Fission yeast Skp1 is required for spindle morphology and nuclear membrane segregation at anaphase

Anna Lehmann, Takashi Toda*

Laboratory of Cell Regulation, Cancer Research UK, London Research Institute, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

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Abstract Skp1 is a core component of the Skp1-Cullin-1-F-box ubiquitin ligase. Here, we show a novel role for fission yeast Skp1 in mitotic progression. Temperature-sensitive *skp1-A7* mutants enter mitosis, but fail to execute anaphase. Time-lapse imaging shows that spindles in this mutant form intranuclear arch-like structures, which eventually collapse abruptly. The two spindle poles are also seen to move backward to the cell centre rather than towards the cell ends. These abnormal phenotypes appear to stem from defects in nuclear membrane segregation. Our results show that Skp1 is required for coordinated structural alterations of mitotic spindles and nuclear membranes.

Keywords: Fission yeast; Mitotic spindle; Nuclear envelope; Skp1-Cullin-1-F-box ubiquitin ligase; Skp1

1. Introduction

The ubiquitin-mediated proteolysis pathway plays a major role in regulated intracellular protein degradation. The pathway consists of a series of enzymatic reactions. The enzymes that carry out these reactions are known as the ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3) [1,2]. The Skp1-Cullin-1-F-box (SCF) is a conserved E3 ubiquitin ligase that consists of the three components listed above and a fourth, the RING finger protein Rbx1/Roc1/Hrt1 [3–7]. Among these components Skp1 has a unique function, it acts as a bridge between the F-box protein and Cullin-1 [8]. The F-box proteins are substrate-specific receptors, whilst Cullin-1 is a structural platform that binds Skp1 (and indirectly F-box proteins/substrates) and Rbx1 (and E2/ubiquitin) [8].

The microtubule cytoskeleton displays cell-cycle-dependent dynamic behaviour in all eukaryotic cells. Upon entry into mitosis, cytoplasmic arrays of interphase microtubules disassemble and are reorganized into mitotic spindles. During mitosis, spindle dynamics alter dramatically at both plus and minus ends [9,10]. In fungi, unlike animal cells in which the nuclear envelope is broken down upon mitotic entry, the nuclear envelope remains intact (called closed mitosis). This implies that the dynamics of mitotic spindles and nuclear membranes must be coordinately regulated during mitosis. In particular, during anaphase B when spindle elongation occurs following sister chromatid separation, structural alterations of the nuclear envelope, such as extension and segregation into the two daughter cells, should take place simultaneously. This coordination between spindle dynamics and nuclear architecture ensures faithful segregation of chromosomes and the nuclear organelle at telophase. Despite this, mechanisms underlying this harmonious correlation remain largely elusive, particularly compared to the recent advancement in the study of sister chromatid segregation [11,12]. The molecular knowledge of how the nucleus and the nuclear membrane segregate is, therefore, limited.

In this study, we describe an unexpected finding on the role for Skp1. We show that fission yeast Skp1 is required for anaphase execution. Skp1 is essential for the proper morphology of anaphase spindles and the nucleus. Remarkably, in temperature-sensitive (ts) *skp1* mutants, anaphase spindles form arch-like structures rather than straight ones. The middle of the arch eventually undergoes physical collapse. Detailed kinetic analysis has led us to propose that Skp1 plays a crucial role in segregation of nuclear membrane structures at anaphase.

2. Materials and methods

2.1. Strains, media, genetic methods and nomenclature

Strains used in this study are the following. *h⁻leu1ura4⁻skp1-A7*, *h⁺leu1ura4cut11⁺-GFP-ura4⁺*, *h⁻leu1cut11⁺-GFP-ura4⁺skp1-A7*, *h⁻leu1cut12⁺-CFP-ura4⁺skp1*-A7, and *h⁻leu1ura4pof3::ura4⁺*. YE5S was used as rich media and modified synthetic EMM2 as minimal media. Standard techniques were used as described [13] for growth and maintenance of strains.

2.2. C-terminal epitope-tagging

C-terminal tagging of genes of interest was performed by a PCR-based gene targeting method [14].

2.3. Indirect immunofluorescence microscopy

Cells were fixed with methanol or formaldehyde and primary antibodies (TAT-1 1/50) were applied, followed by Cy3 conjugated sheep anti-mouse IgG (Amersham). Immunofluorescence images were viewed with a chilled video CCD camera (C4742-95, Hamamatsu Photonics Ltd.) and a PC computer containing kinetic image AQM software (Kinetic Imaging Ltd.) or with a laser scanning confocal microscope LSM510 (Zeiss Co.) and processed by use of Adobe® Photoshop (version 5.5).

* Corresponding author. Fax: +44-20-7269-3258.
E-mail address: toda@cancer.org.uk (T. Toda).

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2.4. Time-lapse live imaging

Plasmids carrying *nmt1-GFP-atb2*$^+$ were introduced into a *skp1-A7* strain. Cultures were grown to mid log phase (5×10^6 cells/ml) in liquid minimal medium in the presence of thiamine at 26 °C, filtered and washed with YE5S, transferred to YE5S media at 36 °C and incubation continued for 4 h. In the case of a *skp1* strain containing the integrated *cut12*$^+$*-CFP* gene, rich YE5S medium was used. Cells were then mounted on a thin layer of rich media agar beneath a coverslip, which was preincubated at 36 °C. The microscope stage was also preheated and stabilized at 36 °C. Live images were taken using Axioplan 2 imaging microscope (Zeiss) attached to a Hamamatsu ORCA-ER I digital camera and Pentium III processor. The images were processed using Kinetic Imaging Advance 6 software and Adobe® Photoshop (version 5.5).

3. Results and discussion

3.1. Temperature-sensitive skp1 mutants display mitotic phenotypes

A collection of ts mutant alleles of *Schizossacharomyces pombe skp1*$^+$, isolated by PCR-based mutagenesis, shows a G2 cell cycle delay phenotype at the restrictive temperature, accompanied by cell elongation [15]. Careful observation of these ts strains showed that mutant cells also display mitotic defects after prolonged G2 phase. As shown in Fig. 1A and B, after 8 h incubation at 36 °C, septation without chromosome segregation ('cut') was observed, with 8% of cells showing this phenotype (also see Table 1). In addition, many cells (>30%) show chromosome mis-segregation (a cell marked with an arrowhead in Fig. 1B). In contrast, wild-type cells incubated under the same condition at 36 °C or mutant cells grown at 26 °C never displayed these abnormal mitoses. This observation suggests that Skp1 is required for mitotic progression in addition to entry into mitosis from G2.

3.2. skp1 mutant cells show abnormal bent spindles that are formed independently of Pof3 F-box protein function

In order to clarify in more detail those mitotic defects occurring in a ts *skp1* strain, immunofluorescence microscopy was performed with anti-tubulin antibody. Morphology of the mitotic spindles was examined with a laser scanning confocal microscope. This analysis revealed that the spindle of *skp1-A7* mutant cells bends in mitosis and forms an arched or circular structure (Fig. 1C).

We have previously shown that deletion of the F-box protein, Pof3, leads to G2 cell cycle delay [16], which is also seen in *skp1-A7* mutants and furthermore this G2 delay in *skp1-A7* is, at least in part, dependent upon Pof3 function [15]. We, therefore, wished to assess if cells deleted for *pof3*$^+$ showed similar bent spindle phenotypes. To address this, *pof3* cells were grown in rich medium at 36 °C for 4 h, fixed and stained with anti-tubulin antibody. As shown in Fig. 1D, no bent spindles were observed in any *pof3* cells, instead normal spindle elongation was evident. This suggests that, contrary to the delay observed in mitotic entry, the spindle defects occurring in *skp1-A7* cells are unrelated to loss of Pof3 function.

3.3. The spindle arches and eventually collapses in skp1-A7 cells undergoing live mitosis at 36 °C

We next sought to follow the in vivo dynamics of bent spindles in *skp1-A7* cells. For this purpose, cells were observed live in mitosis with GFP-tubulin. *skp1-A7* mutant cells, carrying a plasmid containing the α-tubulin gene *atb2*$^+$, tagged with GFP, under the control of a thiamine-inducible promoter [17,18], were grown to mid-log phase at 26 °C, shifted to 36 °C for 4 h and examined undergoing mitosis. Cells were filmed with frames every 30 s, brightness and contrast were adjusted to compensate for photobleaching of the GFP signal. Time-lapse photographs of *skp1-A7* showed that a live cell undergoing mitosis appears to proceed normally until phase 2 (prophase to anaphase A) [19] (from 0 to 600 s in Fig. 2), but during phase 3 (anaphase B equivalent, starting at 690 s),

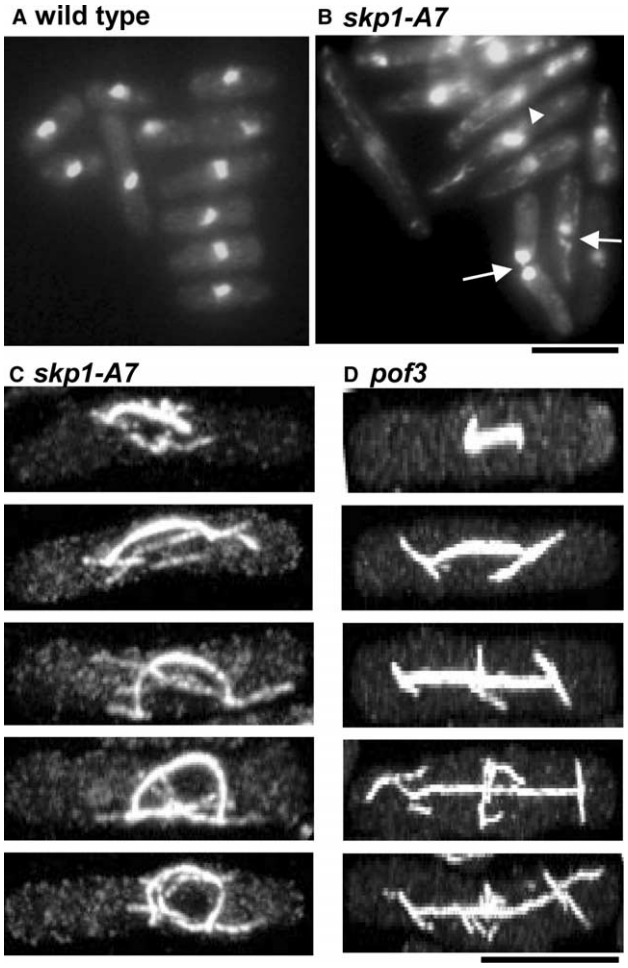

Fig. 1. ts *skp1-A7* mutants show chromosome segregation defects with bent spindles but *pof3* cells do not. (A,B) Wild-type (A) or *skp1-A7* cells (B) were grown at 26 °C and shifted to 36 °C for 8 h. Cells were fixed with formaldehyde and stained with DAPI. 'Cut' cells are marked with arrows, whilst a cell displaying unequal chromosome segregation is shown with arrowhead. (C,D) *skp1-A7* mutant (C) or *pof3* cells (D) were grown at 36 °C for 4 h, fixed with methanol and stained with anti-tubulin antibody. Cells were examined with a laser scanning confocal microscope. 3D composites of 10 slices through each cell were made. Bar indicates 10 μm.

Table 1
Percentage of 'cut' cells in ts *skp1* strains

Alleles	Wild-type	*skp1-A2*	*skp1-A3*	*skp1-A4*	*skp1-A7*
Cut (%)	<0.5	9	16	5	8

Each ts *skp1* strain was shifted from 26 to 36 °C and incubated for 6 h. Cells were fixed with formaldehyde and stained with DAPI. At least 200 cells were counted. At 26 °C, no cut cells were observed (<0.5%).

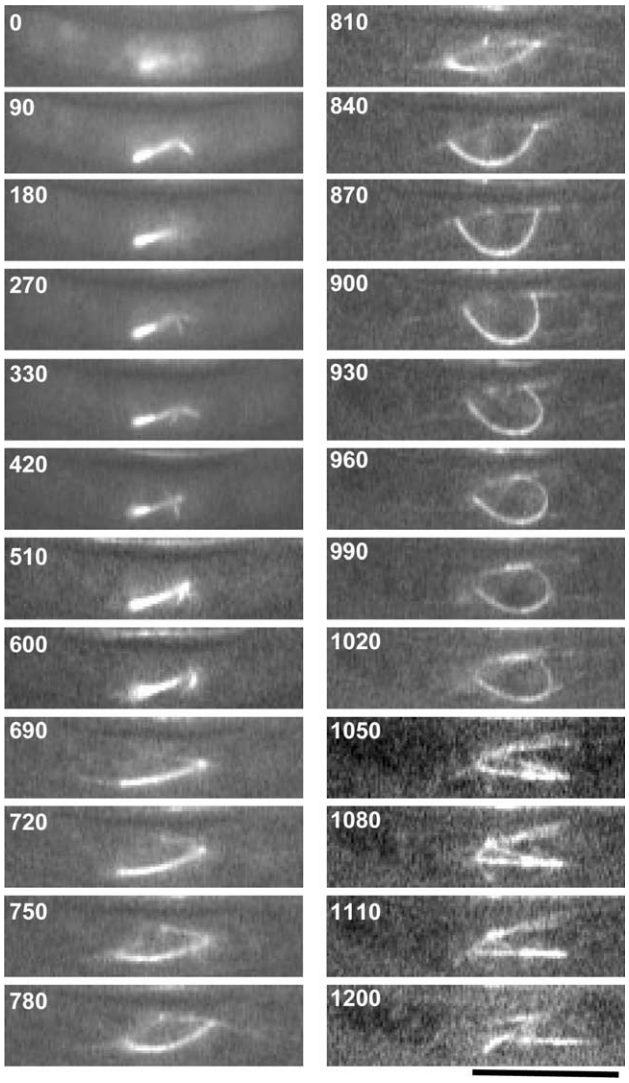

Fig. 2. Time-lapse live analysis of anaphase spindles in *skp1-A7* mutant cells. *skp1-A7* mutant cells, containing a plasmid copy of *atb2*$^{+}$*-GFP* under a thiamine-repressible promoter, were grown at 36 °C for 4 h. Cells were mounted on a thin layer of agar on the surface of a pre-warmed slide and examined on a pre-heated microscope stage undergoing live mitosis. Frames were taken every 30 s (time shown in the upper left corner). The spindle was observed to bend during anaphase (690 s) and eventually collapses completely (1050 s). Bar indicates 10 μm.

suffers from spindle curvature. The spindle then collapses abruptly after it has formed a complete circle (1020 and 1050 s). This abnormal spindle phenotype was observed in four out of every 10 mitotic *skp1* cells ($n = 20$). This result indicates that Skp1 function is required for mitotic progression, in particular spindle elongation and morphology during anaphase B.

3.4. Reverse spindle pole movement of skp1-A7 cells

The bending of the spindle in *skp1-A7* mutant cells appears to show that the ends of the spindle move together towards the centre, instead of separating towards the cell tips, which happens during normal anaphase B [20]. In wild-type cells, one end (minus) of microtubules nucleates from the spindle pole bodies (SPBs). The spindle elongates during anaphase B probably by a sliding motion of overlapping microtubules in the central spindle region. This pushes the two SPBs apart increasing the distance between them [20]. We wondered if the SPBs also showed backward movement. To investigate this we used a *skp1-A7* mutant strain containing *cut12*$^{+}$*-CFP*, the SPB marker [20]. Cells were treated in the same manner as those carrying GFP-tagged α-tubulin. Cells with the two SPBs that were already on adjacent sides of the nucleus were chosen for examination and followed through mitosis. Time-lapse pictures were taken every 15 s and adjustments were made to maintain both SPBs in the photographic plane. SPBs were observed to move apart initially (Fig. 3A, 0–135 s, corresponding to phases 1 and 2). At 150 s (indicated by an arrow), the SPBs began to move back towards one another and this movement continued until ~300 s. Once the distance of the two SPBs became very close (the distance is <2 μm), they appeared to drift to one end of the cell, but did not move any closer together (from 315 to 585 s).

The inter-SPB distance was measured and plotted as a function of time (Fig. 3B). As the spindle was observed to be elongating, albeit in an unusual direction, the inter-SPB distance was used as a measure of the rate of elongation of the spindle. The spindle was seen to elongate at a rate of 1.4 μm/min. This is equivalent to the velocity previously measured for wild-type spindles undergoing anaphase B of *S. pombe* mitosis at 36 °C [19].

We previously showed that one of the other major phenotypes seen in *skp1-A7* is prolonged G2 cell cycle period and that the delay is dependent upon activation of the DNA damage checkpoint [15]. It is of note that this cell cycle delay prior to mitotic entry is necessary for appearance of an abnormal anaphase in *skp1-A7* mutants as the *skp1-A7rad3* double mutants, in which G2 delay phenotype is abolished, no longer show anaphase defects. We therefore concluded that the molecular defects, which eventually result in aberrant spindle behaviour during anaphase B, would stem from the delay during preceding G2 phase, and furthermore that spindle elongation per se occurs with normal kinetics in *skp1-A7* mutants.

3.5. Abnormal nuclear membrane and its colocalization with bent spindles

The bent spindles of the *skp1-A7* mutant cells seem to form a circle around the region of the nucleus. Unlike animal cells, yeast cells exhibit closed mitosis, in which the nuclear envelope never disassembles during mitosis. Given this fact, we asked whether this circular structure was a result of the spindle being constrained by the nuclear envelope. If abnormality of spindle morphology was the primary defect, the nuclear envelope would display normal separation. Alternatively, if the nuclear membranes were altered structurally, this would result in an inability to elongate the spindle and force it around the edge of the nuclear envelope. In order to distinguish these possibilities, we crossed a *skp1-A7* mutant strain with a strain containing *cut11*$^{+}$ [21] tagged with GFP. Cut11-GFP serves as a nuclear membrane marker. *skp1-A7 cut11*$^{+}$*-GFP* strains were grown at 36 °C for 4 h and fixed with methanol. Fixed cells were stained with anti-tubulin antibody and both microtubules and the nuclear membranes were examined in a single cell. Merged images of these cells showed that where the spindle was bent, it was colocalized with the nuclear envelope, the tubulin and GFP staining overlapping (Fig. 4A). This result suggests that it is the nuclear membrane, which primarily behaves abnormally in *skp1-A7* cells.

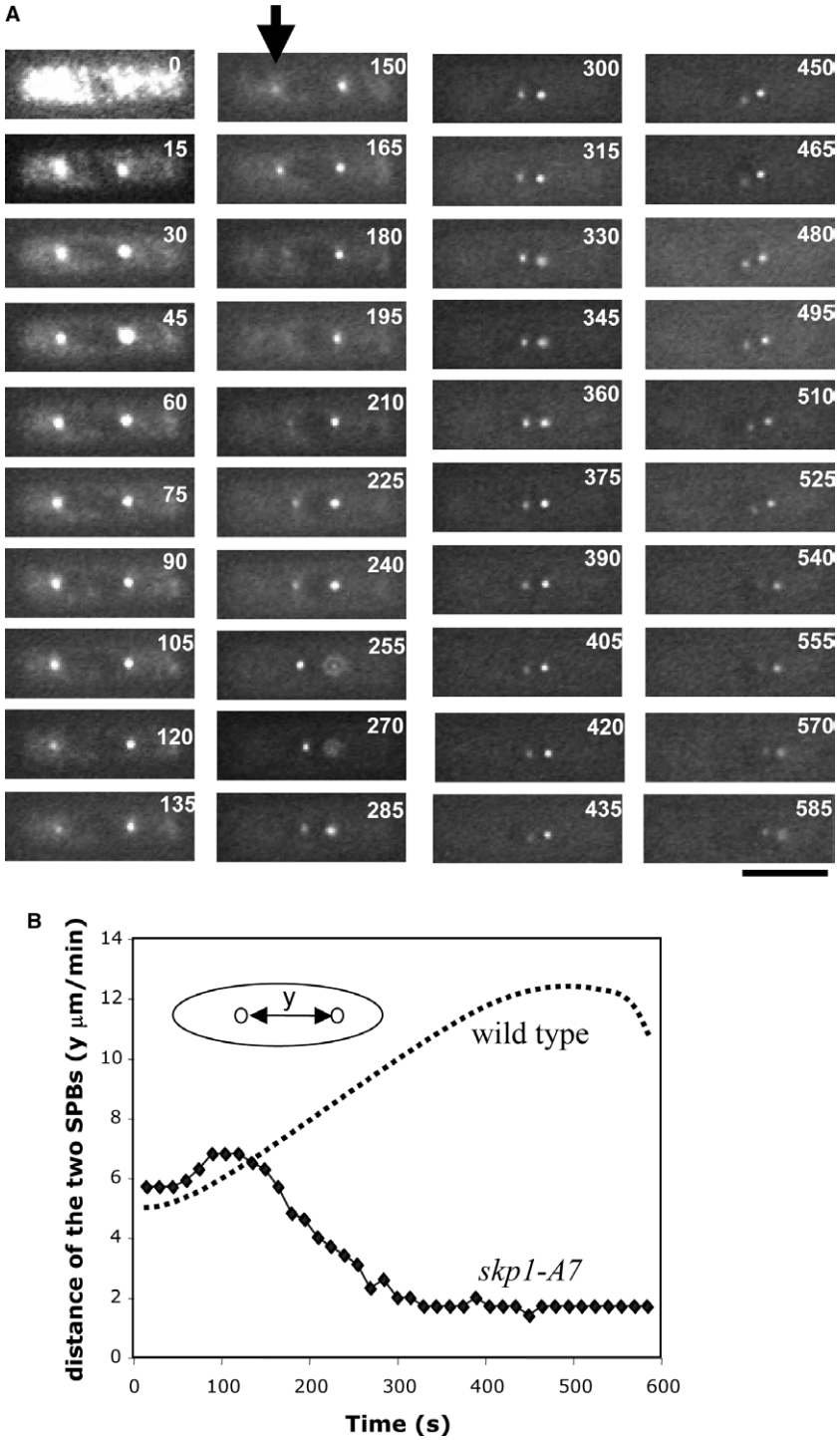

Fig. 3. SPB movement during anaphase B in *skp1-A7* mutant cells. (A) *skp1-A7* mutant cells, containing the integrated *cut12*$^{+}$*-CFP* gene, were grown for 4 h at 36 °C before being transferred to a thin layer of agar on a pre-heated microscope slide and examined on a pre-warmed microscope stage. Frames were taken every 15 s (shown in the upper right corner). The SPBs were observed to move away from each other and then return towards each other from 150 s (arrow). The SPBs stop moving towards each other and drift as a pair to the side of the cell (~300 s). (B) The inter-SPB distance (*y*) was measured and plotted as a function of time. The normal behaviour of SPBs in wild-type cells at 36 °C [19] is indicated as a black dotted line for comparison. The velocity of SPB movement was calculated from the gradient of the line at its steepest point. Bar indicates 10 μm.

To further examine the nuclear membrane structure during mitosis in *skp1-A7* cells, time-lapse live analysis was performed in a *skp1-A7* strain, containing *cut11*$^{+}$*-GFP*. Although Cut11-GFP signals faded more rapidly at 36 °C, particularly when compared with those of tubulin or Cut12, we could manage to record Cut11-GFP in *skp1-A7*. As shown in Fig. 4B, the nuclear membrane appeared to separate into two portions (135 s, shown by two yellow arrowheads), suggesting attempted nuclear division. Later, the signal fused again to display one structural body (240 s, shown by one yellow arrow). We have

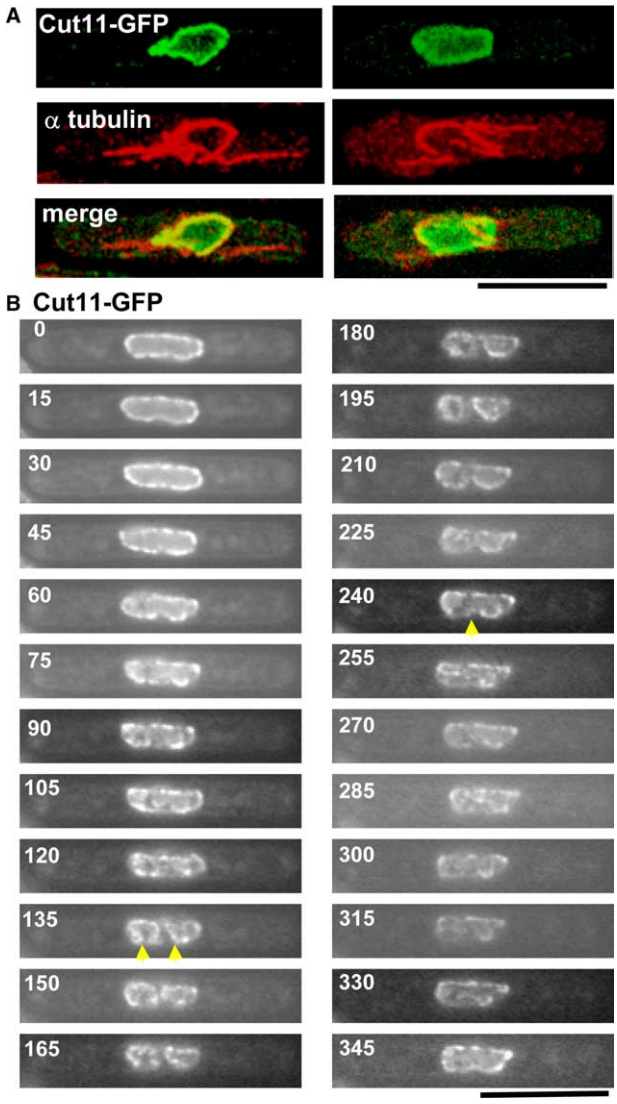

Fig. 4. The nuclear envelope of *skp1-A7* mutant cells during anaphase B. (A) *skp1-A7* mutant cells, containing a chromosomally integrated copy of *cut11*$^{+}$*-GFP*, were grown at 36 °C for 4 h. The cells were fixed with methanol and stained with anti-tubulin antibody, followed by Cy3-conjugated secondary antibody. Fixed cells were mounted and examined with a laser scanning confocal microscope. 10 slices through each cell were visualized and 3D composites made. The cells showed bent spindles and deformed nuclear membranes. The spindle and nuclear membranes colocalized as seen by the yellow regions where GFP (green) and Cy3 signals (red) overlap (merged images at the bottom). (B) *skp1-A7* mutant cells, containing the integrated *cut11*$^{+}$*-GFP* gene, were grown for 4 h at 36 °C before being transferred to a thin layer of agar on a pre-heated microscope slide and examined on a pre-warmed microscope stage. Frames were taken every 15 s (shown in the upper left corner). Points, when the nuclear membrane started to separate into two bodies (135 s) and again fused into one body (240 s), are shown by yellow arrows. Bar indicates 10 μm.

observed abnormally segregating nuclear membranes in the majority of the live cell (9 out of 10). It is also of note that, in addition to segregation defects, mitotic-specific SPB structures [21] were never evident in mitotic *skp1-A7* cells. These results imply that Skp1 function is required for structural alterations and segregation of the nuclear membrane in yeast anaphase.

3.6. An essential role for Skp1 in yeast anaphase B

We have shown here that fission yeast Skp1 is essential for execution of anaphase B. In ts *skp1* mutants, the spindle elongates with normal velocity, however instead of elongating towards the cell ends, it moves towards the cell centre. This reverse movement leads the spindles to forming arched structures with the two SPBs being located in a close vicinity to one another. Surprisingly, the arched spindles then physically collapse at the middle in late anaphase. As far as we know, this type of spindle breakage has not been reported in any mitotic mutants previously characterized. There are several possible ways in which the spindle curvature observed in *skp1-A7* cells could be envisaged to occur. From a physical point of view, the appearance of the arched spindles suggests that some kind of anti-elongation force is being applied. This force must act in the opposite direction to poleward forces exerted by the spindle. Several mechanisms of producing such a force are conceivable, such as latent connections between the SPBs, inappropriate mitotic motor behaviour, hyperstabilized astral microtubules or unreplicated chromatin bridges.

One hypothesis, which we would like to propose here, is that in *skp1-A7* mutant cells the nuclear envelope fails to obtain plasticity during anaphase B, thereby preventing segregation of the nuclei and nuclear membrane. In this model the spindle defects of this mutant are explained as secondary phenotypes, induced by the physical constraints of nuclear envelope structures. Skp1 is a central component of the SCF, an ubiquitin ligase. We would therefore like to propose that some substrate of the SCF needs to be degraded for execution of proper anaphase. In wild-type cells this substrate protein would be involved in a change in the elasticity of the nuclear membrane, and its proteolysis via Skp1-SCF would result in making the nucleus more susceptible to stretching during anaphase B. In *skp1-A7* defective cells, on the other hand, this substrate would accumulate, inhibiting structural changes in the nuclear membrane and resulting in a resistant barrier to the elongating spindle. It should be noted that G2 cell cycle delay is a prerequisite for an abnormal anaphase in *skp1-A7* mutants, suggesting that some proteins accumulate during prolonged G2 phase.

At anaphase, sister chromatid segregation occurs via activation of another E3 ubiquitin ligase anaphase promoting complex/cyclosome (APC/C), which is responsible for securin destruction, followed by separase-dependent cohesin cleavage [11,12]. As the dynamic behaviour of chromosome and the nucleus has to be regulated coordinately at anaphase, it would be attractive to speculate that APC/C-dependent and SCF-dependent ubiquitin proteolysis would act in a harmonious manner at this stage towards different substrates to ensure timely segregation of sister chromatids and nuclear envelopes.

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