Erratum

Appelgren, H., Kniola, B. and Ekwall, K. (2003). Distinct centromere domain structures with separate functions demonstrated in live fission yeast cells. *J. Cell Sci.* **116**, 4035-4042.

We apologize for the errors that occurred in the Summary of both the online and print versions of this paper.
The corrections are detailed below.

In line 1 of the Summary, *Schizosaccaromyces pombe* is incorrectly referred to as '*Saccharomyces pombe*'.
Correct version: Fission yeast (*Schizosaccaromyces pombe*) centromere DNA is organized in a central core region flanked on either side by a region of outer repeat (*otr*) sequences.

In line 29 of the Summary, the word 'of' is missing.
Correct version: The declustering of centromeres in *mis6* cells correlated with loss of the Ndc80 kinetochore marker protein from the centromeres.

In line 35 of the Summary, mis-segregation has been incorrectly changed to 'misaggregation'.
Correct version: Time-lapse microscopy of live *mis6* and *nuf2-1* mutant cells in mitosis showed similar severe mis-segregation phenotypes whereas the *rik1* mutants showed a mild cohesion defect.

Distinct centromere domain structures with separate functions demonstrated in live fission yeast cells

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Summary

Fission yeast (*Saccharomyces pombe*) centromere DNA is organized in a central core region flanked on either side by a region of outer repeat (*otr*) sequences. The *otr* region is known to be heterochromatic and bound by the Swi6 protein whereas the central core region contains an unusual chromatin structure involving the histone H3 variant Cnp1 (*S. pombe* CENP-A). The central core is the base for formation of the kinetochore structure whereas the flanking region is important for sister centromere cohesion. We have previously shown that the ultrastructural domain structure of *S. pombe* centromeres in interphase is similar to that of human centromeres. Here we demonstrate that *S. pombe* centromeres are organized in cytologically distinct domains even in mitosis. Fluorescence in situ hybridization of fixed metaphase cells revealed that the *otr* regions of the centromere were still held together by cohesion even after the sister kinetochores had separated. In live cells, the central cores and kinetochores of sister chromosomes could be distinguished from one another when they were subjected to mitotic tension. The function of the different centromeric domains was addressed. Trans-acting mutations affecting the kinetochore (*nuf2*) central core domain (*mis6*) and the heterochromatin domain (*rik1*) were analyzed in live cells. In interphase, both *nuf2* and *mis6* caused declustering of centromeres from the spindle pole body whereas centromere clustering was normal in *rik1* despite an apparent decondensation defect. The declustering of centromeres in *mis6* cells correlated with loss the Ndc80 kinetochore marker protein from the centromeres. Interestingly the declustered centromeres were still restricted to the nuclear periphery thus revealing a kinetochore-independent peripheral localization mechanism for heterochromatin. Time-lapse microscopy of live *mis6* and *nuf2-1* mutant cells in mitosis showed similar severe misaggregation phenotypes whereas the *rik1* mutants showed a mild cohesion defect. Thus, *S. pombe* centromeres have two distinguishable domains even during mitosis, and our functional analyses support the previous observations that the kinetochore/central core and the heterochromatin domains have distinct functions both in interphase and mitosis.

Movies available online

Key words: Fission yeast, Centromere, Mitosis, Live analysis

Introduction

The centromere in most species can be observed as a localized primary constriction of the metaphase chromosome. Centromeric DNA harbors a large protein complex, the kinetochore, which mediates the attachment of the chromosomes to the mitotic and meiotic spindle thereby ensuring proper chromosome segregation. Although centromeric DNA sequences have little homology between organisms, structurally the centromeres and kinetochores are similar between such distantly related species as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila melanogaster* and humans (reviewed by Pidoux and Allshire, 2000; Bjerling and Ekwall, 2002; Blower et al., 2002). Centromeric DNA in fission yeast occupies 40-100 kb on each chromosome, organized with a unique central core sequence (*CC/cnt*) (Chikashige et al., 1989; Clarke and Baum, 1990) flanked by inner (*imr/B*) and outer (*otr/K+L)* repeat arrays (Fig. 1A). The use of chromatin immunoprecipitation (ChIP) has established that the central core region is bound by proteins Cnp1 (fission yeast homologue of human CENP-A) Mis6 and Mis12 bind (Takahashi et al., 2000; Saitoh et al., 1997) and XMAP215 homologues, Dis1 and Mtc1/Alp14 (Garcia et al., 2001; Nakaseko et al., 2001). In contrast the outer repeated region is occupied by the chromodomain proteins Swi6 and Chp1 that are structural components of centromeric heterochromatin over the *imr* and *otr* repeats (Partridge et al., 2000). The passenger protein Bir1/Cut17 transiently associates with the *imr* and *otr* repeats and not with the central core region in mitosis (Morishita et al., 2001). Thus, the DNA sequence analysis that first revealed the symmetrical organization of *S. pombe* centromeres (Clarke et al., 1986; Chikashige et al., 1989) and the pattern of protein binding (established by ChIP analysis) to the central core and *otr/imr* regions suggested that the fission yeast centromeres are composed of two different domains. The contrasting phenotypes of mutants such as *rik1* and *swi6,* with defects in heterochromatin (Ekwall et al., 1995; Ekwall et al., 1996), as compared to mutants such as *mis6,* with defects in the central core (Saitoh et al., 1997), indicated that these two domains are functionally distinct. Interphase centromeres in *S. pombe* are

Table 1. Yeast strains used in this study

Strain	Genotype	Source
Fy2214	*h⁻ leu1-32 ura4-D18 ade6-210 GFP-Swi6-LEU2*	Pidoux et al., 2000
Hu334	*h⁺ ade6-210 leu1-32 ura4-D18 Pom152-GFP::kanMX6*	This study
Hu500	*h⁻ Ndc80-GFP::kanMX6 ura4-Bub1-HA::ura4+ leu1-32 ade6*	Wigge and Kilmartin, 2001
Hu607	*rik1::LEU2 Ndc80-GFP::kanMX6 ura4 ade6*	This study
Hu633	*leu1-32 mis6-302 ura4-DSE/D18 ade6-210/NCO GFP-Swi6-LEU2*	This study
Hu634	*leu1-32 mis6-302 ura4-DSE/D18 ade6-210/NCO Ndc80-GFP::kanMX6*	This study
Hu810	*h⁺ Cut12-GFP::ura4+ CFP-Cnp1::kanMX6 leu-132 ura4-DSE/D18*	This study
Hu849	*h⁻ Cut12-CFP::kanMX6 Ndc80-GFP::kanMX6 ade6 leu-132*	This study
Hu891	*h⁺ leu1-32 ura4 Cut12-CFP::kanMX6 lacO (LYS1)/dis1 GFP-LacI (HIS7)*	This study
Hu899	*mis6-302 leu1-32 ura4-DSE/D18 Cut12-CFP::kanMX6 lacO (LYS1)/nmt1 GFP-LacI-NLS (HIS7)*	This study
Hu900	*mis6-302 ade6-210/NCO leu1-32 ura4-DSE/D18 Pom152-GFP::kanMX6*	This study
Hu901	*rik1::LEU2 leu1-32 ura4 Cut12-CFP::kanMX6 lacO (LYS1)/nmt1 GFP-LacI-NLS (HIS7)*	This study
Hu903	*rikI::LEU2 ade6-210 leu1-32 ura4-DSE/D18 Pom152-GFP::kanMX6*	This study
Hu908	*nuf2-1 leu1-32 ura4 Cut12-CFP::kanMX6 lacO (LYS1)/nmt1 GFP-LacI-NLS (HIS7)*	This study
Hu981	*Cut12-CFP::kanMX6 Ndc80-GFP::kanMX6 CFP-Cnp1::kanMX6 leu1-32 ade6 ura4*	This study
Hu1048	*h⁺/h⁻ diploid Cut12-CFP::kanMX6 Cut12-GFP::ura4+*	This study

discretely clustered at the nuclear periphery, adjacent to the spindle pole body (SPB) that is located on the cytoplasmic side of the nuclear envelope (Funabiki et al., 1993). This clustering facilitated the description of cytological structural domains in fission yeast, and revealed a structural conservation between fission yeast and humans (Kniola et al., 2001). These results have both paved the way for the current study in which we present analyses of the structure of fission yeast centromeres during mitosis and of the structure of fission yeast centromeres in live cells, and also for further investigation into the putative specific functions of the centromere *otr/imr* and kinetochore central core domains in interphase and mitosis.

Materials and Methods

Strains and media

Media were prepared according to standard methods (Moreno et al., 1991). Strains used are listed in Table 1. Strains carrying genes fused to green and cyan fluorescent proteins (GFP and CFP) were constructed using the methods described previously (Bahler et al., 1998) with the following modifications. Plasmids containing CFP were constructed by replacing the entire coding region of GFP with that of CFP using standard techniques. Briefly, CFP was amplified by PCR using the cameleon pYC2 as template (Miyawaki et al., 1997) and subsequently ligated into pFA6 in which GFP had been released. Expression of N-terminally tagged genes was controlled by the thiamine repressed *nmt1* promoter.

Microscopy

We performed GFP and CFP fluorescence microscopy on live cells using Openlab software version 3.1 and a ZEISS Axioplan 2 imaging microscope equipped with a Hamamatsu C4792-95 camera. Cells were prepared as described previously (Facanha et al., 2002; Pidoux et al., 2000). Cells were analyzed at 30°C with the exception of *mis6-302* and *nuf2-1* cells, which were analyzed at 36°C. The temperature during cultivation of *mis6-302* and *nuf2-1* cells was raised from 25°C to 36°C 6 hours and 4 hours, respectively, prior to analysis. Electron microscopy was carried out according to Kniola et al. (Kniola et al., 2001) and fluorescence in situ hybridization (FISH) was performed as described by Ekwall et al. (Ekwall et al., 1995) using the pRS140 probe to detect *otr* (Chikashige et al., 1989).

Movies

Frames were collected every 25 seconds and are displayed at a rate of 2 frames/second. Movies are available at http://jcs.biologists.org/supplemental and selected images are presented in Fig. 1B,C.

Results and Discussion

Distinct centromere domains during live mitosis

To obtain a more comprehensive understanding of centromere function and dynamics we first tested if mitotic fission yeast centromeres were organized into cytologically separable domains. FISH was used to detect centromeric outer repeat (*otr*) sequences in *S. pombe* cells immunolabeled for the kinetochore protein Ndc80. Ndc80 is a kinetochore marker protein that forms an evolutionarily conserved protein complex with Nuf2 and Spc24 (Wigge and Kilmartin, 2001). Digital deconvolution analysis (z=0.2 μm) revealed that cells in metaphase had a single unseparated cen (*otr*)-FISH signal on paired sister chromatids that were flanked by two Ndc80-GFP signals facing opposite cellular poles (Fig. 1B). The distance measured (0.48-0.53 μm) between the paired kinetochore signals correspond to the published kinetochore to kinetochore distances of 3-dimensional metaphase spindle models, based on EM serial sectioning (Ding et al., 1993). We therefore concluded that the heterochromatin (*otr*) domain was clearly distinct from the kinetochore domain in metaphase.

Using green and cyan fluorescent proteins (GFP and CFP) we were then able to explore the centromere/kinetochore dynamics and multilayered structure in real time. We were able to simultaneously detect two tagged proteins by using strains expressing chromosomal copies of a combination of both green-tagged and cyan-tagged proteins. As a reference point for spindle movement, the spindle pole body (SPB) marker Cut12-GFP (Bridge et al., 1998) or Cut12-CFP was used. We also CFP tagged the *S. pombe* central core binding protein Cnp1 (Takahashi et al., 2000). Cnp1 is the *S. pombe* homologue of human CENP-A (Warburton et al., 1997; Vafa and Sullivan, 1997), *Drosophila* CID (Blower and Karpen, 2001), budding yeast Cse4 (Meluh et al., 1998) and *C. elegans* HCP3 (Buchwitz et al., 1999). CENP-A is a histone H3 variant that creates a specialized chromatin structure, which determines the site of kinetochore assembly.

We began an investigation of centromere domain movements in live cells by examining the spindle growth using the Cut12-CFP marker (see Movie 1 at http://jcs.biologists.org/supplemental). As seen in earlier studies (Nabeshima et al., 1998), we found that the SPBs moved in three

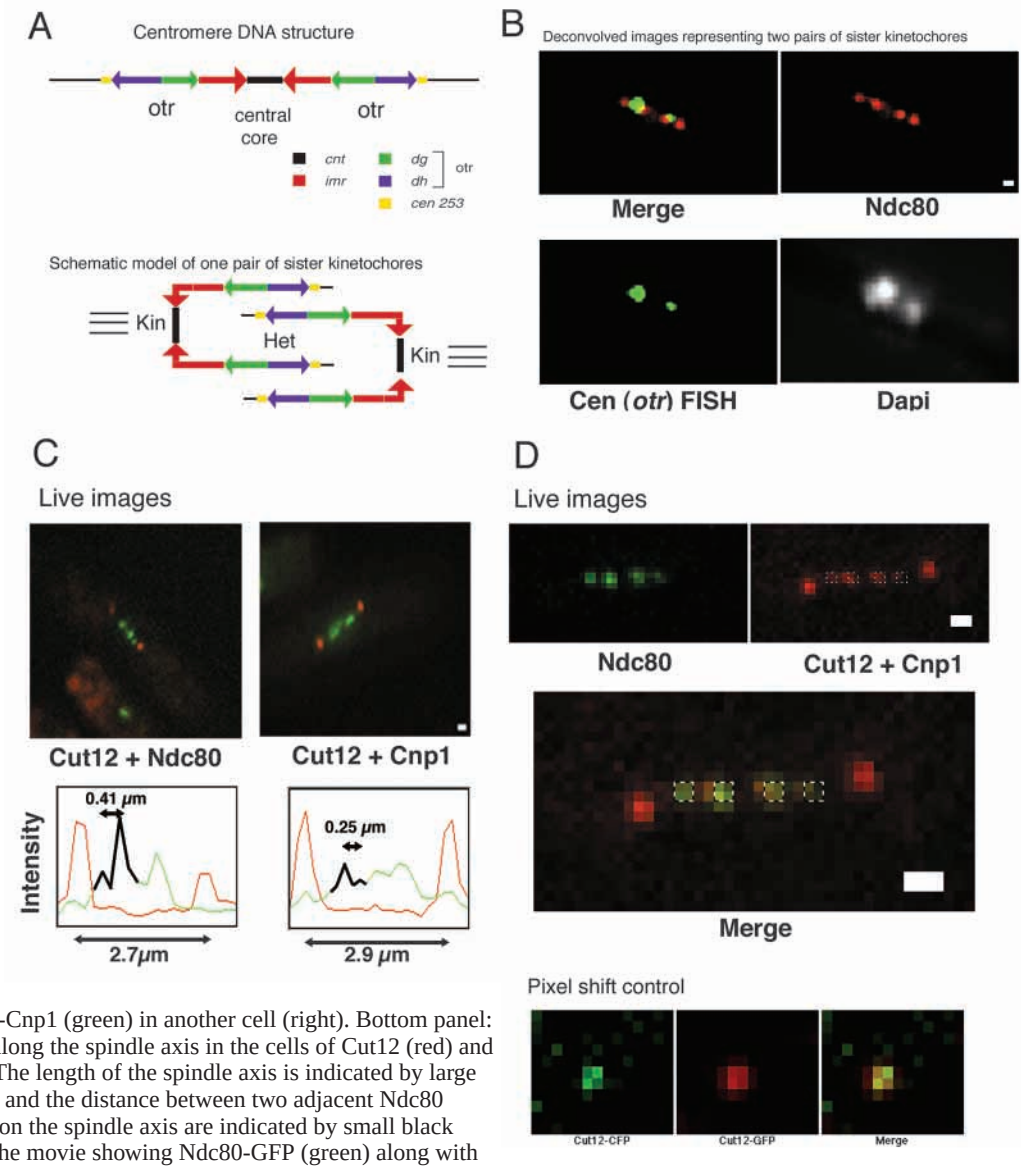

Fig. 1. Mitotic fission yeast centromeres are organized in cytologically separable domains. (A) Schematic diagrams of *S. pombe* centromere structures. Centromere 1 is shown as an example but centromeres 2 and 3 have similar symmetric organization. Top: centromere 1 DNA consists two *otr* regions containing a region of centromeric outer repeats (*dg* and *dh*) flanking a single central core region containing the *cnt* sequence. Bottom: a putative structure of centromere 1 in sister centromeres at metaphase (based on data in Fig. 1B,C,E and supplemental data at http://jcs.biologists.org/supplemental/). Kinetochores (Kin) are separated and facing opposite spindle poles whereas the *otr* regions are held together by sister-chromatid cohesion. The central core regions are the bases for Kin structures. (B) Deconvolved IF images of fixed metaphase cells showing two pairs of sister kinetochores. The *otr* DNA sequences were detected by FISH (green) and are flanked by the kinetochore protein (Ndc80) signal (red). (C) Frames from live imaging movies (see Movies 1 and 2 at http://jcs.biologists.org/supplemental) showing the spindle pole body protein Cut12-CFP (red) along with Ndc80-GFP (green) in one cell (left) and Cut12-GFP (red) along with CFP-Cnp1 (green) in another cell (right). Bottom panel: the graphs show the pixel intensities along the spindle axis in the cells of Cut12 (red) and Cnp1 or Ndc80 as indicated (green). The length of the spindle axis is indicated by large black arrowed lines below each graph and the distance between two adjacent Ndc80 (0.41 μm) or Cnp1 (0.25 μm) signals on the spindle axis are indicated by small black arrowed lines. (D) Top: frames from the movie showing Ndc80-GFP (green) along with CFP-Cnp1 and Cut12-CFP (red) in one cell. The dotted squares represent the pixel position of the Ndc80 signals (see Movies 1 and 2 at http://jcs.biologists.org/supplemental). Bottom: pixel shift control of the Hu1048 strain expressing the same protein in dual colors: Cut12-CFP (green) and Cut12-GFP (red). The perfect colocalization of the two colors of Cut12 (bottom) shows that the pixel differences between Cnp1 and Ndc80 (top) are significant. (A-C) Scale bar: 0.50 μm.

distinct phases. In the first phase, the SPB was duplicated. In the second phase the spindle length remained nearly constant (2.5-3.0 μm) while the sister kinetochores made oscillatory movements back and forth. In the third phase, which corresponds to anaphase B, the spindle rapidly extended (up to 10 μm). By taking advantage of the nearly constant spindle length of the second stage of spindle growth we were able to establish the relative position on the metaphase spindle of our marker proteins. The central core domain of the centromere detected with CFP-Cnp1, and the kinetochore detected with Ndc80-GFP can be seen occupying slightly different positions along the spindle axis when compared to the localization of Cut12-CFP/GFP in cells with equal axis lengths (2.7-2.9 μm distance between SPBs) (Fig. 1C; Movies 1 and 2 at http://jcs.biologists.org/supplemental). The slightly different localizations of Cnp1 and Ndc80 during phase two were directly visualized in triple-tagged live cells expressing Ndc80-GFP, CFP-Cnp1 and Cut12-CFP (Fig. 1C, top; see Movie 3 at http://jcs.biologists.org/supplemental). The perfect colocalization of the dual color of the control strain expression of Cut12 (Fig. 1C, bottom) shows that the pixel differences between Cnp1 and Ndc80 (Fig. 1C, top) are significant. This finding reinforced the evidence for a multilayered domain structure in mitotic fission yeast centromeres. A schematic model illustrating our observations on *S. pombe* centromere structures at metaphase is presented in the bottom panel of Fig. 1A.

Different centromere domains and different centromere subfunctions

Kinetochore assembly, binding of kinetochore microtubules, orientation of sister kinetochores to opposite poles and their poleward movements are separable centromere functions

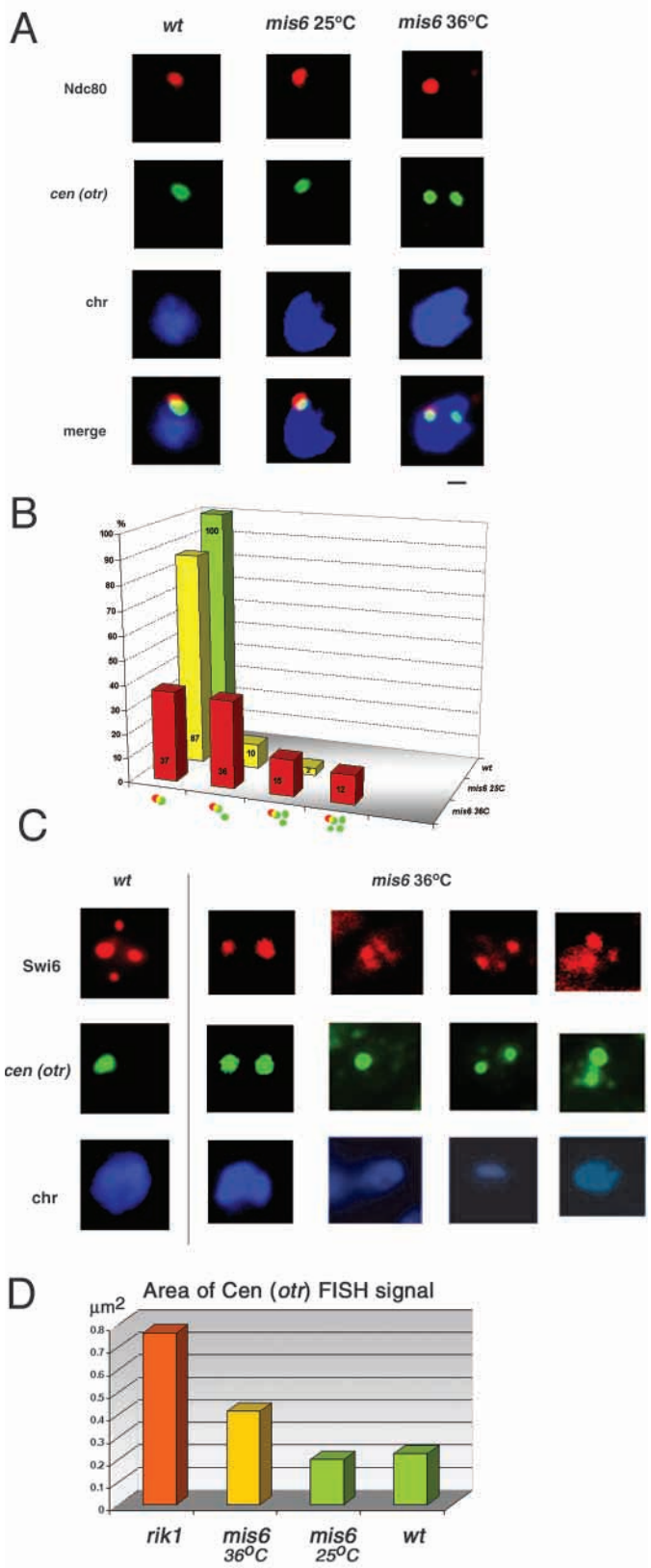

Fig. 2. Interphase centromere DNA clustering is impaired in *mis6* cells and declustered centromeres contain the heterochromatin marker Swi6 but lack the kinetochore component Ndc80. (A) Images showing *cen* (*otr*)-FISH and Ndc80 localization in wild-type and *mis6* cells at the permissive and restrictive temperatures. Scale bar: 0.40 µm. (B) Diagram showing the percentage of declustered centromeres. Symbols below the diagram show that the four categories of bars represent cells with normal centromere clusters, cells with one declustered centromere, cells with two declustered centromere FISH signals and finally cells with three declustered centromere FISH signals. (C) Images showing the location of the chromodomain protein GFP-Swi6 in wild-type cells and *mis6* cells, respectively. (D) The area of cen(*otr*)-FISH signals represented as a bar diagram. The area in *rik1-302* at 25°C (strain Hu607) was 0.8±0.4 µm, *mis6* (Hu633) at 36°C was 0.4±0.2 µm, *mis6* at 25°C was 0.2±0.1 µm and wild type (Fy2214) at 25°C was 0.2±0.1 µm (*n*=50).

which may be organized by different structural domains of the centromere. Evidence from *Drosophila* shows that cohesion function is independent from kinetochore assembly (Lopez et al., 2000) and the results of RNAi inhibition of CID (*Drosophila* CENP-A) also argues that the centromere and flanking heterochromatin are physically and functionally separable protein domains that are required for different centromere functions (Blower and Karpen, 2001). In fission yeast, mutations in cohesin, which is normally enriched in the flanking heterochromatin, do not seem to affect Cnp1 loading in the central core region (Toyoda et al., 2002). To further test if the different domains we characterized had different centromere functions, we analyzed trans-acting mutations that affect each of the domains. The *rik1-304* mutation specifically disrupts centromeric heterochromatin (Allshire et al., 1995; Ekwall et al., 1996). The *mis6* mutation abolishes Cnp1 loading to the central core region (Saitoh et al., 1997; Takahashi et al., 2000). The *nuf2-1* mutation disrupts the kinetochore (Nabetani et al., 2001).

We began by analyzing the effect of *mis6* mutation on interphase centromere clustering and kinetochore behavior using *otr* FISH combined with immunofluorescence microscopy (IF) of Ndc80-GFP. It has previously been shown that the first mitosis is normal when synchronized *mis6* mutant cells are shifted to the restrictive temperature but the second mitosis is severely defective and lethal (Saitoh et al., 1997). Furthermore it was shown that a high proportion of *mis6* interphase cells in a unsynchronized culture, 6 hours after being shifted up to the restrictive temperature, had scattered centromere FISH signal, not clustered at the SPB (Saitoh et al., 1997). These results were confirmed in our study where the control wild-type interphase cells invariably showed one large centromere FISH signal overlapping with the Ndc80-GFP signal (Fig. 2A,B) whereas 63% of *mis6* interphase cells had multiple dispersed after 6 hours at the restrictive temperature. One of the cen (*otr*) FISH signals usually overlapped with the Ndc80 signal. At the permissive temperature, 13% of *mis6* cells gave multiple cen (*otr*) spots. Our interpretation of the loss of the Ndc80 kinetochore marker that correlates with scattered cen (*otr*) signals is that the central core region affected by *mis6* was required to connect the kinetochore component Ndc80 to the centromere, and that failure in this process led to declustering of the centromeres.

The chromodomain proteins Swi6 (fission yeast homologue of human HP1) localizes to centromeres mating type region and telomeres (Ekwall et al., 1995). Swi6 is required for proper formation of heterochromatin as well as sister chromatid

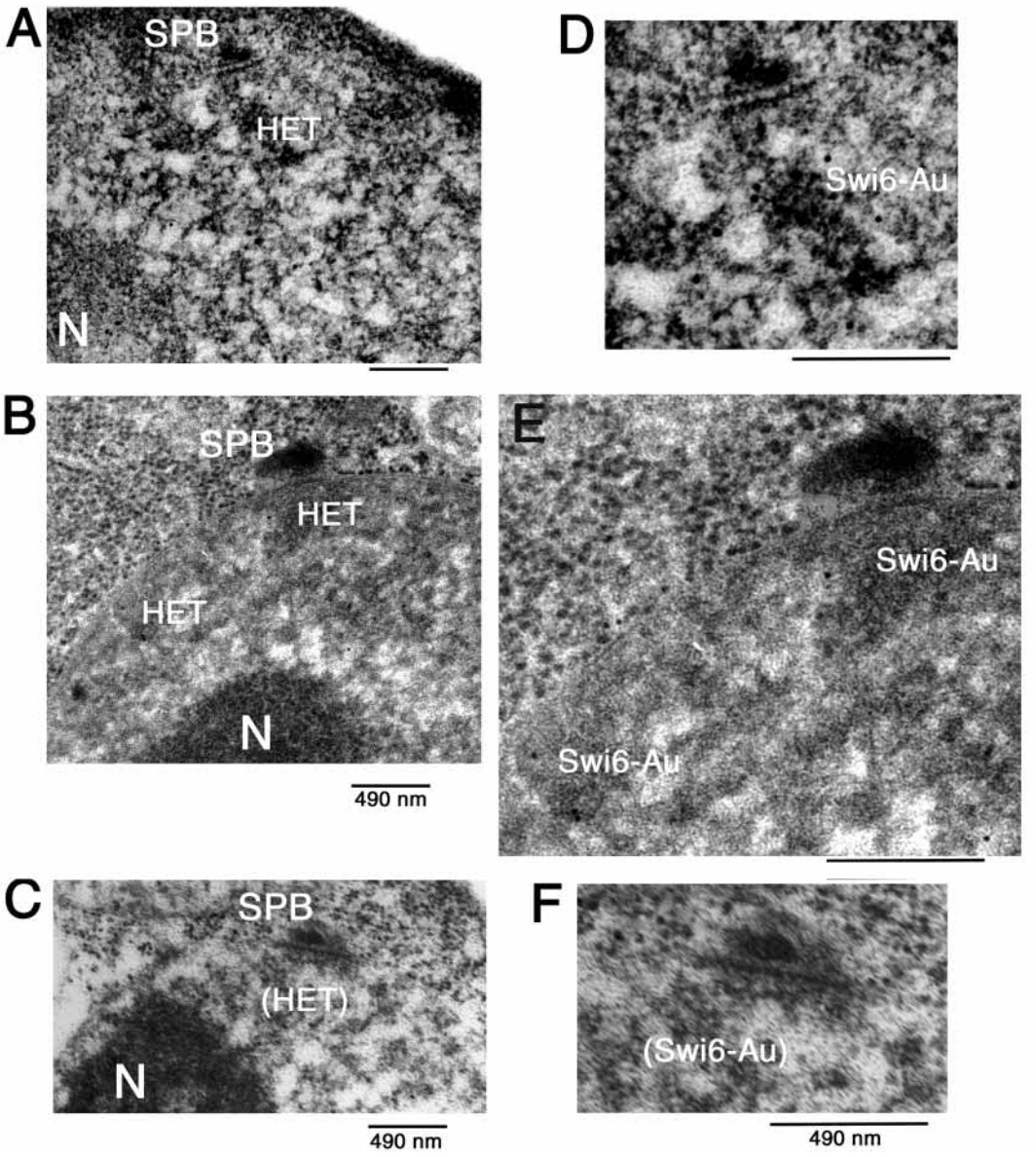

Fig. 3. EM analysis of centromeres in *mis6* and *rik1* cells. Cells were subjected to HPF EM and immuno-gold staining for GFP-Swi6. (A,D) Different magnifications of a wild-type cell labeled with GFP-Swi6 immuno-gold. The strain used was Fy2214. (B,E) Two different magnifications of a *mis6* cell grown at 36°C prior to fixation and labeled with GFP-Swi6 immuno-gold. Declustering of centromeric heterochromatin is seen. The strain used was Hu633. (C,F) Two different EM magnifications of a *rik1* cell where electron dense heterochromatin is not readily detectable. Immuno-gold staining of GFP-Swi6 was not performed on this sample. The strain used was Hu607. HET, heterochromatin; N, nucleolus; SPB, spindle pole body. Swi6-Au, 10 nm gold labeling shows the presence of GFP-Swi6. Scale bar: 490 nm.

cohesion, and the recruitment of Swi6 to the *otr* centromeric DNA is dependent on Clr4 and Rik1 (Ekwall et al., 1996; Bernard et al., 2001; Nonaka et al., 2002). To investigate if centromeric heterochromatin was affected by a mutation in *mis6*, we immunostained fixed *mis6* cells with anti-Swi6 and found that Swi6 was still localized on the declustered centromeres (Fig. 2C). While centromeric Swi6 spots were declustered the telomere and mating type spots of Swi6 still appeared relatively normal in *mis6* cells. To verify that heterochromatin was indeed separated from the previously identified anchor structures and plate like structures near the SPB (Kniola et al., 2001) we fixed wild-type and *mis6* cells using high pressure freezing (HPF) and examined them by Swi6 immunoelectron microscopy (EM). Centromeric electron-dense centromeric heterochromatin was normal in wild-type controls cells but was detected at distances far away from the SPB and anchor structure in *mis6* cells, suggesting that the loss of Cnp1 played a role in anchoring the centromere DNA to the SPB via the kinetochores (Fig. 3B,E). The structure of heterochromatin in *rik1* cells was also analyzed by HPF-EM. Using this method, the heterochromatin domain was not readily detectable and appeared less electron dense than in wild-type control cells, whereas the plate-like 'anchor' structures indeed appeared to be intact (Fig. 3C,F). This indicated that the kinetochore assembly mediated by the central core domain was functionally independent of the heterochromatin domain. To test this further, we analyzed the *rik1* mutant by cen (*otr*) FISH, and as previously demonstrated (Ekwall et al., 1996) we found that the clustering of centromeres in interphase was intact. Furthermore, the colocalization with kinetochore component Ndc80 was normal in 100% of *rik1* cells (data not shown). However, the area of the cen (*otr)* FISH signal in interphase was enlarged from 0.2±0.1 in wild-type cells to 0.8±0.2 μm^2 in *rik1* cells (n=50). This indicated that the centromeric heterochromatin was decondensed in cells with compromised *rik1* (Fig. 2D). In contrast, the *mis6* mutant cells showed a cen (*otr)* FISH area of 0.2±0.1 μm^2 at 25°C and 0.4±0.2 μm^2 at 36°C (n=50). The

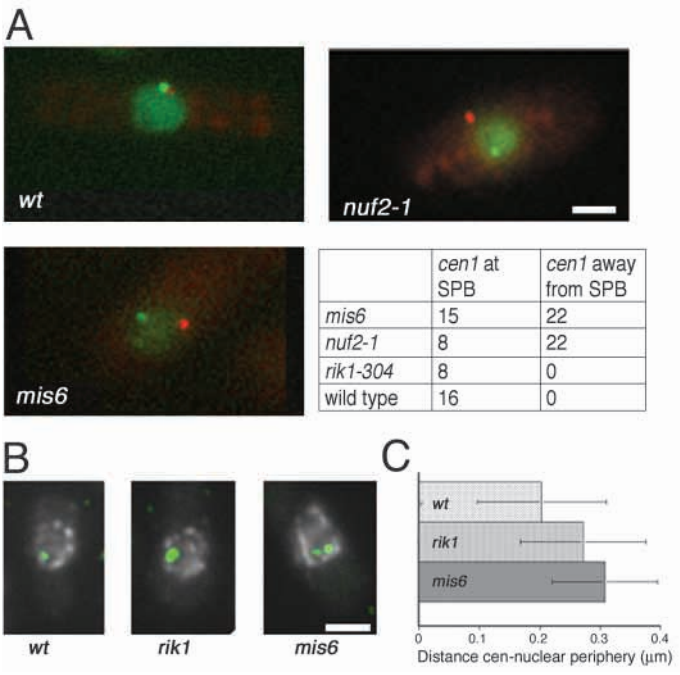

Fig. 4. Declustering of centromeres in *mis6* and *nuf2-1* cells and localization of declustered centromeres to the nuclear periphery. (A) Images and statistical analysis of live cells showing the location of *cen1::lacO*-LacI-GFP (green) signals and the SPB marker Cut12-CFP (red) in wild-type, *mis6* and *nuf2-1* cells as indicated. Scale bar: 1.0 μm. *cen1* at SPB indicates that *cen1::lacO*-LacI-GFP is within 0.2 μm from Cut12-CFP and *cen1* away from SPB indicates that *cen1::lacO*-LacI-GFP is >0.2 μm from Cut12-CFP. (B) Images showing the location of centromeres (green) with respect to the nuclear periphery marker Pom152-GFP (white). Scale bar: 1.0 μm. See also deconvolved stacks of images (see supplemental Fig. S1 at http://jcs.biologists.org/supplemental). (C) Diagram showing the distance in one focal plane between the declustered centromere cen(*otr*)-FISH signal and the nuclear periphery as marked by Pom152-GFP (strains were Hu334 wild type (*n*=26), Hu903 *rik1* (*n*=36) and Hu900 *mis6* (*n*=16).

slight increase in the area of the *otr* signal in *mis6* cells at 36°C can be accounted for by the scattering of centromeres rather than a decondensation effect. These findings substantiated the notion that heterochromatin and central core/kinetochore domains have separate functions in interphase cells.

Microtubules are not required for centromere clustering

To investigate centromere clustering in live interphase cells we first tested whether clustering was dependent on microtubules by treating cells expressing Cut12-GFP and CFP-Cnp1 with 10 μg/ml the microtubule poison thiabendazole (TBZ). The resulting live-analysis movies (see Movies 4 and 5 at http://jcs.biologists.org/supplemental) revealed that, although TBZ suppressed the SPB movements, there was no detectable declustering of centromere (CFP-Cnp1) signals in the TBZ-treated cells.

Centromere declustering effects of nuf2 and mis6

In order to visualize what happens to declustered centromeric DNA in live cells, an integrated lacI-GFP fusion protein that binds lacO repeats integrated in the vicinity of centromere 1 (Nabeshima et al., 1998) was used in combination with Cut12-CFP and the mutants *rik1*, *nuf2-1* and *mis6*. 73% and 59% respectively of *nuf2* and *mis6* mutant cells showed an abnormal positioning of the *cen1::lacO*-LacI-GFP signals i.e. cen1 far away from SPB, whereas cen1 positioning was normal, at the SPB, in all wild-type and *rik1* cells (Fig. 4A). To test if the declustered centromeres in the *mis6* mutant maintained attachments to the nuclear periphery, we immunostained the Pom152-GFP nuclear envelope marker (Bjerling et al., 2002) and FISH stained cen (*otr*), which detects all three centromeres. This experiment showed that most of the declustered centromeres in *mis6* cells were still preferentially found at the nuclear periphery, on average 0.30 μm from the nuclear rim (Fig. 4B,C; see also supplemental Fig. S1 at http://jcs.biologists.org/supplemental/). Thus, since *mis6* is required for association of Ndc80 to the centromere (Fig. 2A) we conclude that kinetochore components are normally required for anchoring centromeres to the SPB, but an unknown, independent mechanism may be responsible for peripheral positioning of the declustered centromeric heterochromatin domains, even in the absence of kinetochore components.

Table 2. Characteristics of anaphase *cen1* movements in live wild-type, *mis6-302* and *nuf2-1* cells

	SPB-SPB (μm)	SPB-*cen1* (μm)	SPB-*cen1** (μm)
wt	3.0-4.0 (*n*=7)	0.24±0.09	2.87±0.58
	4.0-5.0 (*n*=7)	0.5±0.31	3.85±0.78
mis6-302	3.0-4.0 (*n*=12)	0.95±0.61	1.13±0.6
	4.0-5.0 (*n*=8)	1.72±0.42	1.93±0.46
nuf2-1	3.0-4.0 (*n*=7)	0.91±0.22	1.34±0.3
	4.0-5.0 (*n*=4)	0.74±0.08	1.1±0.36

Column 1 shows two categories of SPB-SPB distance in wild-type (wt) *mis6-302* and *nuf2-1* cells, respectively. Columns 2 and 3 show the distance from one SPB to the centromeres in the two segregating chromosome 1 (*cen1* and *cen1**, respectively).

Consequences in mitosis

Mis6 has previously been shown to influence spindle length, as shown by the increase of metaphase spindle length in *mis6* mutants, which may be caused by an imbalance between the kinetochore forces that are pulling and the kinetochore-independent forces that are pushing on the centromeres (Goshima et al., 1999). To visualize mitotic defects in *mis6* and *nuf2-1* cells we monitored *cen1::lacO*-LacI-GFP and the SPB marker Cut12-CFP by time-lapse microscopy. We found that both *mis6* and *nuf2-1* mutant cells displayed severe defects in movement of sister-centromeres in relation to the SPB compared with wild-type control cells (Fig. 5). In contrast, the majority of *rik1* mutant cells displayed similar movements of sister-centromeres to those of wild-type cells (data not shown). To characterize the behavior of centromeres in *mis6* and *nuf2-1* cells we measured the distance from one SPB to the other and the distance from one SPB to the centromeres of the two segregating *cen1::lacO*-LacI-GFP signals (*cen1* and *cen1**, respectively). Table 2 shows that on similar length, 4.0-5.0 μm, spindles the average distance between the one SPB and the

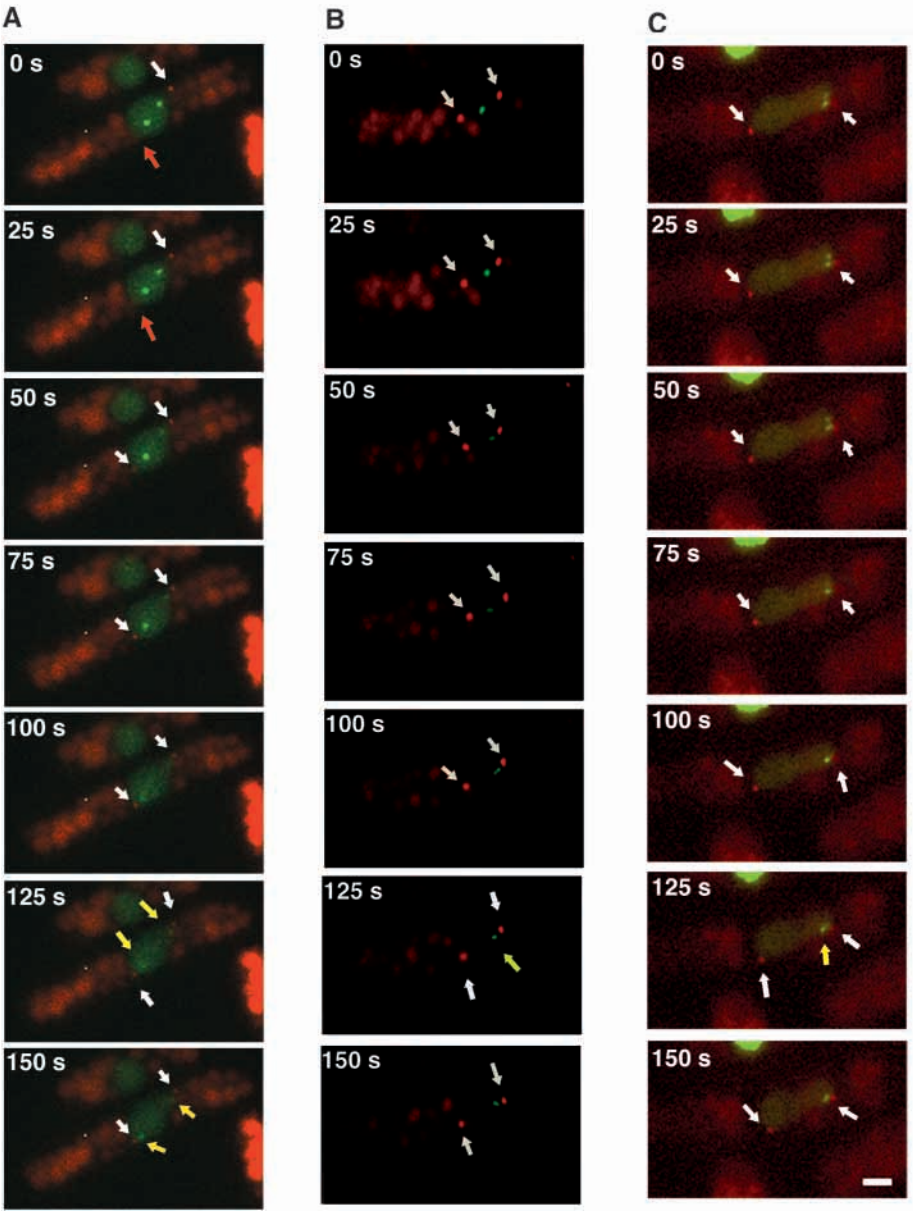

Fig. 5. Analysis of *cen1::lacO*-LacI-GFP and SPBs during mitosis in live wild type, *nuf2* and *mis6* cells. Images from time lapse analysis of live cells showing the location of *cen1::lacO*-LacI-GFP (green) and the SPB marker Cut12-CFP (red) in (A) wild-type (B) *mis6* and (C) *nuf2-1* cells. Scale bar: 0.2 μm. The white and red arrows indicate the positions of the SPB Cut12-CFP signals (red arrows indicate inferred SPB positions from other images in the time course series) and the yellow arrows indicate the position of *cen1::lacO*-LacI-GFP in cases where labeling was weak. The cytoplasmic red signal is nonspecific and due to autofluorescence detected with the CFP filter. Statistical analysis of anaphase *cen1::lacO*-LacI-GFP movements in live wild-type, *mis6-302* and *nuf2-1* cells is shown in Table 2.

closest centromere (*cen1*) was relatively similar in wild-type *mis6* and *nuf2-1* cells (0.5±0.31, 1.72±0.42 and 0.74±0.08 μm, respectively). However, on similar length 4.0-5.0 μm spindles the furthest-away sister centromere, *cen1**, was 3.85±0.78 μm from the same SPB in wild-type cells, 1.93±0.46 μm in *mis6* cells and 1.1±0.36 μm in *nuf2-1* cells. Thus, whereas wild-type centromeres were segregated by at least 3.5 μm, centromeres had not significantly segregated from each other in *mis6* and *nuf2-1* cells. This severe mis-segregation phenotype is in accordance with the phenotypes reported earlier for *mis6* and *nuf2-1* (Saitoh et al., 1997; Nabetani et al., 2001). The fact that the two phenotypes are so similar indicates that loss of kinetochore components from centromeres in *mis6* is functionally equivalent to the compromised kinetochore structures in *nuf2-1*. The consequence in both mutants is likely the occurrence of monotelic attachments in which only one functional sister kinetochore binds kinetochore microtubules or completely unattached sister kinetochores, resulting in spindle-kinetochore force imbalance and rapid spindle elongation as previously observed (Goshima et al., 1999). In contrast, the defects in the heterochromatin domain caused by *rik1* lead to decondensation of the heterochromatin. This decondensation had no effect on clustering and caused no dramatic effect on sister kinetochore behavior (data not shown) except for the previously observed lagging chromosome phenotype (Ekwall et al., 1996). This phenotype is likely caused by a cohesin defect, since Rik1 is required to localize Swi6 to centromeres (Ekwall et al., 1996) and Swi6 is required in its turn for the recruitment of cohesins to the centromeric heterochromatin (Bernard et al., 2001; Nonaka et al., 2002). Interestingly, we also observed a decondensation of centromeric heterochromatin in *rik1* cells (Fig. 2D). In budding yeast, cohesins have been shown to regulate condensin function after condensins are associated with chromatin (Lavoie et al., 2002). Therefore, we speculate that the decondensation observed in *rik1* is explained by loss of Swi6 causing a loss of cohesins and hence misregulation of condensins. In our experiments decondensation or cohesion defects were not observed in trans-acting mutations that affect central core or kinetochore. In contrast, these mutants showed defects in clustering of centromeres at the SPB and severe mitotic defects. Thus, the kinetochore and the central core are functionally distinct from the flanking heterochromatin domain, both with respect to the centromere function in interphase and the mitotic behavior of sister centromeres.

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