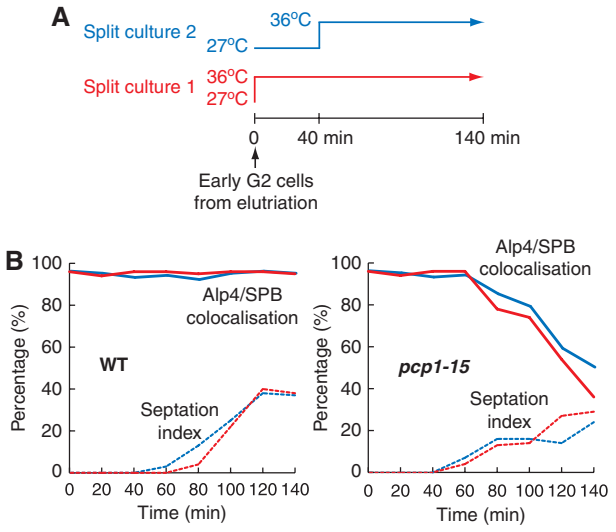

Figure 4 Pcp1 is required for γ-TuC recruitment to the SPB specifically during mitosis. (**A**) Experimental diagram. Synchronous cultures of early G_2 cells (grown at 27°C) were divided into two at time 0. One culture was immediately shifted to 36°C (culture 1, red), while the other was kept at 27°C for another 40 min, followed by shift to 36°C (culture 2, blue). (**B**) Alp4–GFP localisation at the SPB in synchronous culture experiments. Alp4–GFP signals were monitored in wild-type (left) or pcp1-15 cells (right) at each time point ($n > 100$).

mitotic SPB, but also suggest that γ-TuC recruitment during interphase is mediated by a Pcp1- and Mto1-independent mechanism. Given that centromeres are clustered in close proximity to the SPB during interphase (Funabiki *et al*, 1993), γ-TuC might be tethered to this location via interaction with some kinetochore components (Appelgren *et al*, 2003).

pcp1-18 is defective in NE reorganisation and SPB insertion during mitosis

As noted earlier, while both *pcp1-15* and *pcp1-18* cells display similar mitotic defects, unlike *pcp1-15*, γ-TuC localisation is not significantly impaired in the *pcp1-18* mutant (Figure 3A). This implies that Pcp1 plays an essential role independent of γ-TuC recruitment to the mitotic SPB. To uncover this additional role of Pcp1, we performed a multi-copy suppressor screen. Among the suppressor genes that specifically rescued *pcp1-18* were those encoding the nuclear pore complex (NPC) components—Cut11 (orthologue of Ndc1; West *et al*, 1998) and Pom152 (potential orthologue of GP210; Tran and Wente, 2006) (Figure 5A; see Table I for a complete list of suppressors). Both these proteins are transmembrane nucleoporins that constitute the inner membrane rings of the NPC (Alber *et al*, 2007b). In fission yeast that undergoes closed mitosis, Cut11 is not only an essential NPC component, but also accumulates specifically to the SPB upon mitotic onset (West *et al*, 1998). Furthermore, this protein is required for SPB insertion into the NE during mitosis (West *et al*, 1998; see Supplementary Figure S4 for Pom152 localisation).

Given the role of Cut11 in SPB insertion, we investigated whether *pcp1-18* cells fail to reorganise the NE and the SPB in mitosis. To this end, the ultrastructures were examined by electron microscopy (EM) in high-pressure-frozen cells. In wild-type mitotic cells, as reported previously (Ding *et al*, 1997), the electron-sparse NE discontinued underneath the electron-dense SPB, indicating that the NE opened up to form a fenestra for SPB insertion (Figure 5B). By striking contrast, in *pcp1-18* cells the NE underneath one of the SPBs remained intact (the SPB on the left hand side in Figure 5D and E). The examined cells are indeed in mitosis, as indicated either by NE herniation (the result of monopolar spindles prodding on the NE; Figure 5D, and see also Figure 6A) or the presence of spindle microtubules (Figure 5E).

We also observed NE and SPB structures in *pcp1-15* mitotic cells. In clear contrast to *pcp1-18*, the NE formed a fenestra normally in this mutant (Figure 5C), consistent with our suppression data showing that an increased dosage of Cut11 does not restore growth of *pcp1-15* mutants (Figure 5A). However, we noticed that the SPB was placed obliquely, rather than in parallel, above the NE, indicating that despite the apparent NE invagination, SPB insertion into the NE is somewhat compromised in this mutant. These results indicate that Pcp1 is required for NE invagination and SPB insertion during mitosis.

Plo1 recruitment to the mitotic SPB is impaired in pcp1-18

We next sought to examine whether Cut11 localisation is disrupted in *pcp1-18* cells. However, we observed no abnormalities in Cut11–GFP localisation (Figure 6A). Probing further into the underlying defect in the *pcp1-18* mutant, we inspected the list of allele-specific multi-copy suppressors again and noticed that overproduction of the essential SPB component Cut12 (Bridge *et al*, 1998) also suppressed the temperature sensitivity of the *pcp1-18* mutant (Figure 6B). We accordingly examined whether Pcp1 is involved in Cut12 recruitment to the SPB, but again Cut12–GFP localised normally to the SPB in *pcp1-18* cells at 36°C (Figure 6C). These data imply that functional activities, rather than localisation, of Cut11 and Cut12 are impaired in the *pcp1-18* mutant.

Previous work showed that Cut12 plays a role in promoting G_2/M transition by activating polo kinase Plo1 upon mitotic entry (Bridge *et al*, 1998; MacIver *et al*, 2003; Petersen and Hagan, 2005). Given this notion, we examined Plo1–GFP localisation in *pcp1-18* cells. Notably we found a significant reduction in its signal at the SPB in this mutant (Figure 6D and E). This result was confirmed by an additional experiment in which a *pcp1-15* or *pcp1-18* strain (each carrying Plo1–GFP, Sfi1–mRFP, and mCherry–Atb2) was mixed with wild-type cells (carrying only Plo1–GFP) in the same culture and shifted to the restrictive temperature. Under this condition, *pcp1-18* cells specifically lost the GFP (Plo1) signals from the SPB (the top right panel in Supplementary Figure S5), while either wild-type or *pcp1-15* cells retained the GFP–Plo1 signals (top two panels). These results demonstrate an essential role for Pcp1 in Plo1 recruitment to the mitotic SPB.

We consequently asked if potentiation of Plo1 recruitment to the SPB could suppress the *pcp1-18* mutant. For this purpose, we used a hypermorphic allele of *cut12*, *cut12.s11*, that enhances Plo1 recruitment to the mitotic SPB (Hudson *et al*, 1990; MacIver *et al*, 2003; Petersen and Hagan, 2005). Indeed, *pcp1-18cut12.s11* double mutants were capable of forming colonies at 34°C, the semi-restrictive temperature for *pcp1-18* (Figure 6F). Furthermore it was found that Plo1 is recruited to the SPB in *pcp1-18cut12.s11* cells (Figure 6H

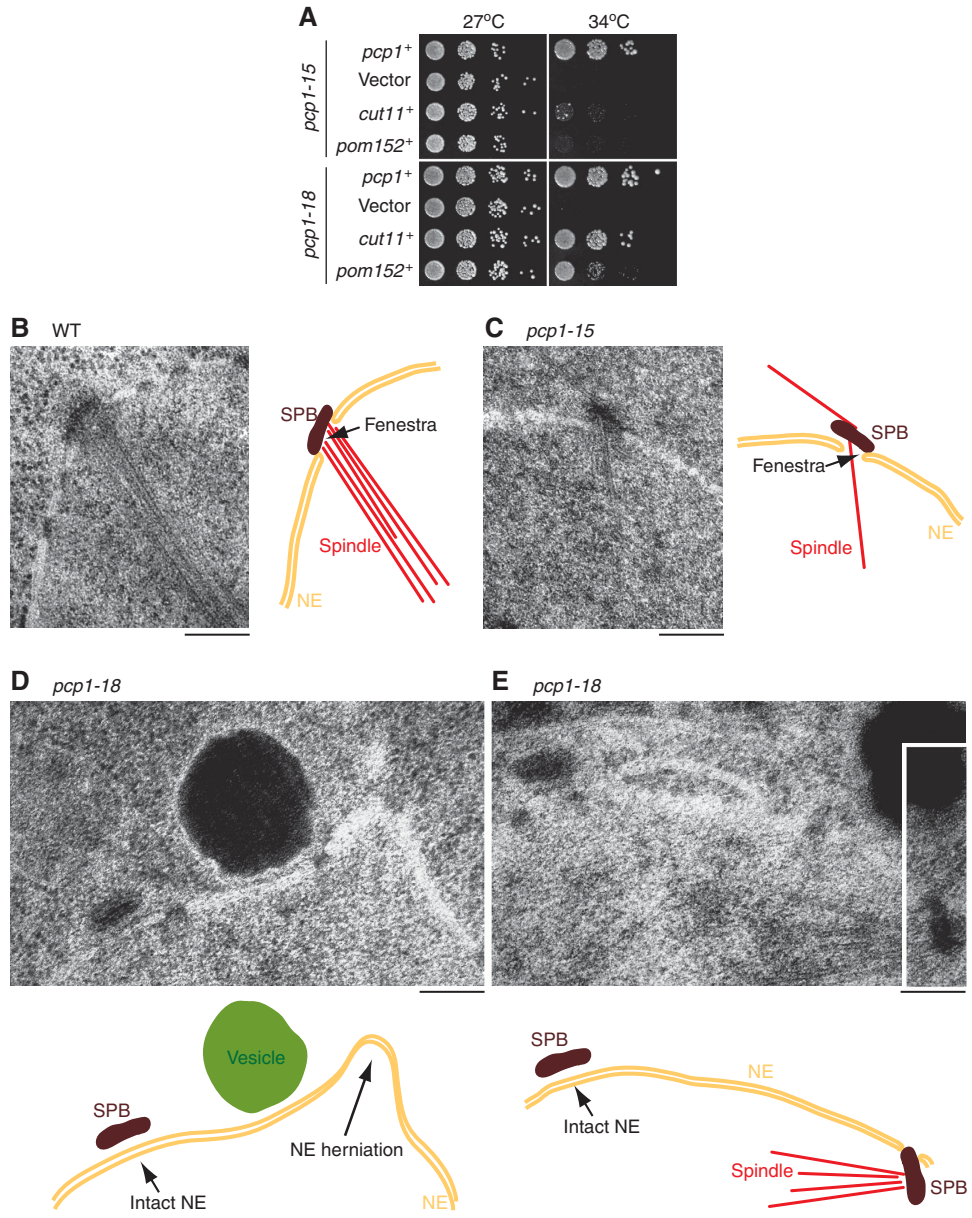

Figure 5 In the *pcp1-18* mutant, formation of a fenestra in the NE and SPB insertion are impaired. (**A**) Ten-fold serial dilutions of the *pcp1* mutants harbouring the indicated multi-copy suppressor plasmids. Cells were grown overnight in selective media at 27°C prior to spotting onto YES media and incubated for 3 days at 27 or 34°C. (**B–E**) Electron micrographs showing ultrastructures of the SPB, the NE, and spindle microtubules. Synchronised cultures (incubated at 36°C for 100 min, enriched mitotic cells) were processed for EM. Schematic diagrams are included next to or below each electron micrograph. Monopolar spindles in panel **E** originate from the SPB on the right (overlaid from adjacent section, presumably the mother SPB) that is abnormally inserted into the NE ($n = 5$) (four out of five *pcp1-18* cells showed SPB insertion defects). Scale bars, 200 nm.

Table I List of multi-copy suppressor genes of the *pcp1* mutants

Genes	Suppression	
	pcp1-15	*pcp1-18*
pcp1$^+$	Yes	Yes
cut11$^+$	No	Yes
pom152$^+$	No	Yes
cut12$^+$	No	Yes
kms2$^+$	No	Yes
tcg1$^+$	No	Yes

kms2$^+$ encodes an SPB component (Miki *et al*, 2004), while *tcg1*$^+$ encodes a potential homologue of budding-yeast ribonucleoprotein Rnp1 (Cusick, 1994). Functions of either protein are unknown at the moment.

and G). It is of note that not only *cut12.s11* but also multi-copy *cut11*$^+$ restored Plo1 recruitment to the SPB in *pcp1-18* cells (Supplementary Figure S6A and B). Together, these findings demonstrate that Pcp1 is required for Plo1 recruitment to the SPB during mitosis.

Promotion of mitotic entry rescues defects in growth and SPB recruitment of Plo1 in the pcp1-18 mutant

Polo kinase plays pivotal roles in a variety of processes in cell-cycle control (Nigg, 2007; Takaki *et al*, 2008; Archambault and Glover, 2009). Among them, one of its established functions at the centrosome and SPB is the advancement of mitotic entry (MacIver *et al*, 2003; Petersen

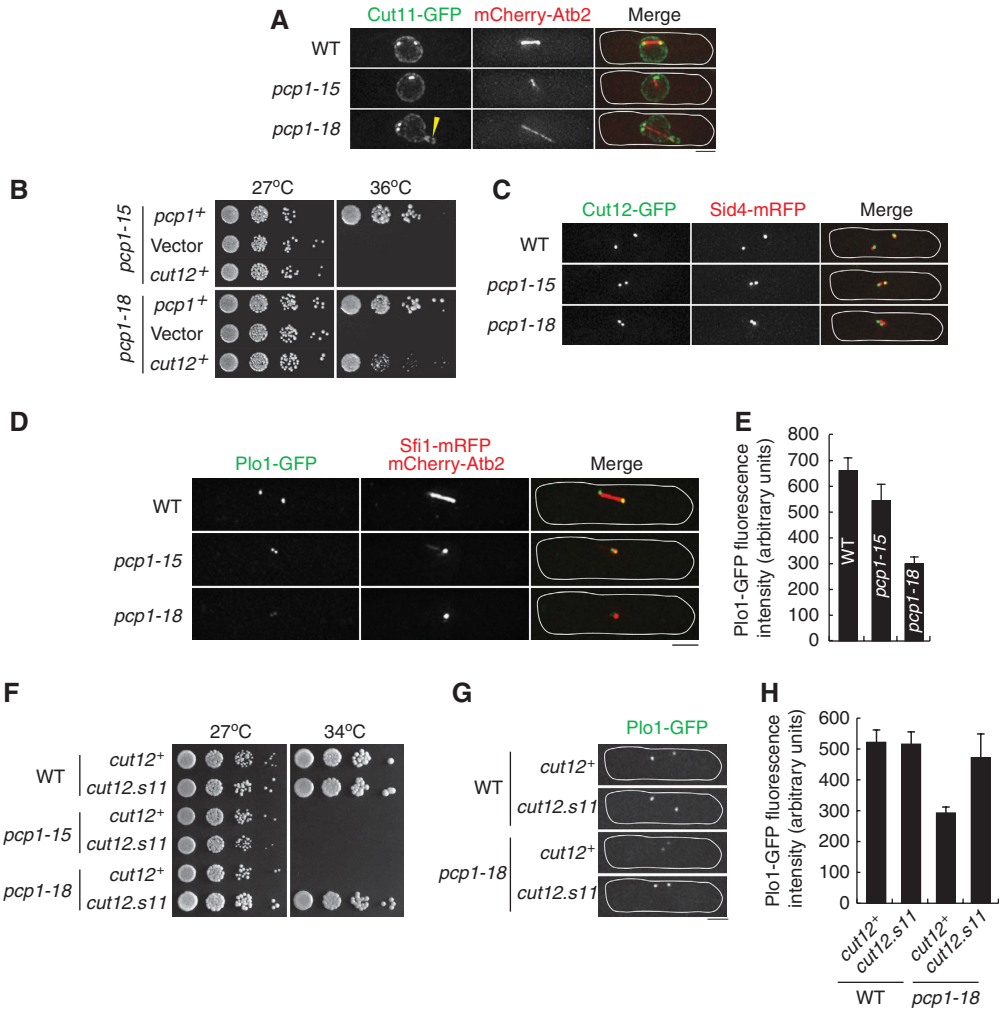

Figure 6 Pcp1 is required for recruitment of Plo1 to the SPB. (**A**) Localisation of Cut11–GFP in mitotic cells at 36°C. NE herniation (arrowhead), probably corresponding to the EM picture shown in Figure 3D for *pcp1-18*, was observed in which Cut11 appears to somewhat accumulate ($n>50$). (**B**) Ten-fold serial dilutions of the *pcp1* mutants harbouring the indicated multi-copy suppressors. (**C**) Normal localisation of Cut12–GFP to the SPB during mitotic at 36°C ($n>100$). (**D**) Plo1–GFP delocalises from the mitotic SPB specifically in *pcp1-18* mutants. Fluorescence images (**A**, **C**, and **D**) were taken from live cells. (**E**) Quantification of Plo1–GFP signal intensity at the SPB in panel D ($n>50$); the error bars indicate standard error. (**F**) Ten-fold serial dilution assays on YES media. (**G**) Increased recruitment of Plo1–GFP at the SPB in the *pcp1-18cut12.s11* double mutant at the semi-permissive temperature of 34°C. (**H**) Quantification of Plo1–GFP signal intensity at the SPB in panel G ($n>50$); the error bars indicate standard error. Fluorescence images (**A**, **C**, **D**, and **F**) were taken from live cells. Scale bars, 2 μm.

and Hagan, 2005; Takaki *et al*, 2008). If Pcp1's role in Plo1 recruitment lies in promoting mitotic entry, forced advancement in the onset of mitosis independently of Plo1 would suppress this mutant. To test this idea, we constructed a double mutant between *pcp1-18* and *wee1-50*, a mutation that causes premature entry into mitosis due to untimely activation of Cdc2 (CDK1) (Nurse, 1990). Remarkably, *pcp1-18wee1-50*, but not *pcp1-15wee1-50*, exhibited improved growth at the semi-permissive temperature of 32°C (Figure 7A). These results, therefore, have uncovered a novel role for Pcp1 in the recruitment of Plo1 to the SPB to promote mitotic entry, and at the same time established a link between Plo1 recruitment and mitotic NE reorganisation for SPB insertion.

Pcp1 interacts with the γ-TuC and Plo1

Results presented above suggest that Pcp1 physically interacts with the γ-TuC and Plo1, thereby recruiting these two factors directly to the SPB. To address this point, cell extracts were prepared from wild type and *pcp1* mutants, and co-immunoprecipitation experiments were performed. As shown in Figure 7B, we did detect an interaction between Pcp1 and either the γ-TuC or Plo1 (lane 13). This interaction appeared fairly tight, as binding was sustainable against washing with high-salt buffers (lanes 14 and 15, 0.3 and 0.5 M NaCl, respectively). Co-immunoprecipitation was specific as no Plo1 or γ-TuC was pulled down when preimmune sera were used for precipitation (lanes 4–6). Interestingly, budding-yeast Spc110 is also reported to interact with both γ-TuC and Cdc5 (budding-yeast polo kinase; Knop and Schiebel, 1997, 1998; Snead *et al*, 2007).

Next we performed immunoprecipitation using mutant extracts prepared from cultures incubated under restrictive conditions (4 h, at 36°C). Contrary to a simple scenario predicting impaired interaction in mutants, the amount of precipitated γ-TuC or Plo1 did not appear noticeably different between the wild type and *pcp1-15* or *pcp1-18* even under stringent high-salt conditions (Figures 7B, lanes 16–21). We

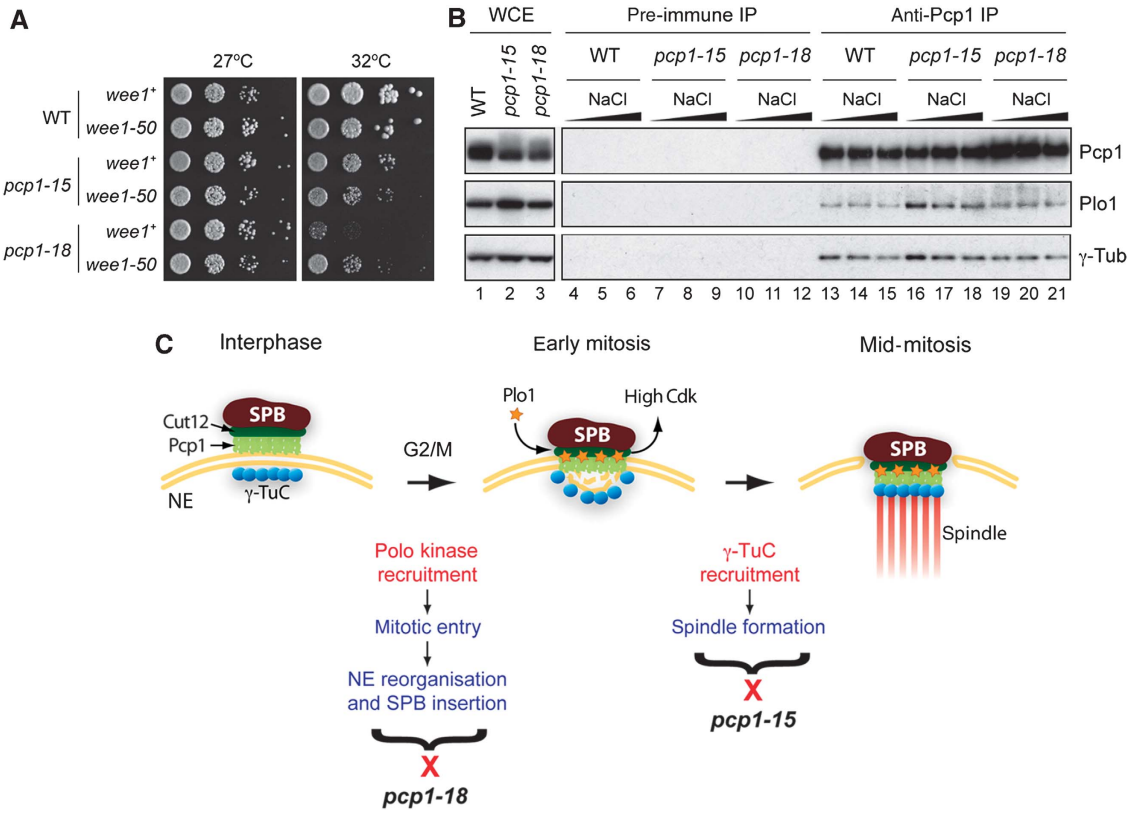

Figure 7 Rescue of *pcp1-18* by premature mitotic entry, physical interaction between Pcp1 and Plo1 or γ-tubulin, and a working model. (**A**) *pcp1-18*, but not *pcp1-15*, is suppressed by premature mitotic entry. Ten-fold serial dilution assays on YES media. (**B**) Pcp1 physically interacts with both γ-TuC and Plo1. Wild-type, *pcp1-15*, or *pcp1-18* cells containing Plo1–GFP–HA were grown at 27°C and shifted up to 36°C for 4 h. Extracts were prepared and immunoprecipitation was performed with affinity-purified anti-Pcp1 antibodies. Precipitates were subjected to SDS–PAGE and immunoblotting was performed with anti-Pcp1, anti-HA, and anti-γ-tubulin antibodies (lanes 13, 16, and 18). As negative control, preimmune sera were used (lanes 4, 7, and 10). Immunoprecipitates on beads were further washed with high-salt buffers containing 0.3 or 0.5 M NaCl (lanes 5, 8, 11, 14, 17, and 20, or lanes 6, 9, 12, 15, 18, and 21, respectively). Note that the salt concentration of original buffers is 0.15 M NaCl; 0.5-mg equivalents of immunoprecipitates and 30 μg of whole-cell extracts (WCE, lanes 1–3) were loaded. (**C**) A model of Pcp1's function during cell division. Pcp1 recruits Plo1 to the SPB at G_2/M transition, leading to full activation of Cdk (Cdc2) and mitotic entry. Note that activated Cdc2 in turn activates Plo1 via a positive-feedback loop. This drives directly or indirectly NE reorganisation and SPB insertion. At the same time Pcp1 recruits γ-TuC to the mitotic SPB to initiate spindle formation. The nature of interactions between Pcp1 and Cut11 (not depicted) or Cut12 remains unclear at this moment. SPB: brown; Cut12: dark green; Pcp1: light green; γ-TuC: blue circles; NE: yellow; Plo1: orange star; and spindle: red lines.

envision that only a small proportion of γ-TuC or Plo1 forms a complex with Pcp1 at the SPB during mitosis (see Discussion). Nonetheless, this result points towards a possibility that Pcp1 directly interacts with γ-TuC and Plo1, thereby playing a structural role as a platform for these factors loading to the mitotic SPB. In sum, our work verifies Pcp1's plausible role in γ-TuC-mediated spindle assembly and unveils its unanticipated function in Plo1-dependent mitotic entry and structural reorganisation of the NE.

Discussion

Our work presented here shows that a single SPB component Pcp1 plays multiple roles in mitosis by independently recruiting two essential components, γ-TuC and Plo1. The *pcp1-15* mutant is defective in γ-TuC recruitment to the mitotic SPB, while *pcp1-18* fails to target Plo1 to this site. We have shown that these defects are allele-specific. Despite these differences, both mutants display similar monopolar spindles, in which only the mother SPB could nucleate spindle microtubules, albeit with reduced intensity. Importantly EM analysis has revealed that *pcp1-18* is further impaired in mitosis-specific NE invagination beneath the daughter SPB, accompanied by failure in SPB insertion into the NE. We believe that this asymmetrical SPB defect is the main reason for monopolar spindle phenotypes observed in this mutant allele. Our study, therefore, illuminates that SPB-mediated mitotic processes, including spindle formation, NE reorganisation, and SPB insertion converge via Pcp1 (see Figure 7C for a model); on one hand, Pcp1 recruits γ-TuC, thereby promoting nucleation and assembly of spindle microtubules; on the other Pcp1 interacts with Plo1 and is responsible for NE invagination and subsequent SPB insertion. Another SPB component, Cut12, plays a corroborative role with Pcp1 in Plo1 regulation at the SPB (Figure 7C). Importantly the core cell-cycle machinery that activates CDK1/Cdc2, thereby determining the timing of mitotic commitment, cooperates at the SPB with the Pcp1–Plo1 pathway by forming a positive-feedback loop (Hagan, 2008).

A conserved γ-TuC-binding domain has recently been identified in a family of pericentrin-related proteins, including centrosomin, Spc110, Mto1, and Pcp1 (Zhang and Megraw,

2007; Samejima *et al*, 2008). It is, therefore, conceivable that Pcp1 directly recruits γ-TuC to the SPB during mitosis. Physical binding between Pcp1 and γ-tubulin supports this notion. Interestingly, *pcp-15*, which fails to recruit the γ-TuC to the mitotic SPB, harbours a mutation within this γ-TuC-binding domain (Supplementary Figure S1). In budding yeast, the temperature-sensitive *spc110-221* allele that carries multiple mutations in the N-terminal region was rescued by overproduction of Spc98 (Alp6 homologue; Nguyen *et al*, 1998), which might be in parallel with our finding described in this study.

Although we are so far unable to recapitulate a reduced recruitment of the γ-TuC to the mitotic SPB by co-immunoprecipitation in the *pcp1-15* mutant, a population of Pcp1 that colocalises with the γ-TuC to the mitotic SPB could be too small to detect by pull-down methods. It is of note that even in wild-type cells, we have not succeeded in showing enhanced interaction between Pcp1 and the γ-TuC or Plo1 during mitosis as compared with the interphase (data not shown). Alternatively, as SPB components expectedly remain insoluble upon protein extractions, we might not be able to show impaired recruitment to the SPB in the mutants under conditions currently used. Further biochemical analyses would determine the underlying mechanism in more detail.

With regard to Plo1 localisation to the mitotic SPB, to our knowledge, this is the first study in any organisms to identify genetically a spindle pole component that plays a critical role in polo kinase recruitment to the SPB/centrosome. It is known that CDK1 and cyclin-B localise to the mitotic centrosome in animal cells (Jackman *et al*, 2003) and to the SPB in fission yeast (Alfa *et al*, 1990; Decottignies *et al*, 2001), implying a universal role for the centrosome/SPB in the temporal regulation of mitotic onset. Precedent work using fission yeast indicated that Cut12 potentiates Plo1 activity upon mitotic onset; however, it should be noted that Cut12 is not required for Plo1 recruitment to the SPB *per se*; in other words Plo1 is still recruited apparently normally to the SPB during mitosis even in the *cut12*-null mutant (Mulvihill *et al*, 1999). This indicates that Cut12, albeit physically interacting with Plo1 (MacIver *et al*, 2003), is not a receptor that is directly responsible for recruitment of this kinase to the mitotic SPB, but instead it is Pcp1 that performs this task. Although at the moment it remains to be determined how Pcp1 and Cut12 functionally and physically interact, it might be stated that Pcp1 is involved in mitosis-specific activation of Cut12 through a hitherto unknown mechanism. Alternatively, as Cut12 is capable of localising to the SPB in *pcp1-18* mutants, Cut12 acts either upstream or independently of Pcp1 (see below).

We also demonstrate that Pcp1 controls SPB insertion and NE invagination during mitosis. Importantly, these processes could be under the control of SPB-localising Plo1, as the defect is only observed in *pcp1-18*, but not *pcp1-15*, cells. Furthermore, the enhancement of Plo1 localisation to the mitotic SPB using gain-of-function *cut12.s11* is sufficient to suppress the *pcp1-18* mutant. Very recently it was shown that the temperature-sensitive, loss-of-function *cut12.1* mutant also displays SPB insertion defects (Tallada *et al*, 2009) as that we observed in *pcp1-18*. This indicates that Pcp1, Cut12, and Plo1 form a functional network via physical binding in SPB-mediated mitotic entry and progression. It would be of interest to examine whether *plo1* mutants display similar SPB insertion defects.

We isolated Cut11 and Pom152, as well as Cut12, as dosage-dependent, allele-specific suppressors of *pcp1-18*. The first two proteins are conserved membrane-integral nucleoporins (Cut11/Ndc1 and Pom152/GP210) and form a stoichiometric subcomplex in both human and budding yeast, thereby constituting an internal membrane ring (Madrid *et al*, 2006; Mansfeld *et al*, 2006; Stavru *et al*, 2006; Alber *et al*, 2007a, b; Galy *et al*, 2008). As Cut11 is enriched at the SPB during fission-yeast mitosis (West *et al*, 1998), it would be tempting to speculate that this integral membrane protein is directly involved in mitotic NE invagination and SPB insertion. Indeed it is shown that the temperature-sensitive *cut11.1* mutant has monopolar spindles with asymmetric SPB defects, as in the *cut12.1* or *pcp1* mutants analysed here (West *et al*, 1998; Tallada *et al*, 2009). An intriguing possibility is that Cut11 and/or Pom152 are downstream effectors of Plo1, including phospho-substrates, although suppression of Plo1 recruitment to the SPB by *cdc12.s11* or multi-copy *cut11*$^+$ implies a more complicated scenario such as a positive-feedback loop rather than a simple linear pathway. We have addressed whether or not it would be possible to genetically differentiate distinct functions of Pcp1 using *pcp1-15* and *pcp1-18* mutants, but so far these two mutant alleles behave similarly in terms of non-complementation and synthetic lethal interactions with mutations in other related genes (see Supplementary Notes 1 and 2, and Supplementary Figure S7).

Although NE-mediated processes such as NE invagination and SPB insertion may be regarded as a close mitosis-specific event, it is known that in animal cells the NE undergoes dynamic structural alterations upon mitotic entry, in which the small restricted regions of the NE below the centrosome indeed invaginate prior to mitotic NE breakdown (Beaudouin *et al*, 2002; Salina *et al*, 2002; Hetzer *et al*, 2005; Tran and Wente, 2006; D'Angelo and Hetzer, 2008), which might well be analogous to the formation of NE fenestra observed in fission yeast (Ding *et al*, 1997). It would be of instant interest to see whether NE invagination in animal cells is regulated by polo kinase. In conclusion, our work has unearthed that Pcp1 and possibly the analogous pericentrin-like proteins play a more central and integral role in the orchestration of cell-cycle events than previously anticipated, and may provide insight into tumorigenesis and other types of human diseases attributed to centrosomal dysfunction.

Materials and methods

Yeast genetics and strains

Strains used in this study are listed in Supplementary Table S1. Standard methods for yeast genetics and gene tagging were used (Moreno *et al*, 1991; Bähler *et al*, 1998b; Sato *et al*, 2005, 2009). For isolation of temperature-sensitive *pcp1* mutant, *pcp1*$^+$ fragments containing C-terminally tagged HA epitope and *hph*^{r} gene (*pcp1*$^+$*-HA-hph*^{r}) were randomly mutagenised during error-prone PCR amplification using Vent DNA polymerase (New England Biolabs Ltd.) supplemented with 10 × deoxyguanosine triphosphate (dGTP). PCR fragments were ethanol-precipitated and transformed into a wild-type strain (513; Supplementary Table S1). Approximately 5000 transformants were screened for temperature sensitivity at 36°C. The isolated temperature-sensitive mutants were backcrossed to *pcp1*$^+$*-mRFP-kan*^{r} to ensure proper integration and to remove potential off-site mutations.

Multi-copy suppressor screening

The *pcp1* mutants were transformed with a genomic DNA library. Approximately 15 000 transformants growing on selection plates at 27°C were replica-plated onto YES containing phloxin-B and the plates were incubated at 36 or 34°C. Plasmids were isolated from the growing colonies and sequenced to identify the suppressor genes. Plasmids were retransformed into the *pcp1* mutants to reconfirm suppression.

Microscopy

For fluorescence microscopy an AxioplanII microscope (Zeiss Ltd.) with Volocity software (Improvision Co.) and the Deltavision-SoftWoRx system (Olympus and Applied Precision Co.) were used. Images were taken at 15 sections along the *z*-axis at 0.2- to 0.4-mm intervals; they were then deconvolved and merged into a single projection. For immunofluorescence microscopy, TAT-1 (α-tubulin monoclonal antibody provided by K Gull, Oxford University, UK) was used. The captured images were processed with Adobe Photoshop CS2 (version 9.0).

Electron microscopy

Samples were cryofixed by high-pressure freezing using a Leica EM PACT2. Freeze substitution was performed in anhydrous acetone containing 0.01% osmium tetraoxide, 0.1% glutaraldehyde, and 0.25% uranyl acetate for 3 days at −90°C using a Leica EM AFS. Freeze-substituted cells were gradually warmed to −20°C and were finally infiltrated with Epon. Serial thin sections of ∼60 nm were cut using a Leica Ultracut UCT microtome and placed on pioloform-coated slot grids. Sections were post-stained with 2% uranyl acetate in 70% methanol for 4 min and with lead citrate for 1 min. Images were acquired with a FEI Tecnai Biotwin electron microscope and Gatan DigitalMicrograph. The captured images were processed with Adobe Photoshop CS2 (version 9.0).

Immunochemistry

Immunofluorescence microscopy was performed with methanol-fixed samples. Cells were processed in PEM-based buffers (200 mM Pipes, 2 mM EGTA, 2 mM $MgSO_4$, adjusted to pH 6.9). TAT-1 (anti-α-tubulin antibody) and anti-Pcp1 antibodies were used at 1:200 and 1:5000, respectively.

Immunoprecipitation was performed using standard procedures as briefly described below. Proteins were extracted in lysis buffer (50 mM Tris–HCl (pH 7.4); 1 mM EDTA (pH 8.0); 150, 300, and 150 mM NaCl; 0.05% NP-40; 10% glycerol) by breaking the cells at 4°C with glass beads (setting 5.5, 25 s, 2 ×) in a FastPrep FP120 apparatus (Savant. Co., MN, USA). The protein extracts were collected after 15 min of centrifugation at 13 000 *g* at 4°C. The co-immunoprecipitations were performed by adding 4 mg of protein extract to protein-A dynabeads (Invitrogen Ltd., CA, USA) bound with anti-Pcp1 antibody or preimmune serum. For immunoblotting anti-Pcp1, anti-HA (16B12, Covance Inc., CA, USA), and anti-γ-tubulin (Sigma) antibodies were used at 1:5000, 1:200, and 1:500, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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