Charakterisierung der essentiellen Kinetochorkomponenten Spc7 und Fta2 der Spalthefe *Schizosaccharomyces pombe*

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1 Abkürzungsverzeichnis

Abb.	Abbildung
Bp	Basenpaare
C. elegans	*Caenorhabditis elegans*
cen1	Zentromer 1
C-terminal	Carboxy-terminal
d. h.	das heißt
DNA	Desoxyribonukleinsäure
Kbp	Kilobasenpaare
MDa	Megadalton
min	Minuten
N-terminal	Amino-Terminal
RNAi	Ribonukleinsäure-Interferenz
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
s. u.	siehe unten
+TIP	microtubule plus end–tracking protein
tRNA	transfer-Ribonukleinsäure
u. a.	unter anderem
z. B.	zum Beispiel
z. T.	zum Teil
µm	Mikrometer

2 Zusammenfassung

Die akkurate Verteilung der zuvor duplizierten Chromosomen auf beide Folgezellen in der Mitose ist für das Überleben der Zelle essentiell. Die Anheftung der Schwesterchromatiden über einen auf der Zentromer-DNA assemblierten Multiproteinkomplex, dem Kinetochor, an die Spindel-Mikrotubuli ist hierbei ein kritischer Schritt. Es sind bis dato eine Vielzahl von Proteinen identifiziert worden, die in diesen Prozess involviert sind, jedoch sind die genauen Interaktionen nur teilweise geklärt.

In der vorliegenden Arbeit wurden die beiden neu isolierten und zu unterschiedlichen Kinetochorkomplexen gehörenden Spc7- und Fta2-Proteine der Spalthefe *Schizosaccharomyces pombe* untersucht. Es konnte gezeigt werden, dass diese konstitutiv am Kinetochor assoziierten Proteine für den wildtypischen Aufbau des Kinetochors, für die Anbindung der Kinetochore an die Spindel-Mikrotubuli sowie für die notwendige bipolare Ausrichtung der Chromosomen auf der Spindel benötigt werden.

Weiterhin ergaben meine Analysen, dass das essentielle Spc7-Protein, eine Komponente des NMS-Komplexes, physikalisch mit dem Mikrotubuli-assoziierten EB1-Familienmitglied Mal3 interagiert und somit eine Verbindung zwischen Kinetochor und Mikrotubuli herstellt. *spc7*-Mutanten weisen massive Chromosomenfehlverteilungen sowie starke Spindeldefekte auf. Somit ist Spc7 das bisher einzige konstitutive Kinetochorprotein, das durch das Einwirken auf die Mikrotubuli-Dynamik und/oder auf die bipolare Anordnung der Mikrotubuli den Aufbau und die Funktionalität der Spindel beeinflusst.

Die Untersuchung der essentiellen Sim4-Komplexkomponente Fta2 führte zu der Identifizierung einer neuen Subgruppe innerhalb dieses Komplexes, die aus Fta2 und Mal2 besteht. Beide Proteine üben ähnliche, jedoch nicht identische Funktionen aus. Mutationen in *fta2*$^+$ führen zu starken Chromosomensegregationsdefekten. Ferner ist Fta2 das bisher einzige Protein der Sim4-Gruppe, für das gezeigt werden konnte, dass es einen Einfluss auf die Assoziation des Kinetochors mit der Spindel hat.

Entgegen der biochemisch separaten Aufreinigung der NMS- und Sim4-Kinetochorkomplexe zeigten meine Analysen, dass diese Komplexe über Spc7, Fta2 und Mal2 miteinander verknüpft sind. Mal2 interagiert physikalisch mit Spc7 und zusätzliches *mal2*$^+$ supprimiert durch die Stabilisierung des Mutantenproteins partiell *spc7*-Mutanten. Spc7 wiederum ist spezifisch für die wildtypische Kinetochor-Assoziation von Fta2 notwendig.

3 Einleitung

Chromosomenfehlverteilungen führen zu schweren Defekten

Für die Weitergabe der genetischen Erbinformation ist eine akkurate Verteilung der duplizierten Chromosomen auf die Folgezellen in der Mitose und Meiose notwendig. Eine Fehlverteilung des Chromatins resultiert in Aneuploidie, einem Ungleichgewicht der Chromosomenanzahl, und führt zu krankhaften Veränderungen oder gar zum Tod der Zelle (Übersichtsartikel: Cimini und Degrassi, 2005; Cahill *et al.*, 1999). Beim Menschen beispielsweise gibt es nur wenige, während der Meiose zustande kommende Aneuploidien, die mit dem Leben vereinbar sind. Das wohl bekannteste Beispiel ist das Down-Syndrom, das aus einer Trisomie des Chromosoms 21 resultiert (Jacobs *et al.*, 1959; Lejeune *et al.*, 1959). Aber auch Aneuploidien in somatischen Zellen führen zu schwerwiegenden Erkrankungen. So findet man in Krebszellen häufig eine veränderte Chromosomenanzahl, wobei allerdings bisher noch nicht geklärt ist, ob dies eine Voraussetzung oder eine Folge der Tumorentstehung ist (Übersichtsartikel: Rajagopalan und Lengauer, 2004; Dey, 2004; Marx, 2002). Spontane Aneuploidie ist ebenfalls in Zellen von Alzheimer-Patienten zu beobachten (Migliore *et al.*, 1997). Weiterhin hat man einen Zusammenhang zwischen Aneuploidie und der vorzeitigen Zellalterung in Mäusen festgestellt, wofür es auch beim Menschen einige Anhaltspunkte gibt (Übersichtsartikel: Baker *et al.*, 2005).

Für eine präzise Vererbung des genetischen Materials müssen die Chromosomen in der S-Phase (DNA-Synthese) des Zellzyklus vollständig dupliziert und in der M-Phase (Mitose) mit Hilfe der Teilungsspindel präzise auf die beiden Folgezellen aufgeteilt werden. Mehrere Kontrollpunkte im Zellzyklus stellen sicher, dass nur fehlerfrei replizierte Schwesterchromatiden ohne DNA-Schäden in der Mitose segregiert werden (Pasero *et al.*, 2003; Zhou und Elledge, 2000; Zhou *et al.*, 2002).

Ein kritischer Punkt der Mitose ist die Verknüpfung der Chromosomen mit der mitotischen Spindel. Diese Bindung wird über einen Multiproteinkomplex, dem auf dem Zentromer-Chromatin assemblierten Kinetochor, gewährleistet (Übersichtsartikel: Kline-Smith *et al.*, 2005; Gadde und Heald, 2004).

Der Zentromer-DNA-Bereich variiert innerhalb der Eukaryoten

Das Zentromer-Chromatin verschiedener Organismen ist von großer Variabilität, kann jedoch prinzipiell in zwei Klassen unterteilt werden: in die Punkt- und die regionale Zentromer-DNA. Die Punkt-Zentromere existieren nur in einigen Hefen und zeigen teilweise eine Organismus-übergreifende Sequenzkonservierung. Innerhalb eines Organismus haben sie eine

geringe, genau definierte Länge sowie konservierte Sequenzbereiche (Pluta *et al.*, 1995; Hegemann und Fleig, 1993; Meraldi *et al.*, 2006; Übersichtsartikel: Cleveland *et al.*, 2003). So besteht beispielsweise die Punkt-Zentromer-DNA der Bäckerhefe *Saccharomyces cerevisiae* aus einer 125 Bp langen Sequenz (Fitzgerald-Hayes *et al.*, 1982). Diese wird in drei konservierte Bereiche unterteilt, die mit jeweils spezifischen Proteinen interagieren (Übersichtsartikel: Cleveland *et al.*, 2003).

Dagegen ist die Organisation der Zentromer-DNA in anderen Organismen, wie z. B. in der Spalthefe *Schizosaccharomyces pombe*, in *Arabidopsis thaliana*, in *Drosophila melanogaster* oder auch im Menschen, wesentlich komplexer. Die DNA-Sequenz dieser regionalen Zentromere ist innerhalb eines Organismus nur teilweise und zwischen verschiedenen Organismen gar nicht konserviert und besteht häufig aus wiederkehrenden Sequenzelementen (Übersichtsartikel: Carroll und Straight, 2006). Bei der Bildung eines funktionellen regionalen Zentromers spielen epigenetische Faktoren eine große Rolle (Übersichtsartikel: Carroll und Straight, 2006). So wurde z. B. die Ausbildung von sogenannten Neozentromeren an DNA-Regionen in menschlichen Zellen oder auch in Pflanzen beobachtet, die normalerweise keine Zentromerformation aufweisen und somit keine Zentromer-DNA-spezifischen Sequenzen tragen (Übersichtsartikel: Carroll und Straight, 2006; Amor und Choo, 2002).

Trotz der Unterschiede in der Zentromer-DNA-Sequenz verschiedener Organismen haben die Zentromere aller bisher untersuchten Eukaryoten neben dem in den meisten Organismen vorkommenden Heterochromatin eine spezifische Chromatinstruktur gemeinsam, die essentiell für die Funktionalität des Zentromer-Kinetochor-Elements ist (Pidoux und Allshire, 2005). Diese resultiert aus dem Einbau einer speziellen, konservierten Histon H3-Variante, genannt CENP-A, in die Nukleosomen (Übersichtsartikel: Choo, 2001).

Das Kinetochor verbindet die DNA mit der mitotischen Spindel

Auf der Zentromer-DNA wird das aus mehreren Untereinheiten bestehende Kinetochor assembliert. Der einfachste Kinetochorkomplex wurde bis dato in *S. cerevisiae* beschrieben und besteht aus 17 Subkomplexen mit über 70 Proteinen und einer Masse von mindestens 5 MDa (McAinsh *et al.*, 2003; De Wulf *et al.*, 2003; Nekrasov *et al.*, 2003; Meraldi *et al.*, 2006). Abhängig vom Organismus existiert der Kinetochorkomplex während des gesamten Zellzyklus oder wird sukzessive in diesem aufgebaut (Übersichtsartikel: Maiato *et al.*, 2004).

Viele Kinetochorproteine sind konserviert und finden sich entweder ausschließlich in Punkt-Zentromeren, nur in regionalen Zentromeren oder aber in beiden wieder (Meraldi *et al.*, 2006).

Die Kinetochorproteine können in zwei verschiedene Gruppen eingeteilt werden: in die inneren, direkt an die Zentromer-DNA bindenden Kinetochorproteine und in die äußeren, die Verbindung zur Spindel herstellenden Proteine. Zu diesen äußeren Kinetochorproteinen gehören vier, in bisher fast allen untersuchten Eukaryoten konservierte Einzelproteine oder Komplexe: die Proteine Mcm21/Mal2, Spc105/Spc7, der Ndc80- sowie der MIND-Komplex (Meraldi *et al.*, 2006). Die äußeren Kinetochorproteine interagieren wiederum mit transient an das Kinetochor bindende Proteine. Diese assoziieren entweder Mikrotubuli-unabhängig oder werden über die Spindel an das Kinetochor gebracht. Die essentielle Mikrotubuli-Kinetochor-Verbindung wird durch Vertreter der transienten Proteine mit den äußeren Kinetochorkomponenten hergestellt (Übersichtsartikel: Rieder und Salmon, 1998; Amor *et al.*, 2004; Maiato *et al.*, 2004).

An die Kinetochore bindet im Zuge der Mitose die Teilungsspindel, die aus Mikrotubuli sowie Mikrotubuli-assoziierten Proteinen besteht (Übersichtsartikel: Tanaka *et al.*, 2005b). Die dynamischen Mikrotubuli durchlaufen Phasen des Wachsens und des Schrumpfens, deren Rate zellzyklusabhängig gesteuert wird (Nogales *et al.*, 1999). Die bipolare Spindel wird hauptsächlich von den beiden gegenüberliegenden Zentrosomen (bei Hefen den Spindelpolkörpern) der Zelle polymerisiert. Weiterhin können vom Kinetochor aus Mikrotubuli gebildet werden, die dann in die Spindel integrieren (Übersichtsartikel: Gadde und Heald, 2004; Khodjakov *et al.*, 2003; Tulu *et al.*, 2006). In *Drosophila*-S2-Zellen konnte gezeigt werden, dass auch nicht-depolymerisierte Mikrotubuli aus der vorangegangenen Interphase in die Spindel eingefügt, sowie weitere Mikrotubuli aus der Spindel heraus gebildet werden (Mahoney *et al.*, 2006).

Die Mikrotubuli der mitotischen Spindel können in drei verschiedene Gruppen unterteilt werden: zum einen in die vom Spindelpolkörper zum Kinetochor eines Chromatids reichenden Kinetochormikrotubuli, des Weiteren in die interpolaren Mikrotubuli, die von beiden Spindelpolkörpern her polymerisiert werden und mit ihren Plusenden in der Spindelmitte überlappen sowie in die ebenfalls vom Spindelpolkörper ausgehenden, aber zum Zellkortex hin orientierten Astralmikrotubuli. Letzteren wird eine Funktion bei der Spindelpositionierung zugeschrieben (Übersichtsartikel: Kline-Smith und Walczak, 2004). Die Anzahl der an ein Kinetochor bindenden Kinetochormikrotubuli ist vom Organismus abhängig. *S. cerevisiae* Kinetochore werden beispielsweise von nur einem Mikrotubulus gebunden, die Kinetochore von *S. pombe* hingegen von 2-4 Mikrotubuli und menschliche Kinetochore von 20-30 Mikrotubuli (Übersichtsartikel: Maiato und Sunkel, 2004; Ding *et al.*, 1993). Binden mehrere Kinetochormikrotubuli an ein Kinetochor, so liegen die Mikrotubuli

gebündelt vor und werden als Kinetochorfasern bezeichnet (Übersichtsartikel: Maiato und Sunkel, 2004; Kline-Smith und Walczak, 2004).
Es ist bis dato nicht geklärt, ob in Organismen, in denen mehrere Mikrotubuli mit den Kinetochorproteinen assoziieren, jeder Mikrotubulus von dem gleichen, am Kinetochor mehrfach vorhandenen Set an Proteinen gebunden wird. Hiervon wird allerdings ausgegangen (Rieder und Salmon, 1998; Emanuele *et al.*, 2005; Zinkowski *et al.*, 1991).

Der Mechanismus der Chromosomensegregation

Es ist von essentieller Bedeutung für das Überleben der Zelle, dass die beiden Schwesterkinetochore jedes duplizierten Chromosoms an die Mikrotubuli unterschiedlicher Spindelpolkörper binden (Bi-Orientierung). Werden ein oder beide Schwesterchromatiden von nur von einem Spindelpolkörper ausgehende Mikrotubuli gebunden, so spricht man von mono-orientierten Chromosomen (Übersichtsartikel: Tanaka *et al.*, 2005a).
In Vertebraten wie auch in *S. cerevisiae* wurde gezeigt, dass zunächst eine Verknüpfung zwischen dem Kinetochor und der lateralen Fläche des Mikrotubulus, gefolgt von einem schnellen polwärts gerichteten Transport, stattfindet (Übersichtsartikel: Biggins und Walczak, 2003; Tanaka *et al.*, 2005a). In Säugerzellen heften sich diese nun mono-orientierten Chromosomen mit Hilfe eines Motorproteins an benachbarte Mikrotubuli, die bereits bi-orientierte Chromosomen binden. Dieses Motorprotein transportiert das mono-orientierte Chromosom Richtung Zellmitte, womit die Wahrscheinlichkeit einer bipolaren Verknüpfung steigt (Kapoor *et al.*, 2006). Nach der bipolaren Verbindung der Schwesterkinetochore mit der Spindel bleiben die in Richtung Kinetochor orientierten Plusenden der Mikrotubuli weiterhin dynamisch. Es erfolgt die Anordnung der Chromosomen auf der Metaphasenplatte (Metaphase). Nach der Deaktivierung des Spindelkontrollpunktes, der sicherstellt, dass alle Schwesterkinetochore korrekt mit der Spindel verbunden sind, werden die Schwesterchromatiden getrennt und durch Verkürzung der Kinetochormikrotubuli zu den beiden entgegengesetzt gelegenen Spindelpolkörpern der Zelle gezogen (Anaphase A). Dies geschieht wahrscheinlich zum einen durch den vom Kinetochor aus induzierten Abbau des Mikrotubulus-Plusendes (pac-man Modell) und zum anderen durch den polwärts gerichteten Abbau des Mikrotubulus am Minusende (Flux) (Übersichtsartikel: Gadde und Heald, 2004; Kline-Smith und Walczak, 2004). Hierbei unterscheiden sich jedoch die Organismen voneinander. So findet man beispielsweise keinen Flux in *S. pombe* Anaphasen-Spindeln (Kline-Smith und Walczak, 2004; Sagolla *et al.*, 2003; Maiato und Sunkel, 2004). Die Spindelelongation in der Anaphase B separiert die beiden Spindelpolkörper zusammen mit

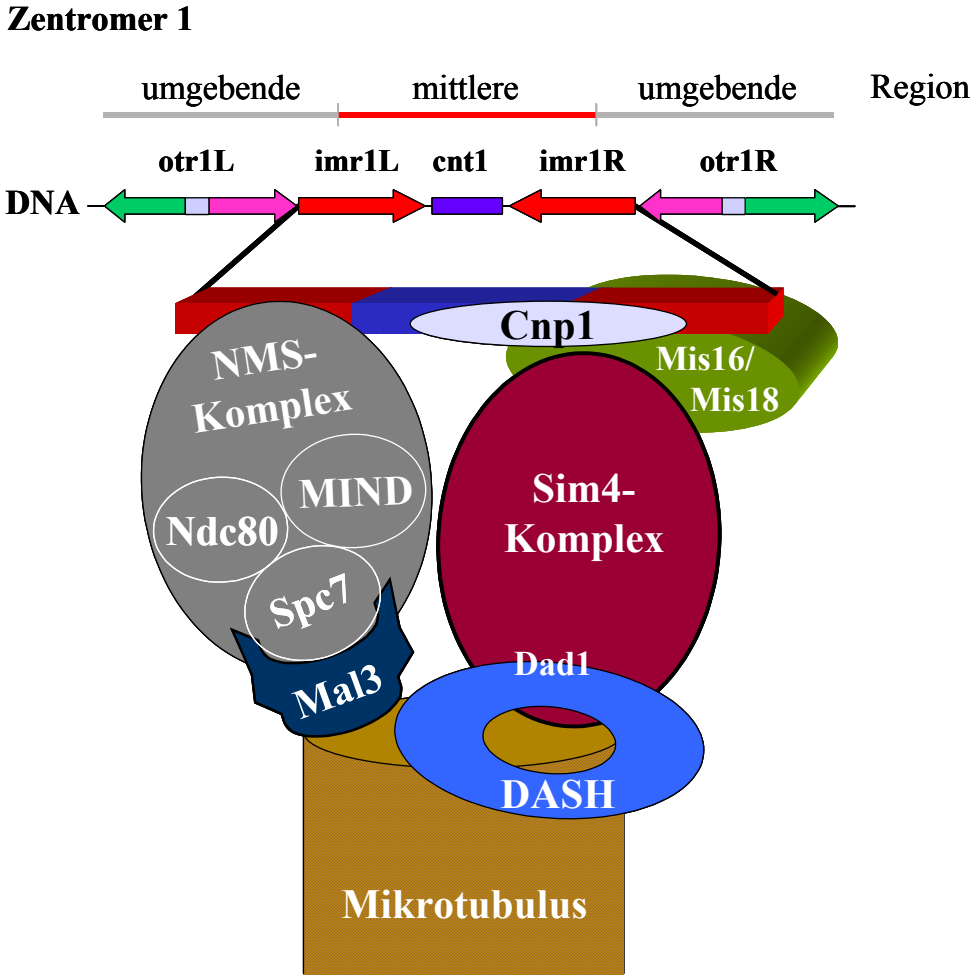
der angehefteten DNA zu den beiden Polen der Zelle. Dies geschieht durch die Polymerisation von Tubulindimeren an das Plusende resultierend in dem Aneinander-Vorbei-Gleiten der interpolaren Mikrotubuli in der Spindel-Überlappungszone. Mikrotubuli-assoziierte Proteine (Nicht-Motor- sowie Motorproteine) sind in diesen Prozess involviert (Kline-Smith und Walczak, 2004). Nach Separation der DNA wird in der Zelle ein Septum eingezogen und die Zelle geteilt. Somit sind zwei Folgezellen mit identischer DNA-Information entstanden (Übersichtsartikel: Kline-Smith und Walczak, 2004).

In der hier vorliegenden Arbeit wurde die Spalthefe *S. pombe* als Modellorganismus für die Aufklärung mitotischer Fragestellungen verwendet. Dieser bietet die Möglichkeit guter experimenteller Erforschung und weist zugleich eine große Ähnlichkeit im mitotischen Prozess zu höheren Organismen auf.

Das Zentromer in *S. pombe* besteht aus drei Sequenzelementen

Die regionale Zentromer-DNA der drei Chromosomen der Spalthefe *S. pombe* hat eine Größe von 40-110 Kbp und besteht jeweils aus einer konservierten, zentralen Kernregion (*cnt*), die rechts und links von invertierten, Chromosomen-spezifischen Sequenzwiederholungen umgeben ist (*imr*). Diese werden wiederum durch repetitive, konservierte Bereiche, die *otr*-Regionen, flankiert (Abb. 1). Die ungleiche Größe der *S. pombe* Zentromere ist auf eine unterschiedliche Anzahl an *otr*-Elementen zurückzuführen (Übersichtsartikel: Pidoux und Allshire, 2004). Das Zentromer in *S. pombe* wird in zwei Bereiche eingeteilt, die durch eine tRNA-enthaltene Chromatinbarriere voneinander abgegrenzt werden (Abb. 1; Kniola *et al.*, 2001; Scott *et al.*, 2006; Partridge *et al.*, 2000). Dies ist zum einen der mittlere Bereich (*cnt* sowie ein großer Teil von *imr*), welcher die oben genannte spezielle Chromatinstruktur aufweist und auf dem der Kinetochorproteinkomplex aufgebaut wird, und zum anderen der heterochromatische umgebende Bereich (die restliche *imr*-Region und *otr*), der für die Kohäsion der beiden Schwesterchromatiden notwendig ist (Pidoux und Allshire, 2004). Wildtypische Zellen, bei denen Gene in die Zentromer-DNA eingefügt werden, zeigen ein transkriptionelles Stilllegen dieser Gene (Pidoux und Allshire, 2000). So haben die mit der mittleren Zentromerregion assoziierten Kinetochorproteine eine Funktion bei dem transkriptionellen Ausschalten von Genen in diesem Bereich (Allshire *et al.*, 1995; Jin *et al.*, 2002; Pidoux *et al.*, 2003; Kerres *et al*., 2006a).

Im Folgenden wird nur auf die mit dem mittleren Bereich assoziierten Proteine eingegangen.



NMS-Komplex

Ndc80	MIND	Spc7
Ndc80	Mis12	
Nuf2	Mis13	
Spc24	Mis14	
Spc25	Nnf1	

Sim4-Komplex

Sim4	Fta1
Mal2	Fta2
Mis6	Fta3
Mis15	Fta4
Mis17	Fta5
Dad1	Fta6
	Fta7

Abbildung 1: Das Zentromer und der Kinetochorkomplex in *S. pombe*.
Schematische Abbildung der mittleren und umgebenden Zentromer-DNA von Chromosom 1 mit den bisher identifizierten, an den mittleren Bereich bindenden Kinetochorkomplexen sowie dem Mikrotubuli-assoziiertem Mal3-Protein und dem DASH-Komplex. Zur Vereinfachung ist nur die Assoziation eines Mikrotubulus dargestellt. Die Komponenten untereinander sind nicht maßstabsgetreu abgebildet.

Die auf der mittleren Zentromerregion assemblierten Kinetochorproteine sind in *S. pombe* in verschiedene Komplexe unterteilt

Bisher sind in *S. pombe* drei konstitutive Kinetochorkomplexe des mittleren Zentromer-Bereichs identifiziert worden: Dies sind der Mis16/Mis18-Komplex, der Sim4-Komplex sowie der NMS-Komplex (Abb. 1; Hayashi *et al.*, 2004; Pidoux *et al.*, 2003; Obuse *et al.*, 2004; Liu *et al.*, 2005). Bis auf eine Ausnahme (s. u.) sind in *S. pombe* alle bis jetzt charakterisierten Kinetochorproteine des mittleren Bereichs essentiell für das Überleben der

Zelle (Goshima *et al.*, 1999; Jin *et al.*, 2002; Pidoux *et al.*, 2003; Kerres *et al.*, 2004; Kerres *et al.*, 2006a).

Für den Einbau des Zentromer-spezifischen CENP-A Homologs Cnp1 in die Nukleosomen werden mindestens zwei Kinetochorkomplexe benötigt. Dies ist zum einen der Sim4- und zum anderen der aus zwei Komponenten bestehende Mis16/Mis18-Komplex, der ebenfalls für die Kinetochorlokalisierung des Sim4-Komplexes verantwortlich ist (Hayashi *et al.*, 2004; Pidoux *et al.*, 2003; Takahashi *et al.*, 2000). Der Sim4-Komplex besteht aus 13 Proteinen, u. a. aus den von mir untersuchten Mal2- und Fta2-Proteinen (Liu *et al.*, 2005; Fleig *et al.*, 1996; Jin *et al.*, 2002; Kerres *et al.*, 2006a). Die Lokalisierungsabhängigkeiten sowie die genaue Funktion und die Interaktion dieser Komponenten untereinander und mit anderen Kinetochorproteinen war zu Beginn meiner Doktorarbeit weitgehend unklar und konnten von mir weiter aufgeklärt werden (Kerres *et al.*, 2006a). Interessanterweise gehört zu dem Sim4-Komplex auch das konstitutiv Kinetochor-lokalisierte, nicht-essentielle Dad1-Protein (Abb. 1; Liu *et al.*, 2005; Sanchez-Perez *et al.*, 2005). Dad1 wurde zusätzlich als eine Komponente des Mikrotubuli-assoziierten, zehn Komponenten umfassenden DASH-Komplexes isoliert. Dieser gehört zu der Gruppe der transient mit dem Kinetochor assoziierten Proteine (Liu *et al.*, 2005; Sanchez-Perez *et al.*, 2005). In *in vitro* Studien mit *S. cerevisiae* DASH-Komplexen wurde gezeigt, dass dieser Ringe um Mikrotubuli bildet (Westermann *et al.*, 2006). Die Kinetochor-Assoziation des DASH-Komplexes, der in *S. pombe* nicht essentiell ist, benötigt einen funktionellen Sim4-Komplex (Liu *et al.*, 2005). Da der Sim4-Komplex essentiell ist, muss er neben der Assoziation des DASH-Komplexes mit dem Kinetochor noch andere, bisher unbekannte Funktionen ausüben.

Des Weiteren wurde in *S. pombe* kürzlich der konservierte NMS-Komplex als eine biochemisch separat aufgereinigte Einheit identifiziert (Abb. 1; Liu *et al.*, 2005). Dieser besteht aus dem Ndc80-Komplex, dem MIND-Komplex sowie dem Spc7-Protein (Abb. 1; Kerres *et al.*, 2004; Obuse *et al.*, 2004; Liu *et al.*, 2005). Der MIND-Komplex wie auch der Ndc80-Komplex sind in *S. pombe* nur anfänglich untersucht. Diese konservierten Komplexe wurden jedoch in anderen Organismen bereits charakterisiert, so dass auch Rückschlüsse auf die Funktion in *S. pombe* gezogen werden können (Goshima *et al.*, 2003; Goshima und Yanagida, 2000; Übersichtsartikel: Kline-Smith *et al.*, 2005). Funktionsbeeinträchtigungen dieser Komplexe resultieren in Chromosomen-Fehlsegregationen (MIND, Ndc80) sowie in Spindeldefekten oder sogar in Nicht-Assoziation des Kinetochors mit der Spindel (Ndc80) (Übersichtsartikel: Kline-Smith *et al.*, 2005; Goshima *et al.*, 1999). Es wird postuliert, dass in Vertebraten die Aneinanderlagerung von ca. 30 Ndc80-Komplexen entlang ihrer Längsseite

eine geöffnete, zylindrische Andockstelle für einen ans Kinetochor bindenden Mikrotubulus darstellt (Emanuele *et al.*, 2005).

Das bis zu Beginn meiner Doktorarbeit noch unbekannte Spc7-Protein wurde in unserem Labor identifiziert und charakterisiert (Vietmeier-Decker, 2004; Kerres *et al.*, 2004; Kerres *et al.*, 2006b). So waren die Lokalisierungsabhängigkeiten zwischen Spc7 und den anderen NMS-Subkomplexen vollkommen unbekannt und konnten von mir weitgehend aufgezeigt werden (Kerres *et al.*, 2006b). Des Weiteren konnte ich den ersten Mikrotubuli-assoziierten Interaktionspartner eines konstitutiven Kinetochorproteins identifizieren und dadurch die Verbindung zwischen dem Kinetochor und den Mikrotubuli weiter aufklären (Kerres *et al.*, 2004).

Mikrotubuli-assoziierte Proteine lokalisieren teilweise transient am Kinetochor

Zu den Mikrotubuli-assoziierten Proteinen gehört die Gruppe der sogenannten +TIPs, die entlang der Mikrotubuli lokalisieren, jedoch präferentiell an den Plusenden gebunden sind. Sie haben eine Funktion bei der Regulation der Mikrotubuli-Dynamik oder bei der Assoziation der Mikrotubuli mit anderen Strukturen (Übersichtsartikel: Akhmanova und Hoogenraad, 2005).

So stabilisieren beispielsweise die *S. pombe* +TIPs Alp14 und Dis1, die zur konservierten TOG/XMAP215-Familie gehören, Mikrotubuli durch Erhöhung der Wachstumsrate am Plusende. Diese Proteine binden während der Mitose an das Kinetochor und stellen daher vermutlich eine Verknüpfung des Kinetochors mit der mitotischen Spindel her (Übersichtsartikel: Ohkura *et al.*, 2001; Gard und Kirschner, 1987; Vasquez *et al.*, 1994; Garcia *et al.*, 2001; Garcia *et al.*, 2002). Allerdings sind die Interaktionspartner am Kinetochor bisher unbekannt.

Das Mikrotubuli-Plusende-assoziierte Peg1-Protein ist das CLASP-Familienmitglied in *S. pombe* und stabilisiert in der Mitose Mikrotubuli, während es diese in der Interphase destabilisiert (Grallert *et al*., 2006). Peg1 lokalisiert ebenfalls am Kinetochor (Karig, 2004).

Ein weiterer Vertreter der Spalthefen +TIPs ist Mal3, ein Mitglied der konservierten EB1-Proteinfamilie, welches in die Regulation der Mikrotubuli-Dynamik involviert ist (Beinhauer *et al.*, 1997). Eine Fehlfunktion des Mal3-Proteins resultiert neben Mikrotubuli-Abnormalitäten während der Interphase auch in mitotischen Defekten (Beinhauer *et al.*, 1997). Das humane EB1-Protein wurde als Interaktionspartner des Tumorsuppressors „Adenomatous Polyposis Coli“ (APC) isoliert (Su *et al.*, 1995). EB1 sowie APC lokalisieren am Kinetochor und spielen eine Rolle bei der Bi-Orientierung der Chromosomen in der

Metaphase, jedoch werden sie nicht für die Interaktion zwischen Mikrotubuli und Kinetochor *per se* benötigt (Draviam *et al.*, 2006).

Zielsetzung dieser Arbeit war es, zum besseren Verständnis der mitotischen Prozesse beizutragen. Durch die Funktionsanalyse spezifischer Kinetochorkomponenten im Modellorganismus *S. pombe*, insbesondere der evolutionär konservierten Mal2- und Spc7-Proteine, konnten wichtige Fragestellungen weiter aufgeklärt werden. So war von Interesse, welche Kinetochorproteine die Verknüpfung mit der Teilungsspindel vermitteln und welche Proteine auf der Seite der Spindel in diesen Prozess involviert sind. Spielt das an den Mikrotubuli-Plusenden lokalisierte Mal3-Protein hier eine Rolle?
Weiterhin stellte sich die Frage, welche Proteine für den wildtypischen Aufbau eines Kinetochors und dessen Subkomplexe verantwortlich sind und wie sich diese gegenseitig beeinflussen.
Unklar war auch, ob es funktionelle Verbindungen zwischen verschiedenen Komplexen wie dem Sim4- und dem NMS-Komplex gibt.

4 Ergebnisse und Diskussion

Spc7 verbindet das Kinetochor mit den Spindel-Mikrotubuli

Ein Multikopien-Suppressorscreen einer *mal3*-Mutante führte zu der Isolierung des Spc7-Proteins (Vietmeier-Decker, 2004). Die gefundene Interaktion zwischen Spc7 und Mal3 ist nicht nur genetisch, sondern auch physikalisch, da beide Proteine miteinander ko-immunopräzipitieren (Kerres *et al.*, 2004). Somit sind das Mikrotubuli-Plusende-assoziierte Mal3-Protein und die konstitutive Kinetochorkomponente Spc7 die zuerst identifizierten Interaktionspartner in *S. pombe*, die eine direkte Funktion bei der Verbindung zwischen dem Kinetochor und der Spindel haben. Spc7 ist ein essentielles Protein und während des gesamten Zellzyklus am Kinetochor, genauer an der mittleren Zentromerregion, lokalisiert (Kerres *et al.*, 2004). Es ist das erste bekannte konstitutive Kinetochorprotein, für das gezeigt werden konnte, dass es scheinbar keine Rolle bei dem transkriptionellen Stilllegen von Genen, die in die Zentromerregion eingefügt wurden, spielt (Kerres *et al.*, 2006b). Spc7 lokalisiert vermutlich am äußeren Kinetochor, räumlich weiter von der Zentromer-DNA entfernt, in der Interaktionszone zwischen dem Kinetochor und den Mikrotubuli.

Die Funktion von Spc7 wurde mit Hilfe von Mutanten untersucht (Kerres *et al.*, 2004; Kerres *et al.*, 2006b). In ihnen ist der Spindelkontrollpunkt, der die Zellen bei auftretenden Verknüpfungsfehlern zwischen Kinetochor und Spindel-Mikrotubuli in *S. pombe* nur transient in der Metaphase zurückhält, aktiviert (Kerres *et al.*, 2006b). Der Kontrollpunkt erfasst zwei verschiedene Zustände: Zum einen ist dies die Bindung von Mikrotubuli an das Kinetochor *per se*, was von einer das Kontrollpunktprotein Mad2-beinhaltenden Untereinheit registriert wird. Zum anderen wird die aus der bipolaren Verknüpfung resultierende Spannung zwischen den beiden Schwesterkinetochoren durch den die Kontrollpunktkomponente Mph1-enthaltenen Subkomplex wahrgenommen (Übersichtsartikel: Lew und Burke, 2003; Skoufias *et al.*, 2001; He *et al.*, 1997; He *et al.*, 1998). Beide Spindelkontrollpunkt-Untereinheiten sind in den *spc7*-Mutanten aktiviert, da eine Deletion von $mad2^{+}$ oder $mph1^{+}$ in einer temperatursensitiven *spc7*-Mutante zu einem verschlechterten Wachstum führt (Kerres *et al.*, 2006b). Die Aktivierung des Kontrollpunktes zeigt sich auch durch eine verlängerte Metaphasenspanne bei Überexpression einer dominant-negativen *spc7*-Mutante (Kerres *et al.*, 2006b).

Durch Fluoreszenzmarkierung beider Schwesterkinetochore eines Chromosoms sowie der mitotischen Mikrotubuli konnte visuell gezeigt werden, dass in den *spc7*-Mutanten tatsächlich nicht alle Kinetochore mit der Spindel verbunden sind (Kerres *et al.*, 2004; Kerres *et al.*, 2006b). Diese Nicht-Verknüpfung führt zu massiven Fehlern bei der DNA-Segregation. In bis

zu 60 % der mitotischen Zellen in den verschiedenen *spc7*-Mutanten findet man nicht getrennte DNA trotz elongierter Spindel (Kerres *et al.*, 2004; Kerres *et al.*, 2006b). Dies kann aus der fehlenden Anknüpfung des Kinetochors mit den Spindel-Mikrotubuli und somit dem Verbleiben der DNA in der Zellmitte während der Spindelverlängerung resultieren. In geringerem Maße (bis zu 22 %) findet man DNA, die über die gesamte Spindel geschmiert ist, was auf nur teilweise segregierte Chromosomen hindeutet. Eventuell hat sich hier die Verbindung zwischen bereits anfänglich zu den Polen gezogenen Chromatiden und der Spindel wieder gelöst. Eine partielle Verteilung der DNA oder ungleichmäßig getrennte Chromosomen, entstanden aus einer Segregation beider Schwesterchromatiden zu demselben Pol, treten ebenfalls in bis zu 17 % der mitotischen *spc7*-Mutanten bei der restriktiven Temperatur auf (Kerres *et al.*, 2004; Kerres *et al.*, 2006b). Hier war die notwendige Bi-Orientierung der Schwesterchromatiden auf der Spindel nicht gewährleistet, so dass beide Schwesterkinetochore mit von einem Spindelpolkörper ausgehende Mikrotubuli verbunden wurden (Kerres *et al.*, 2004; Kerres *et al.*, 2006b).

Die Nichtverknüpfung der Kinetochore mit der Spindel scheint keineswegs durch eine Nicht-Assemblierung des Kinetochors *per se* und somit dem fehlenden Anheftungspunkt erklärt werden zu können, da zumindest die zu unterschiedlichen Komplexen gehörigen Kinetochorkomponenten Mal2 und Spc24 in den Mutanten richtig lokalisiert sind (Kerres *et al.*, 2004; Kerres *et al.*, 2006b).

Spc7 wird demzufolge für die korrekte Anknüpfung der Kinetochore und somit der Schwesterchromatiden an die Spindel-Mikrotubuli benötigt. Die oben beschriebenen Phänotypen resultieren allerdings nicht nur aus einer fehlenden Interaktion zwischen Spc7 und Mal3, da eine *mal3*-Mutation oder gar -Deletion wesentlich schwächere Phänotypen hervorruft (Beinhauer *et al.*, 1997).

Spc7 wird für den Aufbau und die Funktionalität der Spindel benötigt

Die mitotische Spindel der Spalthefe wird wie in höheren Eukaryoten erst zu Beginn der Mitose assembliert (Nabeshima *et al.*, 1998). Es werden in *S. pombe* drei Spindelstadien unterschieden (Abb. 2): In der 1. Phase, einem Prophase ähnlichen Stadium, wird die bipolare Spindel zwischen den zwei Spindelpolkörpern aufgebaut. Phase 2 zeichnet sich durch eine nahezu konstante Spindellänge von 2,5 µm mit sehr dynamischen Mikrotubuli aus. Am Ende dieser Phase werden die Schwesterchromatiden zu den entgegengesetzten Spindelpolkörpern separiert. Sie beinhaltet somit die Meta- sowie die Anaphase A. Die Spindelelongation und folglich die Segregation der Schwesterchromatiden zu den beiden Zellenden findet in Phase 3,

der Anaphase B, statt (Nabeshima *et al.*, 1998). Beim Eintritt in diese Phase wird die Dynamik der einzelnen Mikrotubuli verringert, was in einer Stabilisierung der Spindel resultiert (Mallavarapu *et al.*, 1999; Nabeshima *et al.*, 1998; Sagolla *et al.*, 2003). Die Elongationsrate der Mikrotubuli steigt hingegen von Stadium 2 zu 3 an (Nabeshima *et al.*, 1998).

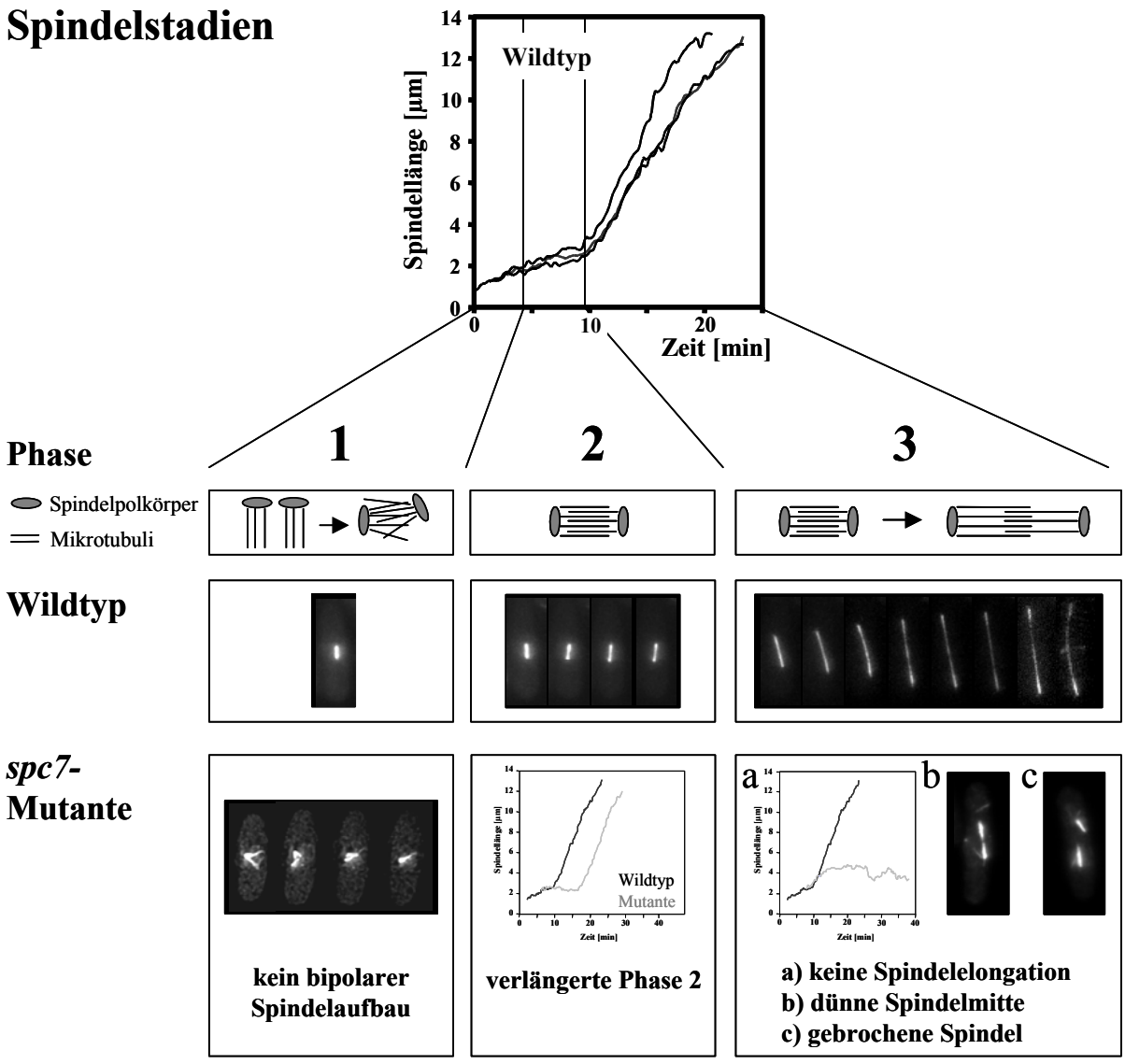


Abbildung 2: Alle drei Spindelstadien sind in *spc7*-Mutanten beeinträchtigt.
Gezeigt sind die drei *S. pombe* Spindelstadien im Wildtyp sowie die möglichen Veränderungen in dominanten *spc7*-Mutanten. Für den Wildtyp sind neben einem Diagramm, in dem die Veränderung der Spindellänge über die Zeit zu sehen ist, schematische Abbildungen der Spindel sowie Fluoreszenzaufnahmen der mitotischen Mikrotubuli in den verschiedenen Stadien gezeigt. Dabei stellen die gezeigten Fluoreszenzbilder Zeitaufnahmen derselben Zelle dar. Für die *spc7*-Mutanten sind Fluoreszenzaufnahmen der Spindel oder aber graphische Darstellungen der Spindellänge über die Zeit zu sehen. Zur Vereinfachung ist hier zum Vergleich der Graph von nur einer Wildtypzelle aus dem obigen Diagramm in schwarz und der Graph für die Mutante in grau abgebildet.

Die Analyse von *spc7*-Mutanten zeigte, dass Spc7 für alle Spindelstadien benötigt wird (Abb. 2; Kerres *et al.*, 2006b). In wildtypischen Zellen werden während des Spindelaufbaus in Phase 1 die Mikrotubuli, die von zwei unterschiedlichen Spindelpolkörpern polymerisiert werden, untereinander verbunden und somit bipolar angeordnet (Hagan und Yanagida, 1995; Hagan und Yanagida, 1990). In *spc7*-Mutanten schlägt teilweise die Bildung einer bipolaren Spindel vermutlich durch fehlerhafte oder ausbleibende Verknüpfungen fehl (Abb. 2; Kerres *et al.*, 2006b). Dieser Phänotyp tritt auch bei einer Fehlfunktion des Motorproteins Cut7 auf. Dieses Kinesin ist in der Spindelmitte lokalisiert und verbindet Mikrotubuli, die von unterschiedlichen Spindelpolkörpern ausgehen (Hagan und Yanagida, 1990).

Des Weiteren ist auch z. T. das Spindelstadium der Phase 2 in *spc7*-Mutanten verändert, da die Dauer dieser Phase, wahrscheinlich durch die Aktivität des Spindelkontrollpunktes, massiv verlängert ist (Abb. 2; Kerres *et al.*, 2006b).

spc7-Mutanten weisen ebenfalls Probleme bei der Spindelelongation in Phase 3 auf, was sich durch Zyklen einer anfänglichen Elongation, gefolgt von einem Zusammenfallen der Spindel, durch eine verdünnte Spindelmittelzone oder durch das Auseinanderbrechen der Spindel zeigt (Abb. 2; Kerres *et al.*, 2006b). Die Phasen des Wachstums und des Schrumpfens der Anaphasen-Spindel können durch den Beginn der Phase 3 ohne die wildtypische Verminderung der Mikrotubuli-Dynamik erklärt werden. Die Spindel-Mikrotubuli unterlaufen hierbei weiterhin in hoher Frequenz Phasen der Polymerisation und Depolymerisation. So wurde in *S. cerevisiae* gezeigt, dass die Abwesenheit der regulatorischen Cdc14-Phosphatase zu keiner Veränderung der Mikrotubuli-Dynamik zwischen den Phasen 2 und 3 und somit zu keiner Spindelelongation führt (Higuchi und Uhlmann, 2005). Auch die häufig in *spc7*-Mutanten zu beobachtende dünne Spindelmitte kann u. a. durch eine zu hohe Dynamik einzelner Mikrotubuli zustande kommen.

In wildtypischen Zellen überlappen die von beiden Spindelpolkörpern ausgehenden Mikrotubuli in der frühen Anaphase zu zwei Dritteln der Mikrotubuli-Länge in einer ungeordneten Weise und später abwechselnd angeordnet in einem Drittel ihrer Länge (Ding *et al.*, 1993). Die Ausmaße der Überlappungsbereiche unterscheiden sich zwischen den individuellen Mikrotubuli (Ding *et al.*, 1993). Diese sind durch in elektronenmikroskopischen Aufnahmen sichtbare Brücken von bisher unbekannter Natur miteinander verbunden (Ding *et al.*, 1993). Vermutlich resultiert die dünne Spindelmitte in *spc7*-Mutanten aus einer Überlappung von nur noch einem Teil der Mikrotubuli, wofür auch die beobachteten zusätzlichen Mikrotubuli in den *spc7*-Mutanten sprechen (Kerres *et al.*, 2006b). Ase1, der Spalthefen-Vertreter der konservierten PRC1-Familie, bündelt Interphasen sowie mitotische

Mikrotubuli in der Überlappungszone (Loiodice *et al.*, 2005; Yamashita *et al.*, 2005). Dieses Protein wird in der Mitose verstärkt zu Beginn der Phase 3 und somit bei der Verringerung der Mikrotubuli-Dynamik an die Spindelmittelzone rekrutiert (Loiodice *et al.*, 2005; Yamashita *et al.*, 2005). Die Abwesenheit des nicht-essentiellen Proteins führt in mitotischen Zellen zu z. T. mit den *spc7*-Mutanten vergleichbaren Phänotypen. Dies sind zusätzliche Mikrotubuli und Brüche in der Spindelmitte (Loiodice *et al.*, 2005; Yamashita *et al.*, 2005). Eine verminderte Anzahl an untereinander verknüpften Mikrotubuli könnte daher auch in *spc7*-Mutanten die Ursache für die dünne Spindelmitte sein. Der in 10 % der mitotischen Zellen einer *spc7*-Mutante beobachtete Bruch der Spindel ist wahrscheinlich eine Folge einer instabilen, sehr dünnen Mittelzone. Weiterhin deuten Versuche, in denen die Spindelmitte von *S. pombe*-Zellen künstlich mit Hilfe von Laserstrahlen beeinträchtigt wurde, ebenfalls durch ähnliche Konsequenzen auf eine Funktion von Spc7 bei der Anordnung und Verknüpfung der Mikrotubuli innerhalb der Spindel hin. Als Folge der Schädigung findet hier eine Verkürzung der Spindellänge, d. h. ein teilweises Kollabieren oder aber ein Bruch der Spindel statt (Khodjakov *et al.*, 2004; Tolic-Norrelykke *et al.*, 2004).

Weitere Hinweise auf das Einwirken von Spc7 auf Mikrotubuli ergaben sich aus genetischen Interaktionsstudien von Spc7 mit der α2-Tubulinuntereinheit sowie mit den Mikrotubuli-assoziierten Proteinen Dis1, Mal3 und Peg1 (Kerres *et al.*, 2006b).

Meine Daten deuten darauf hin, dass Spc7 die bipolare Spindelstruktur über die Mikrotubuli-Dynamik und/oder die Mikrotubuli-Bündelung beeinträchtigt. Dabei ist für das konstitutive Kinetochorprotein sowohl ein direkter als auch ein indirekter Einfluss auf die Spindel vorstellbar. Eine mögliche Interaktion von Spc7 mit einem regulatorischen Protein am Kinetochor, das in der späteren Mitose an die Spindelmitte lokalisiert, kann sich indirekt auf die Spindel-Dynamik und die Verknüpfung der Mikrotubuli untereinander auswirken. Eine Vielzahl von Proteinen, wie z. B. die sogenannten „chromosomal-passenger“ Proteine und Mikrotubuli-assoziierte Proteine, ändern ihre Lokalisierung während der Mitose und können daher regulatorisch auf unterschiedliche Proteine oder Prozesse einwirken (Petersen *et al.*, 2001; Kerres, unveröffentlicht; Rajagopalan und Balasubramanian, 2002; Sanchez-Perez *et al.*, 2005; Liu *et al.*, 2005; Garcia *et al.*, 2001; Nakaseko *et al.*, 2001). In *S. cerevisiae* ist u. a. das „chromosomal-passenger“ Protein Bir1 für die Assoziation der konstitutiven Kinetochorkomponente Ndc10 an die Spindelmittelzone erforderlich (Bouck und Bloom, 2005). Ndc10 ist hier für die Spindelstabilität notwendig (Bouck und Bloom, 2005). In *ndc10*-Mutanten sind, wie in *spc7*-Mutanten, in der Anaphase B Abfolgen einer Elongation und

eines Zusammenfallens der Spindel zu sehen (Bouck und Bloom, 2005). Auch scheint die Spindelmitte sehr dünn zu sein.

In der Bäckerhefe sind bereits mehrere konstitutive Kinetochorkomponenten identifiziert worden, die mit der Spindel assoziieren (Müller-Reichert *et al.*, 2003; Goh *et al*., 1993). Es ist nicht auszuschließen, dass geringe Mengen von Spc7 in *S. pombe* ebenfalls mit der Spindelmitte ko-lokalisieren, die allerdings aus technischen Gründen oder aufgrund einer sehr kurzen Spindellokalisierung bisher unentdeckt blieben. Spc7 könnte so einen direkten Einfluss auf den Ablauf der Prozesse in der Mittelzone nehmen. In diesem Zusammenhang ist interessant, dass eine N-terminale Variante von Spc7 mit der Spindel zu ko-lokalisieren scheint (Kerres *et al.*, 2006b). Hingegen zeigt das Volllängenprotein keine Mikrotubuli-Assoziation, so dass die vermutliche Spindellokalisierung anscheinend über den C-Terminus reguliert wird.

Spc7 ist somit die einzige bisher beschriebene konstitutive Kinetochorkomponente in *S. pombe*, deren Beeinträchtigung sich unmittelbar auf den Aufbau, die Struktur und die Funktionalität der Spindel-Mikrotubuli auswirkt. Die beobachteten Spindelphänotypen ziehen wahrscheinlich Defekte in der Chromosomensegregation nach sich, die letztendlich zum Tod der Zelle führen.

Spc7 wird für die Lokalisierung des MIND- aber nicht des Ndc80-Komplexes benötigt

Spc7 bildet zusammen mit den vier Komponenten Ndc80, Nuf2, Spc24 und Spc25 (Ndc80-Komplex) sowie den vier Komponenten Mis12, Mis13, Mis14 und Nnf1 (MIND-Komplex) den übergeordneten NMS-Komplex (Abb. 3; Nekrasov *et al.*, 2003; Liu *et al.*, 2005; Obuse *et al.*, 2004). Die Verbindung zwischen Spc7 und dem Ndc80-Komplex konnte durch Ko-Immunopräzipitationen von Spc7 und Spc24 gezeigt werden (Kerres *et al.*, 2004). Des Weiteren können Spc7 und Mis12 miteinander ko-immunopräzipitieren (Obuse *et al.*, 2004). Es stellte sich die Frage, ob Spc7 und die MIND- und Ndc80-Komponenten für die gegenseitige Kinetochorlokalisierung benötigt werden. Durch Expression von GFP-Fusionsproteinen in den spezifischen Kinetochormutanten wurde gezeigt, dass die MIND- und Spc7-Lokalisierungen voneinander abhängig sind, die Assoziation der Ndc80-Komponenten und Spc7 jedoch unabhängig voneinander stattfinden (Abb. 3; Kerres *et al.*, 2006b). Auch Mis12 assoziiert unabhängig vom Ndc80-Komplex am Kinetochor (Saitoh *et al.*, 2005). Weiterhin kann zusätzliches *spc7*$^{+}$ oder zusätzliches *mis12*$^{+}$ eine Mutante des jeweils anderen Proteins partiell supprimieren, wobei die Suppression von *mis12*-Mutanten durch *spc7*$^{+}$ stärker ist als andersherum. Die Überexpression von *spc7*$^{+}$ in einer *nuf2*-Mutante

führt hingegen zu verschlechtertem Wachstum (Kerres *et al.*, 2006b; Obuse *et al.*, 2004). Interessanterweise ist eine Doppelmutante zwischen *spc7* und *nuf2* bei allen getesteten Temperaturen synthetisch letal (Kerres *et al.*, 2006b). Demzufolge wird für das Überleben von *spc7*-Mutanten bei der permissiven Temperatur der wildtypische Ndc80-Komplex benötigt. Das Spc7-Protein scheint insgesamt enger mit dem MIND-Komplex als mit dem Ndc80-Komplex zu interagieren. Dennoch unterscheiden sich die Phänotypen zwischen den MIND-Komplex- und den *spc7*-Mutanten. Die MIND-Mutanten weisen ungleiche Chromosomenverteilung sowie Probleme bei der bipolaren Anordnung der Chromosomen in der Metaphase auf (Goshima *et al.*, 1999; Obuse *et al.*, 2004). Zusätzlich zu diesen Phänotypen zeigen *spc7*-Mutanten keine Segregation der DNA sowie massive Spindeldefekte. Das deutet auf eine gegenseitige Abhängigkeit des Spc7-Proteins und des MIND-Komplexes sowie auf eine gemeinsame, verwandte Funktion neben weiteren, unterschiedlichen Rollen hin. Durch die Beeinträchtigung dieser zusätzlichen Funktionen kommen die ungleichen Phänotypen zustande.

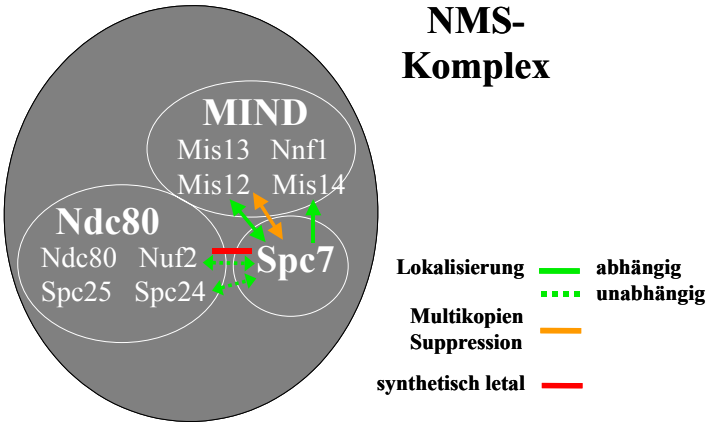


Abbildung 3: Die *S. pombe* NMS-Subkomplexe interagieren miteinander. Schematisch dargestellt sind die den NMS-Komplex-bildenden Proteine, die in die entsprechenden Subkomplexe eingeordnet sind. Die Lokalisierungsabhängigkeiten sowie die genetischen Interaktionen zwischen den untersuchten Proteinen sind eingezeichnet.

Der NMS-Komplex ist konserviert (Nekrasov *et al.*, 2003; Obuse *et al.*, 2004; Cheeseman *et al.*, 2004; Meraldi *et al.*, 2006). In RNAi Studien in *C. elegans* wurde für das Spc7-Homolog KNL-1 eine zentrale Rolle in der Kinetochorassemblierung festgestellt. Es ist für die wildtypische Lokalisierung des *C. elegans* MIND- sowie absolut für die Assemblierung des *C. elegans* Ndc80-Komplexes notwendig (Desai *et al.*, 2003; Cheeseman *et al.*, 2004). In *S. cerevisiae* sind nahezu alle untersuchten Doppelmutanten zwischen den Ndc80-, den MIND-Komplexkomponenten und Spc7 synthetisch letal (Nekrasov *et al.*, 2003). Diese Ergebnisse stimmen nicht mit den Befunden in *S. pombe* überein. Die Analyse der Spc7-Homologen aus weiteren Organismen wird zeigen, ob die Funktionen und Wirkungsmechanismen des NMS-Komplexes evolutionär konserviert sind.

Fta2 bildet zusammen mit Mal2 eine Subgruppe innerhalb des Sim4-Komplexes

Neben dem NMS-Komplex ist vor kurzem der Sim4-Komplex isoliert worden. Dieser besteht aus den sechs, bereits zuvor identifizierten Proteinen Sim4, Mal2, Mis6, Mis15, Mis17 und Dad1 sowie aus sieben, bis dahin noch nicht charakterisierten Komponenten namens Fta1-7 (Pidoux *et al.*, 2003; Jin *et al.*, 2002; Goshima *et al.*, 1999; Hayashi *et al.*, 2004; Liu *et al.*, 2005). Das Fta2-Protein wurde zeitgleich in unserem Labor als Suppressor einer *mal2*-Mutante identifiziert (Kerres *et al.*, 2006a). Das für die akkurate Chromosomensegregation benötigte Mal2 ist ein konserviertes Protein und existiert ebenfalls im Menschen (Fleig *et al.*, 1996; Jin *et al.*, 2002; Meraldi *et al.*, 2006; Foltz *et al.*, 2006; Okada *et al.*, 2006). Die Interaktionen zwischen den einzelnen Proteinen des Sim4-Komplexes untereinander sind bisher nur teilweise untersucht worden (Pidoux *et al.*, 2003; Jin *et al.*, 2002; Goshima *et al.*, 1999). Wir konnten zeigen, dass Mal2 zusammen mit dem essentiellen, konstitutiv an der mittleren Zentromerregion lokalisierten Fta2 eine Subgruppe innerhalb des Sim4-Komplexes bildet, wobei die Funktionen beider Proteine nicht identisch, jedoch sehr eng miteinander verwandt sind (Kerres *et al.*, 2006a). Die Lokalisierung beider Proteine ist absolut abhängig von der Funktionalität des jeweils anderen. Sie sind ferner die bisher einzigen Mitglieder des Sim4-Komplexes, die eine vollständige gegenseitige Suppression der Mutantenphänotypen aufweisen (Kerres *et al.*, 2006a; Pidoux *et al.*, 2003; Hayashi *et al.*, 2004). Doppelmutanten zwischen *mal2* und *fta2* sind nicht überlebensfähig. Somit ist bei Beeinträchtigung des einen Proteins das andere notwendig für das Überleben der Zelle bei der permissiven Temperatur (Kerres *et al.*, 2006a).

Fta2 ist für die bipolare Ausrichtung der Chromosomen sowie für die Assoziation der Kinetochore mit der Spindel notwendig

Temperatursensitive *fta2*-Mutanten zeigen bei der restriktiven Temperatur nahezu keine Abnormalitäten der Spindel, weisen jedoch massive DNA-Verteilungsdefekte in der Mitose auf. Dies sind Fehlsegregationen und sich nicht trennende DNA, die in der Zellmitte zurück bleibt, während die Spindel elongiert (Abb. 4a; Kerres *et al.*, 2006a). Der letztere Phänotyp tritt in keiner anderen bisher untersuchten Mutante des Sim4-Komplexes auf. In *S. pombe* ist er innerhalb der konstitutiven Kinetochorproteine bislang nur in *nuf2*- und in *spc7*-Mutanten beschrieben worden (Nabetani *et al.*, 2001; Kerres *et al.*, 2006b). Die in der Anaphase B in der Zellmitte zurückbleibende DNA entsteht offensichtlich durch Defekte bei der Verknüpfung zwischen dem Kinetochor und den Mikrotubuli. Dementsprechend zeigen 58 % der Zentromere von Chromosom 1 in *fta2*-Mutanten mit diesem Phänotyp keine Assoziation

mit der Spindel (Abb. 4b). Ob in einer Mutante des Subgruppen-Partners Mal2 alle Kinetochore mit den Spindel-Mikrotubuli assoziiert sind, bleibt zu untersuchen, wobei es bereits Hinweise auf eine Funktion von Mal2 bei der Verbindung zwischen Kinetochor und der Spindel gibt (Jin *et al.*, 2002). Auch für andere Sim4-Komplexmutanten ist noch nicht analysiert worden, ob die Mikrotubuli-Kinetochor-Bindung korrekt stattfindet.

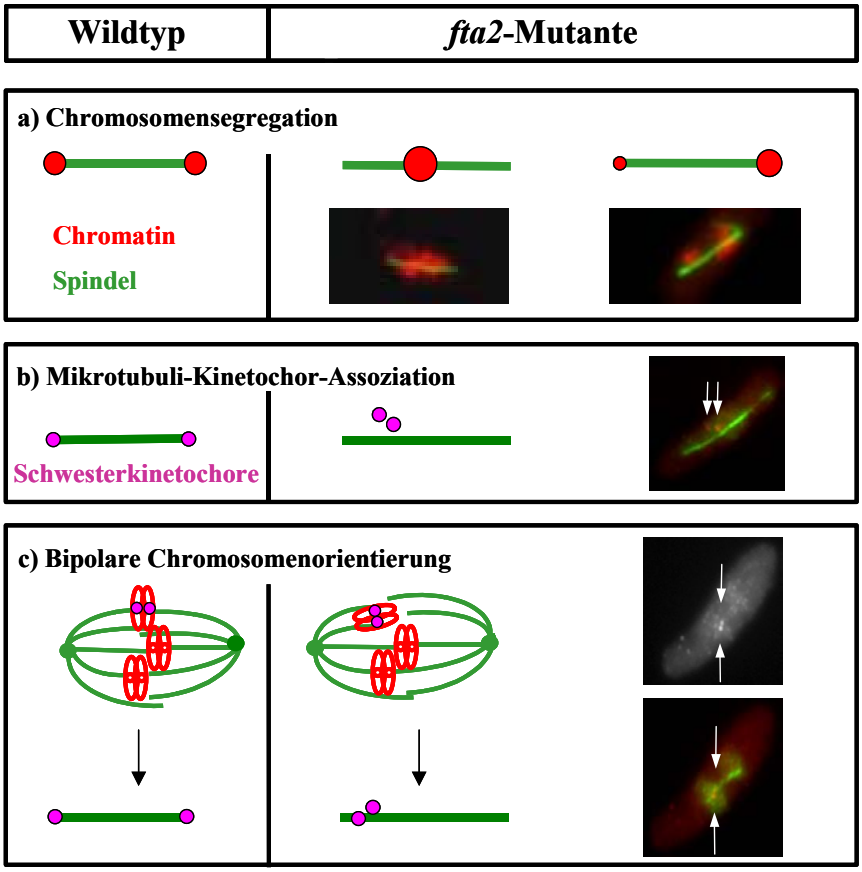


Abbildung 4: *fta2*-Mutanten weisen unterschiedliche Defekte in der Mitose auf.
Schematische Darstellung der fehlerhaften Chromosomensegregation von *fta2*-Mutanten im Vergleich zum Wildtyp. Für die *fta2*-Mutanten sind zusätzlich Immunofluoreszenzabbildungen gezeigt. In den Schemata sind in grün die Spindel, in rot das Chromatin und in violett die Schwesterkinetochore von Chromosom 1 dargestellt. In den Immunofluoreszenzfotos ist in a) das Chromatin und die Spindel angefärbt, in b) und c) sind die Schwesterkinetochore von Chromosom 1, die zusätzlich mit Hilfe von weißen Pfeilen verdeutlicht sind, sowie die Spindel angefärbt.

Des Weiteren scheint die bipolare Verknüpfung der Kinetochore mit den Mikrotubuli durch Mutationen in *fta2*$^+$ beeinträchtigt zu sein, da in 42 % der *fta2*-Mutanten mit elongierter Spindel beide Schwesterchromatiden von Chromosom 1 zu einem Zellende segregieren (Abb. 4c; Kerres *et al.*, 2006a). Die bipolare Anordnung der Chromosomen auf der Spindel wird u. a. durch den DASH-Komplex vermittelt (Janke *et al.*, 2002; Sanchez-Perez *et al.*, 2005). Hierbei ist die Kinetochor-Bindung des DASH-Komplex-Proteins Dad1 notwendig für die Kinetochorlokalisierung der übrigen DASH-Komponenten (Liu *et al.*, 2005). Es konnte

gezeigt werden, dass in *fta2*-Mutanten die Dad1-Komponente und somit vermutlich der gesamte DASH-Komplex nicht korrekt lokalisiert ist. Hiermit übereinstimmend liegt der Mph1-Spindelkontrollpunktweg, der die Spannung am Kinetochor und somit die bipolare Verknüpfung detektiert, in *fta2*-Mutanten aktiviert vor (Kerres *et al.*, 2006a; He *et al.*, 1998). Weiterhin ist Fta2 für die wildtypische Assemblierung anderer Komponenten des Sim4-Komplexes, der offensichtlich hierarchisch aufgebaut wird, notwendig (Kerres *et al.*, 2006a).

Die Sim4- und NMS-Kinetochorkomplexe interagieren über Spc7 miteinander

In *S. pombe* wurden die Sim4- und NMS-Komplexe separat voneinander isoliert (Obuse *et al.*, 2004; Liu *et al.*, 2005). Diese interagieren jedoch genetisch miteinander (Kerres *et al.*, 2006b). In einem Screen zur Aufdeckung von Suppressoren der NMS-Komplexmutante *spc7* wurde interessanterweise das Sim4-Komplexprotein Mal2 als partieller Suppressor isoliert (Abb. 5; Kerres *et al.*, 2006b). Zusätzliches Mal2 reduziert in *spc7*-Mutanten die Anzahl der abnormalen DNA-Segregationsereignisse in der Anaphase um 19 %, vermutlich durch die beobachtete Stabilisierung des Spc7-Mutantenproteins (Kerres *et al.*, 2006b). Übereinstimmend mit diesem Befund konnte eine physikalische Interaktion von Mal2 mit Spc7 gezeigt werden, wobei allerdings nur ein geringer Teil dieser Proteine miteinander ko-immunopräzipitiert (Kerres *et al.*, 2006b). Die Verbindung zwischen beiden Proteinen ist entweder sehr instabil oder aber sie interagieren nur in einem begrenzten Zeitraum im Zellzyklus miteinander. Es ist nicht geklärt, ob die Proteine während der Wechselwirkung mit ihren jeweiligen Komplexen assoziiert sind und somit die beiden Komplexe miteinander verbinden oder aber, ob sie unabhängig von ihren Komplexen miteinander interagieren.

Interessanterweise scheint die Interaktion zwischen Mal2 und Spc7 konserviert zu sein. So ist in der Bäckerhefe bei einer Aufreinigung von Mcm21 (*S. cerevisiae* Mal2) -assoziierten Proteinen Spc105, das *S. cerevisiae* Spc7, isoliert worden (De Wulf *et al.*, 2003). Des Weiteren wurden ebenfalls Komponenten des Ndc80- sowie des MIND-Komplexes zusammen mit Mcm21 aufgereinigt (De Wulf *et al.*, 2003). Somit konnte eine Verbindung aller Subkomplexe aus dem auch in *S. cerevisiae* bestehenden NMS-Komplex mit dem Mal2-Homolog gezeigt werden. Hiermit übereinstimmend interagiert in 2-Hybrid-Analysen von *S. cerevisiae*-Proteinen Mcm21 mit der Ndc80-Komponente Spc24. Diese Verbindung konnte jedoch nicht mit Hilfe von Ko-Immunopräzipitationen bestätigt werden (Janke *et al.*, 2001). Die *S. pombe*-Proteine Mal2 und Mis12 weisen ebenfalls eine 2-Hybrid-Interaktion auf (Jin *et al.*, 2002). Allerdings zeigt der Mal2 Subgruppen-Partner Fta2 mit Mis12 keine physikalische Interaktion in Ko-Immunopräzipitationen (Kerres, unveröffentlicht). Weiterhin wurde vor

kurzen auch in menschlichen Zellen bei einer Affinitätsaufreinigung des Mal2-Homologen CENP-O das humane Spc7 namens AF15q14 isoliert (Okada *et al.*, 2006; Meraldi *et al.*, 2006). Hier war analog zu *S. pombe* die aufgereinigte Proteinmenge sehr gering.

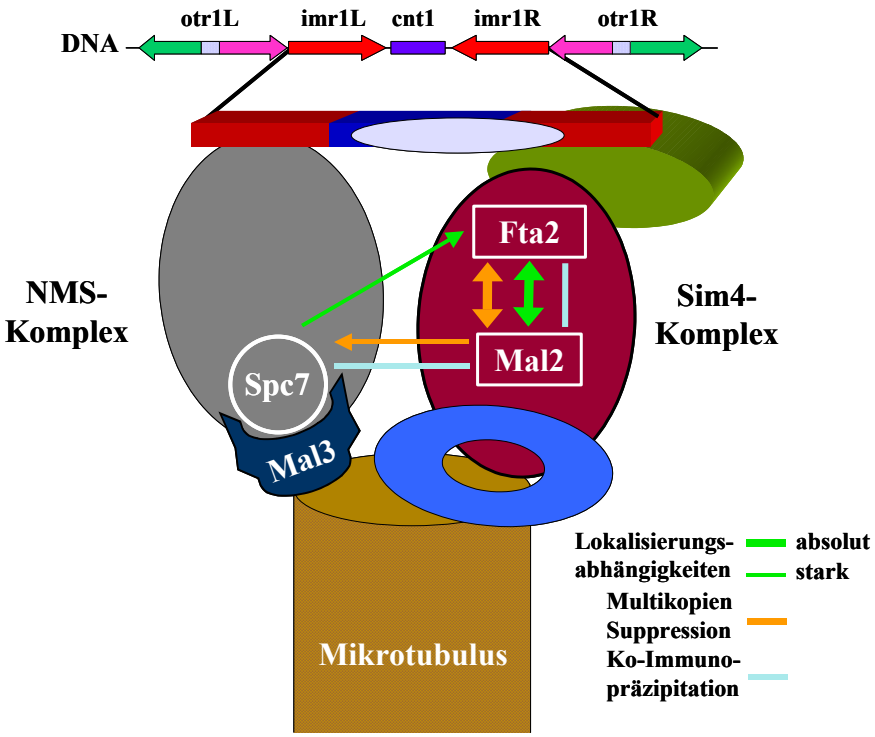


Abbildung 5: Der NMS- und der Sim4-Komplex interagieren miteinander.
Schematische Abbildung der Interaktion zwischen dem NMS- und dem Sim4-Komplex über das Spc7-Protein. Mal2 supprimiert *spc7*-Mutantenphänotypen. Spc7 ist für die wildtypische Fta2-Lokalisierung notwendig. Fta2 und Mal2 bilden zusammen eine Subgruppe innerhalb des Sim4-Komplexes.

Die NMS-Komponente Spc7 wird für die wildtypische Lokalisierung des Sim4-Komplexproteins Fta2 benötigt

Da eine Interaktion zwischen zwei Komponenten des NMS- und des Sim4-Komplexes gezeigt werden konnte, wurde analysiert, ob die Funktionalität dieser Proteine für die Lokalisierung von Komponenten des anderen Komplexes notwendig ist. Die Kinetochorlokalisierung von Spc7 hängt nicht von einem funktionellen Mal2 oder umgekehrt ab, jedoch ist die Assoziation des Mal2 Subgruppen-Partners Fta2 in 76 % der mitotischen *spc7*-Mutantenzellen stark reduziert oder nicht detektierbar (Abb. 5; Kerres *et al.*, 2006b). Diese Reduktion kommt nicht durch eine Destabilisierung des Fta2-Proteins in der *spc7*-Mutante zustande (Kerres *et al.*, 2006a). Überraschenderweise sind jedoch Proteine, die in *fta2*-Mutanten beeinträchtigt sind, trotz der Fta2-Reduktion in *spc7*-Mutanten anscheinend wildtypisch lokalisiert (Kerres *et al.*, 2006b; Kerres *et al.*, 2006a). Die Fta2-Kinetochor-Assoziation ist spezifisch von Spc7 und nicht von anderen bisher getesteten NMS-

Komplexkomponenten abhängig, da eine korrekte Lokalisierung von Fta2 in *nuf2*- und *mis12*-Mutanten stattfindet (Kerres *et al.*, 2006b). Bis dato ist nur ein weiteres Beispiel für eine Lokalisierungsabhängigkeit zwischen den NMS- und Sim4-Komplexen bekannt. So benötigt die korrekte Kinetochor-Assoziation von Sim4 während der Mitose ein funktionelles Nuf2-Protein (Saitoh *et al.*, 2005).

In der vorliegenden Arbeit wurden die Kinetochorkomponenten Spc7 und Fta2 näher charakterisiert. Sie haben jeweils eine Funktion bei der Assoziation der Spindel-Mikrotubuli mit den Kinetochoren sowie eine Funktion bei der bipolaren Ausrichtung der Chromosomen auf der Spindel. Mutationen in beiden Proteinen führen zu massiven DNA-Verteilungsdefekten. Für Spc7 wurde als erstes konstitutives Kinetochorprotein eine Funktion bei der Spindelintegrität festgestellt. Es konnte weiterhin gezeigt werden, dass entgegen der gegenwärtigen Annahme Proteine aus den NMS- und Sim4-Komplexen miteinander interagieren. Zukünftige Arbeiten werden klären müssen, welche genaue Rolle die einzelnen Proteine und Komplexe bei der Spindel-Assoziation spielen. Vermitteln sie nur den ersten Kontakt der Kinetochore mit den Mikrotubuli und/oder sind sie für die anschließende stabilere Bindung notwendig? Wie beeinflusst Spc7 als konstitutive Kinetochorkomponente den Spindelaufbau und die Spindelstruktur? Weiterhin bleibt zu untersuchen, ob die Interaktion zwischen den zuvor separat angesehenen Sim4- und NMS-Komplexen nur zwischen den einzelnen Proteinen und somit Komplex-unabhängig ist oder, ob sie wirklich die beiden Komplexe miteinander verbindet.

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The Fission Yeast Kinetochore Component Spc7 Associates with the EB1 Family Member Mal3 and Is Required for Kinetochore–Spindle Association

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A critical aspect of mitosis is the interaction of the kinetochore with spindle microtubules. Fission yeast Mal3 is a member of the EB1 family of microtubule plus-end binding proteins, which have been implicated in this process. However, the Mal3 interaction partner at the kinetochore had not been identified. Here, we show that the *mal3* mutant phenotype can be suppressed by the presence of extra Spc7, an essential kinetochore protein associated with the central centromere region. Mal3 and Spc7 interact physically as both proteins can be coimmunoprecipitated. Overexpression of a Spc7 variant severely compromises kinetochore-microtubule interaction, indicating that the Spc7 protein plays a role in this process. Spc7 function seems to be conserved because, Spc105, a *Saccharomyces cerevisiae* homolog of Spc7, identified by mass spectrometry as a component of the conserved Ndc80 complex, can rescue *mal3* mutant strains.

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Introduction

Segregation of chromosomes requires the association of spindle microtubules and chromosomes. Attachment of the mitotic spindle fibres occurs at a multicomponent protein complex, the kinetochore, that is assembled on centromeric DNA. This DNA region differs greatly in structure and size among various organisms (reviewed in Pidoux and Allshire, 2000; Cleveland *et al.*, 2003). The budding yeast centromere DNA is the simplest one described and consists of a very well defined 125-bp region while in higher eucaryotes, centromeric DNA is made up of highly repetitive sequences encompassing up to millions of base pairs. The centromere DNA of the fission yeast *S. pombe* lies in between these two extremes: it occupies between 40-100 kb on each chromosome and is composed of a central region flanked by inner and outer repetitive sequences. To date, proteins found to be associated with these regions either bind to the central core region or to the outer repeats thus pointing to the existence of two distinct domains in the fission yeast centromere (reviewed in Pidoux and Allshire, 2000). The heterochromatic outer repeats are required for centromere cohesion (reviewed in Bernard and Allshire, 2002) while the central region is needed for the assembly of the kinetochore *per se* (Saitoh *et al.*, 1997; Goshima *et al.*, 1999; Jin *et al.*, 2002; Pidoux *et al.*, 2003). However in spite of the different *cis*-acting DNA requirements, a substantial number of kinetochore proteins have been conserved from yeast to humans among them the four-component Ndc80 complex. This complex is required for kinetochore-microtubule association and spindle checkpoint signalling (He *et al.*, 2001; Janke *et al.*, 2001; Wigge and Kilmartin, 2001; Bharadwaj *et al.*, 2004; McCleland *et al.*, 2004).

The spindle microtubules that attach to kinetochores are highly dynamic structures that alternate between phases of growth and shrinkage (Kirschner and Mitchison, 1986). This dynamic behaviour is also observed after microtubules are attached to kinetochores and is co-regulated by components of the kinetochore complex and microtubule plus-end associated proteins. The role of kinetochore motors such as CENP-E and Kin1-related proteins in this process has been amply documented (reviewed in McIntosh *et al.*, 2002; Cleveland *et al.*, 2003; Mimori-Kiyosue and Tsukita, 2003). Microtubule plus-end proteins also have been implicated in the local control of microtubule dynamics and in the attachment of microtubules to kinetochores. Members of the CLIP170 family are associated transiently with prometaphase kinetochores and are required for kinetochore-microtubule attachment (Dujardin *et al*., 1998; Lin *et al*., 2001). Recently the nonmotor microtubule-associated protein (MAP) family CLASP has been identified as CLIP170/CLIP115 interaction partners (Akhmanova *et al*., 2001; Maiato *et al*., 2003). CLASP1 has been shown to localize near

growing spindle microtubule plus-ends and at the outer corona kinetochore region and is required for microtubule dynamics at the microtubule-kinetochore interface (Akhmanova *et al*., 2001; Maiato *et al*., 2003).

Another functionally conserved group of plus-end MAPs is the EB1 family which includes the *S. pombe* Mal3 protein (reviewed in Tirnauer and Bierer, 2000; Beinhauer *et al.*, 1997). Human EB1 was originally identified as an interaction partner of the adenomatous polyposis coli tumor suppressor APC (Su *et al*., 1995). Members of this family localize along microtubules, but are preferentially associated with microtubule plus-ends, regulate microtubule dynamics and have an important role in the interaction of the microtubule cytoskeleton with other cellular structures (reviewed in Tirnauer and Bierer, 2000; Schuyler and Pellman, 2001; Gundersen and Bretscher, 2003; Mimori-Kiyosue and Tsukita, 2003). Recently, EB1 and APC have been shown to localize to kinetochores indicating an involvement in chromosome capture (Fodde *et al.*, 2001; Kaplan *et al.*, 2001). This association of EB1 is restricted to polymerising microtubules pointing to a role for EB1 in regulating microtubule dynamics at the microtubule-kinetochore interface (Tirnauer *et al.*, 2002a). Fission yeast Mal3 was identified in a screen for components required for genome stability and although loss of the protein is not lethal it leads to increased chromosome loss and altered microtubule dynamics (Beinhauer *et al*., 1997). In addition, *mal3* mutant cells showed a significant increase in the number of cells with condensed chromosomes indicating defects in early aspects of mitosis (Beinhauer *et al.*, 1997). To better understand the role of Mal3 in mitosis we conducted a screen for extragenic suppressors of the *mal3* mutant phenotype. We identified a total of 10 suppressors that were able to rescue the chromosome loss phenotype of the *mal3* mutant strain. The most frequently isolated extragenic suppressor, the *spc7*$^{+}$ gene, codes for an essential kinetochore protein that appears to interact with Mal3 at the microtubule-kinetochore interface.

Materials and Methods

Strains and media

Genotypes of strains are listed in Table 1. For determination of genetic interaction at least three double mutants were tested per cross. *S. pombe* strains were grown in rich or minimal medium (YE5S, or EMM and MM) with appropriate supplements (Moreno *et al*., 1991). G418 resistance was scored on 100mg/liter G418 (Calbiochem). EMM with 5μg/ml thiamine repressed the *nmt1*$^+$ promoter. For high-level expression from the *nmt1*$^+$ promoter cells were grown in thiamine-less liquid EMM for 18 to 22h at 30 °C. Suppression of TBZ hypersensitivity was monitored on selective MM with 5-7.5 μg/ml TBZ. Suppression of minichromosome loss was assayed as described (Beinhauer *et al*., 1997). *S. cerevisiae* strains were grown in rich medium (YPD) or selective medium (SD) with appropriate supplements (Kaiser *et al*., 1994). Suppression of TBZ hypersensitivity was tested on selective SD with 75 μg/ml TBZ or YPD with 50-100 μg/ml TBZ; resistance to G418 on YPD containing 200mg/liter G418. For repression of the *tetO-CYC1* promoter cells were grown in non-selective SD containing 100μg/ml Doxycycline.

Table 1. Yeast strains used in this study

Name	Genotype	Source
UFYS135	*h$^+$ mal3Δ::his3$^+$ ade6-M210 leu1-32 ura4-D18 his3Δ*	U. Fleig
UFYS0203	*h$^-$ mal3-1 ade6-M210 leu1-32 ura4-D6 Ch^{16}[ade6-M216]*	U. Fleig
UFY177	*h$^+$ mal2-GFP/kan^R ade6-M210 leu1-32 ura4-D18*	U. Fleig
UFY25CX	*h$^+$ spi1-25 leu1-32 ade6-M210 ura4-D6 his3Δ*	U. Fleig
KG425	*h$^-$ ade6-M210 leu1-32 his3Δ ura4-D18*	K. Gould
KG554	*h$^+$ ade6-M216 leu1-32 his3Δ ura4-D18*	K. Gould
	h$^-$ mal3-pkGFP/ura4$^+$ ade6-M210 leu1-32 his3Δ ura4-D18	H. Browning
YUG37	*MATa ura3-52 trp1Δ63 GAL2 LEU2-tTA(leu2::pCM149)*	J. Hegemann
YSH12	*MATa ura3-52 trp1-289 leu2-3112 his3Δ1 bim1:: kan^R*	J. Hegemann
UFY155	*h$^-$ spc7-GFP/Kan^R ade6-M210 leu1-32 ura4-D6*	This study
UFY617	*h$^-$ spc7-HA/Kan^R ade6-M210 leu1-32 ura4-D6 Ch^{16}[ade6-M216]*	This study
UFY498	*h$^+$ spc7-HA/Kan^R mal2-GFP/Kan^R ade6-M210 leu1-32 ura4-D6*	This study
UFY496	*h$^-$ spc7-GFP/Kan^R mal3Δ::his3$^+$ ade6-M210 leu1-32 ura4 his3Δ*	This study
UFY466	*h$^+$ spc7-GFP/Kan^R nda3-KM311 ade6 leu1-32 ura4-D6*	This study
UFY637	*h$^+$ mal3-pkGFP/ura4$^+$ spc7-HA/Kan^R adeM-210 leu1-32 ura4$^-$*	This study
UFY639	*h$^-$ mal3-pkGFP/ura4$^+$ spc7-HA/Kan^R cut9-665 ade6-M210 leu1-32*	This study
UFY724	*h$^-$ mal2–1 spc7-GFP/Kan^R ade6-M210 ura4$^-$ Ch^{16}[ade6-M216]*	This study
UFY693	*h$^-$ spc7-GFP/Kan^R spi1-25 ade6-M210 leu1-32 ura4-D6*	This study
YJO359	*MATa ade2–101 trp1Δ63 leu2-Δ1 ura3-52 his3-Δ200 lys2-801 SPC105-ProA::His3MX6 sst1::loxP*	This study
UFY699	*MATa ura3 trp1 bim1:: kan^R LEU2-tTA (leu2:: pCM149) SPC105(-50, -1):: tetO-CYC1/Kan^R*	This study
YCJ341	*ade2-101 trp1-Δ63 leu2-Δ1 ura3-52 his3-Δ200 lys2-801 SPC24-ProA::His3MX6 cdc16-1::LYS2*	This study

Identification of *spc7*$^+$ and DNA methods

Multicopy extragenic suppressors of the *mal3* mutant phenotypes were isolated by transformation of the *mal3-1* strain with a *S. pombe* genomic bank (Barbet *et al*., 1992). Ura$^+$ transformants were replica-plated twice onto MM plates containing 7.5 μg/ml TBZ. Plasmids were isolated from transformants able to grow on TBZ-containing media and tested for the ability to rescue the increased minichromosome loss phenotype of the *mal3-1* strain by visual screening for the suppression of colony sectoring (Niwa *et al.*, 1986; Beinhauer *et al.*, 1997).

The 2.7 Kb long *SPC105* ORF was expressed from the modified *nmt1* promoter in *S. pombe* plasmid pJR2-41XU or from the *MET25* promoter in the 2 micron containing *S. cerevisiae* plasmid pRS473MET25. Correct annotation of the SPCC1020.02 ORF was confirmed by amplification of this ORF via PCR using a *S. pombe* cDNA Bank (Clontech).

A *spc7* null allele (*Δspc7*) was generated by replacing the internal 3.06 Kb of the 4.09 Kb *spc7*$^+$ ORF with the *his3*$^+$ marker in the diploid strain KG425xKG554.Tetrad analysis of 28 heterozygous *Δspc7*/*spc7*$^+$ diploids revealed that only the 2 his$^-$ spores/tetrad grew.

Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) using *S. pombe* strains was performed as described (Jin *et al*., 2002). Strains were shifted to 18 °C for 2 hours prior to fixation with 3 % paraformaldehyde. Strains containing GFP-tagged Spc7 or Mal2 were used for ChIP. 2 μl rabbit anti-GFP antibody (Molecular Probes) was used in the ChIP and 25 μl Protein A agarose (Roche). Multiplex PCR with Taq DNA polymerase (Roche) was used for analysis of centromeric chromatin in crude extracts and immunoprecipitates, using the primer pairs for *cnt* (central core), *otr* (outer repeat) and *fbp1*$^+$ (euchromatic control) as described (Jin *et al.*, 2002) and the *imr* (inner most repeat) pair: 5'-GGATATATGTATTCTTGCACTC -3' and 5'-GGCTACCAGCATTGTTATTCATAACC-3'. PCR reactions contained 1.25 mM $MgCl_2$, 0.25 mM dNTPs, 2 μl ChIP sample or 2 μl 1/10 dilution of crude sample, with 50 ng of each primer in a 25 μl reaction.

For co-immunoprecipitation wild-type strains or a *cut9-665* strain incubated for 4 hours at 36 °C were washed once with STOP buffer (0.9 % NaCl, 1mM NaN_3, 10mM EDTA, 50 mM NaF). Approximately 1 x 10^9 cells were resuspended in 80 μl Hepes-Lysis buffer (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % Na-deoxycholate, 1 mM PMSF, Complete protease inhibitor (Roche)) and lysed using glass beads. Then 450 μl Hepes buffer was added and samples were centrifuged twice for 30

minutes in a microfuge at 4°C. A 150 µl amount of each sample was incubated on ice for 1 hr with 50 µl anti-HA MicroBeads or anti-GFP MicroBeads (Miltenyi Biotec). Immunoprecipitates were isolated using the µMACS epitope tagged protein isolation kit according to the manufacturers instructions (Miltenyi Biotec) except that the immune complexes were washed for maximally 5 minutes. After elution, the immune complexes were boiled, resolved on SDS-7 % polyacrylamide gels and blotted onto Immobilon-P (Millipore). As Spc7-HA is a 158 kDa protein and Mal3-GFP a 62 kDa protein blots were cut in half and the top half (greater than 85 kDa proteins) probed with anti-HA antibody (monoclonal mouse; Roche); the bottom half with anti-GFP antibody (polyclonal rabbit, Molecular Probes). Immobilised antigens were detected using the ECL Advance Western Blotting Kit (Amersham Biosciences).

Affinity purification and MALDI-TOF analysis

4000 OD of cells in 15 ml lysis buffer (50 mM Tris, pH 8.0; 140 mM KCl; 5mM $MgCl_2$; 10 % glycerol; 1 % Triton X-100 ; 2mM DTT, 10mM NaF, 100mM glycerophosphate and protease inhibitors) were lysed with glass beads. The cleared lysate was incubated with 200 µl IgG-Agarose (Sigma) for 4h at 4 ^{o}C. Subsequently the agarose was washed with 20 ml lysis buffer, 20 ml wash buffer (10 mM Tris pH 8.0; 250 mM LiCl, 5 mM $MgCl_2$, 0.5 % sodium deoxycholate, 0.5 % NP 40) and 1 ml of 5 mM ammonium acetate (pH 5.0). The sample was eluted with 2 ml of 500 mM ammonium acetate (pH 3.4), dried and subjected to PAGE plus MALDI-TOF analysis a described (Shevchenko *et al*., 1996).

Microscopy

For *S. pombe* cells, photomicrographs were obtained using a Zeiss Axiovert200 fluorescence microscope coupled to a CCD camera (Hamamatsu Orca-ER) and Openlab imaging software (Improvision). Immunofluorescence microscopy was done as described (Hagan and Hyams, 1988; Bridge *et al.*, 1998). For tubulin staining, primary monoclonal anti-tubulin antibody TAT1 (Woods *et al*., 1989) followed by FITC or Cy3-conjugated secondary sheep anti-mouse antibodies (Sigma-Aldrich) were used. Strains expressing HA- and GFP-fusion proteins were observed in fixed cells by indirect immunofluorescence with mouse anti-HA antibody (Covance) followed by Cy3-conjugated sheep anti-mouse antibodies (Sigma) and rabbit anti-GFP (Molecular Probes) followed by FITC-conjugated goat anti-rabbit antibodies (Sigma). Processed cells were stained with DAPI before mounting.

Results

Identification of *spc7*$^+$ as a suppressor of the *mal3* mutant phenotype

S. pombe strains carrying a mutant *mal3* allele are hypersensitive to microtubule destabilizing drugs such as thiabendazole (TBZ) and show a 400-fold increase in the loss of a non-essential minichromosome (Beinhauer *et al*., 1997). Furthermore a *Δmal3* (mal3 deletion) cell population showed a fourfold increase in the number of undivided condensed chromosomes compared to a wild-type strain (Beinhauer *et al*., 1997). In addition the spindle checkpoint which monitors the correct alignment of chromosomes on the spindle appears to be activated in *Δmal3* cells. Double-mutant strains of *Δmal3* with a null allele of *mph1*$^+$, which is an evolutionarily conserved component of the spindle checkpoint pathway (He *et al*., 1998), showed reduced growth compared to the single mutant strains (data not shown). To clarify the role of the Mal3 protein in mitosis we conducted a search for multicopy suppressors of the *mal3* mutant mitotic phenotypes (Materials and Methods). Apart from the wild-type *mal3*$^+$ ORF, we identified a total of 10 genes, that were able to suppress the hypersensitivity to TBZ and the increased minichromosome loss of the *mal3-1* strain (Vietmeier-Decker and Fleig, unpublished). The screen was saturating as most suppressors were isolated several times.

A previously uncharacterised ORF with the systematic name SPCC1020.02 (*S. pombe* genome project at the Sanger Institute) was isolated most frequently, namely 26 times, in this screen. We named this ORF *spc7*$^+$ (**S**. **p**ombe **c**entromere). According to the sequence annotation of the *S. pombe* genome the *spc7*$^+$ ORF is 4095 bp in length and codes for a 153.5 kDa protein. We confirmed the annotation of this ORF by identification of a cDNA clone that contained the entire coding sequence (see Materials and Methods). The presence of extra *spc7*$^+$ rescued the TBZ hypersensitivity and the increased loss of a non-essential minichromosome of the *mal3-1* strain (Figure 1A and 1B respectively). Extra *spc7*$^+$ also rescued the TBZ hypersensitivity of *Δmal3* and all other *mal3* mutant strains tested (Figure 1A, data not shown) implying that suppression by *spc7*$^+$ is not allele specific and can occur even in the absence of *mal3*$^+$. The original genomic clone that suppressed the *mal3* phenotypes only contained the last 2028 bp of the 4095 bp long *spc7*$^+$ ORF. This fragment rescued as well as full length *spc7*$^+$ (data not shown). The first in frame ATG was predicted to give rise to a Spc7 variant without the N-terminal 820 amino acids. To test if this prediction was correct, the C-terminal 1635 bp region of the *spc7*$^+$ ORF was expressed from the modified *nmt1* promoter in *S. pombe* plasmid pJR2-41XU (Moreno *et al*., 2000). This Spc7 variant (Spc7-C) was able to fully suppress the *mal3* phenotypes (data not shown).

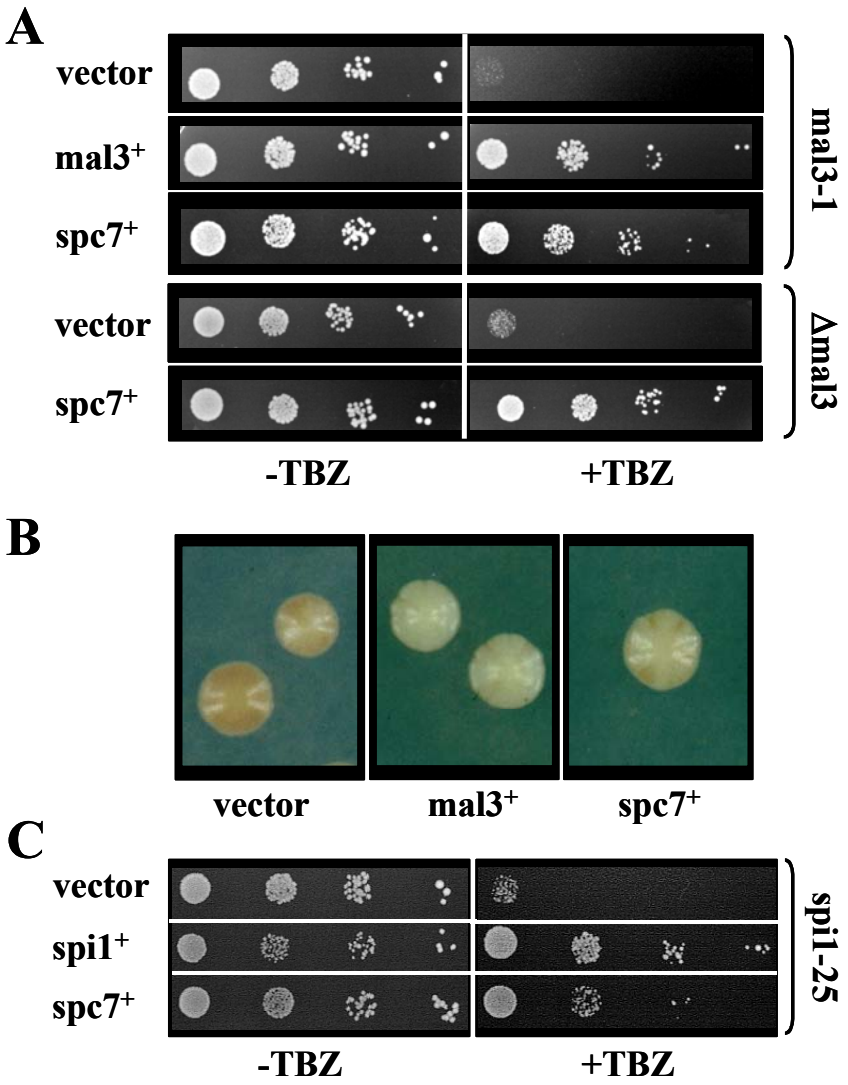


Figure 1. ***spc7***$^{+}$ **suppresses the phenotypes of** ***mal3*** **and** ***spi1-25*** **mutant strains**.
(A) The TBZ hypersensitivity of the *mal3-1* and *Δmal3* strains is rescued by extra *spc7*$^{+}$. Left and right panels show serial dilution patch tests (10^{4} to 10^{1} cells) of *mal3-1* and *Δmal3* transformants grown under selective conditions in the absence (-TBZ) or presence (+TBZ) of 7 μg/ml Thiabendazole for 5 days at 24 °C. Vector control indicates plasmid without insert; mal3^{+} denotes the presence of wild-type *mal3*$^{+}$ on a plasmid. **(B)** The minichromosome loss phenotype of the *mal3-1* strain, indicated by adenine auxotrophy and numerous red sectors in a white colony (vector), is rescued by the presence of *mal3*$^{+}$ or *spc7*$^{+}$ on a plasmid. Plates were incubated at 24 °C. **(C)** The TBZ hypersensitivity of the *spi1-25* strain is rescued by extra *spc7*$^{+}$. Panels show serial dilution patch tests of *spi1-25* transformants grown under selective conditions in the absence (-TBZ) or presence (+TBZ) of 7 μg/ml TBZ for 5 days at 24 °C. Vector control indicates plasmid without insert, while spi1^{+} denotes the presence of wild-type *spi1*$^{+}$ on a plasmid.

We had tested for genetic interaction between *mal3*$^{+}$ and other genes encoding microtubule plus-end associated proteins such as *dis1*$^{+}$ and *alp14*$^{+}$/*mtc1*$^{+}$ which encode members of the TOG/XMAP215 family (Ohkura *et al.*, 1988; Nabeshima *et al.*, 1995; Garcia *et al.*, 2001; Nakaseko *et al.*, 2001). No genetic interaction was observed between *Δmal3* and the mutant *dis1-288* allele. However *Δalp14 Δmal3* double mutants were synthetically lethal at all temperature tested thus indicating that the two proteins have a role in the same essential

process. We therefore assayed if extra *spc7*$^+$ could also rescue the mutant phenotypes of an *Δalp14* strain (Garcia *et al*., 2001) but found that *spc7*$^+$ was unable to rescue the temperature-sensitivity or the TBZ hypersensitivity of the *Δalp14* strain (data not shown).

Identification of *spc7*$^+$ as a suppressor of *SpRan* mutant phenotype

We have previously reported the characterization of a partial loss of function mutant of the *S. pombe* Ran GTPase Spi1 that led to aberrant mitosis and severe genome instability (Fleig *et al*., 2000). Strains carrying this specific mutation, named *spi1-25*, were hypersensitive to TBZ. This mutant phenotype was suppressed partially by the presence of extra Mal3 (Fleig *et al*., 2000). A multicopy suppressor analysis identified several other genes that could rescue the *spi1-25* TBZ hypersensitivity (Karig and Fleig, unpublished). Among them was the *spc7*$^+$ ORF. As shown in Figure 1C plasmid-borne expression of *spc7*$^+$ partially suppressed the TBZ hypersensitivity of the *spi1-25* strain. In addition *spc7*$^+$ also rescued partially the temperature-sensitivity of the *spi1-25* strain (data not shown).

Spc7 is an essential component of the fission yeast kinetochore

To determine if *spc7*$^+$ was essential for vegetative growth one copy of the *spc7*$^+$ ORF was replaced with the *his3*$^+$ marker in a diploid strain (Materials and Methods). Sporulation and subsequent tetrad analysis of this strain revealed that only 2 of the 4 spores in a tetrad could grow into a colony and these were always his$^-$ indicating that *spc7*$^+$ is an essential gene. To determine the subcellular localization of Spc7, a fluorescence-improved version of GFP was fused to the COOH-terminal end of the endogenous *spc7*$^+$ coding region. The strain expressing the Spc7 fusion protein was indistinguishable in phenotype from the isogenic wild-type strain, indicating that the tagged gene was functional. Immunofluorescence analysis of interphase cells expressing Spc7-GFP revealed a single fluorescent dot near the nuclear periphery, while early mitotic cells showed up to six dots associated with the condensed chromatin (Figure 2A, panels a and b respectively). Anaphase cells showed two Spc7-GFP dots that cosegregated with the separated chromatin (Figure 2A, panel c). Cells arrested in mitosis by overexpression of the spindle checkpoint component Mph1 (He *et al*., 1998) showed up to 6 fluorescent signals: two signals co-localized with each of the three duplicated chromosomes positioned on a short spindle (Figure 2B). As this type of localization is characteristic of *S. pombe* kinetochore proteins, we compared the intracellular localization pattern of the Spc7 fusion protein with that of the known kinetochore protein Mal2 (Jin *et al*., 2002). For this purpose a strain was used in which the endogenous *spc7*$^+$ ORF had been

tagged with the HA epitope and the endogenous *mal2*$^+$ ORF fused to the *GFP* ORF. Co-localization of the Spc7 and Mal2 fusion proteins was observed in interphase and mitotic cells (Figure 2C, panels e-f) in all cells analysed (n=150). Interestingly in interphase cells 71 % of cells had the Mal2 signal closer to the nuclear periphery than the Spc7 signal (n= 76) (Figure 2C, panel e). Taken together these data imply that the Spc7 protein is a component of the fission yeast kinetochore, that localizes at centromeres throughout the cell cycle.

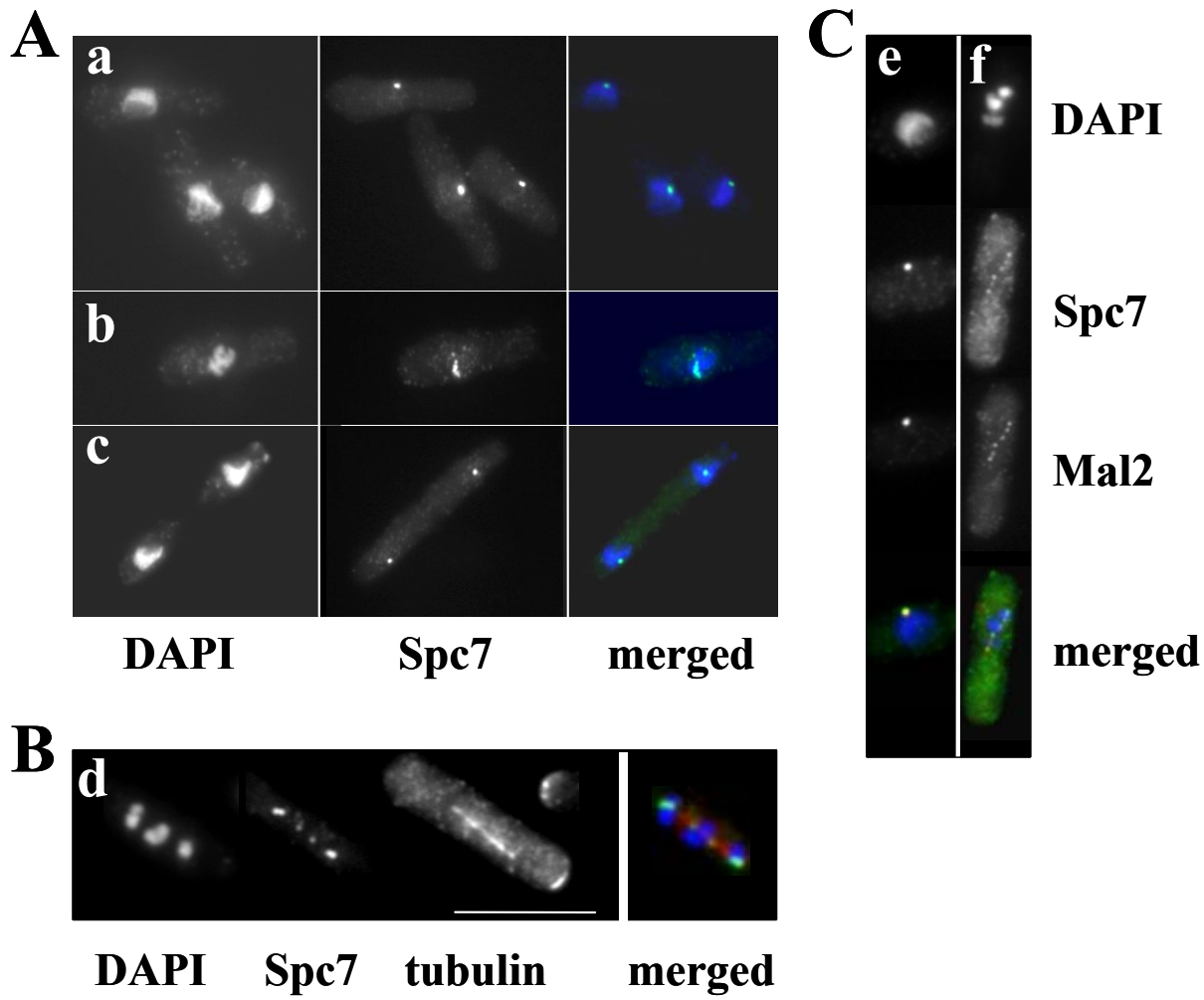
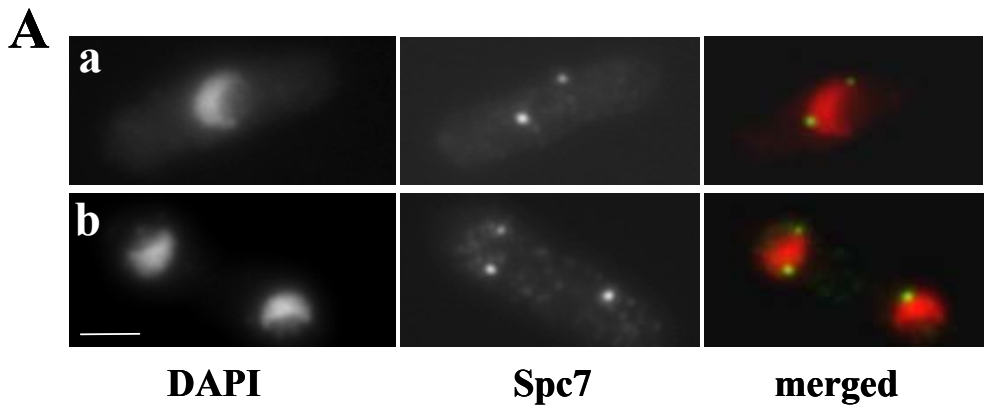


Figure 2. Spc7 localizes to the kinetochore.
(A) Localization of the Spc7-GFP protein in wild-type cells in interphase (a) early (b) or late (c) stages of mitosis. Fixed cells were stained with DAPI and anti-GFP antibody. **(B)** Localization of the Spc7 fusion in a wild-type cell arrested by overexpression of *mph1*$^+$. GFP signals, spindles and condensed chromosomes were simultaneously observed by staining with anti-GFP antibody, anti-tubulin antibody and DAPI. **(C)** Co-localization of Spc7-HA (green) and Mal2-GFP (red) fusion proteins in interphase cells (e) and cells arrested by overexpression of *mph1*$^+$ (f). Chromosomes, HA- and GFP-signals were simultaneously observed by staining with anti-GFP antibody, anti-HA antibody and DAPI. Bar, 10 µm.

We next determined by immunofluoresence analysis if the subcellular localization of Spc7-GFP was affected in the temperature-sensitive *spi1-25* and *mal2-1* strains, the cold-sensitive *nda3-KM311* (β-tubulin) strain and the *Δmal3* strain (Umesono *et al.*, 1983; Fleig *et al.*, 1996; Beinhauer *et al.*, 1997; Fleig *et al.*, 2000). For this purpose the endogenous *spc7*$^{+}$*-GFP* ORF was crossed into these strains. At the restrictive temperature Spc7-GFP was localized correctly in the Ran mutant strain *spi1-25* and the *mal2-1* strain, which encodes a defective kinetochore component (Jin *et al*., 2002). Furthermore kinetochore localization of Spc7 was not affected in the *nda3-KM311* mutant strain at the non-permissive temperature implying that an intact microtubule-cytoskeleton is not required for Spc7 localization (data not shown).
As described previously the *Δmal3* population showed a fourfold increase in the number of cells with condensed chromosomes indicating an early mitotic defect. Immunofluoresence analysis of these fixed cells revealed that the vast majority (>90 %, n=76) showed 6 fluorescent Spc7-GFP dots on a metaphase spindle (3x2 sister centromeres; data not shown). No severe anaphase defects were observed. However clustering of the Spc7 protein at the spindle pole body (SPB) was affected severely in post-mitotic *Δmal3* cells. In wild-type cells centromeres and the associated kinetochore proteins cluster adjacent to the SPB in interphase cells and this association with each other and the SPB is only disrupted in M-phase (Funabiki *et al.*, 1993; Ekwall *et al.*, 1995; Saitoh *et al.*, 1997). 7.5 % of *Δmal3* G2 interphase cells showed instead of the expected single Spc7-GFP signal two dots associated with the nuclear pheriphery (Figure 3A, panel a). The brighter one of these dots co-localized with the SPB using the Cut12 SPB marker (data not shown; Bridge *et al*., 1998). Interestingly, more than 35 % of *Δmal3* post-anaphase cells showed 2 or rarely 3 Spc7-GFP signals per nucleus (Figure 3A, panel b and 3B). These data demonstrate that the Mal3 protein is required for SPB clustering of Spc7 especially in post-anaphase cells. However Mal3 is not *per se* required for clustering of centromeres/ kinetochores at the SPB. Microscopic examination of *Δmal3* cells expressing the GFP-tagged Mal2 kinetochore protein always revealed a single fluorescent dot in G2 cells and two Mal2-GFP dots that co-localized with the separated chromatin in post-anaphase cells (data not shown; Jin *et al*., 2002). As Spc7 clustering does not require an intact microtubule cytoskeleton, it is possible that SPB-associated Mal3 is required for Spc7 clustering. A *Dictyostelium* member of the EB1 family has been shown to be a genuine component of the centrosome (Rehberg and Graf, 2002).



B

defective Spc7 clustering	wild type	Δmal3
interphase	0	7.5
post-anaphase	0	35.8

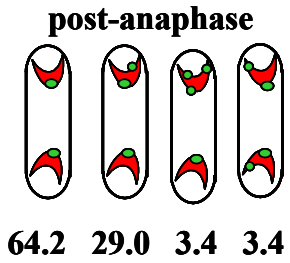


Figure 3. **Clustering of Spc7 is defective in a *Δmal3* strain.**
(A) Localization of the Spc7-GFP fusion protein in *Δmal3* cells in G2 (a) and post-anaphase (b) cells. Fixed cells pregrown at 24 °C were stained with DAPI and anti-GFP antibody. Bar, 5 μm. **(B)** Shown are the percentages of interphase cells (G2) (n= 120) and post-anaphase cells (n=174) that have more than one Spc7 signal per nucleus. The right most panel shows the various distributions observed for the Spc7 signal in *Δmal3* post-anaphase cells. Red, chromatin; green, Spc7-GFP signal.

Spc7 is associated with the central core region of the fission yeast centromere

The centromeric DNA of *S. pombe* is 40-100 kb in size and consists of a central core (*cnt*) flanked by arrays of inner (*imr*/B) and outer repeats (*otr*/K+L) (Figure 4 shows the centromeric DNA of chromosome I) (reviewed in Clarke, 1998). To identify the centromere region with which the Spc7 protein associates, chromatin immunoprecipitation (ChIP) was carried out (Partridge *et al*., 2000). Cells expressing either Mal2-GFP or Spc7-GFP fusion proteins were analysed in ChIP assays using anti-GFP antibodies. DNA present in crude extracts or immunoprecipitates using anti-GFP antibodies were analysed by multiplex PCR, using primers to amplify the *cnt*, *imr*, and *otr* regions in the centromere of chromosome I and an unrelated euchromatic control region *fbp*. As previously reported the Mal2 protein associates with the central core region as shown by a specific enrichment of the *cnt* and *imr* sequences in the Mal2-GFP ChIP (Jin *et al.*, 2002) (Figure 4). The Spc7 ChIPs also showed an enrichment of the *cnt* and *imr* sequences. These DNA sequences were enriched 2.5 and 2.4 fold, respectively, relative to the *fbp* DNA in Spc7 ChIPs in comparison to the input control. The *otr* sequence was not enriched in Spc7 ChIPs. These data indicate an association of the

Spc7 fusion protein with the central domain of the centromere and imply an involvement of this domain in kinetochore-microtubule attachment. Interestingly the enrichment of the *cnt* and *imr* sequences in Spc7 ChIPs was repeatedly and reproducibly approximately twofold lower compared to Mal2 ChIPs (Figure 4, data not shown).

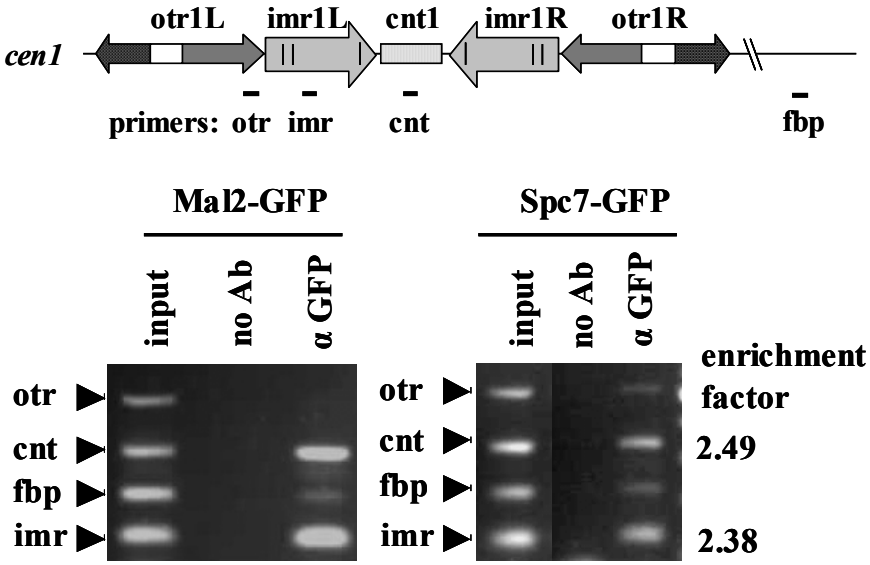


Figure 4. Spc7 is associated with the central domain of *cen1*.
Cells expressing Spc7-GFP or Mal2-GFP were fixed and processed for ChIP using anti-GFP antibodies. Chromatin in immunoprecipitates and crude extracts was analysed by multiplex PCR, using primers to amplify regions in *cen1*: *cnt*, *imr*, *otr* and an euchromatic negative control locus, *fbp*. *cnt* and *imr* sequences are specifically enriched in ChIPs of proteins associated with the central domain such as the Mal2 fusion protein. The *cnt* and *imr* sequences are also enriched in Spc7-GFP ChIPs, indicating association of the Spc7 fusion protein with the central centromere domain.

Spc7 affects kinetochore-microtubule interactions

We had found that wild-type cells expressing full-length Spc7 from the modified *nmt1* promoter (pREP41; moderate overexpression) had no visible effect on the growth of the cells, nor did overproduction from the wild-type *nmt1*$^{+}$ promoter (pREP3; strong overexpression) (data not shown). Moderate overproduction of the C-terminal part of Spc7 (Spc7-C), which is sufficient to suppress the *mal3* mutant phenotypes, also showed no effect on cell growth (Figure 5A, middle panel). However strong overproduction of this Spc7 variant, which is still able to associate with the kinetochore (data not shown), led to severe growth inhibition (Figure 5A, bottom panel). The growth inhibition was not due to a simple displacement of the full length Spc7 protein by Spc7-C at the kinetochore. The Spc7-GFP fusion protein was properly localized in wild-type cells overexpressing *spc7-c* (data not shown). To assay the consequences of Spc7-C overproduction we looked at immunofluorescence staining of a wild-type strain overexpressing *spc7-c* for 18-22 h. While interphase cells showed no obvious

abnormalities, cells undergoing mitosis were severely affected. After 18 hours of induction 24 % of the cells in a population were in mitosis and this number increased to 40 % at later time points (22h). 67 % of mitotic cells showed severe chromosome segregation defects and cells with a "cut" phenotype became more frequent (Figure 5B, data not shown).

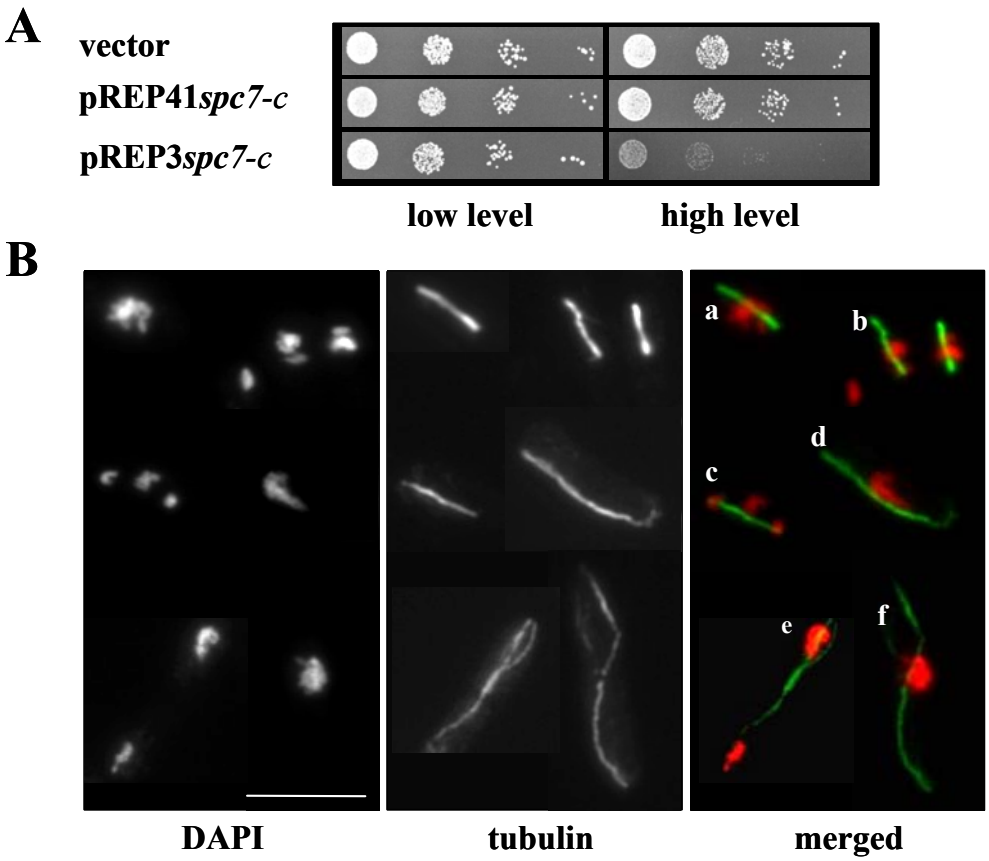


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Figure 5. Overexpression of a Spc7 variant in a wild-type strain leads to growth inhibition and severe mitotic defects.
(A) Spc7-C was expressed from two different versions of the regulatable *nmt1*$^{+}$ promoter. pREP3 gives rise to strong and pREP41 to moderate overexpression. Shown are serial dilution patch tests (10^4 to 10^1 cells) of transformants grown under selective conditions for 4 days at 30 °C. **(B)** Photomicrographs of wild-type cells overexpressing *spc7-c* from pREP3. Fixed cells were stained with anti-tubulin antibody and DAPI. Shown is a composite of cells displaying various mitotic defects: non-separated chromatin (a, d, f) or unequally/partially divided chromatin on elongating spindles (b, c, e). Bar is 10 µm

Two predominant defective chromosome resolution phenotypes were observed: (i) no separation of highly condensed chromatin on an elongating spindle (Figure 5B, cells a, d, f) and (ii) unequal segregation of the chromatin (Figure 5B, cells b, c, e). In the first phenotypic class chromosomes were not separated on an elongating spindle. Using the Mal2 protein as a kinetochore marker we found (i) that association of Mal2 with the kinetochore was unaffected by Spc7-C overexpression and (ii) that not all kinetochores in these cells were associated with the mitotic spindle (Figure 6 panel c, non-attached kinetochore marked by an arrow). In cells,

where the chromatin had an arrow like appearance, kinetochores were clustered at the "tip" of the arrow (Figure 6 panel d). The second phenotypic class consisted of chromatin that was segregated asymmetrically. Part of the chromosomes were found at one end of a short anaphase spindle (Figure 5B, cell b) or only part of the chromosomal material had been segregated to the two ends of the spindle while the rest remained unseparated at the equatorial plate (Figure 5B, cell c). Rarely we also observed fully elongated spindles showing asymmetric segregation of the chromosomes (Figure 5B, cell e). Cells belonging to the second phenotypic class often contained kinetochores that did not appear to be attached to spindle microtubules (Figure 6, panels a and b). We repeated the above experiment using a second kinetochore marker, namely Spc24, and obtained similar results (data not shown; Wigge and Kilmartin, 2001).

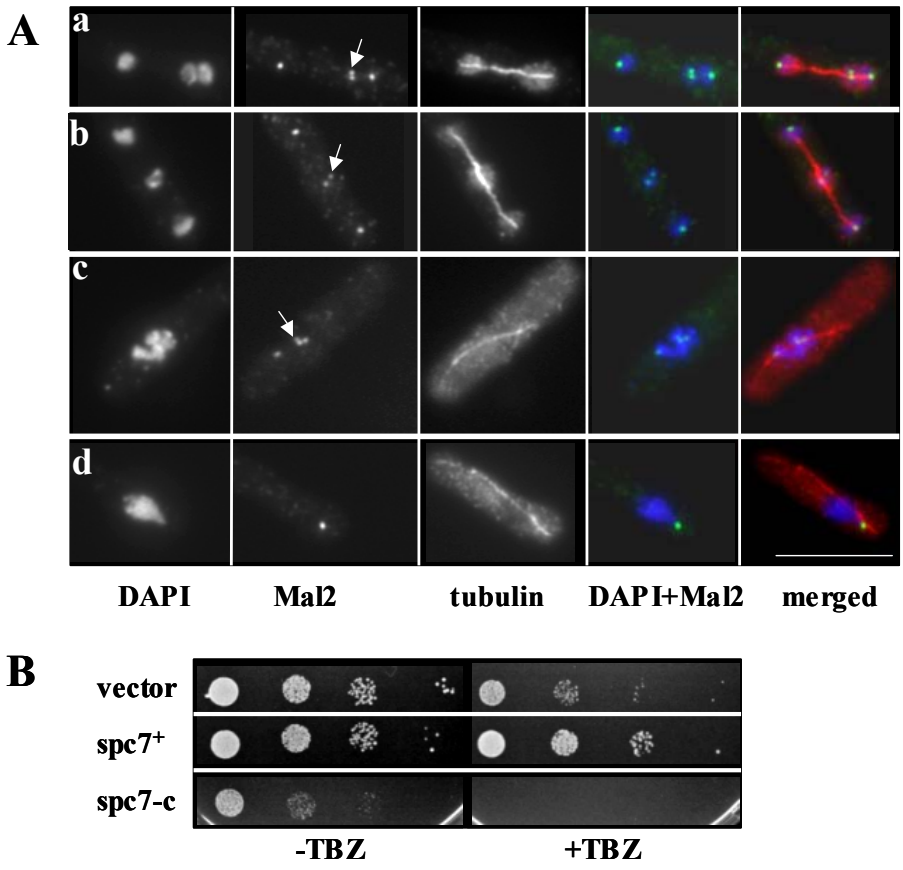

Figure 6. The association between spindle and kinetochore is altered by Spc7-C overproduction.
(A) Photomicrographs of *mal2*$^{+}$*-GFP* cells overexpressing *spc7-c*. Fixed cells were stained with DAPI, anti-GFP and anti-tubulin antibody. Shown are elongating spindles without or with unequal segregation of the chromatin. Merged images show chromatin, Mal2-GFP and the spindle. The arrows indicate kinetochores not attached to the spindle. Bar is 10 µm. (**B**) Overexpression of Spc7 or Spc7-C from pREP3 alters sensitivity to microtubule destabilizing drug TBZ. Left and right panels show serial dilution patch tests (10^4 to 10^1 cells) of wild-type transformants expressing high levels of *spc7*$^{+}$ or *spc7-c* in the absence or presence of TBZ. Vector control indicates plasmid without insert.

Interestingly overexpression of full length Spc7 or Spc7-C from the wild-type *nmt1*$^+$ promoter in a wild-type strain led to differences in the sensitivity to TBZ. As shown in Figure 6B overproduction of full length Spc7 leads to increased resistance to the microtubule destabilizing drug TBZ while overexpression of the Spc7 variant Spc7-C gave rise to the opposite effect.

Spc7 co-immunoprecipitates with Mal3

Given the finding that extra *spc7*$^+$ could suppress the mitotic phenotypes of *mal3* mutant strains we investigated if the Mal3 and Spc7 proteins interacted. For this purpose we tested if Spc7 co-immunoprecipitated Mal3 and vice versa. Immunoprecipitation with anti-GFP or anti-HA antibodies was carried out with protein extracts from strains expressing endogenous Mal3-GFP and/or Spc7-HA. These immunoprecipitates were then analysed by Western blotting using anti-GFP and anti-HA antibodies. As shown in Figure 7 immunoprecipitation of Spc7-HA clearly co-immunoprecipitates Mal3-GFP in wild-type cell extracts. We could also co-immunoprecipitate Mal3-GFP with anti-HA antibodies from mitotically arrested *cut9-665* extracts (Figure 7). *cut9*$^+$ codes for a subunit of the anaphase promoting factor (Yamada *et al*., 1997). Furthermore immunoprecipitation of Mal3-GFP also co-immunoprecipitated Spc7-HA although the amount of Spc7-HA that was co-immunoprecipitated was low (Figure 7). Western blot analysis showed the existence of two Spc7-HA specific bands. Our preliminary analysis indicates that the slower migrating form is phosphorylated and the predominant form in *cut9-665* arrested cells (data not shown, Figure 7). Both forms could be co-immunoprecipitated by Mal3. Our data thus imply that the Mal3 and Spc7 proteins interact with each other. Interestingly, we were unable to co-immunoprecipitate Spc7 and Mal3 upon prolonged washing steps indicating that the interaction between Mal3 and Spc7 is not a stable one.

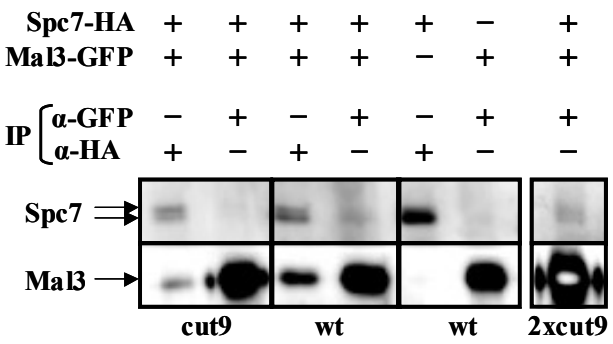

Figure 7. Spc7 and Mal3 co-immunoprecipitate.
Protein extracts were prepared from wild-type (wt) and *cut9-665* (cut9) strains expressing Spc7-HA, Mal3-GFP or both. Lysates were halved: one half was used for immunoprecipitation (IP) with an anti-HA antibody, the other with an anti-GFP antibody. The immunoprecipitates were resolved by SDS-PAGE, blotted to Immobilon-P and cut in half at the 85 kDa size marker. The top half was probed with anti-HA antibody; the bottom half with anti-GFP antibody. Immobilized antigens were detected using the ECL kit. Positions of Spc7 and Mal3 are indicated by arrows. The right most lane (2xcut9) is identical to lane 2 except that twice the amount of immunoprecipitate was used. This illustrates more clearly the co-immunoprecipitation of Spc7-HA by Mal3-GFP.

Spc7 belongs to a conserved protein family that co-purifies with the conserved Ndc80 complex

Database searches using PEDANT at MIPS identified 9 potential homologs from other fungal organisms. Although the overall sequence identity between these proteins is only around 22 % the amino acid comparison showed a number of conserved sequence motifs of yet unknown function (data not shown). Among these homologs is the 105 kDa *S. cerevisiae* Spc105 protein, which shares a number of sequence motifs with the C-terminal part of the 153 kDa Spc7 protein (data not shown; Wigge *et al*., 1998; Nekrasov *et al*., 2003). To determine whether members of this family have a similar function we tested the ability of the *S. cerevisiae* Spc105 protein to suppress phenotypes of *mal3* mutant strains. Spc105 is part of the *S. cerevisiae* kinetochore as shown by ChIP and immunofluoresence analysis of a Spc105 fusion protein (data not shown, Nekrasov *et al*., 2003). We identified the Spc105 protein as a component that co-purified with the centromere associated, highly conserved Ndc80 complex. Spc24, a known Ndc80 complex component was Protein A-tagged and the fusion protein isolated from cell extracts using affinity chromatography with IgG-Sepharose. The proteins that co-purified with Protein A-tagged Spc24 were identified by peptide mass fingerprints (MALDI-TOF mass spectrometry). In addition to the previously identified components of the Ndc80 complex, namely Ndc80, Nuf2, Spc24 and Spc25 (Janke *et al*., 2001; Wigge and Kilmartin, 2001) we also identified Spc105 in this analysis (Figure 8A). We thus conclude that Spc105 is associated closely with the evolutionarily conserved Ndc80 complex. Next, we protein A-tagged Spc105 and identified the Ydr532c protein as a co-purification partner (data not shown). The 44 kDa Ydr532c protein has been identified as a component of the SPB and very recently as a new component of the budding yeast kinetochore (Giaever *et al*., 2002; Huh *et al*., 2003; Nekrasov *et al*., 2003).

To test if Spc105 and Spc7 might have a similar function, we tested if Spc105 could suppress the TBZ hypersensitivity of *mal3* mutant strains. As shown in Figure 8B, *SPC105* expressed from the regulatable *S. pombe nmt1*$^{+}$ promoter was able to partially suppress the TBZ hypersensitivity of the *S. pombe mal3-1* and *Δmal3* strains. In addition *SPC105* expressed from the *MET25* promoter on a plasmid was able to partially suppress the TBZ hypersensitivity of the *S. cerevisiae Δbim1* strain (Figure 8B). Bim1 is the *S. cerevisiae* member of the EB1 family (Schwartz *et al*., 1997).

We next tested if *SPC105* and *BIM1* interacted genetically. For this purpose we used a strain where *SPC105* was expressed from an ectopic promoter. The endogenous *SPC105* promoter was replaced by the Doxycycline regulatable *tetO-CYC1* promoter in a strain with an

integrated *tetR*-VP16(tTA) activator allowing regulated expression from the *tetO-CYC1* promoter (Gari *et al*., 1997). Cells carrying this construct are viable, but show a strong increase in the loss of a GFP-marked chromosome fragment and increased sensitivity to TBZ (data not shown; Figure 8C). The TBZ-hypersensitivity of the *tetO-CYC/SPC105* strain was increased by the absence of $bim1^+$ as a *tetO-CYC/SPC105 Δbim1* double mutant strain was more sensitive to TBZ than the single mutant strains (Figure 8C). Furthermore the double mutant strain showed reduced growth at 36 °C and at 18 °C compared to the single mutant strains (data not shown) thus implying that *BIM1* and *SPC105* show genetic interaction.

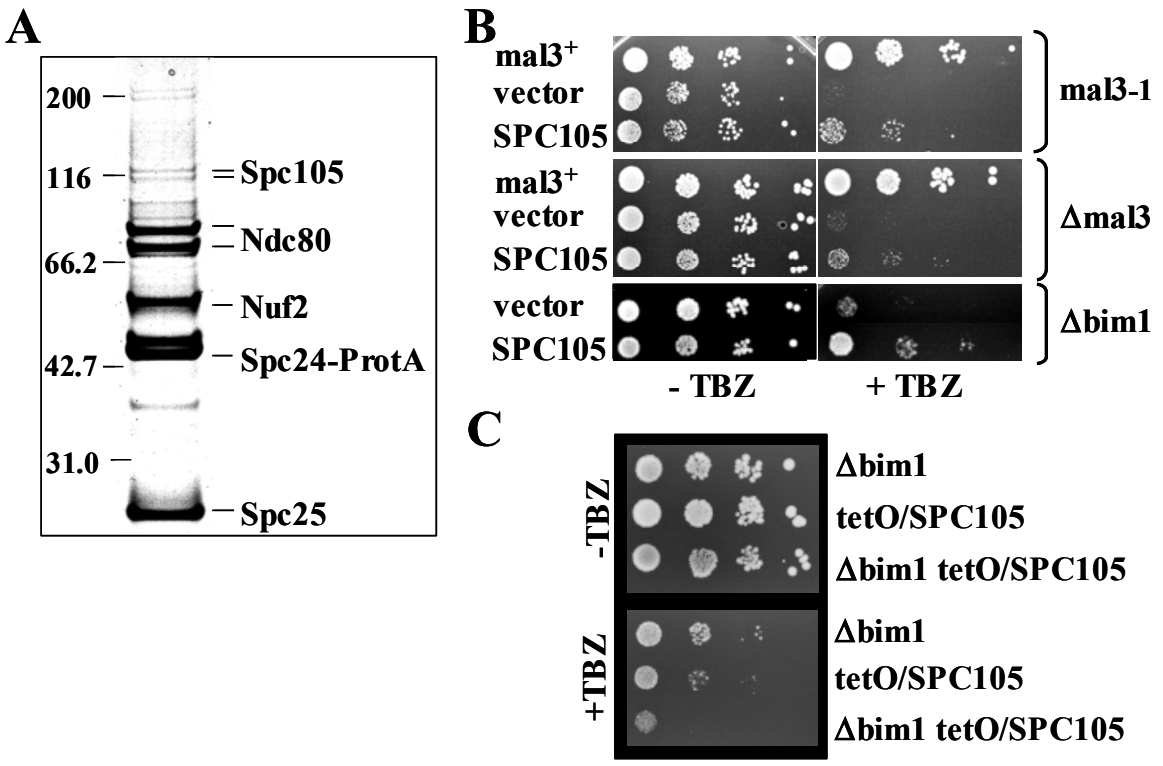

Figure 8. The *S. cerevisiae* Spc105, a component of the conserved Ndc80 kinetochore complex, rescues *mal3* mutants.
(A) Spc105 co-purifies with the Ndc80 complex. Protein A-tagged Spc24 was purified from 4000 OD_{578} of cells by affinity chromatography with IgG-Agarose and the complete preparation was subjected to SDS PAGE. Co-purifying proteins were identified from Coomassie-stained bands by peptide mass finger printing (MALDI-TOF). Bands that are not labelled represent contaminants. **(B)** The TBZ-hypersensitivity of the *S. pombe mal3-1* and *Δmal3* strains and the *S. cerevisiae Δbim1* strain is partially rescued by overexpression of *SPC105*. Panels show serial dilution patch tests (10^4 to 10^1 cells) of *mal3-1*, *Δmal3*and *Δbim1* transformants grown under selective conditions in the absence (-TBZ) or presence (+TBZ) of TBZ . Plates were incubated for 5 days at 24 °C (*mal3* strains) or 3 days at 30 °C (*Δbim1* strain). Vector control indicates plasmid without insert. **(C)** Serial dilution patch tests of *Δbim1*, *tetO-CYC1/SPC105* single and double mutant strains on YPD with Doxycycline with or without 50 μg/ml TBZ. Strains were grown at 30 °C for 48 h.

Discussion

We investigated the role of *S. pombe* EB1 family member in mitosis by screening for suppressors that were able to rescue the *mal3* mutant phenotypes, namely TBZ hypersensitivity and increased chromosome loss. The most frequently isolated suppressor, *spc7*$^{+}$, codes for an essential component of the kinetochore thus demonstrating that Mal3 plays a role at the spindle-kinetochore interface. Members of the EB1 family have been shown to be associated with kinetochores (Juwana *et al.*, 1999; Fodde *et al.*, 2001; Kaplan *et al.*, 2001; Rehberg and Graf, 2002; Tirnauer *et al.*, 2002a) but the interaction partner at the kinetochore has remained unclear. The finding that the *S. cerevisiae* Spc105 protein appears to be a homolog of Spc7 implies that this interaction at the spindle-kinetochore interface has been conserved.

The Spc7 protein is an essential component of the *S. pombe* kinetochore that associates specifically with the central centromere region implying that this region is required for kinetochore-microtubule association. Although phenotypic analysis of various *S. pombe* kinetochore mutants had suggested that this region is needed for kinetochore-microtubule association (Saitoh *et al.*, 1997; Goshima *et al.*, 1999; Jin *et al.*, 2002; Pidoux *et al.*, 2003), Spc7 is the first central centromere region protein for which a direct involvement at the kinetochore-microtubule interface has been demonstrated. In support of this finding, Dis1, a member of the TOG/XMAP215 family of microtubule-associated proteins, is associated with this region in mitosis, although the other XMAP215 homolog in *S. pombe*, Alp14/Mtc1, shows a somewhat different localization (Garcia *et al*., 2001; Nakaseko *et al*., 2001). Association of Mal3 with a specific centromere region needs yet to be demonstrated. Our attempts to map the centromere region with which Mal3 interacts using ChIP followed by multiplex PCR were not successful (data not shown). However given the finding that Spc7 and Mal3 could be co-immunoprecipitated it is feasible that Mal3 is associated with the central centromere region.

Enrichment of the central region DNA sequences in Spc7 ChIPs was repeatedly twofold lower than that of another central region associated protein, namely Mal2. This might imply that in an assembled kinetochore Spc7 is physically further away from the centromeric DNA than Mal2. Ultrastructural analysis has shown that *S. pombe* centromeres are multilayered structures (Kniola *et al*., 2001). In particular the Ndc80 complex, with which Spc7 is probably associated, was shown to be part of an “anchor structure” close to the SPB and distinct from the localization of the *S. pombe* CENP-A homolog Cnp1 (Kniola *et al*., 2001). Consistent

with this observation we found that in the majority of interphase cells the Spc7 signal was further away from the nuclear periphery than the Mal2 signal.

Our experimental data indicate that the role of the Spc7 protein at the kinetochore is that of linking the kinetochore to microtubule-plus ends and possibly influencing the dynamics of kinetochore microtubules. Firstly, *spc7*$^{+}$ rescued the increased chromosome loss phenotype of a *mal3* mutant strain and can be co-immunoprecipitated with the microtubule-plus-end protein Mal3. Secondly, overexpression of an Spc7 variant (Spc7-C) led to a dominant negative phenotype and kinetochores that were not associated with the mitotic spindle implying that Spc7 affects kinetochore-microtubule interactions. Thirdly, preliminary evidence indicates that Spc7 might have a role in microtubule dynamics. Extra *spc7*$^{+}$ is able to rescue the TBZ-hypersensitivity of specific strains such as *mal3* mutants and the Ran mutant *spi1-25*. Overexpression of full length Spc7 increases the resistance of cells to the microtubule destabilizing drug TBZ, while overproduction of Spc7-C has the opposite effect. Fourthly, the *S. cerevisiae* kinetochore protein Spc105 can partially suppress the phenotypes of *S. pombe mal3* mutant strains suggesting that Spc7 and Spc105 might be functionally homologous. We identified Spc105, as a co-purification partner of the Ndc80 complex. The highly conserved Ndc80/HEC1 kinetochore complex is required for the establishment and maintenance of kinetochore-microtubule interactions and plays a role in spindle checkpoint activity (He *et al.*, 2001; Janke *et al.*, 2001; Wigge and Kilmartin, 2001; DeLuca *et al.*, 2002; Bharadwaj *et al.*, 2004; McCleland *et al.*, 2004). Spc105 has also been found recently in affinity purifications of *S. cerevisiae* kinetochore proteins that define the Mtw1p complex (De Wulf *et al*., 2003; Nekrasov *et al*., 2003). Furthermore, Spc105 affinity purification has been described to contain components of the Ndc80 complex (Nekrasov *et al*., 2003). Our finding that Spc105 co-purifies with the Ndc80 complex component Spc24 confirms that Spc105 is in proximity to the Ndc80 complex. The fact that Spc105 was not detected in Ndc80 preparations (Nekrasov *et al*., 2003) might reflect the fact that Spc105 is more closely associated with Spc24 than Ndc80. Alternatively, this might be due to differences in the experimental conditions.

Homologs of the Ndc80 complex also exist in *S. pombe* and have been shown to be part of the kinetochore (Kniola *et al*., 2001; Wigge and Kilmartin, 2001). It is at present unclear if Spc7 is closely associated with or part of this complex. However Spc7 can be co-immunoprecipitated by the *S. pombe* Spc24 protein and vice versa (Kerres and Fleig, unpublished). Furthermore Spc24, like Spc7, is associated with the central centromere region

(Kerres and Fleig, unpublished). It is thus feasible to propose that Spc7 is associated with the *S. pombe* Ndc80 complex.

The identification of Spc7/Spc105, as a suppressor of the *mal3* mutant phenotype implies that the Ndc80 complex or proteins in close proximity to this complex might possibly play a more direct role in association with microtubule plus-ends than previously envisaged (reviewed in Cheeseman *et al*., 2002). Interestingly components of the Ndc80 complex as well as Spc105 but not inner kinetochore proteins like Ndc10 were identified in enriched SPB preparations (Wigge *et al*., 1998).

Our experimental data suggest that one of the factors required for kinetochore-microtubule association is an interaction between the kinetochore protein Spc7 and the microtubule plus-end associated protein Mal3. The Ran GTPase seems to play a role in this specific association as extra Spc7 can rescue a *spi1* mutation, which results in a decrease in the amount of active Ran in cells that are competent for nucleocytoplasmic transport (Fleig *et al.*, 2000; Salus *et al.*, 2002). Recently Ran has been implicated in kinetochore function as the GTPase activating proteins RanGAP1 and RanBP2 are associated with metaphase kinetochores and *C. elegans* Ran was localized to kinetochores, where it appeared to play a role in the association of kinetochore microtubules to chromosomes (Bamba *et al.*, 2002; Joseph *et al.*, 2002). Furthermore in *Xenopus* egg extracts the spindle checkpoint and the kinetochore association of spindle checkpoint proteins is directly regulated by Ran-GTP levels (Arnaoutov and Dasso, 2003).

What is the function of Mal3 at the kinetochore-microtubule interface? Our co-immunoprecipitation analysis suggests that the interaction between Spc7 and Mal3 is not a stable one thus making it unlikely that the main role of Mal3 is that of continuous microtubule-kinetochore attachment. In support of this is the finding that the kinetochore localization of EB1 was restricted to a subset of early mitotic kinetochores that were associated with polymerising microtubule plus-ends (Tirnauer *et al*., 2002a). It is thus more feasible that Mal3 has a role in regulating microtubule dynamics at the kinetochore. The effect of EB1 family members on microtubule dynamics has been amply documented (Beinhauer *et al.*, 1997; Tirnauer *et al.*, 1999; Nakamura *et al.*, 2001; Busch and Brunner, 2004). Absence of EB1 family members leads to reduced microtubule length while overexpression of EB1 bundles microtubules (Bu and Su, 2001; Nakamura *et al.*, 2001; Rogers *et al.*, 2002; Tirnauer *et al.*, 2002b; Ligon *et al.*, 2003). Furthermore Mal3 affects microtubule dynamics by initiating microtubule growth and inhibiting catastrophe events in interphase microtubule arrays (Beinhauer *et al.*, 1997; Busch and Brunner, 2004). As overexpression of Spc7 makes

wild-type cells more resistant to microtubule-destabilizing drugs we would like to suggest that Mal3 and Spc7 are part of the complex protein machinery that modulates the dynamic behaviour of microtubules at the kinetochore. In this context it is interesting to note that the ability of EB1 to promote microtubule polymerisation was dependent on the presence of the C-terminal part of APC suggesting that EB1 function is modulated by other proteins (Nakamura *et al*., 2001). If Spc7 has a similar effect on the function of Mal3 or if it acts in this process but independent of Mal3 remains to be determined.

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Molecular Biology of the Cell

The conserved Spc7 protein links kinetochore complexes and is required for spindle integrity in fission yeast

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The Spc7 kinetochore protein was identified as an interaction partner of Mal3, a member of the EB1 family of microtubule plus-end binding proteins. Spc7 associates with the central centromere region of the chromosome but does not affect transcriptional silencing. Here, we show that Spc7 is required for the integrity of the spindle as well as for targeting of MIND but not of Ndc80 complex components to the kinetochore. Spindle defects in *spc7* mutants were severe ranging from the inability to form a bipolar spindle in early mitosis to broken spindles in mid-anaphase B. *spc7* mutant phenotypes were partially rescued by extra α-tubulin or extra Mal2 which associates with Spc7 and stabilizes the defective Spc7 protein. This interaction demonstrates that Spc7 physically links two independent kinetochore complexes: the Ndc80-MIND-Spc7 complex and the Mal2-containing Sim4 complex.

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Introduction

The precise segregation of chromosomes is a complex process that requires the coordinated interaction between spindle and kinetochores. Kinetochores are macromolecular structures that assemble on centromeric DNA and fulfil multiple functions: they mediate the bipolar attachment of sister chromatids to spindle microtubules, maintain this attachment during dynamic microtubule behaviour and generate the spindle checkpoint signalling required for anaphase onset. These functions are essentially conserved although the composition and morphology of kinetochores can differ greatly among various organisms. In particular, the centromeric DNA requirements vary dramatically from the simple 125 bp ´point` centromeres of the budding yeast *Saccharomyces cerevisiae* with the three CDEI-III protein binding motifs to the ´regional` centromeres which are more complex, carry repetitive sequences and can encompass millions of base pairs. Such centromeres exist in plants, metazoans and fungi like the fission yeast *Schizosaccharomyces pombe* (reviewed in: Pidoux and Allshire, 2000; Cleveland *et al.*, 2003). The centromere DNA of *S. pombe* is 40 to 100 kb in size and is composed of a central core region (*cnt*) that is flanked by inverted repeat elements (*imr*). These elements are surrounded by outer repeat elements (*otr*) (reviewed in: Clarke, 1998). The heterochromatic outer repeats are needed for sister centromere cohesion and might help in the orientation of the multiple kinetochore microtubule attachment sites (reviewed in: Pidoux and Allshire, 2004) while the central region acts as a platform for the association of kinetochore complexes required for microtubule-kinetochore interaction (Saitoh *et al.*, 1997; Pidoux *et al.*, 2003; Hayashi *et al.*, 2004; Kerres *et al.*, 2004).

The simplest and best studied kinetochore, that of *S. cerevisiae*, contains more than 60 proteins while mammalian kinetochores are predicted to contain a 100 or more protein components (McAinsh *et al.*, 2003; Fukagawa, 2004). These proteins, which exist in subcomplexes, can be classed as regulatory or structural components. The latter group of proteins are required as a connecting bridge between the centromeric DNA and the microtubules of the spindle. Interestingly, *S. cerevisiae* kinetochore subcomplexes that link centromere DNA binding proteins to microtubule binding proteins have been conserved in evolution (Meraldi *et al.*, 2006). Kinetochore components found in ´point` and ´regional` centromeres include the COMA complex member Mcm21p, the Ndc80 and MIND complexes and the Spc105p protein implying the central importance of these components in kinetochore function (Ortiz *et al.*, 1999; Wigge and Kilmartin, 2001; Euskirchen, 2002; De Wulf *et al.*, 2003; Nekrasov *et al.*, 2003; Westermann *et al.*, 2003; Meraldi *et al.*, 2006). In *S. cerevisae*

Ndc80, COMA and MIND complexes share a function in kinetochore capture by the side of spindle microtubules (Tanaka *et al.*, 2005).

In *S. pombe* these proteins are constitutive kinetochore components and exist in two biochemically separable complexes: the Mcm21p ortholog Mal2 is part of the 13 component Sim4 complex, while the 4 component MIND subcomplex, the 4 member Ndc80 complex and the Spc105p ortholog Spc7 make up the Ndc80-MIND-Spc7 kinetochore complex (Obuse *et al.*, 2004; Liu *et al.*, 2005). The essential Mal2 protein associates with the central centromere region and is required for the transcriptional silencing and the specialized chromatin structure of this region (Jin *et al.*, 2002). Mutations in *mal2*$^{+}$ and other components of the Sim4 complex give rise to extreme missegregation of chromosomes (Saitoh *et al.*, 1997; Jin *et al.*, 2002; Pidoux *et al.*, 2003; Hayashi *et al.*, 2004; Kerres *et al*., 2006). Interestingly, the Sim4 complex plays a role in the incorporation of the kinetochore-specific histone H3 variant CENP-A and functions as a loading dock for the DASH complex (Takahashi *et al.*, 2000; Pidoux *et al.*, 2003; Liu *et al.*, 2005; Sanchez-Perez *et al.*, 2005). The non-essential fission yeast DASH complex is required for biorientation of sister chromatids (Liu *et al.*, 2005; Sanchez-Perez *et al.*, 2005). Members of the Ndc80-MIND-Spc7 complex are also associated with the central centromere and maintain the special chromatin architecture of this region but are not involved in CENP-A targeting (Goshima *et al.*, 1999; Hayashi *et al.*, 2004; Kerres *et al.*, 2004; Liu *et al.*, 2005). The Ndc80 complex in fission yeast and other organisms plays an important role in kinetochore-microtubule association and is needed for spindle checkpoint signaling (He *et al.*, 2001; Janke *et al.*, 2001; Nabetani *et al.*, 2001; Wigge and Kilmartin, 2001; McCleland *et al.*, 2003; Saitoh *et al.*, 2005). We have shown recently that Spc7 also plays an important part in the interaction between microtubules and kinetochore as *spc7*$^{+}$ was isolated as a suppressor of a *mal3* mutant (Kerres *et al.*, 2004). Mal3 is the fission yeast member of the EB1 microtubule-plus-end-tracking protein family, which regulates microtubule dynamics and mediates the interaction between different cellular complexes (reviewed in: Gundersen and Bretscher, 2003; Mimori-Kiyosue and Tsukita, 2003; Vaughan, 2005). Mal3 is required for genome stability amongst others by preventing monopolar attachment of sister chromatids (Beinhauer *et al.*, 1997; Asakawa *et al.*, 2005). EB1 family members are targeted to kinetochores on polymerising microtubules and play a role in kinetochore capture (Fodde *et al.*, 2001; Kaplan *et al.*, 2001; Tirnauer *et al.*, 2002; Tanaka *et al.*, 2005). Overexpression of the constitutive Spc7 kinetochore protein rescued all mitotic phenotypes of *mal3* mutants and the Spc7 and Mal3 proteins interact physically. However, in contrast to the loss of the non-essential *mal3*$^{+}$, loss of *spc7*$^{+}$ results in inviability due to severe

chromosome missegregation (Beinhauer *et al.*, 1997; Kerres *et al.*, 2004). This finding implies that the interaction with Mal3 is just one of the tasks of the Spc7 protein. We have thus extended our analysis of Spc7 function in mitosis.

Materials and Methods

Strains and Media

The yeast strains used in this study are listed in Table 1. All new strains were obtained by crossing the appropriate strains followed by tetrad or random spore analysis and genotyping. At least 3 double mutants were tested per cross. Tetrad analysis of 16 tetrads of the cross *nuf2-1* x *spc7-23* revealed that spores carrying both mutations were able to germinate and divide twice indicating synthetic lethality. Double mutants between *spc7-23* and a null allele of a component of the DASH complex, namely *duo1Δ* were inviable. Tetrad analysis of 16 tetrads revealed that double mutants germinated and then died. Strains were grown in rich media (YE5S) or minimal media (EMM or MM) with supplements (Moreno *et al.*, 1991). MM with 5 µg/ml thiamine repressed the *nmt* promoters. For high-level expression from *nmt* promoters cells were grown in thiamine-less media for 22-48 hrs at 25°C or 18-24 hrs at 30 or 32°C. Resistance to G418 was tested on YE5S plates containing 100 mg/l G418. Transcriptional silencing assays were carried out as described (Jin *et al.*, 2002; Pidoux *et al.*, 2003).

Table 1. Yeast strains used in this study

Name	Genotype	Source
UFY1267	*h⁻ mis12-537 spc7-23/his3⁺ ade6-M216 leu1-32*	this study
UFY1028	*h⁺ spc7-23/his3⁺ his3-D1 ade6-M216 leu1-32 ura4-D18*	this study
UFY1029	*h⁺ spc7-24/his3⁺ his3-D1 ade6-M216 leu1-32 ura4-D18*	this study
UFY1027	*h⁺ spc7-30/his3⁺ his3-D1 ade6-M216 leu1-32 ura4-D18*	this study
UFY1163	*h⁻ nuf2-GFP/ura4⁺ spc7-23/his3⁺ ade6-M216 leu1-32 ura4-D18*	this study
UFY1249	*h⁻ mis12-GFP/LEU⁺ spc7-23/his3⁺ leu1-32*	this study
UFY1307	*h⁺ mis14-GFP spc7-23/his3⁺ ura4⁻ leu1-32*	this study
UFY1266	*h⁺ spc24-GFP/Kan^R spc7-23/his3⁺ ade6-M216 his⁻ leu1-32 ura4⁻*	this study
UFY1069	*h⁺ mal2-GFP/Kan^R spc7-23/his3⁺ his3-D1 ade6-M216 leu1-32 ura4⁻*	this study
UFY1258	*h⁻ sim4-GFP/Kan^R spc7-23/his3⁺ arg3-D4 ade6-M210 ura4- leu1-32 his3-D1*	this study
UFY1187	*h⁻ dad1-GFP/Kan^R spc7-23/his3⁺ leu1-32 ura4-D18*	this study
UFY1260	*h⁻ spc7-23/his3⁺ mis15-68 ade6-M216*	this study
UFY1256	*h⁺ spc7-23/his3⁺ mis17-362 ura4-D18*	this study
UFY1264	*h⁺ spc7-23/his3⁺ sim4-193 his3-D1 ade6-M210 leu1-32 ura4-D18*	this study
UFY1088	*h⁺ spc7-23/his3⁺ mal2-1 ade6-M210 leu1-32 ura4-D18*	this study
UFY1196	*h⁺ spc7-23/his3⁺ mis6-302 leu1-32 ura4-D18*	this study
UFY1085	*h⁻ fta2-291/his3⁺ spc7-23/his3⁺ leu1-32 ade6-M210 ura4-D18 his3-D1*	this study
UFY1048	*h⁻ fta2-291/his3⁺ his3⁻ leu1-32 ura4-D18 ade6-M210*	this study
UFY1175	*h⁺ spc7-23/his3⁺ mad2Δ::ura4⁺ ade6-M216 leu1-32 ura4-D18*	this study
UFY1177	*h⁻ spc7-23/his3⁺ mph1Δ::ura4⁺ ade6-M216 leu1-32 ura4-D18*	this study
UFY1062	*h⁻ spc7-23/his3⁺ cnt1(NcoI):arg3 his3-D1 ade6-M210 leu1-32 ura4⁻ arg3-D4*	this study
UFY1081	*h⁺ spc7-23/his3⁺otr2(HindIII):ura4⁺ ura4-DS/E leu1-32 ade6-M216 arg3-D4*	this study
UFY1222	*h⁺ spc7-23/his3⁺ his7⁺::lacI-GFP lys1⁺::LacOP leu1⁻ ura4⁻*	this study

Name	Genotype	Source
UFY1060	*h⁻ spc7-30/his3⁺ Kan^R-nmt81-GFP-atb2⁺ leu1-32*	this study
UFY1254	*h⁺ mis6-3xHA/ LEU⁺ spc7-23/his3⁺ ade6-M216 leu1-32 ura4-D18*	this study
UFY1228	*h⁻ spc7-HA/Kan^R Kan^R-nmt81-GFP-atb2⁺ leu1-32*	this study
UFY1244	*h⁺ spc7-23-GFP/Kan^R/his3⁺ his3-D1 ade6-M216 leu1-32 ura4-D18*	this study
UFY1248	*h⁻ spc7-GFP/Kan^R nuf2-1/ura4⁺ ura4⁻ ade6-M210 his3-D1*	this study
UFY1269	*h⁺ spc7-GFP/Kan^R mis12-537 leu1-32*	this study
UFY1224	*h⁺ spc7-23/his3⁺ mal3Δ::his3⁺ his3⁻ ade6-M210 leu1-32 ura4-D18*	this study
UFY1288	*h⁻ spc7-23/his3⁺ alp14Δ::kan^R ura4⁻ leu1-32*	this study
UFY1262	*h⁺ spc7-23/his3⁺ peg1-1 ura4-D18 leu1-32*	this study
UFY498	*h⁺ spc7-HA/Kan^R mal2-GFP/Kan^R ade6-M210 leu1-32 ura4-D6*	U. Fleig
UFY597	*h⁺ mal2-GFP/Kan^R ade6-M210 leu1-32 ura4-D6*	U. Fleig
UFY617	*h⁻ spc7-HA/Kan^R ade6-M210 leu1-32 ura4-D6 Ch^16[ade6-M216]*	U. Fleig
UFY852	*h⁻ mal2-1 leu1-32 ade6-M210 ura4-D18*	U. Fleig
UFY724	*h⁻ spc7-GFP/Kan^R mal2-1 ade6-M210 ura⁻ Ch^16[ade6-M216]*	U. Fleig
UFY135	*h⁺ mal3Δ::his3⁺ ade6-M210 leu1-32 ura4-D18 his3Δ*	U. Fleig
UFY336	*h⁺ alp14Δ::kan^R ura4⁻*	T. Toda
IH1563	*h⁻ peg1-1 leu1-32 ura4-D18*	I. Hagan
FY4540	*h⁻ sim4-193 cnt1(NcoI):arg3 cnt3(NcoI):ade6 otr2(HindIII):ura4 tel1L:his3 ade6-M210 leu1-32 ura4-D18 arg3-D4 his3-D1*	R. Allshire
FY648	*h⁺ swi6Δ::his1⁺ otr1R(SphI)::ura4⁺ ura4-DS/E leu1-32 ade6-M210*	R. Allshire
FY5231	*h⁺ sim4-193 arg3-D4 ade6-M210 his3-D1 ura4-D18 leu1-32*	R. Allshire
KG425	*h⁻ ade6-M210 leu1-32 his3Δ ura4-D18*	K. Gould
ANF251-9A	*h⁺ nuf2-1/ura4⁺ ura4-D18*	Y. Hiraoka
SS638	*h⁻ mad2Δ::ura4⁺ leu1-32 ura4-D18 ade6-M210*	S. Sazer
SS560	*h⁻ mph1Δ::ura4⁺ leu1-32 ura4-D18 ade6-M216*	S. Sazer
	h⁻ mis6-302 leu1-32	M. Yanagida
	h⁻ mis15-68	M. Yanagida
	h⁻ mis17-362	M. Yanagida
	h⁻ mis12-537 leu1-32	M. Yanagida
	h⁻ Kan^R-nmt81-GFP-atb2⁺ leu1-32	T. Toda

Generation of *spc7^ts* alleles and DNA methods

A pBSK based plasmid containing the last 2028 bps of the 4095 bps long *spc7⁺* ORF followed 3` by the *his3⁺* gene was used as a template for a mutagenic PCR reaction. A 3896 bp long DNA fragment containing the last 1664 bps of the *spc7⁺* ORF and the *his3⁺* selection marker were transformed into strain KG425. His⁺ transformants that grew at 25°C but not at 36°C were identified and correct integration of the mutagenized DNA fragments was tested via PCR. A *spc7⁺* containing plasmid was able to fully rescue the temperature-sensitivity of these strains, which were backcrossed twice. DNA sequence analysis showed point mutations at the following positions in the ORF: *spc7-24* (3340 (A to T); 3959 (T to G)), *spc7-23* (3887 (T to G); 4079 (G to T)), *spc7-30* (3026 (G to A); 3914 (A to G); 4067 (G to T)). We generated an endogenous *spc7-23-gfp* fusion via PCR based gene targeting using the Kan^R cassette (Bahler *et al.*, 1998). *spc7-n-gfp* and *spc7-c-gfp* fusions were constructed by homologous recombination in *S. cerevisiae*, cut out of vector pRS316 and cloned behind the *nmt1⁺* promoter in plasmid pJR2-3XL (Moreno *et al.*, 2000). *spc7-n* and *spc7-c* contain the first 2460 or the last 1632 bps of the *spc7⁺* ORF.

Microscopy

Photomicrographs of fixed cells were obtained using a Zeiss Axiovert200 fluorescence microscope coupled to a charge-coupled device camera (Hamamatsu Orca-ER) and Openlab imaging software (Improvision, Coventry, UK). Immunofluorescence microscopy was carried out as described previously (Hagan and Hyams, 1988; Bridge *et al.*, 1998). For tubulin staining the monoclonal anti-tubulin antibody TAT1 was used as primary antibody followed by fluorescein isothiocyanate-conjugated goat anti-mouse antibodies (Sigma-Aldrich). HA or GFP fusion proteins were observed by indirect immunofluorescence using mouse anti-HA antibody (Covance, Princeton, NJ) or rabbit anti-GFP antibodies (Invitrogen, Carlsbad, CA), respectively. Cy3-conjugated sheep anti-mouse antibodies or Cy3-conjugated sheep anti-rabbit antibodies (Sigma-Aldrich) were used as secondary antibodies. Before mounting, cells were stained with 4,6-diamidino-2-phenylindole (DAPI). Images of living cells expressing an integrated version of *GFP.nmt81.atb2* were obtained using a DeltaVision RT Imaging System (Applied Precision Instruments) with a Peltier-cooled CCD Coolsnap HQ Camera (Roper Scientific). Optical sections were recorded every 0.3 microns in a volume totalling 6 microns every 10 seconds for 30 minutes. All images were analysed using Imaris (Bitplane) software. Transformed cells were grown in liquid EMM supplemented media without thiamine for 36-48 hours at 25°C before analysis.

Immunoprecipitations

For immunoprecipitations or coimmunoprecipitations strains expressing Spc7-HA, Spc7-23-GFP, Spc7-C-GFP, Mal2-GFP, GFP-Atb2 or a combination of these tagged proteins were grown at 25°C or 30°C in YE5S or MM over night and then shifted to the restrictive temperature followed by protein extraction and immunoprecipitation as described previously (Kerres *et al.*, 2004). Eluates were boiled and resolved on a SDS-8%-polyacrylamid gel and blotted. Blots were probed with anti-HA antibody (monoclonal mouse; Roche Diagnostics) or anti-GFP antibody (polyclonal rabbit, Invitrogen) followed by the secondary antibody (Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L); Jackson ImmunoResearch Laboratories or Peroxidase-conjugated Donkey Anti-Rabbit IgG; GE Healthcare, respectively). Immobilized antigens were detected using the ECL Advance Western blotting kit (GE Healthcare).

Results

Spc7 is not required for transcriptional silencing of the central centromere region

To better understand the function of Spc7 in mitosis we generated temperature sensitive (ts) *spc7* alleles by mutating the 3´ part of the *spc7*$^+$ ORF (Materials and Methods). The three *spc7* mutant strains, named *spc7-23*, *spc7-24* and *spc7-30*, which showed the tightest ts phenotype were analysed in greater detail (Figure 1A). DNA sequence analysis revealed that each of these strains carried several point mutations in the *spc7*$^+$ ORF (Figure 1B). The mutations leading to ts *spc7* alleles lie in two non-conserved regions at the very 3´end of the *spc7*$^+$ ORF implying that these regions are important for Spc7 protein function (Figure 1B; Nekrasov *et al.*, 2003; Cheeseman *et al.*, 2004; Meraldi *et al.*, 2006). To determine if these Spc7 variants were still present at the kinetochore we analysed the subcellular localization of a Spc7-23-GFP fusion protein by immunofluorescence analysis. The mutant Spc7-23 protein is present at the kinetochore at 25°C but not at temperatures above 32°C probably due to compromised protein levels in the cell (Supplementary Figure 1; see later).

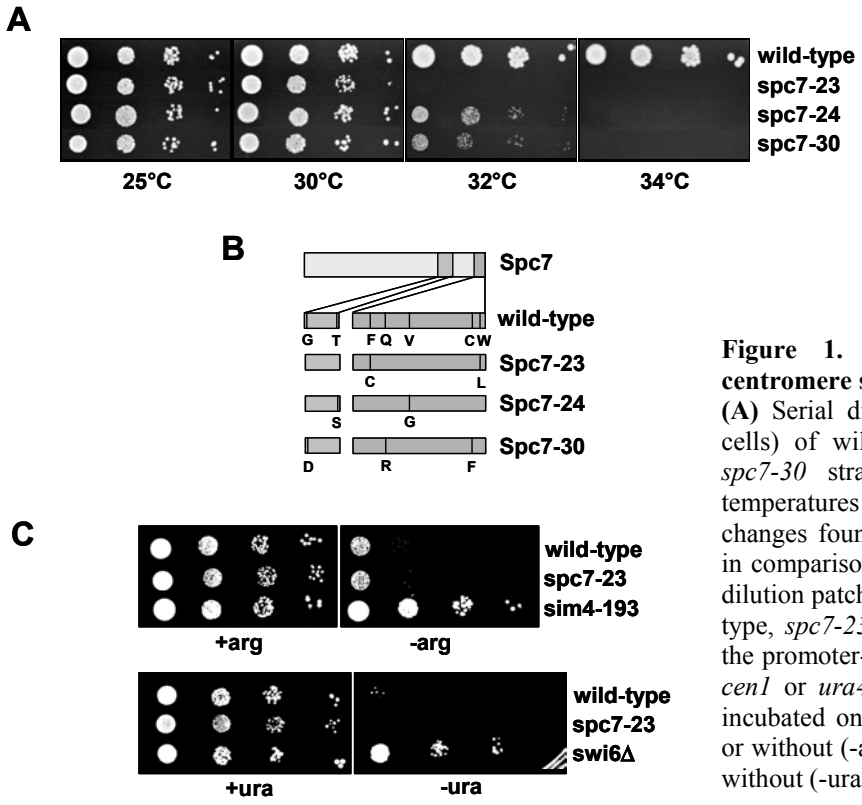

Figure 1. Spc7 is not involved in centromere silencing.
(A) Serial dilution patch tests (10^4 to 10^1 cells) of wild-type, *spc7-23*, *spc7-24* and *spc7-30* strains grown at the indicated temperatures for 3 to 4 days. **(B)** Amino acid changes found in the Spc7 mutant proteins in comparison to wild-type Spc7. **(C)** Serial dilution patch tests (10^4 to 10^1 cells) of wild-type, *spc7-23* and *sim4-193* cells that have the promoter-crippled *arg3*$^+$ gene inserted at *cen1* or *ura4*$^+$ inserted at *otr2*. Cells were incubated on selective medium with (+arg) or without (-arg) arginine or with (+ura) and without (-ura) uracil at 25°C for 6 days.

We have shown previously that Spc7 is a constitutive kinetochore component that is associated with the central core region of the centromere (Kerres *et al.*, 2004). Wild-type strains that carry a marker gene inserted at a centromeric region are auxotroph for this specific

marker due to transcriptional silencing of the centromeric DNA (Pidoux and Allshire, 2000). Defective kinetochore components lead to alleviation of this transcriptional silencing (Allshire *et al.*, 1995; Jin *et al.*, 2002; Pidoux *et al.*, 2003). To analyse if Spc7 was required for transcriptional repression, we tested if marker genes inserted at the *otr2* (otr region of centromere 2) or *cnt1* regions were expressed in the *spc7-23* mutant strain (Partridge *et al.*, 2000; Pidoux *et al.*, 2003).Wild-type strains that contain the *arg3*$^+$ gene inserted at the *cnt1* region show poor growth on arginine minus medium, while kinetochore mutants such as *sim4-193* allow growth on this medium (Figure 1C). Interestingly, the presence of *spc7-23* did not alleviate transcriptional silencing of the central *cnt* region at 25°C (Figure 1C). Raising the incubation temperature to 30°C gave the same result. Furthermore, transcriptional repression of the *otr* regions was unaffected in the *spc7-23* mutant strain (Figure 1C; data not shown). Consistent with this finding, centromere association of the histone H3 variant Cnp1 (CENP-A) was unaffected in *spc7* mutant cells (data not shown). As Spc7 is part of the Ndc80-MIND-Spc7 complex we tested if other members of this complex were required for transcriptional silencing of the central *cnt1* region. We found that in mutant *nuf2-1* (Ndc80 component) and *mis12-537* (MIND component) cells transcriptional silencing at *cnt1* still occurred (data not shown). Thus the constitutive Ndc80-MIND-Spc7 kinetochore complex is not required for transcriptional silencing of the central centromere region.

***spc7* mutants show severe defects in chromosome segregation and spindle attachment**

The mutations in the *spc7*$^+$ ORF lead to aberrant chromosome segregation. At the permissive temperature, the majority of *spc7-24* and *spc7-30* mitotic cells appear to segregate chromosomes equally while expression of the *spc7-23* allele at this temperature leads to a high number of abnormal mitosis (Figure 2A, left panel). Incubation of these strains at the non-permissive temperature gave rise to over 70% of mitotic cells with severe chromosome segregation defects (Figure 2A, right panel). All three mutant *spc7* strains showed the following abnormal chromosome resolution phenotypes: (i) no separation of highly condensed chromatin on an elongating spindle (Figure 2B, b and d), (ii) condensed chromatin that was smeared along the spindle (Figure 2B, a and c) and (iii) unequally or partially separated chromatin (Figure 2B, e and f). Spindle structure was often aberrant (see later). The very high frequency of *spc7* mitotic cells with non-separated chromatin or condensed chromatin smeared along the elongating spindle can be caused by compromised kinetochore-microtubule interactions. We therefore assayed if centromeres were associated with the mitotic spindle by determining co-localization of centromere 1 marked with GFP (*cen1-gfp*)

and the spindle in the *spc7-23* strain (Nabeshima *et al.*, 1998). In cells with an elongating spindle but smeared or non-separated chromatin 46% of the *cen1-GFP* signals were not spindle associated implying that the microtubule-kinetochore interactions were severely affected in these cells (Figure 2C, data not shown; Kerres *et al.*, 2004). Furthermore in 50% of anaphase cells with unequally segregated chromatin the *cen1-GFP* sister centromeres co-segregated indicating that Spc7 is also required for bipolar chromosome orientation. In these cells, we measured the distance between the two GFP signals and found that in 8/15 cells the distance between the signals was significantly greater than 0.6 µm. Our data thus imply that co-segregation of sister chromatids is not simply due to a non-disjunction event (Nabeshima *et al.*, 1998).

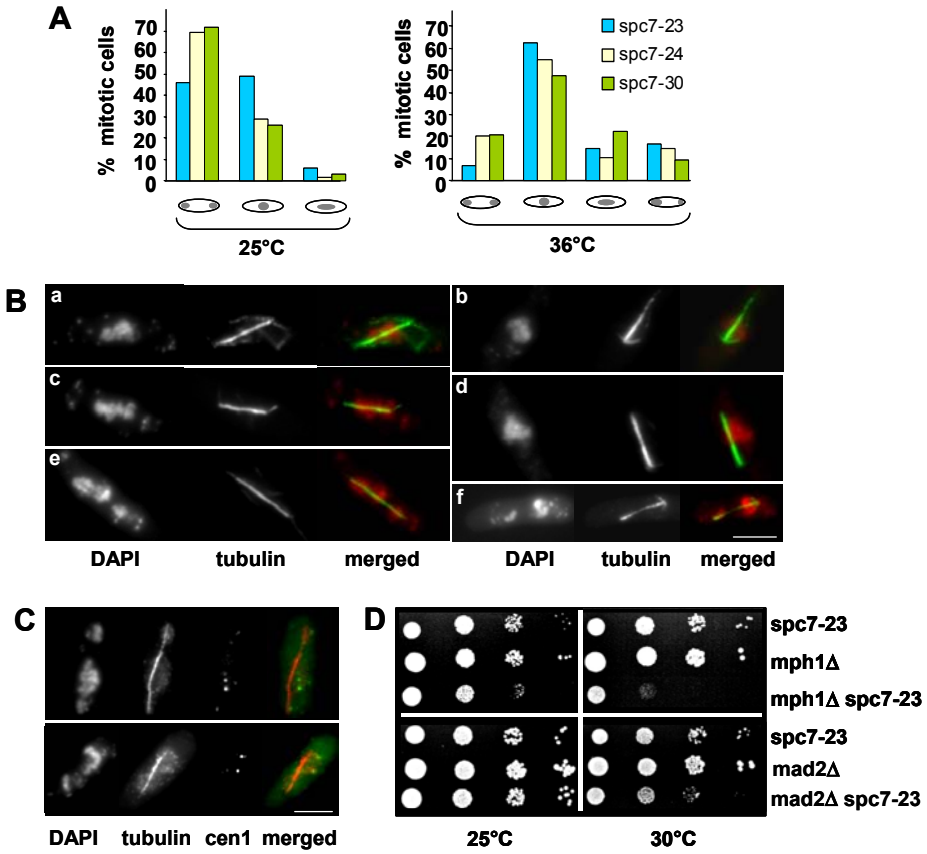

Figure 2. Temperature-sensitive *spc7* mutants have severe mitotic defects.
(A) Chromatin distribution in mitotic *spc7* mutant cells with an elongating spindle incubated at the permissive (25°C) or restrictive temperature (6 hrs at 36°C). N/strain=100. **(B)** Photomicrographs of *spc7-23* cells incubated at 36°C. Fixed cells were stained with DAPI and anti-tubulin antibody. Shown are the 3 main phenotypes observed: smeared chromatin (a, c), non-separated chromatin (b, d) and unequally/partially segregated chromatin (e, f) on an elongating spindle. Bar 5 µm. **(C)** Photomicrographs of *cen1.gfp spc7-23* cells incubated at 36°C for 6 hrs. Fixed cells were stained with anti-GFP antibody, DAPI and anti-tubulin antibody. The merged images show cen1.GFP plus spindle staining. Bar 5 µm. N=28. **(D)** sp*c7-23* interacts genetically with components of the spindle checkpoint pathway. Serial dilution patch tests of *spc7-23*, *mph1Δ*, *mad2Δ* and the respective *spc7-23* double mutants grown at the indicated temperatures for 3 days.

The non-essential 10-subunit DASH complex coordinates bipolar chromosome attachment in *S. pombe* (Liu *et al*., 2005; Sanchez-Perez *et al*., 2005). We therefore attempted to construct double-mutant strains of *spc7-23* with a null allele of *duo1*$^{+}$, which encodes a component of the DASH complex (Materials and Methods). Such double mutants were inviable indicating that *spc7* mutants require the presence of a functional DASH complex for survival at the permissive temperature.

Compromised microtubule-kinetochore interactions should lead to the activation of the spindle assembly checkpoint that detects unattached, monotelic or syntelically attached kinetochores (reviewed in: Musacchio and Hardwick, 2002; Cleveland *et al.*, 2003). Indeed, double mutants of *spc7-23* with null alleles of *mph1Δ* and *mad2Δ*, which code for conserved spindle checkpoint components (He *et al.*, 1997; He *et al.*, 1998) showed reduced growth in comparison to the single mutant strains (Figure 2D).

Spc7 is required for kinetochore targeting of the MIND complex

Spc7 is part of the Ndc80-MIND-Spc7 complex (Obuse *et al.*, 2004; Liu *et al.*, 2005). To analyse the role of Spc7 within this complex, we determined the subcellular localization of the Ndc80 complex components Spc24 and Nuf2 and the MIND complex components Mis14 and Mis12 in the *spc7-23* ts strain (Goshima *et al.*, 1999; Nabetani *et al.*, 2001; Wigge and Kilmartin, 2001; Obuse *et al.*, 2004). Immunofluorescence analysis of the *gfp*-tagged fusion proteins Spc24 and Nuf2 revealed that these proteins were localized correctly in the *spc7-23* ts mutant incubated at the non-permissive temperature (Figure 3A and 3B, data not shown). Furthermore kinetochore association of the Spc7-GFP fusion protein was unaffected in the *nuf2-1* ts strain (Figure 3A), indicating that components of the Ndc80 complex and Spc7 localize to the kinetochore independent of each other. We then analysed kinetochore localization of Mis12-GFP and Mis14-GFP fusion proteins in the *spc7-23* mutant. While Mis12 kinetochore localization was unaffected in a *spc7-23* strain grown at the permissive temperature, kinetochore localization of this proteins was severely reduced or absent in the majority of fixed *spc7-23* cells incubated at the non permissive temperature (Figure 3A and B). However the Mis12 protein was still present in *spc7*^{ts} cells (Supplementary Figure 2). Mis14 kinetochore localization was also affected in a *spc7-23* strain (Figure 3A). Furthermore the Spc7-GFP fusion protein was not localized correctly in the *mis12-527* mutant (Figure 3A) implying that the kinetochore localization of Spc7 and components of the MIND complex are dependent on each other.

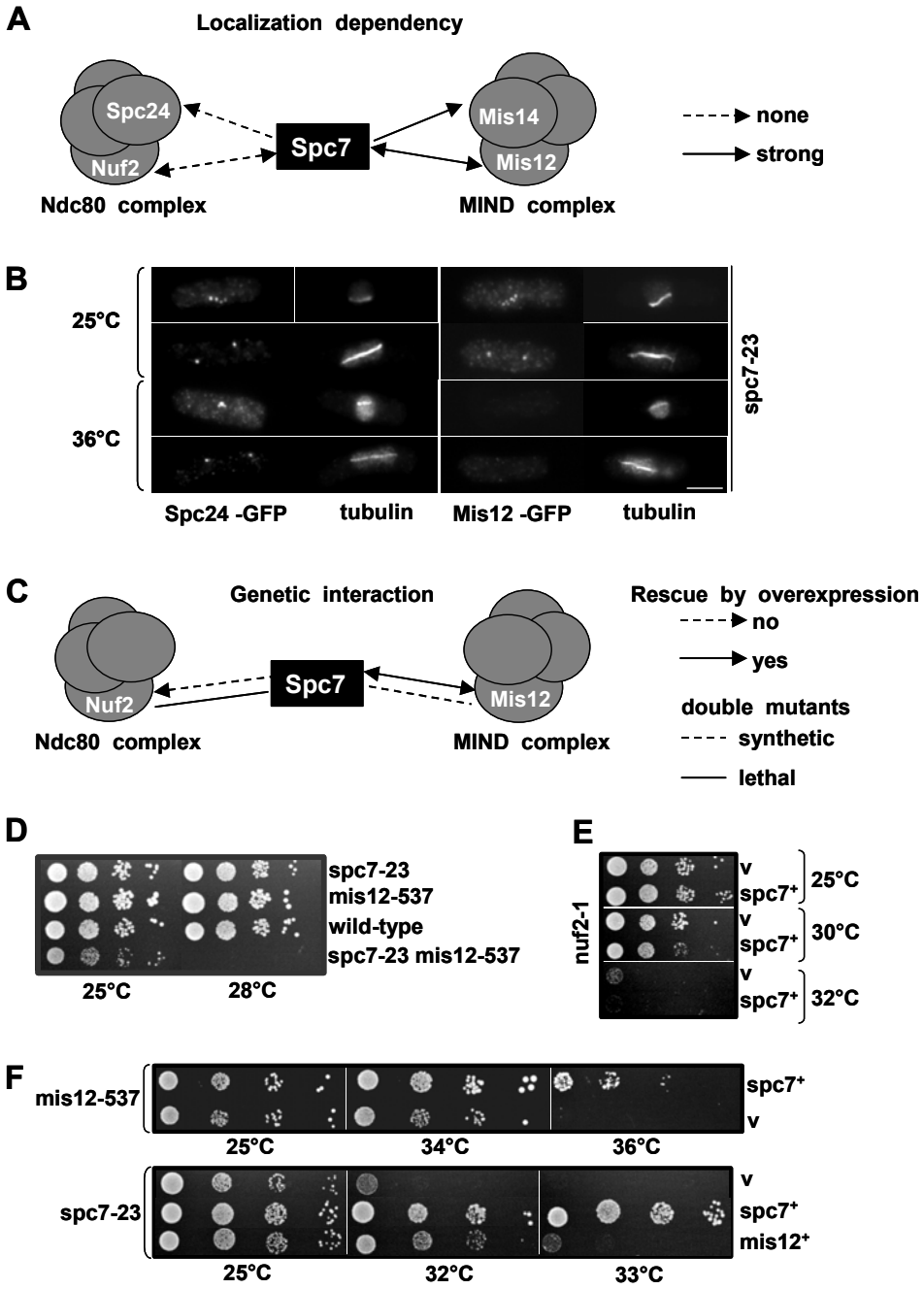

Figure 3. Interaction between *spc7*$^+$ and other components of the Ndc80-MIND-Spc7 kinetochore complex.
(A) Diagrammatic representation of the kinetochore localization of Spc7-GFP in *nuf2-1* and *mis12-537* ts mutants and that of the Spc24-GFP, Nuf2-GFP, Mis12-GFP and Mis14-GFP fusion proteins in the *spc7-23* ts mutant. **(B)** Photomicrographs of *spc7-23* cells expressing Spc24-GFP or Mis12-GFP. The strains were incubated at 25°C or for 8 hours at 36°C, fixed and stained with anti-tubulin antibody and anti-GFP antibody. Bar 5 μm. **(C)** Diagrammatic representation of the genetic interactions between *spc7*$^+$ and *nuf2*$^+$ and *mis12*$^+$. **(D)** Serial dilution patch tests (10^4 to 10^1 cells) of *spc7-23*, *mis12-537* and a *spc7-23 mis12-537* double mutant grown on YE5S for 4 (25°C) or 3 (28°C) days. **(E)** and **(F)** Serial dilution patch tests (10^4 to 10^1 cells) of *nuf2-1* and *mis12-537* transformants grown under selective conditions at the indicated temperatures for 5 to 7 days. v indicates vector control; spc7$^+$ denotes the presence of wild-type *spc7*$^+$ driven by the *nmt41* promoter under derepressed conditions.

Next, we analysed the growth phenotypes of *spc7-23* and *mis12-537* and *nuf2-1* double mutants. *spc7-23 mis12-537* double mutants were viable but showed slightly reduced growth compared to the single mutant strains at temperatures below 28°C. They were inviable at temperatures higher than 28°C (Figure 3C and D). We were unable to construct a *spc7-23 nuf2-1* double mutant by tetrad analysis (Material and Methods; Figure 3C). Microscopic analysis of *spc7-23 nuf2-1* spores showed that such spores germinated and divided twice. We then tested if overexpression of *spc7*$^{+}$ could rescue the ts phenotype of the Ndc80 component *nuf2-1* or the MIND component *mis12-153*. Extra *spc7*$^{+}$ resulted in reduced growth of the *nuf2-1* strain at 30°C and could not suppress the non-growth phenotype of this strain at higher temperatures (Figure 3E). However, overexpression of *spc7*$^{+}$ partially rescued the ts phenotype of the mis*12-537* strain and extra *mis12*$^{+}$ rescued the non-growth phenotype of the *spc7-23* strain at 32°C (Figure 3 F; Obuse *et al.*, 2004).

Spc7 interacts genetically and physically with the Sim4 complex component Mal2

The Sim4 kinetochore complex is a 13-component protein complex that exists independently of the Ndc80-MIND-Spc7 complex (Liu *et al.*, 2005). However, we have shown previously that the kinetochore localization of the Sim4 complex component Fta2 was reduced in a *spc7* mutant strain (Kerres *et al*., 2006). This dependency seems to be specific for Fta2 as other Sim4 complex components such as Mal2-GFP, Mis6-HA, Sim4-GFP and Dad1-GFP were localized correctly in a *spc7-23* mutant background at the restrictive temperature (Figure 4A). Next, we analysed the growth phenotype of double mutants of *spc7-23* with components of the Sim4 complex. All double mutants were viable at 25°C but showed different degrees of growth inhibition at higher temperatures (Figure 4B, Supplementary Figure 3). *spc7-23 mis6-302* double mutants showed the most severe growth reduction, while a slight synthetic effect was observed for *spc7-23 sim4-193* and *spc7-23 mis17-362* double mutant strains. The growth phenotypes of the other double mutants were in between these two extremes (Figure 4B, Supplementary Figure 3). It is not clear at presence if the variability observed for these synthetic effects is of functional significance. However**,** the different growth phenotypes of the tested double mutants is not simply a consequence of different susceptibilities to temperature. For example, at 32°C the *mis6-302* single mutant grows well, the *sim4-193* mutants shows reduced growth while the *mal2-1* single mutant is unable to grow at this temperature.

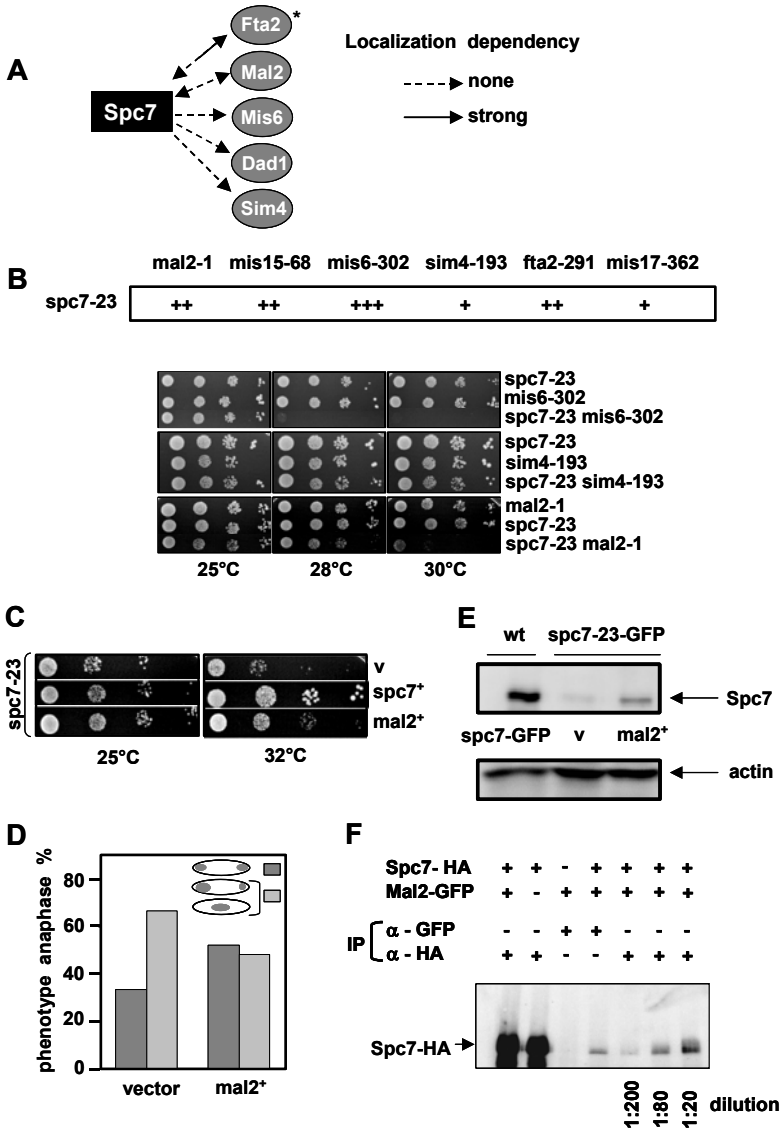

Figure 4. *spc7* interacts with components of the Sim4 complex. **(A)** Diagrammatic representation of the kinetochore localization of Fta2-GFP, Mal2-GFP, Mis6-GFP and Dad1-GFP in the *spc7-23*^{ts} mutant and Spc7-GFP in a *mal2-1* and *fta2-291* mutants incubated at the non-permissive temperature. * Data taken from Kerres *et al.* (2006). **(B)** Representation of the genetic interactions between *spc7-23* and components of the Sim4 complex. + to +++; weak to strong genetic interaction. Serial dilution patch tests (10^4 to 10^1 cells) of *spc7-23*, *mis6-302*, *sim4-193*, *mal2-1* and the respective double mutants grown on YE5S at the indicated temperatures for 3 to 6 days. **(C)** Serial dilution patch test of *spc7-23* transformants grown on selective medium at the indicated temperatures for 3 (32°C) or 4 (25°C) days. Vector control (v) indicates plasmid without insert, *mal2*$^+$ and *spc7*$^+$ denote the presence of wild-type *mal2*$^+$ or *spc7*$^+$ expressed from the wild-type promoter or from the thiamine repressible *nmt41*$^+$ promoter in the absence of thiamine, respectively. **(D)** Diagrammatic representation of anaphases observed in *spc7-23* cells transformed with a vector control or a plasmid overexpressing *mal2*$^+$. Cells were incubated for 6 hrs at 32°C before fixation. N/strain = 50. **(E)** Immunoprecipitations of GFP-tagged Spc7 proteins. Protein extracts prepared from a wild-type (wt) strain expressing Spc7-GFP from a plasmid and a *spc7-23-gfp* strain transformed with a vector control (v) or a plasmid expressing *mal2*$^+$ were analysed by western blot analysis using an anti-GFP antibody. Strains were grown for 6 hours at 32°C. Actin was used as a loading control. **(F)** Spc7-HA and Mal2-GFP coimmunoprecipitate. Protein extracts prepared from strains expressing Spc7-HA, Mal2-GFP or both were used for immunoprecipitation (IP) with an anti-GFP or anti-HA antibody and subjected to western blot analysis using an anti-HA antibody. For lanes1 and 4 one lysate was halved: one half was used for immunoprecipitation with the anti-HA antibody and the other with the anti GFP-antibody. Lanes 5-7 are dilutions of the immunoprecipitate shown in lane 1.

Interestingly, in a first screen for multicopy suppressors of the *spc7* ts phenotype we identified the *mal2*$^+$ ORF. When present on a plasmid *mal2*$^+$ expressed from its wild-type promoter can partially rescue the non-growth phenotype of *spc7* mutant strains (Figure 4C, data not shown) by reducing the number of aberrant mitosis (Figure 4D). The converse, i.e. the rescue of the *mal2-1* ts mutant phenotype by extra *spc7*$^+$ was not observed (data not shown). Overexpression of other components of the Sim4 complex, such as the Mal2-interaction partner Fta2 or the Sim4 protein cannot rescue the ts phenotype of the *spc7-23*

strain at the non-permissive temperature. To understand how extra *mal2*$^{+}$ rescued the *spc7-23* mutant at 32°C we tested if extra *mal2*$^{+}$ affected Spc7-23 protein levels in the cell. In order to detect Spc7 by western blot analysis, the protein needs to be enriched by immunoprecipitation. We therefore immunoprecipitated endogenously expressed mutant Spc7-23-GFP from strains transformed with a vector control or a plasmid expressing extra *mal2*$^{+}$. Overexpression of *mal2*$^{+}$ appears to increase the amount of Spc7-23-GFP in the cell thus suggesting that Mal2 is able to stabilize the mutant Spc7 protein (Figure 4E). We therefore determined if Mal2 and Spc7 could interact physically. Co-immunoprecipitations with anti-GFP or anti-HA antibodies were carried out with protein extracts from strains expressing endogenous Mal2-GFP and/or Spc7-HA. These immunoprecipitations were then analysed by western blotting using anti-HA antibodies to detect Spc7-HA**.** As shown in Figure 4F immunoprecipitations of Mal2-GFP clearly co-immunoprecipitates a fraction of the Spc7-HA protein present in extracts from logarithmically growing cells implying that Spc7 and Mal2 interact.

***spc7* mutants exhibits defects in spindle formation and function**

Immunofluorescence analysis of fixed *spc7*^{ts} mitotic cells revealed that all three *spc7*^{ts} mutants gave rise to abnormal spindle structures. For example, in the *spc7-23* mutant strain grown at the restrictive temperature 47% of all spindles analysed were aberrant. Immunofluorescence staining of *spc7-23* spindles showed the following phenotypes: (i) elongating spindles with very thinly staining midzones (Figure 5A panel a and b, Figure 5B), (ii) disintegration of the spindle evidenced by spindle fraying and/or two separated half-spindles present in one cell (Figure 5A panel c and d), (iii) elongating anaphase spindles that were bent (Figure 5A panel e) and (iv) unequally stained spindles (Figure 5A panel f). The latter phenotype comprised approximately equal proportions of monopolar and bipolar spindles as determined by the subcellular localization of the SPB component Cut12 (Bridge *et al.*, 1998; data not shown). The other *spc7*^{ts} mutants showed similar spindle abnormalities (Figure 5D). We next wanted to analyse these phenotypes in live cells with fluorescence microscopy and thus generated *spc7*^{ts} mutant strains that harboured an integrated version of the α-tubulin *atb2*$^{+}$ ORF tagged with *GFP* and driven by the *nmt81* promoter (GFP.nmt81.atb2) (Garcia *et al.*, 2001). However, presence of the extra α-tubulin partially rescued the non-growth phenotypes of *spc7* mutant strains (Figure 5C, data not shown) and reduced the number of aberrant mitosis and abnormal spindle structures (Figure 5D). We thus tested if these two proteins could interact

physically by performing co-immunoprecipitations in exponentially growing strains expressing Spc7-HA and GFP-Atb2 but failed to find an interaction (Figure 5E).

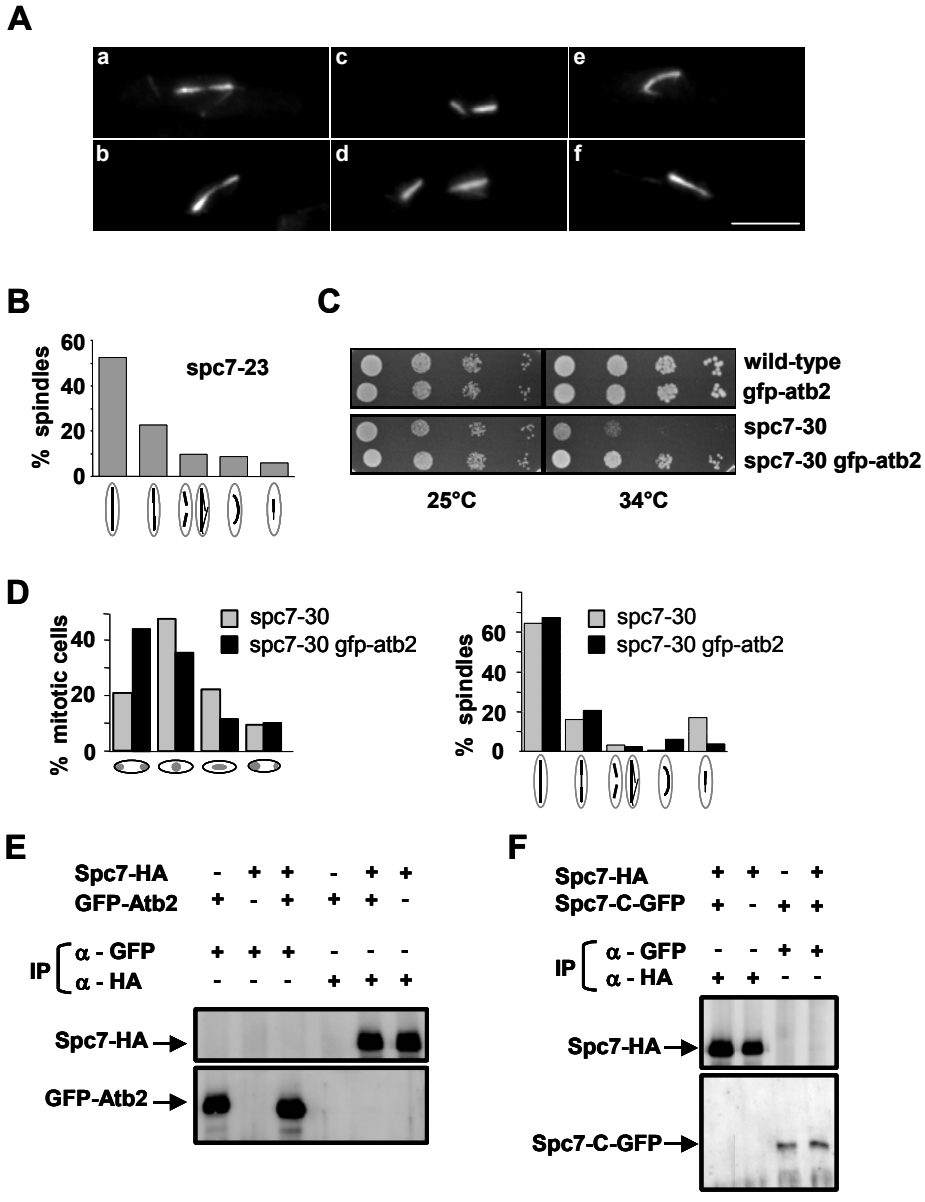

Figure 5. Spc7 is required for the integrity of the spindle. **(A)**Photomicrographs of spindle defects observed in *spc7-23* cells incubated at 36°C for 6 hours: thin spindle midzones (a and b), two half-spindles per cell (c and d), bent spindle (e) and unequally stained spindle (f). Fixed cells were stained with anti-tubulin antibody. **(B)** Diagrammatic representation of the spindle phenotypes observed in *spc7-23* mitotic cells. From left to right: wild-type anaphase spindle, ana-phase spindles with thinly staining mid-zone, disintegrating/ broken anaphase spindles, bent spindles and unequally stained spindles. N= 101. **(C)** Serial dilution patch tests (10^4 to 10^1 cells) of wild-type and *spc7-30* cells carrying the integrated *nmt81-GFP-atb2*. Strains were grown at the indicated temperatures for 5 days under derepressed conditions. **(D)** Chromosome segregation and spindle phenotypes observed in *spc7-30* and *spc7-30 nmt81-GFP-atb2* (spc7-30 gfp-atb2) strains grown at 34°C. N/strain= 100. **(E)** Immunoprecipitations of Spc7-HA and GFP-Atb2 proteins. Protein extracts prepared from strains expressing Spc7-HA, GFP-Atb2 or both were used for immunoprecipitations (IP) with an anti-GFP or anti-HA antibody, followed by western blot analysis using anti-GFP and anti-HA antibodies. **(F)** Immunoprecipitations of Spc7-HA and Spc7-c-GFP proteins. Protein extracts prepared from a wild-type and a Spc7-HA strain expressing plasmid borne Spc7-C-GFP under the control of the $nmt41^+$ promoter were used for immunoprecipitations using anti-GFP or anti-HA antibodies. The immunoprecipitates were halved and analysed by western blot analysis using anti-GFP and anti-HA antibodies.

As aberrant spindle phenotypes in the *spc7^{ts} GFP.nmt81.atb2* mutants were infrequent we analysed spindle structure in wild-type *GFP.nmt81.atb2* cells that overexpressed the dominant negative *spc7-c* variant (Kerres *et al.*, 2004). Overproduction of this C-terminal part of the *spc7^{+}* ORF affects chromosome segregation and leads to similar types of spindle defects as those observed for the *spc7^{ts}* mutants (see later; Kerres *et al.*, 2004). Spc7-C is able to associate with the kinetochore (Figure 7A) and does not appear to interact with wild-type Spc7 as the two proteins cannot be co-immunoprecipitated (Figure 5F). The kinetochore appears to be assembled in *spc7-c* overexpressing cells, as the Mal2 and Spc24 kinetochore proteins are correctly localized (Kerres *et al.*, 2004). Examination of 30 spindles in wild-type *GFP.nmt81.atb2* cells that overexpressed *spc7-c* revealed that 53% of these spindles were abnormal. Defects were found at all stages of spindle formation. In 3 cells formation of a bipolar spindle was defective as microtubules emanated from a single focus or microtubules coming from separated spindle pole bodies were unable to form a stable bipolar spindle (Figure 6B). 7/30 spindles showed a prolonged delay at the metaphase/anaphase A to anaphase B transition probably due to an activated spindle control checkpoint (Figure 6G, spindle marked with an asterisk). This phenotype was observed for only 2/14 wild-type *GFP.nmt81.atb2* cells transformed with the vector control. 3 cells were unable to switch to the phase III spindle stage i.e. spindle elongation in anaphase B in the time frame measured (Figure 6C, 6G; Nabeshima *et al.*, 1998). If spindle phase III was abolished in these cells or just severely delayed is yet unclear. Intriguingly, 2 spindles were assembled and started to elongate with apparently wild-type dynamics and then collapsed in mid-anaphase B. One of these spindles showed rejoining of the two spindle halves and further spindle elongation (Figure 6D). After anaphase the post-anaphase-array (PAA), which is nucleated by the eMTOC, forms at the cell equator (reviewed in: Hagan and Petersen, 2000). In wild-type *GFP.nmt81.atb2* cells transformed with a vector control this structure was observed in 12 /12 cells analysed (Figure 6A). However in 8/11 *GFP.nmt81.atb2* cells overproducing the Spc7 variant the PAA was not observed in the time frame measured although the spindle had elongated fully followed by spindle breakdown (Figure 6E). Intriguingly, extra *spc7-c* also influenced the interphase microtubule cytoskeleton. In wild-type cells, interphase microtubules grow out from the nucleus, continue growth until they reach the cell tip and then depolymerise (Drummond and Cross, 2000). In 20% of *spc7-c* over expressing cells or *spc7^{ts}* mutants the interphase microtubules continued to grow when they reached the cell tip and thus curled around the cell tip (Figure 6F, data not shown).

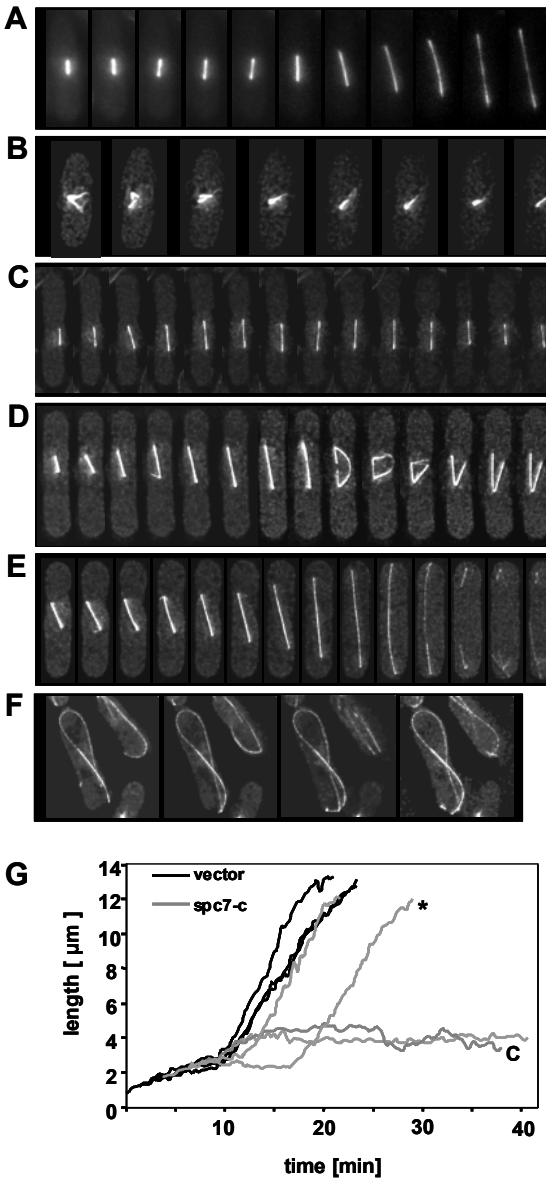

Figure 6. Mitosis in living *spc7-c* expressing cells.
Time-lapse images of mitosis in wild-type *nmt81-GFP-atb2* cells transformed with a vector control (A) or overexpressing *spc7-c* (B-F) from the *nmt1*$^+$ promoter for 36-48 hours at 25°C. Time interval between images: 100 seconds (A to E) or 150 seconds (F). **(A)** Normal spindle elongation. The appearance of the PAA is indicated by an arrow. **(B)** Inability to form a bipolar spindle. The separated spindle pole bodies (second panel) collapse into a single fluorescent signal. Bar 1.5 µm. **(C)** Failure of bipolar spindle elongation. In the time frame measured the spindle shows cycles of spindle elongation (up to 4.7 µm) followed by shrinkage. This spindle is shown diagrammatically in 6G (marked with a C). **(D)** Elongating spindle that collapses in mid-anaphase B, followed by fusion of the elongating spindle fragments and further elongation of the bipolar spindle. **(E)** Normal elongation of the spindle and delay in PAA appearance. The cell shown did not have a PAA in the time measured as shown for the wild-type mitosis in A. **(F)** Aberrant interphase microtubule cytoskeleton in *spc7-c* overexpressing cells. Cells showed fewer microtubule bundles, which often curved around the cell. **(G)** Quantitation of spindle length for *nmt81-GFP-atb2* cells transformed with a vector control (black graphs, 3 spindles) or overexpressing *spc7-c* (grey graphs, 4 spindles). The graph marked with C represents the spindle shown in 6C. The graph marked with an asterisk indicates a delay in spindle elongation possibly caused by an active spindle checkpoint.

Thus, the Spc7 kinetochore protein is required for the integrity of the mitotic spindle and also seems to influence the interphase microtubule cytoskeleton. We therefore determined if Spc7 was able to associate with the microtubule cytoskeleton. Wild-type Spc7 is associated with the kinetochore and we were unable to detect co-localization with the mitotic spindle even in an over expression situation (Kerres *et al.*, 2004; data not shown). The same holds true for the Spc7-C variant. Over expression of a Spc7-C-GFP fusion protein from a plasmid gave rise to a kinetochore specific fluorescence signal (Figure 7A). We then looked at the intracellular localization of a Spc7-N variant (Figure 7A). In contrast to Spc7-C, overexpression of the N-terminal part of the Spc7 protein in a wild-type strain does not lead to growth defects and only results in a very moderate increased sensitivity to the microtubule poison thiabendazole (data

not shown). Spc7-N-GFP expressing cells showed GFP fluorescence present in the entire nucleus possibly because the C-terminal nuclear export sequence is no longer present (Matsuyama *et al.*, 2006). In addition to staining of the nucleus, Spc7-N showed co-localization with the mitotic spindle during mitosis (Figure 7A). Thus Spc7 appears to have the potential to co-localize with the mitotic spindle. This localization is independent of the presence of the microtubule-plus-end associated protein Mal3 (data not shown).

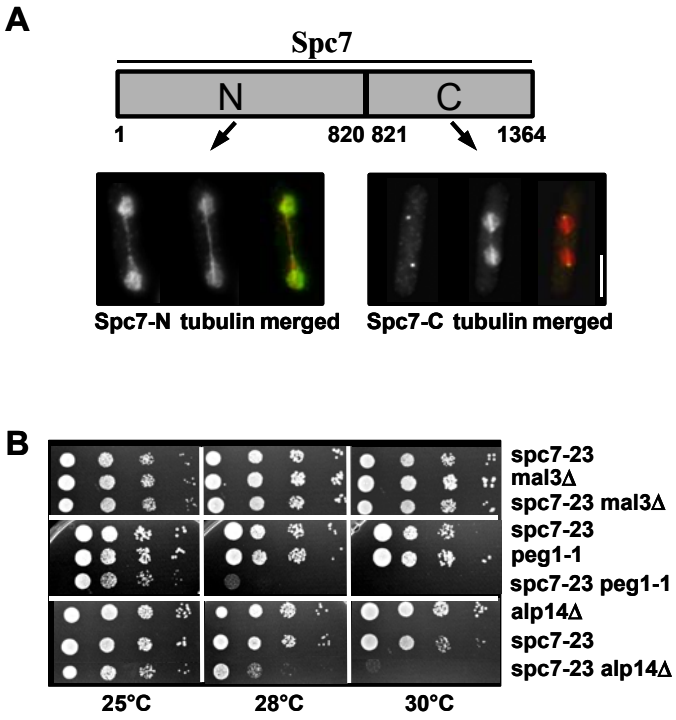

Figure 7. Interaction of *spc7* with components of the mitotic spindle.
(A) Subcellular localization of the Spc7 variants fused to GFP and expressed on a plasmid under the control of the *nmt1*$^+$ promoter. Cells were grown under promoter derepressing conditions for 24 hours at 30°C, fixed and stained with anti-tubulin and anti-GFP antibodies. Bar 5 µm. **(B)** Serial dilution patch tests (10^4 to 10^1 cells) of *spc7-23*, *mal3Δ*, *peg1-1*, *alp14Δ* and the respective double mutants grown on YE5S at the indicated temperatures for 3 to 6 days.

Finally, we looked at the genetic interaction between *spc7*$^+$ and genes coding for microtubule-plus-end associated proteins. *spc7*$^+$ was isolated originally as a suppressor of a *mal3* mutant. This suppression appears to be specific for *mal3* as extra *spc7*$^+$ can not rescue the mutant phenotypes of *dis1-288*, *dis1Δ*, *alp14Δ* or *peg1-1* mutant strains (data not shown; Kerres *et al.*, 2004). Dis1 and Alp14/Mtc1 are members of the TOG/XMAP215 family while Peg1 is the fission yeast member of the CLASP family (Ohkura *et al.*, 1988; Nabeshima *et al.*, 1995; Garcia *et al.*, 2001; Nakaseko *et al.*, 2001; Grallert *et al.*, 2006). Double mutant strains between *spc7* and *dis1-288*, *dis1Δ*, *alp14Δ*, *peg1-1*and *mal3Δ* showed that the absence of

mal3^{+} in a *spc7-23* mutant does not lead to an increased phenotype of that strain implying that the two proteins act in the same pathway (Figure 7B). *spc7-23 alp14Δ* and *spc7-23 peg1-1* double mutants showed no enhanced phenotype at 25°C but were unable to grow at 30°C (Figure 7B). Tetrad analysis at incubation temperatures of 25°C, 28°C or 30°C revealed that *dis1-288 spc7-23* and *dis1Δ spc7-23* double mutants could not be generated. Altogether 52 tetrads with 4 germinating spores were analysed without recovering *dis1spc7-23* double mutants that were able to form a colony. These findings show that *spc7* mutants require the presence of the *dis1*$^{+}$ wild-type gene product for survival at the permissive temperature.

Discussion

During mitosis, the correct interaction of the kinetochore with spindle microtubules is essential for the precise segregation of the duplicated sister chromatids. The conserved Spc7 kinetochore protein is likely to play a direct role in this process, as we have previously shown that it interacts genetically and physically with the EB1 family member Mal3 and is required for spindle-kinetochore association (Kerres *et al.*, 2004). Here, we show that Spc7 is required for proper spindle structure and function. In addition, Spc7 links the Sim4 and Ndc80-MIND-Spc7 kinetochore complexes and is required for kinetochore targeting of the MIND components Mis12 and Mis14 (Figure 3A). Extra Spc7 can partially rescue the temperature sensitivity of the *mis12* and *mis14* mutant strains (Figure 3C and 3F; Obuse *et al.*, 2004). Furthermore, overexpression of *mis12*$^{+}$ can partially suppress the non-growth phenotype of a *spc7*^{ts} mutant (Figure 3F). However, kinetochore targeting of Ndc80 components is unaffected in a *spc7* mutant strain and vice versa (Figure 3B). Overall, these experiments suggest that within the Ndc80-MIND-Spc7 complex Spc7 and the MIND complex interact more closely with each other than Spc7 with the Ndc80 complex. The findings that Spc7 and MIND require each other for kinetochore targeting, while the Ndc80 complex associates independently of Spc7 are in contrast to those observed for KNL-1, the *C. elegans* homologue of Spc7. In that organism the Ndc80 complex components Ndc80 and Spc25^{KBP-3} require KNL-1 for kinetochore association while kinetochore targeting of the MIND complex component Mis12 is only slightly affected in KNL-1 depleted cells (Desai *et al.*, 2003; Cheeseman *et al.*, 2004). Kinetochore targeting of other Spc7 homologues remains to be determined: however independent kinetochore association of MIND and Ndc80 complexes has been shown for a number of organisms (De Wulf *et al.*, 2003; Emanuele *et al.*, 2005; Saitoh *et al.*, 2005).

We also investigated if Spc7 played a role in kinetochore targeting of Sim4 complex components. We found that in *spc7* mutant cells kinetochore localization of the Sim4 complex component Fta2 is significantly reduced while other tested components of this complex do not appear to be affected (Kerres *et al*., 2006; Figure 4A). In particular, kinetochore targeting of Mal2, a very close interaction partner of Fta2, appears unaffected in *spc7* mutants. As kinetochore localization of Fta2 and Mal2 are dependent on each other, our data imply that the severely reduced amount of Fta2 at the kinetochore in *spc7* mutants is sufficient for proper localization of Mal2 in these cells. Interestingly, extra *mal2*$^{+}$ suppressed the *spc7* ts phenotype and Spc7 and Mal2 can interact physically (Figure 4C and 4F). Only a small amount of Spc7 present in the cell extract was co-immunoprecipitated with Mal2, an observation that can be partially explained by the recent finding that the copy number of the *S. cerevisiae* Spc7 ortholog Spc105p at one kinetochore-microtubule attachment site is higher than that of the COMA complex member Ctf19p (Joglekar *et al.*, 2006). In addition, it is possible that the interaction between Spc7 and Mal2 is unstable, limited to a specific cell cycle stage and/or occurs only under certain conditions. Interestingly, the interaction between Spc7 and Mal2 family members appears to be conserved. Affinity purification of proteins interacting with the *S. cerevisiae* Mal2 homologue Mcm21p identified the Spc7 homologue Spc105p (De Wulf *et al.*, 2003). Furthermore, affinity purifications of human CENP-O (Mal2 ortholog) co-purified a small amount of the AF15q14 protein (Spc7 ortholog) (Okada *et al.*, 2006).

The finding that extra *mal2*$^{+}$ partially rescues the *spc7* mutant ts phenotypes implies that the two proteins share some function(s). In accordance with this view, we have found that the genetic interactions between *spc7*$^{+}$ and *mal2*$^{+}$ with microtubule-associated proteins are similar. Cells expressing mutant versions of either protein can only survive in the presence of wild-type *dis1*$^{+}$, show reduced growth in the absence of the other XMAP215 homologue Alp14 and show no increased phenotype in the absence of *mal3*$^{+}$ (Jin *et al.*, 2002; Figure 7B). However, although both proteins are essential for chromosome segregation, the phenotype of a *spc7* mutant is much more severe than that of a *mal2* mutant (Jin *et al.*, 2002; Kerres *et al.*, 2004). In particular, *mal2-1* mitotic cells segregate their chromatin albeit aberrantly, while over 50% of *spc7* mutant mitotic cells show no separation of the condensed chromatin. In addition *mal2-1* mutants have an apparently normal spindle structure, while spindle structure and function is severely affected in *spc7* mutants.

In fission yeast three distinct spindle phases have been defined (Nabeshima *et al.*, 1998). Phase I involves formation of the bipolar spindle in prophase to prometaphase. In phase II, which encompasses metaphase chromosome alignment to the end of anaphase A, the spindle

has a constant length while spindle elongation occurs in the third phase (anaphase B) by sliding apart of antiparallel microtubules in the spindle midzone. Entry into phase III is accompanied by a change in microtubule dynamics leading to more stable microtubules and spindle elongation (Ding *et al.*, 1993; Nabeshima *et al.*, 1998; Mallavarapu *et al.*, 1999; Sagolla *et al.*, 2003). Once the nuclei have been separated towards the cell ends, the spindle breaks down and the PAA appears in the cell middle (reviewed in: Hagan, 1998; Figure 6A). *spc7* mutant cells showed defects in all three spindle phases. We observed monopolar spindles or small aberrant bipolar spindles that collapsed into a single focal point indicating defects in spindle phase I. Such staining patterns have been observed in a wide variety of mutants among them mutants with defects in mitotic motor proteins, spindle pole body components or mitotic regulators such the Ran GTPase and the Aurora-related kinase Ark1 (Hagan and Yanagida, 1990 ,1995; Bridge *et al.*, 1998; West *et al.*, 1998; Fleig *et al.*, 2000; Petersen *et al.*, 2001; Leverson *et al.*, 2002). We were unable to assess defects in spindle phase II directly by microscopy. However the finding that cells overexpressing *spc7-c* showed a prolonged delay at the transition to phase III suggests that this spindle stage is also affected in *spc7* mutants. Spindle stage III involves the rapid elongation of the spindle from 2 to 3 μm to 10-14 μm. Elongation in cells expressing *spc7-c* was discontinous as envisaged by the cycles of spindle elongation and spindle shortening (Figure 6C and 6G) suggesting that the switch in microtubule dynamics that occurs at the onset of spindle phase III was defective in these cells (Mallavarapu *et al.*, 1999). Similar phenotypes have been observed in *S. cerevisae* cells expressing mutant version of the Cdc14p phosphatase or the Ndc10p kinetochore protein (Bouck and Bloom, 2005; Higuchi and Uhlmann, 2005). Cdc14p is required for changing microtubule dynamics at the onset of anaphase and targets the Ndc10p protein, which is needed for spindle stability to the plus-ends of interpolar microtubules at the spindle midzone during anaphase (Goh and Kilmartin, 1993; Bouck and Bloom, 2005; Higuchi and Uhlmann, 2005).

spc7 mutant cells that could execute spindle phase III had a high proportion of anaphase B spindles with abnormal spindle midzones. The spindle midzone, which consists of overlapping antiparallel microtubules (Ding *et al.*, 1993) stained very thinly in living and fixed *spc7* mutants (Figure 5A). Reduced tubulin staining of the spindle midzone has also been observed for a number of *S. cerevisiae* kinetochore mutants among them components of the Ndc80 complex (Wigge and Kilmartin, 2001; Le Masson *et al.*, 2002; McCleland *et al.*, 2003). Consistent with a abnormal spindle midzone, we observed fixed *spc7* mutant cells with two spindle halves and elongating mid-anaphase B spindles that abruptly collapsed in the

middle region in living cells (Figure 5A and 6D). In one case the two spindle halves were able to rejoin and continue spindle elongation. The latter phenotype has also been observed when the middle of medium-length spindles is cut by laser microsurgery or in mutants required for central spindle formation (Mitchison and Salmon, 2001; Khodjakov *et al.*, 2004; Tolic-Norrelykke *et al.*, 2004). Our data thus demonstrate that Spc7 is required for the integrity of the spindle midzone possibly by influencing the dynamics of the microtubule-plus ends. However we can at present not exclude that Spc7 regulates spindle function by some other means such as influencing microtubule bundling as similar phase III phenotypes have been observed in mutants with an *ase1*$^{+}$ null allele (Loiodice *et al.*, 2005; Yamashita *et al.*, 2005). Fission yeast Ase1, which localizes to the spindle midzone in anaphase B, belongs to the conserved Prc1/MAP65 family of microtubule bundling proteins, that is required for central spindle formation and cytokinesis (Schuyler *et al.*, 2003; Verni *et al.*, 2004; Loiodice *et al.*, 2005; Yamashita *et al.*, 2005).

Taken together our results indicate that Spc7 plays a profound role in the formation and function of the spindle. How does Spc7 exert its influence on spindle integrity? Our immunofluorescence analysis of an endogenously expressed wild-type Spc7 fusion protein shows an exclusive kinetochore localization. However, it is possible that Spc7 also associates with the mitotic spindle but we fail to detect it either because the signal is below the threshold sensitivity of our imaging system or due to a highly transient association of the protein with the spindle. In this respect, the co-localization of the Spc7 variant, Spc7-N, with the mitotic spindle might argue that Spc7 has the potential to associate with spindle microtubules and that this association is regulated by the C-terminal part of the Spc7 protein. Interestingly, a component of the Ndc80 kinetochore complex in budding yeast, namely Ndc80p, was shown to be associated with spindle microtubules using immunoelectron microscopy (Muller-Reichert *et al.*, 2003). An alternative, but not mutually exclusive possibility is that Spc7 could exert its influence on spindle microtubules by regulating proteins that localize to kinetochore and spindle. In *S. cerevisiae* the Cdc14p phosphatase is required for spindle localization of a number of proteins that affect spindle function among them the chromosomal passenger proteins aurora kinase Ipl1p and INCENP Sli15p as well as the kinetochore proteins Slk19p and Ndc10p (Pereira and Schiebel, 2003; Bouck and Bloom, 2005). We therefore looked at the localization of the *S. pombe* Cdc14p homologue Flp1/Clp1 (Cueille *et al.*, 2001; Trautmann *et al.*, 2001) in *spc7* mutants and found a wild-type like localization pattern (data not shown). Furthermore Spc7-N showed co-localization with the mitotic spindle in a *flp1Δ* strain (data not shown). We also found that spindle association of the chromosomal passenger

protein Ark1 and the DASH component Dam1 is still possible in the *spc7-23* mutant at the non-permissive temperature (data not shown; Petersen *et al.*, 2001; Leverson *et al.*, 2002; Liu *et al.*, 2005; Sanchez-Perez *et al.*, 2005). However in 40% of *spc7*^{ts} cells with a late anaphase spindle and separated chromatin, Ark1 staining was not confined to the spindle midzone. Instead Ark1.PkC was distributed as a broad signal over most of the spindle (data not shown). If this abnormal Ark1 localization is the cause or a consequence of the aberrant spindle midzone in *spc7*^{ts} cells is at present unclear.

After anaphase, the PAA, nucleated by the eMTOC microtubule organizing structure, is seen by the time the spindle reaches its maximum length (Hagan and Petersen, 2000; Figure 6A). In the majority of *spc7* mutant cells that went through anaphase B this structure was not observed, although breakdown of the spindle occurred (Figure 6E). Thus Spc7 function is needed for PAA formation. Failure to form a PAA has also been observed in mutants that affect γ–tubulin complex function (Sawin *et al.*, 2004; Venkatram *et al.*, 2004; Samejima *et al.*, 2005). Interestingly, components of the γ-tubulin complex, such as Alp4 and Alp6 are also required for a proper interphase microtubule cytoskeleton (Vardy and Toda, 2000). *alp4* mutants have longer interphase microtubules that curve around the cell end: a phenotype very similar to what we have observed for *spc7* mutants (Figure 6F). Intriguingly, a highly overexpressed Spc7-YFP fusion protein localizes to the kinetochore and as a single dot to the periphery at the site of septum formation in the middle of the cell (Matsuyama *et al.*, 2006): thus raising the possibility that Spc7 and the eMTOC co-localize.

Acknowledgements

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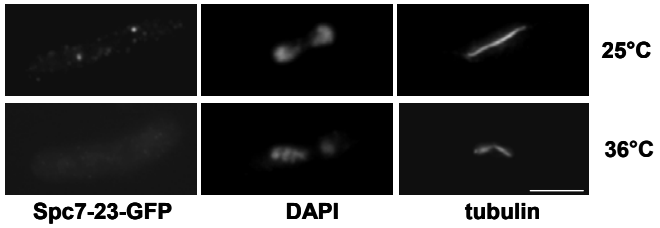
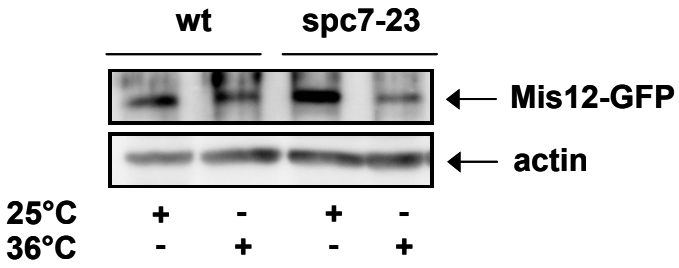
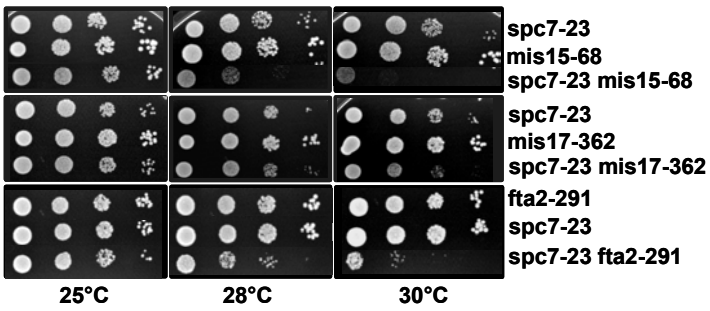
Supplementary Figures



Supplementary Figure 1. Kinetochore localization of Spc7-23.
Cells expressing the mutant Spc7-23-GFP protein were incubated at 25°C or 36°C for 6 hours, fixed, and stained with DAPI, anti-tubulin-antibody and anti-GFP-antibody. Bar 5µm.



Supplementary Figure 2. Mis12-GFP is present in *spc7-23* cells.
Protein extracts prepared from wild-type (wt) or *spc7-23* strains which expressed Mis12-GFP endogenously were used for immunoprecipitations using an anti-GFP-antibody, followed by western blotting using the same antibody. Protein extracts used had a similar protein concentration. Actin was used as a loading control. The strains were incubated at 25°C or 36°C for 6 hours before protein extraction.



Supplementary Figure 3. *spc7-23* interacts genetically with Sim4-complex components.
Serial dilution patch tests of *spc7-23, mis15-68, mis17-362, fta2-291* and the respective s*pc7-23* double mutants grown at the indicated temperatures for 3 to 5 days.

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Fta2, an essential fission yeast kinetochore component, interacts closely with the conserved Mal2 protein

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The fission yeast multiprotein-component Sim4 complex plays a fundamental role in the assembly of a functional kinetochore. It affects centromere association of the histone H3 variant CENP-A as well as kinetochore association of the DASH complex. Here, multicopy suppressor analysis of a mutant version of the Sim4 complex component Mal2, identified the essential Fta2 kinetochore protein, which is required for bipolar chromosome attachment. Kinetochore localisation of Mal2 and Fta2 depend on each other and over expression of one protein can rescue the phenotype of the mutant version of the other protein. *fta2 mal2* double mutants were inviable implying that the two proteins have an overlapping function. This close interaction with Fta2 is not shared by other Sim4 complex components, indicating the existence of functional subgroups within this complex. The Sim4 complex appears to be assembled in a hierarchical way, as Fta2 is localised correctly in a *sim4* mutant. However, Fta2 kinetochore localisation is reduced in a *spc7* mutant. Spc7, a suppressor of the EB1 family member Mal3, is part of the conserved Ndc80-MIND-Spc7 kinetochore complex.

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Introduction

The segregation of the duplicated sister chromatids into two equal sets is achieved by the interaction between spindle microtubules and chromosomes. Attachment of the mitotic spindle fibres occurs at the kinetochore, a multicomponent organelle assembled on centromeric DNA. Kinetochores perform various functions during mitosis: they mediate attachment of the sister chromatids with the plus-ends of spindle microtubules and maintain microtubule attachment during dynamic microtubule behaviour thus generating the physical forces required for chromosome movement. In addition, this complex is needed for spindle checkpoint signalling that regulates anaphase onset. These functions can be carried out by essentially all types of kinetochores, although the centromeric DNA requirements and the composition of the various protein kinetochore complexes can vary greatly between different organisms (Cleveland *et al.*, 2003; Pidoux and Allshire, 2000).

The simplest kinetochore appears to be that of the budding yeast *Saccharomyces cerevisiae* which consists of 125 bp centromeric DNA and more than 60 kinetochore proteins organized into discrete complexes (De Wulf *et al.*, 2003; McAinsh *et al.*, 2003; Westermann *et al.*, 2003). Budding yeast kinetochores exist during most of the cell cycle and the proteins of this organelle are organized into multiple functional subcomplexes, that are assembled hierachically. The outer part of the budding yeast kinetochore associates with a single spindle microtubule (Winey *et al.*, 1995).

Kinetochores from higher eucaryotes, on the other hand, can encompass megabases of highly repetitive DNA sequences, are predicted to contain more than a 100 proteins and are assembled from S-phase to early mitosis (Fukagawa, 2004; Maiato *et al.*, 2004). The association of approximately 20 microtubule plus-ends to the outer plate of a vertebrate kinetochore requires correct orientation of the microtubule attachment sites to one pole to avoid merotelic attachments.

The kinetochores of the fission yeast *Schizosaccharomyces pombe* lie in between these two extremes. The centromeric DNA of *S. pombe* is 35 to 100 kb in size and is composed of a central core region that is flanked by inner and outer repetitive sequences. Marker genes that are placed within the centromeric DNA are transcriptionally silenced (Allshire *et al.,*1994; Allshire *et al.*,1995). *S. pombe* kinetochore proteins identified to date either associate with the central domain or with the heterochromatic outer repeats, thus enforcing the existence of two distinct domains in the fission yeast centromeres. While the outer repeats play an important role in sister centromere cohesion and possibly help to properly orient the multiple kinetochore microtubule

attachment sites (Pidoux and Allshire, 2004), the central region is needed for the assembly of the kinetochore *per se* and the interaction with the mitotic spindle fibres (Goshima *et al.,*1999; Jin *et al.*, 2002; Kerres *et al.,* 2004; Liu *et al.*, 2005; Obuse *et al*., 2004; Pidoux *et al.*, 2003; Saitoh *et al.*, 1997; Sanchez-Perez *et al.,* 2005). The central region has a distinct composition as evidenced by the association of the conserved histone H3 variant Cnp1 and shows an unusual chromatin structure, as a limited micrococcal nuclease digestion gives rise to a smear instead of the expected nucleosomal ladder (Polizzi and Clarke, 1991; Takahashi *et al.*, 2000; Takahashi *et al*., 1992). Kinetochore proteins that associate with the central region are required to maintain this specialized chromatin structure (Goshima *et al.*, 1999; Jin *et al.*, 2002; Saitoh *et al.*, 1997). A substantial number of proteins that associate constitutively with the central region have been described and mutations in genes coding for these proteins lead to extreme missegregation of chromosomes (Goshima *et al.*, 1999; Hayashi *et al.*, 2004; Jin *et al.*, 2002; Pidoux *et al.*, 2003; Saitoh *et al.*, 1997). Recently, using affinity purification, the majority of these proteins have been grouped into two biochemically separable complexes, namely the Ndc80-MIND-Spc7 and the Sim4 complexes (Liu *et al.*, 2005). The MIND complex, made up of 4 conserved, essential proteins, serves in budding yeast as a bridge between kinetochore subunits that associate with the centromeric DNA and those which bind microtubules (De Wulf *et al.*, 2003; Obuse *et al.*, 2004). The 4 component Ndc80 complex is also conserved from yeast to man and is required for kinetochore-microtubule association and spindle checkpoint signalling (He *et al.*, 2001; Janke *et al*., 2001; Wigge and Kilmartin, 2001). Finally, the Spc7 protein was identified as an interaction partner of the Mal3 protein, a member of the EB1 family of microtubule plus end binding proteins (Kerres *et al.*, 2004). Its *C. elegans* homologue KNL-1 is required for targeting a number of components of the outer kinetochore thus directing the assembly of the microtubule-kinetochore interface (Desai *et al.*, 2003). Proteins of the Ndc80-MIND-Spc7 complex have been shown to be required for the special chromatin structure of the central centromere region, however they do not appear to be required for the association of the kinetochore-specific histone H3 variant Cnp1 with this region (Goshima *et al.*, 1999; Hayashi *et al.*, 2004; Takahashi *et al.*, 2000). This is in contrast to Sim4 complex components, which affect the chromatin structure and incorporation of Cnp1 (Pidoux *et al.*, 2003; Takahashi *et al.*, 2000). The Sim4 complex consists of 13 proteins: the previously identified Sim4, Mis6, Mal2, Mis15 and Mis17 proteins as well as the newly identified Fta1-7 proteins and Dad1, a component of the DASH complex (Hayashi *et al.*, 2004; Jin *et al.*, 2002; Liu *et al.*, 2005; Pidoux *et al.*, 2003; Saitoh *et al.*, 1997; Sanchez-Perez *et al.*, 2005). Interestingly, one of the functions of the Sim4 complex is to act as a loading

dock for the transient association of the non-essential fission yeast DASH complex with the kinetochore. It thus plays a role in chromosome biorientation (Liu *et al.*, 2005; Sanchez-Perez *et al.*, 2005). To better understand the function of the Sim4 complex in mitosis, we conducted a screen for extragenic suppressors of one of its members, namely the Mal2 protein (Fleig *et al.,*1996; Jin *et al.,* 2002). Mal2 is a conserved kinetochore component that is essential for faithful chromosome segregation. Its budding yeast counterpart, the Mcm21 protein, is part of the 4 component COMA complex, that links DNA associated kinetochore subcomplexes with those associating with microtubules (De Wulf *et al*., 2003; Ortiz *et al.*, 1999). However the other members of the COMA complex do not appear to exist in fission yeast. Recently orthologs of Mal2 have been identified by computational approaches in a large number of eucaryotes (Meraldi *et al.*, 2006). Furthermore, the human Mal2 homolog, CENP-O, was isolated in screens that identified proteins associated with centromeric chromatin, pointing to the importance of this protein in kinetochore function (Foltz *et al.*, 2006; Okada *et al.*, 2006) In this study, we identified Fta2 as a close interaction partner of Mal2 and provide evidence that the Sim4 complex appears to be assembled in a hierarchical way.

Materials and Methods

Strains and Media

Yeast strains used in this study are listed in Table 1. New strains were obtained by crossing the appropriate strains followed by tetrad or random spore analysis and genotyping. At least 3 double mutants were tested per cross. Strains were grown in rich media (YE5S) or minimal media (EMM or MM) with the required supplements (Moreno *et al*., 1991). MM with 5 μg/ml thiamine repressed the *nmt* promoters. For high-level expression from *nmt* promoters cells were grown in thiamine-less media for 22-24 hrs at 25 °C or 18-20 hrs at 30 °C. Resistance to G418 was tested on YE5S plates containing 100 mg/l G418. Transcriptional silencing assays were carried out as described (Jin *et al.*, 2002; Pidoux *et al.*, 2003).

Table 1. Yeast strains used in this study

Name	Genotype	Source
UFY819	*h$^+$ fta2$^+$/ura4$^+$ his3Δ leu1-32 ura4-D18 ade6-M210*	this study
UFY547	*h$^-$ mal2-1-GFP/Kan^R leu1-32 ade6-M210 ura4-D18*	this study
UFY518	*h$^-$ fta2-GFP/Kan^R leu1-32 ade6-M210 ura4-D6*	this study
UFY544	*h$^-$ fta2-GFP/Kan^R mal2-1 leu1-32 ade6-M210 ura4-D18*	this study
UFY1215	*h$^+$ fta2-292/his3$^+$ cnt1(NcoI):arg3$^+$ cnt3(NcoI):ade6$^+$ otr2(HindIII):ura4$^+$ leu1-32 ura4-D18 arg3$^-$ ade6-M210*	this study
UFY1189	*h$^-$ fta2-292/his3$^+$ imr1R(NcoI):ura4$^+$ ade6-M210 leu1-32 ura4$^-$*	this study
UFY1048	*h$^-$ fta2-291/his3$^+$ his3$^-$ leu1-32 ura4-D18 ade6-M210*	this study
UFY1050	*h$^-$ fta2-292/his3$^+$ his3$^-$ leu1-32 ura4-D18 ade6-M210*	this study
UFY1144	*h$^+$ fta2-291/his3$^+$ his7$^+$::lacI-GFP lys1$^+$::LacOP ura4$^-$ leu1-32*	this study
UFY1179	*h$^+$ fta2-291/his3$^+$ mad2Δ::ura4$^+$ ura4-D18 ade6-M210 leu1-32*	this study
UFY1180	*h$^+$ fta2-291/his3$^+$ mph1Δ::ura4$^+$ ura4-D18 ade6-M210 leu1-32*	this study
UFY1124	*h$^-$ fta2-292/his3$^+$ mal2-GFP/Kan^R ade6-M210 leu1-32 ura4$^-$ his3$^-$*	this study
UFY787	*h$^-$ fta2-HA/Kan^R his3-D1 ade6-M210 leu1-32 ura4-D18*	this study
UFY789	*h$^+$ fta2-HA/Kan^R mal2-GFP/Kan^R ura4$^-$ leu1-32 ade6-M210*	this study
UFY1202	*h$^+$ fta2-291/his3$^+$ mis15-68 leu1-32 ura4-D18*	this study
UFY1204	*h$^-$ fta2-292/his3$^+$ mis15-68 leu1-32*	this study
UFY1198	*h$^-$ fta2-291/his3$^+$ mis6-302 leu1-32 ade6-M210*	this study
UFY1200	*h$^+$ fta2-292/his3$^+$ mis6-302 leu1-32*	this study
UFY1219	*h$^+$ fta2-291/his3$^+$ sim4-193 leu1-32 his3-D1 ura4-D18 ade6-M210*	this study
UFY886	*h$^+$ fta2-GFP/Kan^R sim4-193 ade6-M210 leu1-32 ura4$^-$*	this study
UFY889	*h$^-$ fta2-GFP/Kan^R mis6-302 leu1-32*	this study
UFY1131	*h$^+$ fta2-GFP/Kan^R mis15-68 leu1-32*	this study
UFY1129	*h$^-$ fta2-GFP/Kan^R mis17-362 leu1-32 ura4-D6*	this study
UFY1211	*h$^-$ fta2-291/his3$^+$ sim4-GFP/Kan^R his3-D1 leu1-32 ade6-M210 ura4$^-$*	this study
UFY1239	*h$^-$ fta2-291/his3$^+$ mis6-3HA::leu$^+$ ade6-M210 leu1-32 ura4$^-$*	this study
UFY1054	*h$^-$ fta2-292/his3$^+$ dad1-GFP/Kan^R leu1-32 ura4-D18 ade6-M210*	this study
UFY885	*h$^+$ fta2-GFP/Kan^R mis12-537 leu1-32 ade6-M210*	this study
UFY1184	*h$^+$ fta2-GFP/Kan^R nuf2-1::ura4$^+$ ura4$^-$ leu1-32*	this study
UFY1028	*h$^+$ spc7-23/his3$^+$ his3-D1 ade6-M216 leu1-32 ura4-D18*	this study
UFY1170	*h$^+$ fta2-GFP/Kan^R spc7-23/his3$^+$ leu1-32 ade6-M210 ura4$^-$*	this study
UFY1237	*h$^-$ fta2-291/his3$^+$ spc7-GFP/Kan^R ade6-M210 ura4$^-$*	this study
UFY852	*h$^-$ mal2-1 leu1-32 ade6-M210 ura4-D18*	U. Fleig
UFY597	*h$^+$ mal2-GFP/Kan^R ade6-M210 leu1-32 ura4-D6*	U. Fleig
FY3027	*h$^+$ cnt1(NcoI):arg3 cnt3(NcoI):ade6 otr2(HindIII):ura4 tel1L:his3 ade6-M210 leu1-32 ura4-D18 arg3-D4 his3-D1*	R. Allshire
FY4540	*h$^-$ sim4-193 cnt1(NcoI):arg3 cnt3(NcoI):ade6 otr2(HindIII):ura4 tel1L:his3 ade6-M210 leu1-32 ura4-D18 arg3-D4 his3-D1*	R. Allshire
FY5231	*h$^+$ sim4-193 arg3-D4 ade6-M210 his3-D1 ura4-D18 leu1-32*	R. Allshire

Name	Genotype	Source
KG425	*h⁻ ade6-M210 leu1-32 his3Δ ura4-D18*	K. Gould
KG554	*h⁺ ade6-M216 leu1-32 his3Δ ura4-D18*	K. Gould
ANF251-9A	*h⁺ nuf2-1::ura4⁺ ura4-D18*	Y. Hiraoka
SS638	*h⁻ mad2Δ::ura4⁺ leu1-32 ura4-D18 ade6-M210*	S. Sazer
SS560	*h⁻ mph1Δ::ura4⁺ leu1-32 ura4-D18 ade6-M216*	S. Sazer
	h⁻ mis6-302 leu1-32	M. Yanagida
	h⁻ mis15-68	M. Yanagida
	h⁻ mis17-362	M. Yanagida
	h⁻ mis12-537 leu1-32	M. Yanagida

Identification of *fta2*⁺ and DNA methods

Multicopy extragenic suppressors of the *mal2-1* temperature sensitive (ts) phenotype were isolated by transformation of this strain with a genomic *S. pombe* DNA bank (Barbet *et al.*, 1992). Ura⁺ transformants were isolated at 30.5 °C to 32 °C and the genomic DNA inserts of the plasmids sequenced. At 32 °C only wild-type *mal2*⁺ could be isolated. At 30.5 °C 4/15000 transformants showed better growth. The plasmids of transformants able to grow better at 30.5 °C but not at higher temperatures were analysed further. One plasmid that contained 4 ORFs (cosmid c1783 position:739-6205**)** was subcloned to determine which ORF suppressed the *mal2-1* ts phenotype. This identified the ORF with the systematic name SPAC1783.03, which has recently be named *fta2*⁺ (Liu *et al.*, 2005).

A *fta2*⁺ null allele (Δ*fta2*⁺) was generated by replacing the entire 1056 bp of the *fta2*⁺ ORF plus 7 bp of the 3´ non-coding region with the Kanamycin-resistance (Kan^R) cassette in diploid strain KG425 x KG554 (Bahler *et al.*, 1998). Tetrad analysis of 77 heterozygous Δ*fta2*⁺/*fta2*⁺ diploids revealed that only the 2 Kanamycin-sensitive (Kan^S)spores/tetrad grew. We attempted to delete the *mal2*⁺ ORF in haploid strain KG425 overexpressing *fta2*⁺ from the *nmt1*⁺ promoter as described (Fleig *et al.*, 1996). We generated endogenous *fta2*⁺-*gfp*, *fta2*⁺-HA , *mal2-1-gfp* fusions via PCR based gene targeting using the Kan^R cassette (Bahler *et al.*, 1998). The correct Kan^R transformants were indistinguishable in phenotype from the isogenic parental strain.

Generation of *fta2*^{ts} alleles

A pBSK based plasmid containing the entire 1056 bp long *fta2*⁺ ORF followed 3` by the *his3*⁺ gene was used as a template for a mutagenic PCR reaction that amplified the 3256 bp long *fta2*⁺ *his3*⁺ DNA fragment. This fragment was transformed in strain UFY 819 which contained the *ura4*⁺-marker placed behind the genomic *fta2*⁺ gene. His⁺ transformants that grew at 25 °C but not at 36 °C were identified and tested for loss of the *ura4*⁺ marker on 5-fluoroorotic acid. Correct integration of the mutagenized DNA fragments was tested via PCR.

A *fta2*$^{+}$ containing plasmid was able to fully rescue the temperature-sensitivity of these mutant strains, which were backcrossed twice.

Immunoprecipitations

Chromatin immunoprecipitations (ChIP) were performed as described (Kerres *et al.*, 2004; Pidoux *et al.*, 2003) We performed at least 3 independent ChIP analyses per strain and temperature.

For coimmunoprecipitation, Fta2-HA, Mal2-GFP and Fta2-HA Mal2-GFP expressing strains were grown at 30 °C in YE5S over night followed by protein extraction and immunoprecipitation as has been described (Kerres *et al.*, 2004). Eluates were boiled and resolved on a SDS-9 %-polyacrylamid gel and blotted. Blots were probed with anti-HA antibody (monoclonal mouse; Roche Diagnostics) followed by the secondary antibody (Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L); Jackson ImmunoResearch Laboratories). Immobilized antigens were detected using the ECL Advance Western blotting kit (GE Healthcare, Little Chalfort, Buckinghamshire, United Kingdom).

Microscopy

Photomicrographs were obtained with a Zeiss Axiovert200 fluorescence microscope coupled to a charge-coupled device camera (Hamamatsu Orca-ER) and Openlab imaging software (Improvision, Coventry, UK). Immunofluorescence microscopy was done as described (Bridge *et al.*, 1998; Hagan and Hyams, 1988). Tubulin was stained using monoclonal anti-TAT1 antibodies followed by fluorescein isothiocyanate-conjugated goat anti-mouse antibodies (Sigma-Aldrich). HA or GFP fusion proteins were observed in fixed cells by indirect immunofluorescence with mouse anti-HA antibody (Covance, Princeton, NJ) or rabbit anti-GFP antibodies (Invitrogen, Carlsbad, CA), respectively. Cy3-conjugated sheep anti-mouse antibodies or Cy3-conjugated sheep anti-rabbit antibodies (Sigma-Aldrich) were used as secondary antibodies. Cells were stained with 4,6-diamidino-2-phenylindole (DAPI) before mounting.

Results

Identification of *fta2*$^+$ as a suppressor of the *mal2-1* temperature-sensitive mutant phenotype

The conditionally lethal temperature-sensitive *mal2-1* allele leads to severe missegregation of endogenous chromosomes at the restrictive temperature (Fleig *et al.*, 1996; Jin *et al.*, 2002). To identify Mal2 interaction partners we conducted a multicopy extragenic suppressor screen and identified an ORF with the systematic name SPAC1783.03, that was able to suppress the temperature-sensitivity of the *mal2-1* strain (Materials and Methods; Figure 1A). SPAC1783.03 codes for a 40.5 kDa protein which shows no strong sequence similarity to other proteins in the databases (Sanger Institute, Cambridge). Recently this ORF was named *fta2*$^+$ (Liu *et al.*, 2005). *fta2*$^+$ driven by its own promoter on plasmid pUR19 rescued the temperature-sensitive phenotype of the *mal2-1* strain up to 32 °C, while overexpression of *fta2*$^+$ from the repressible, wild-type *nmt1*$^+$ promoter resulted in suppression of the *mal2-1* non-growth phenotype at all temperatures tested (Figure 1A). We next tested if the essential *mal2*$^+$ ORF could be replaced by high level *fta2*$^+$ expression. To this end we attempted, but failed, to delete the *mal2*$^+$ ORF in haploid strains strongly overexpressing *fta2*$^+$ (Materials and Methods).

The suppression of the *mal2-1* temperature-sensitive phenotype by *fta2*$^+$ is due to suppression of the chromosome missegregation observed in *mal2-1* cells. 53.5 % of *mal2-1* anaphase cells transformed with a vector control and incubated at the non-permissive temperature for 6 hours showed severe chromosome segregation defects. This phenotype was fully suppressed by the presence of wild-type *mal2*$^+$ on a plasmid and reduced to 9.2 % in *mal2-1* cells expressing extra *fta2*$^+$ (Figure 1B).

As shown by the immunofluorescence analysis of a Mal2-1-GFP fusion protein, the mutant Mal2-1 protein is present at the kinetochore at 25 °C. However, Mal2-1 kinetochore association is not observed at the restrictive temperature (Figure 1C). Overexpression of *fta2*$^+$ in this strain rescues this phenotype as Mal2-1-GFP shows kinetochore association at the restrictive temperature in the presence of extra *fta2*$^+$ (Figure 1C). We conclude that extra *fta2*$^+$ is able to rescue the *mal2-1* strain by stabilizing the mutant Mal2-1 protein and/or by helping the mutant Mal2-1 protein to associate with the kinetochore at the non-permissive temperature.

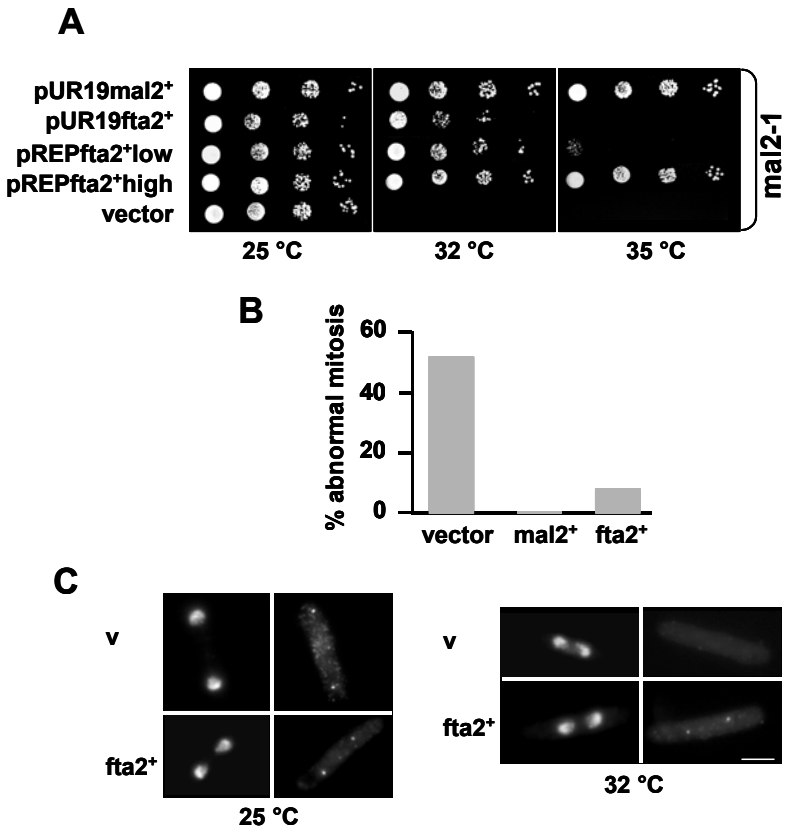

Figure 1. *fta2*$^+$ suppresses the phenotypes of the *mal2-1* mutant strain.
(A) The rescue of the *mal2-1* temperature-sensitive phenotype by *fta2*$^+$ is dosage dependent. Serial dilution patch tests (10^4 to 10^1 cells) of *mal2-1* transformants grown under selective conditions at the indicated temperatures for 4 to 6 days. Vector control indicates plasmid without insert; pUR19mal2$^+$ and pUR19fta2$^+$ denotes the presence of wild-type *mal2*$^+$ or *fta2*$^+$ expressed by their endogenous promoters on plasmid pUR19. pREPfta2$^+$low and pREPfta2$^+$high denote presence of wild-type *fta2*$^+$ driven by the *nmt1* promoter under repressed (low) or derepressed (high) conditions, respectively. **(B)** Overexpression of *fta2*$^+$ suppresses mitotic defects in *mal2-1* cells. The number of abnormal mitosis was determined in *mal2-1* cells transformed with a vector control or plasmids expressing wild-type *mal2*$^+$ or *fta2*$^+$. Cells were shifted from 25 °C to 32 °C for 6 hours, fixed and the number of aberrant anaphases determined. N/strain= 50 **(C)** Kinetochore localisation of Mal2-1-GFP is dependent on *fta2*$^+$ overexpression. *mal2-1-gfp* cells transformed with vector control (v) or a plasmid expressing *fta2*$^+$ under the control of the *nmt1*$^+$ promoter were grown at 25 °C or 36 °C for 6 hrs and the localisation of Mal2-1-GFP determined. Bar 5 μm.

Association of the essential Fta2 protein with the kinetochore is dependent on the presence of functional Mal2

To determine whether *fta2*$^+$ was essential for vegetative growth one copy of the *fta2*$^+$ ORF was replaced with the Kan^R (Kanamycin resistance) marker in a diploid strain (Materials and Methods). Sporulation followed by tetrad analysis of 77 tetrads of this strain revealed that only two of the four spores in a tetrad could grow and these were Kanamycin sensitive indicating that *fta2*$^+$ is an essential gene. To determine the subcellular localisation of Fta2, a fluorescence-improved version of GFP was fused to the COOH-terminal end of the endogenous *fta2*$^+$ ORF (Materials and Methods). Immunofluorescence of interphase and mitotic cells revealed a localisation pattern characteristic of *S. pombe* kinetochore proteins

namely a single fluorescent dot near the nuclear periphery in interphase and late mitotic cells and up to six fluorescent dots in metaphase cells (Figure 2A) (Jin *et al.*, 2002). This localisation pattern is dependent on the presence of a functional Mal2 protein, as no specific Fta2-GFP signal could be detected in a *mal2-1* strain incubated at the restrictive temperature (Figure 2B). Fta2 protein levels in the cell were not affected in the *mal2-1* strain (Supplementary Figure 1). To identify the centromere region with which Fta2 associates and to determine if the association of Fta2-GFP with centromeric DNA was dependent on Mal2 chromatin-immunoprecipitation (ChIP) was carried out (Partridge *et al.*, 2000). Wild-type or *mal2-1* cells expressing Fta2-GFP were analysed in ChIP assays by using anti GFP-antibodies. The DNA present in crude extracts or in the immunoprecipitates was analysed by multiplex PCR analysis using primers to amplify the *cnt*, *imr* and *otr* regions of centromere I and an unrelated euchromatic control (*fbp*) (Figure 2C). In wild-type cells Fta2 associates with the central centromere region as shown by the specific enrichment of the *cnt* and *imr* sequences in the Fta2-GFP ChIP (Figure 2C) (Liu *et al.*, 2005). In *mal2-1* cells Fta2 ChIPs also showed enrichment of the *cnt* and *imr* sequences at the permissive temperature (25 °C), however incubation at the non-permissive temperature led to a severe reduction of the *cnt* and *imr* sequences brought down by chromatin-immunoprecipitation (Figure 2C). These data imply that Fta2 localisation at the kinetochore was dependent on Mal2.

As mutations in genes encoding components of the kinetochore affect centromeric silencing (Pidoux and Allshire, 2000) we tested if marker genes placed within the centromere DNA were still transcriptionally repressed in the *fta2-292* temperature-sensitive mutant strain (see later). To this end we assayed growth of *fta2-292* strains which had the *ura4*$^{+}$ marker gene inserted at *otr2* (otr region of centromere 2) or *imr1*, or the *arg3*$^{+}$ marker gene inserted at *cnt1* (Partridge *et al.*, 2000; Pidoux *et al.*, 2003). Wild-type strains carrying a marker gene inserted at a centromeric region are auxotroph for that particular marker due to transcriptional repression of the centromeric DNA (Pidoux and Allshire, 2000). The presence of *fta2-292* had no influence on transcriptional silencing of the *otr* and *imr* regions but led to alleviation of silencing at the central *cnt* region (our unpublished data; Figure 2D). Wild-type strains containing the promoter-crippled *arg3*$^{+}$ gene inserted into the *cnt1* region grow very poorly on medium that does not contain arginine, while kinetochore mutants such as *sim4-193* alleviate *cnt1* silencing and allow fast growth on arginine minus medium (Figure 2D) (Pidoux *et al.*, 2003). The presence of the mutant *fta2-292* allele led to good growth on medium without arginine implying that transcriptional silencing of the *cnt1* region has been alleviated (Figure 2D).

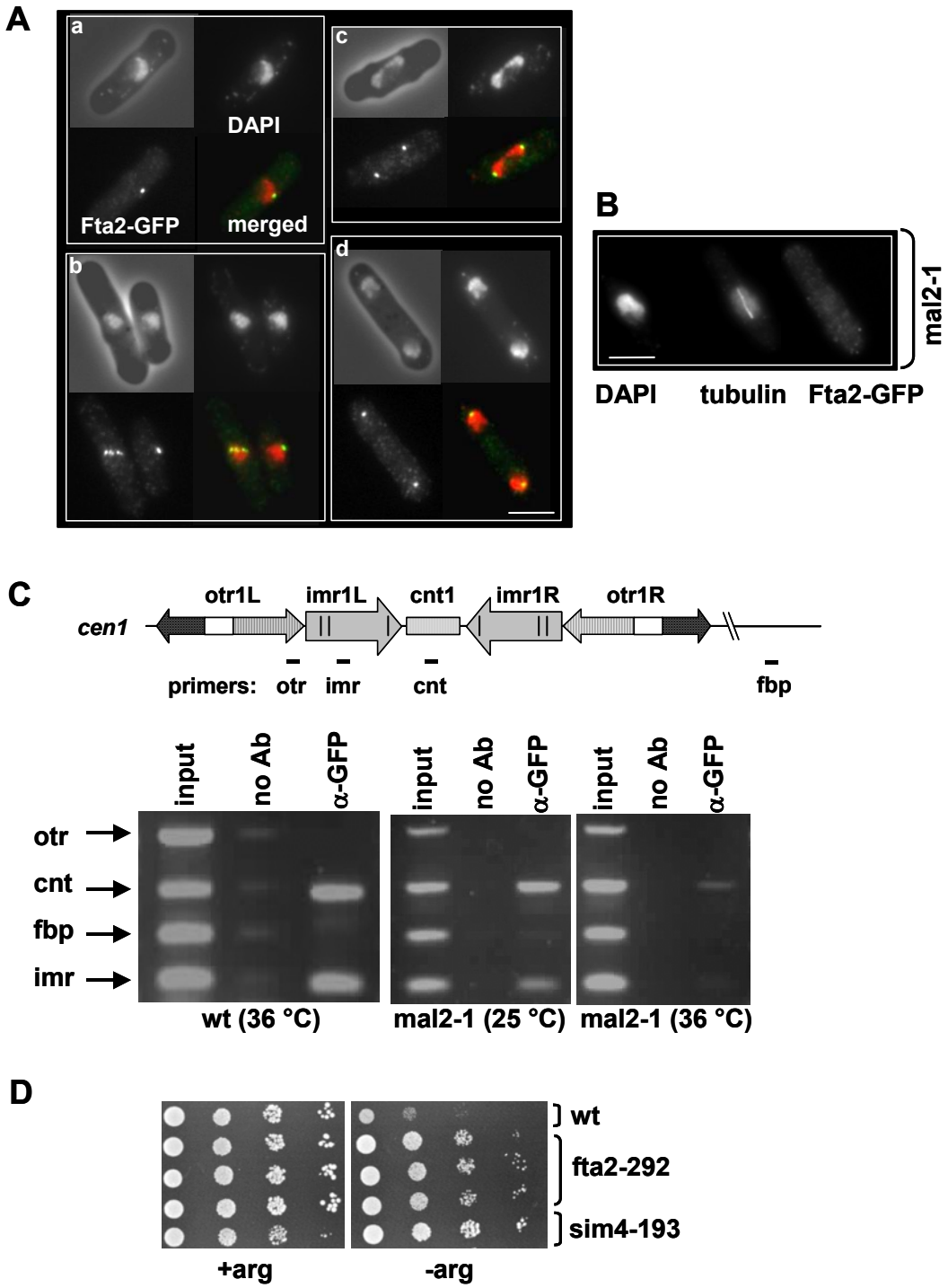

Figure 2. Fta2 kinetochore localisation is dependent on functional Mal2.
(A) Localisation of the Fta2-GFP protein in wild-tpye cells in interphase (a) and early, middle and late mitosis (b (left cell)-d, respectively). Fixed cells were stained with DAPI and anti-GFP antibody. Bar 5 μm. **(B)** Localisation of Fta2-GFP in an early mitotic *mal2-1* cell. Cells were incubated for 6 hrs at 36 °C, fixed and stained with DAPI, anti-tubulin antibody and anti-GFP antibody. Bar 5 μm. **(C)** Fta2 association with the central domain of *cen1* is Mal2 dependent. Wild-type or *mal2-1* cells expressing Fta2-GFP were fixed and processed for ChIP by using anti-GFP antibodies. The chromatin in immunoprecipitates and in the crude extracts was analysed by multiplex PCR. The amplified regions in *cen1* are indicated. *fbp*: euchromatic negative control. The *cnt* and *imr* regions are enriched in Fta2-GFP ChIPs from wild-type cells; the association of Fta2-GFP with these regions is reduced or severely reduced in *mal2-1* cells grown at 25 °C or 36 °C (for 6 hrs), respectively. **(D)** Fta2 is involved in *cnt* centromeric silencing. Serial dilution patch tests (10^4 to 10^1 cells) of wild-type, *fta2-292* and *sim4-193* cells that have the *arg3*$^+$ gene inserted at *cnt1*. Alleviation of silencing leads to growth on arginine minus (-arg) plates. Cells were incubated at 30 °C for 5 days.

***fta2* mutants show severe defects in chromosome segregation and bipolar attachment**

To study the function of Fta2 in mitosis we generated temperature sensitive (ts) *fta2* alleles (Materials and Methods). The two mutant *fta2* strains which showed the tightest ts phenotype were analysed in greater detail. DNA sequence analysis revealed that both strains carried a single point mutation in the *fta2* ORF, one at position 871 (G to A), the other at position 874 (T to C). The single base pair changes resulted in single amino acids changes at position 291 (a change from glycine to serine) and position 292 (a change from phenylalanine to leucine) of the 351 amino acid long Fta2 protein. The mutants were therefore named *fta2-291* and *fta2-292*, respectively (Figure 3A). As the entire *fta2*$^{+}$ ORF was mutagenized but the two mutants with the most prominent ts phenotype had mutations in close proximity to each other, we reasoned that the C-terminal region of Fta2 played an important role in its function. We therefore conducted another database search using WU-BLAST2 for putative Fta2 homologs using the last 81 C-terminal amino acids only (Altschul *et al.*, 1997) . We found a very limited homology to the *S. cerevisiae* kinetochore protein Ctf13 (36 % identical, 50 % similar amino acids in a 63 amino acid long region) (Supplementary Figure 2) (Doheny *et al.*, 1993). Ctf13 is one of the four proteins of the CBF3 kinetochore complex, which is the essential centromere DNA binding complex in budding yeast (Doheny *et al*., 1993; Russell *et al.*, 1999).

Interestingly, in this alignment the amino acids at position 291 and 292 of Fta2 were conserved (Supplementary Figure 2). However, at present it is unclear if Ctf13 and Fta2 share a common domain.

To analyse the reason for the non-growth phenotype of the *fta2* mutant strains at higher temperatures, *fta2-291* and *fta2-292* strains were incubated at the non-permissive temperature for 6 hrs and analysed by immunofluorescence. Although interphase cells showed no obvious abnormalities, mitotic cells were severely affected. All *fta2-291* mitotic cells and 86 % of *fta2-292* mitotic cells showed severe chromosome segregation defects (Figure 3B and 3C). The two predominant abnormal chromosome resolution phenotypes were: 1) unequally or partially separated chromatin (Figure 3B, a and c) and 2) no separation of highly condensed chromatin on an elongating spindle (Figure 3B, b and d). The chromatin was separated, albeit unequally, in the majority of *fta2-292* mitotic cells, while nearly 50 % of *fta2-291* mitotic cells were unable to separate their chromatin (Figure 3C). Aberrant spindle phenotypes were observed rarely. To further characterize the severe chromosome segregation defects observed in *fta2* mutant cells, we analysed the segregation behaviour of sister centromeres by monitoring the segregation behaviour of centromere 1 marked with GFP (*cen1-gfp*)

(Nabeshima *et al.*, 1998). In interphase cells, the distribution of the cen1-GFP signals was very similar to that observed for wild-type cells, indicating that premature sister chromatid separation was not the cause of the aberrant chromatin distribution seen in *fta2* mutants. In mitotic cells, only 26.7 % of *fta2-291* cells with an elongating spindle showed correct separation of *cen1* sister centromeres, although the chromatin in these cells was distributed unequally (Figure 3 D). In 42.3 % of cells the sister centromeres segregated together possibly due to syntelic microtubule attachment (Figure 3D and E). In 3.8 % of the cells only one of the sister centromeres segregated to the end of the cell. Finally, in 26.7 % of cells, sister chromatids were not segregated and remained in the middle of the cell (Figure 3D).

We next asked if all centromeres were associated with the mitotic spindle by assaying co-localisation of the cen1-GFP signal and the spindle (Figure 3E). We found that in cells with segregated chromatin 75 % of cen1-GFP signals were spindle associated while 25 % were not. In cells with an elongating spindle but unseparated chromatin, the majority (58 %) of cen1-GFP signals did not co-localise with the mitotic spindle. Our findings imply that the Fta2 protein is required for correct bipolar chromosome orientation and also plays a role in linking the kinetochore to spindle microtubules.

The observed *fta2* mutant phenotypes should lead to activation of the spindle checkpoint. This checkpoint regulates entry into anaphase by inhibiting the anaphase promoting complex until proper spindle microtubules association of the kinetochores. The absence of spindle microtubules will activate the attachment response while the tension response will be activated in response to the absence of tension between sister kinetochores (Cleveland *et al.*, 2003; Musacchio and Hardwick, 2002). We tested if the spindle checkpoint was active in *fta2* mutant cells by constructing double mutants of *fta2-291*with null alleles of *mad2*$^{+}$ and *mph1*$^{+}$, which encode conserved components of the spindle checkpoint pathway (He *et al.*, 1998; He *et al.*, 1997). The growth properties of *mad2Δ* (*mad2*$^{+}$ deletion) *fta2-291* double mutants were indistinguishable from that of the single *fta2-291* mutant (Figure 3F). In contrast, *mph1Δ fta2-291* strains showed growth defects at 30 °C (Figure 3F). We analysed the phenotypic consequences of an *mph1*$^{+}$ deletion in *fta2-291* strain by incubation of the single and double mutants for 8 hrs at 30 °C followed by DAPI staining. At this temperature 47.1 % of *fta2-291* anaphase cells showed aberrant chromosome segregation, while in the *mph1Δ fta2-291* double mutant this number rose to 64.1 % (our unpublished data). Our results indicate that the *mph1*$^{+}$ spindle checkpoint branch is required for survival of the *fta2-291* strain at 30 °C.

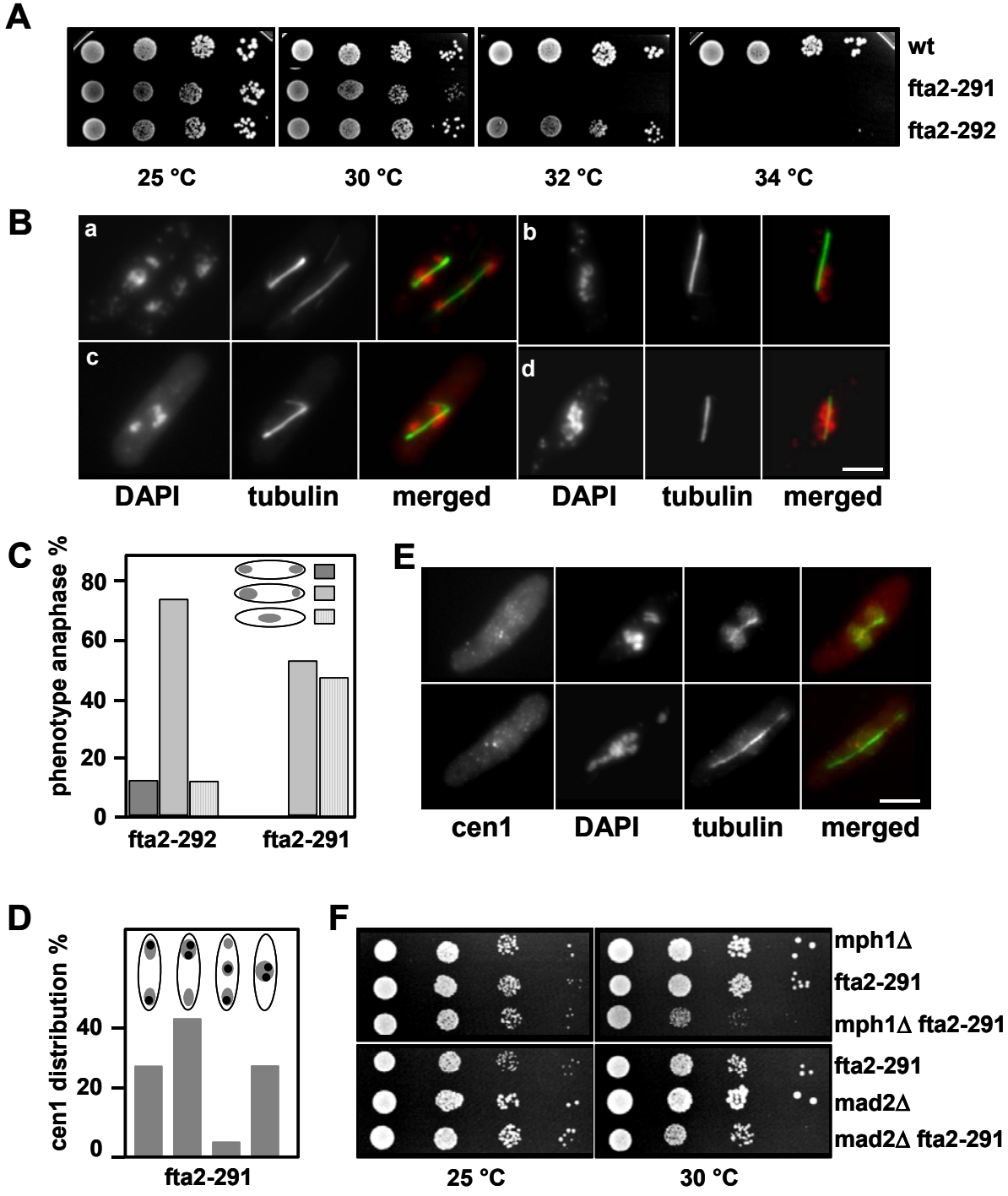

Figure 3. Temperature-sensitive *fta2* mutants have severe mitotic defects.
(A) Serial dilution patch tests (10^4 to 10^1 cells) of wild-type (wt), *fta2-291* and *fta2-292* strains grown at the indicated temperatures for 3 days. **(B)** Photomicrographs of mitotic *fta2-291* (a and b) and *fta2-292* (c and d) cells incubated for 6 hrs at 36 °C. Fixed cells were stained with DAPI and anti-tubulin antibody. Shown are the main two phenotypes: unequally/partially segregated (a,c) or non-separated (b,d) chromatin on an elongating spindle. **(C)** Diagrammatic representation of anaphases observed in *fta2-291* and *fta2-292* strains incubated for 6 hrs at 36 °C. N/strain= 60 **(D)** Diagrammatic representation of *cen1* distribution in mitotic *fta2-291* cells with an elongating spindle (cen1 signal, black dot). **(E)** Distribution of GFP-marked *cen1* in mitotic *fta2-291* cells. Cells were incubated for 6 hrs at 36 °C, fixed and stained with anti-GFP antibody, DAPI and anti-tubulin antibody. **(F)** *fta2-291* interacts genetically with a component of the spindle checkpoint pathway. Serial dilution patch tests of *mph1Δ*, *mad2Δ*, *fta2-291* and the respective *fta2-291* double mutants grown at the indicated temperatures for 3 days.

Extra Mal2 can suppress the *fta2* temperature-sensitive mutant phenotypes

As *fta2*$^+$ was isolated as a suppressor of the *mal2-1* mutant phenotypes, it was of interest to see if extra *mal2*$^+$ could suppress the *fta2* mutant phenotypes. *mal2*$^+$ driven by its own promoter on plasmid pUR19 rescued the ts phenotype of the *fta2-292* strain at all temperatures tested (Figure 4A, left panel) while it could only rescue the ts phenotype of the *fta2-291* strain up to 34 °C. However overexpression of *mal2*$^+$ from the repressible, wild-type *nmt1*$^+$ promoter resulted in suppression of the *fta2-291* non-growth phenotype at all temperatures tested (Figure 4A, right panel). Thus, suppression of the *fta2* ts phenotype by *mal2*$^+$ occurs in the same dosage-dependent manner as has been observed for the suppression of *mal2-1* by *fta2*$^+$. It was therefore not surprising that the kinetochore localisation of a Mal2-GFP fusion protein was affected in *fta2* mutants (Figure 4B). The kinetochore association of Mal2-GFP was not affected in *fta2* mutants grown at the permissive temperature, but was reduced severely in *fta2* ts cells incubated at the restrictive temperature (Figure 4B). Mal2-GFP protein levels were not affected in *fta2* mutant strains incubated at the restrictive temperature (Supplementary Figure 4).

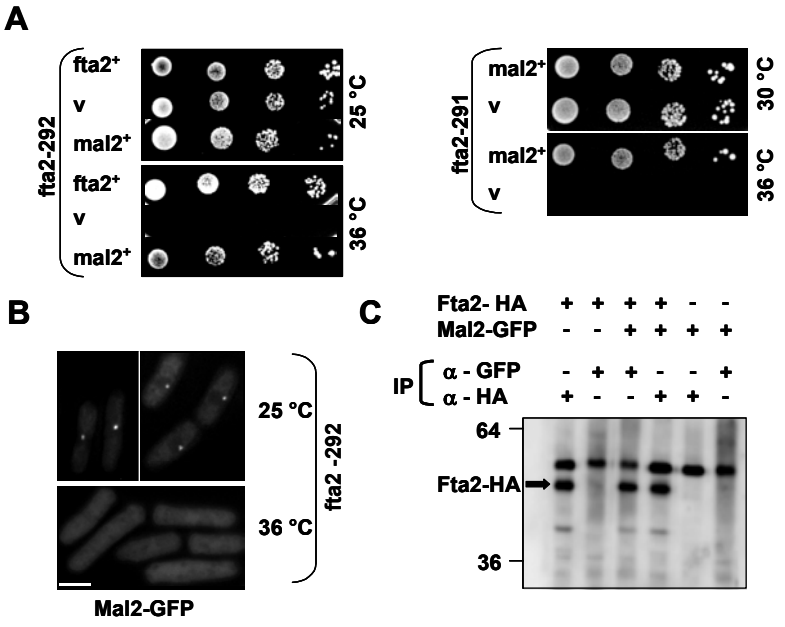

Figure 4. Extra *mal2*$^+$ suppresses the temperature-sensitive non-growth phenotypes of the *fta2* mutant strains.

(A) Serial dilution patch tests (10^4 to 10^1 cells) of *fta2-292* and *fta2-291* transformants grown under selective conditions at the indicated temperatures for 4 days. Vector control (v) indicates plasmid without insert; mal2$^+$ denotes the presence of wild-type *mal2*$^+$ expressed by the endogenous promoters on plasmid pUR19 (for *fta2-292*) or from the thiamine-repressible *nmt1*$^+$ promoter in the absence of thiamine (for *fta2-291*). **(B)** Kinetochore localisation of Mal2-GFP is abolished in the *fta2-292* strain at the non-permissive temperature. *fta2-292* cells expressing Mal2-GFP were grown at 25 °C or shifted for 6 hrs to 36 °C and analysed by fluorescence microscopy. **(C)** Co-immunoprecipitation of Fta2 and Mal2. Protein extracts from strains expressing Mal2-GFP, Fta2-HA or both were used for immunoprecipitation (IP) with an anti-HA or anti-GFP antibody. The immunoprecipitates were resolved by SDS-Page and probed with an anti-HA antibody. KDa size markers are indicated.

Given the close genetic interaction between *mal2*$^+$ and *fta2*$^+$, we investigated whether the Mal2 and Fta2 proteins interacted. For this purpose, we tested whether HA-tagged Fta2 could be co-immunoprecipitated by GFP-tagged Mal2. Immunoprecipitation with anti-HA or anti-GFP antibodies was carried out with protein extracts from strains that expressed endogenous Fta2-HA and /or Mal2-GFP. Analysis of these immunoprecipitates via Western blotting using anti-HA antibodies strongly co-immunoprecipitated Fta2-HA with Mal2-GFP (Figure 4C). Fta2-HA could also co-immunoprecipitate Mal2-GFP (Supplementary Figure 3).
Given the close physical and genetic interaction between *fta2*$^+$ and *mal2*$^+$ we were interested to analyse the phenotype of *fta2 mal2* double mutants. However, we were unable to construct *mal2-1 fta2-291* or *mal2-1 fta2-292* mutants by tetrad analysis at 25 °C. The *mal2-1 fta2* double mutant spores germinated and cells divided 2-3 times before dying (Materials and Methods).

Interaction between Fta2 and other components of the Sim4 kinetochore subcomplex

Recently Fta2 and Mal2 were identified as components of the Sim4-kinetochore complex (Liu *et al.*, 2005). The Sim4 complex consists of the previously identified proteins Sim4, Mal2, Mis6, Mis15, Mis17, the DASH component Dad1 and seven novel proteins Fta1-7 (Goshima *et al.*, 1999; Hayashi *et al.*, 2004; Jin *et al.*, 2002; Liu *et al.*, 2005; Pidoux *et al.*, 2003). Given the close interaction between Mal2 and Fta2 for all parameters tested, we assayed the interaction between Fta2 and other components of the Sim4 complex. We first tested suppression of all existing conditional lethal alleles of the Sim4 complex components by overexpression of *fta2*$^+$. *fta2*$^+$ expressed from the repressible, wild-type *nmt1*$^+$ promoter was unable to suppress the non-growth phenotype of the ts *sim4-193*, *mis6-302*, *mis15-68* or *mis17-362* strains even at semi-permissive temperatures (Figure 5A). In fact, overexpression of *fta2*$^+$ in *mis6-302* was synthetic lethal at 31 °C, a temperature that has only a slight effect on the growth of the *mis6* mutant transformed with a vector control (Figure 5A).
Next, double mutants of *fta2-291* and/or *fta2-292* with *sim4-193*, *mis6-302*, *mis15-68* were constructed by tetrad analysis. In contrast to the *fta2 mal2-1* double mutants, which were inviable, all of these double mutants were able to grow normally at 25 °C. However at higher temperatures, synthetic effects were observed that resulted in poor growth at that particular temperature (Figure 5B). As an example single and double mutants strains of *fta2-291* and *mis15-68* and *sim4-193* are shown (Figure 5C). To determine if kinetochore localisation of Fta2 was affected in mutants of the Sim4 complex, we assayed localisation of a Fta2-GFP fusion protein in *sim4-193*, *mis6-302*, *mis15-68* and *mis17-262* mutants. At the permissive

temperature (25 °C), no difference in Fta2-GFP staining was observed between a wild-type strain and the Sim4 complex mutants, with the exception of the *mal2-1* mutant, which showed less intense Fta2-GFP signals (Figure 5D, our unpublished data). At the non-permissive temperature, no Fta2-GFP signal was observed in *mal2-1* cells (Figure 2B), and severely reduced signals in *mis6-302*, *mis15-68* and *mis17-362* cells (Figure 5D). For example, only 10 % of *mis6-302* cells incubated for 6 hrs at 36 °C showed a wild-type like Fta2-GFP signal, all other cells had a severely reduced or no GFP signal (Figure 5E). This phenotype was irrespective of the cell cycle phase, although interphase cells showed a higher percentage of cells with no GFP signal/versus reduced signal than mitotic cells. Analysis of a Mis6-HA fusion protein in the *fta2-291* mutant gave a similar result (Figure 5F).

Surprisingly, the presence of the mutant *sim4* allele had only mild effects on the correct localisation of Fta2-GFP. After 6-8 hrs at the restrictive temperature, 86 % of *sim4-193* cells showed wild-type like Fta2-GFP signals (Figure 5E). However, the correct localisation of a Sim4-GFP fusion protein was dependent on Fta2 as kinetochore localisation of Sim4-GFP in the *fta2-291* mutant was reduced severely (Figure 5F, our unpublished data).

These results imply that the Sim4 kinetochore complex is build up hierarchically. Mal2 is absolutely required for kinetochore localisation of Fta2. Mis6, Mis15 and Mis17 are also needed for Fta2 localisation, but to a somewhat lesser degree, while Sim4 does not appear to be required. Fta2 is absolutely required for kinetochore localisation of Mal2 and plays an important role in the correct localisation of Mis6 and Sim4 proteins.

Finally, it has been shown previously that Sim4 complex components are required for association of the DASH complex (Sanchez-Perez *et al.*, 2005). We therefore tested if Dad1, a constitutive component of the DASH complex was localised correctly in *fta2-292* mutant cells and found that kinetochore localisation of Dad1-GFP was dependent on functional $fta2^+$ (Figure 5F and 5G).

Figure 5. Interaction between $fta2^+$ and other components of the Sim4 kinetochore complex.
(A) Plasmid-borne expression of $fta2^+$ from the $nmt1^+$ promoter rescues the ts phenotype of a *mal2-1* mutant (+), leads to synthetic lethality (sl) of a *mis6-302* mutant at the semi-permissive temperature and has no effect (-) on the growth of the *sim4-193*, *mis15-68* and *mis17-362* strains at temperatures below the restrictive temperature. Serial dilution patch tests of *mis6-302* transformants grown under selective conditions at the indicated temperatures for 4 days. v, vector control. **(B)** Growth of double mutants of *fta2-291* and/or *fta2-292* with other components of the Sim4 complex. sl, synthetic lethal; s, reduced growth; -, no effect; nd, not done. **(C)** Serial dilution patch tests of *fta2-291*, *mis15-68*, *sim4-193* and double mutants grown on YE5S for 4 (25 °C) or 3 (30 °C) days. **(D)** Kinetochore localisation of a Fta2-GFP fusion protein in the indicated ts strains. All strains were incubated for 6 hrs at the non-permissive temperature before fixation. Wt, wild-type; ++, wild-type like localisation to -, no localisation. N/strain = 140 **(E)** Diagrammatic representation of Fta2-GFP kinetochore localisation in *sim4-193* and *mis6-302* cell populations. N/strain = 140 **(F)** Kinetochore localisation of Sim4-GFP, Mis6-HA in a *fta2-291* mutant and Dad1-GFP in a *fta2-292* mutant grown for 6 hrs at the restrictive temperature. ++, wild-type like localisation to -, no localisation. N/strain = 140. **(G)** Dad1-GFP localisation was analysed in living *fta2-292* cells grown at 25 °C or 36 °C for 6 hours.

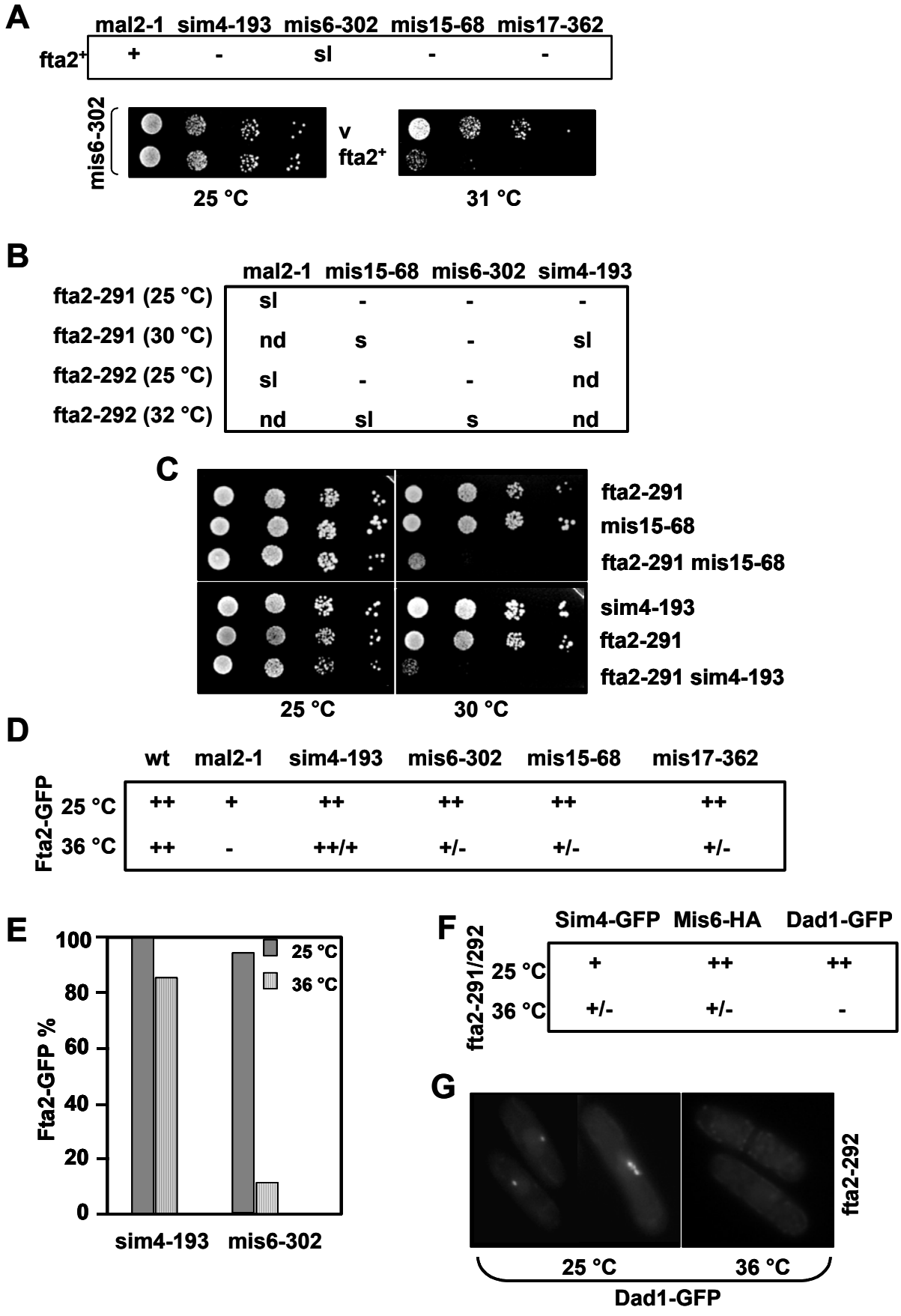

Figure 5. Interaction between *fta2*$^+$ and other components of the Sim4 kinetochore complex.
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Wild-type like Fta2 localisation requires Spc7, a component of the Ndc80-MIND-Spc7 kinetochore complex

Recently, the Ndc80-MIND-Spc7 kinetochore complex has been described (Liu *et al.*, 2005; Obuse *et al.*, 2004). This complex appears to exist independently of the Sim4 complex (Liu *et al.*, 2005).

To analyse a possible interaction between Fta2 and components of the Ndc80-MIND-Spc7 complex, we overexpressed *fta2*$^{+}$ in *mis12-537*, *nuf2-1* and *spc7-23* mutant strains at various temperatures up to the maximally permissive temperatures. The Mis12 protein is part of the MIND complex, while Nuf2 is a component of the Ndc80 complex. Extra *fta2*$^{+}$ had no effect on the growth of a *nuf2-1* mutant, a slight negative effect on the *spc7-23* mutant and gave rise to reduced growth of the *mis12-537* mutant strain (Figure 6A).

To test if Fta2 kinetochore localisation was dependent on the Ndc80-MIND-Spc7 complex, subcellular localisation of the Fta2-GFP fusion protein was determined in *mis12-537*, *nuf2-1* and *spc7-23* temperature-sensitive strains (Goshima *et al*., 1999; Kerres *et al*., 2004; Nabetani *et al.*, 2001). Kinetochore localisation of Fta2-GFP was unaffected in *mis12-537* and *nuf2-1* mutant strains incubated at the non-permissive temperature (Figure 6B, our unpublished data). However, surprisingly, Fta2-GFP localisation was affected in the *spc7-23* strain at the restrictive temperature (Figure 6B). *spc7-23* encodes a ts mutant Spc7 protein, that has a severely reduced kinetochore association at the restrictive temperature, leading to spindle defects and massive chromosome missegregation (Kerres, Jakopec and Fleig, unpublished). While Fta2-GFP kinetochore localisation was unaffected in a *spc7-23* strain grown at 25 °C (Figure 6B), the signal intensity of the fusion protein was reduced or absent in the majority of *spc7-23* cells incubated at the restrictive temperature (Figure 6B and 6C). Fta2 proteins levels were similar in *spc7-23* and wild-type cells (Supplementary Figure 1). Thus, Spc7 is required for correct localisation of the Sim4 complex component Fta2. However a Spc7-GFP fusion protein is localised correctly in a *fta2* mutant (our unpublished data).

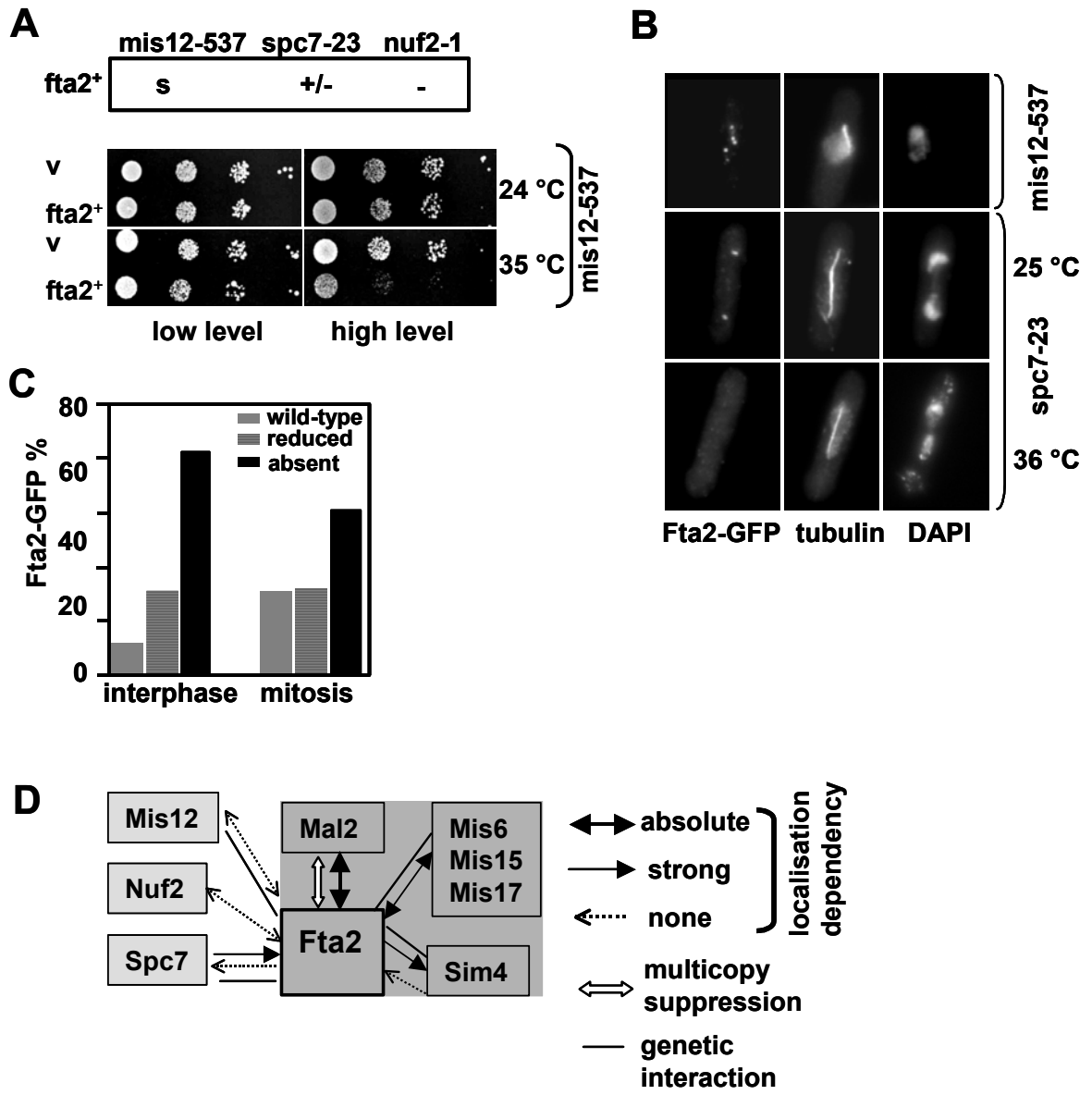

Figure 6. Interaction of *fta2*$^+$ with components of the Ndc80-MIND-Spc7-kinetochore complex.
(A) Growth phenotype of *mis12-537*, *spc7-23* and *nuf2-1* strains transformed with pREPfta2$^+$. s, reduced growth; s/-, slightly reduced growth; -, no effect. The serial dilution patch tests (10^4 to 10^1 cells) show a *mis12-537* strain transformed with pREPfta2$^+$ grown under selective conditions and under *nmt1*$^+$ promoter repressing (low) or derepressing (high) conditions for 6 days. **(B)** Photomicrographs of *mis12-537* and *spc7-23* cells expressing Fta2-GFP. The *spc7-23* strain was incubated at 25 °C or for 6 hrs at 36 °C, the *mis12-537* strain for 6 hrs at 36 °C, fixed and stained with DAPI, anti-tubulin antibody and anti-GFP antibody. Bar 5 μm.**(C)** Diagrammatic representation of Fta2-GFP in a *spc7-23* cell population incubated for 6 hrs at 36 °C. N /cell cycle phase = 55. **(D)** Summary of the interactions between Fta2 and components of the Sim4 and Ndc80-MIND-Spc7 complexes.

Discussion

We investigated the role of the conserved Mal2 protein in mitosis by screening for extragenic suppressors that were able to rescue the *mal2-1* ts phenotype at a semi-permissive growth temperature and thus identified the essential kinetochore component Fta2. Both proteins were recently shown to be members of the Sim4 kinetochore complex (Liu *et al.*, 2005).

Our characterisation of Fta2 shows that it localises to the central domain of the fission yeast centromere, where it is required for transcriptional silencing. This specific alleviation of central core silencing has also been documented for other mutant components of the Sim4-complex, such as Mis6, Mal2 and Sim4 and probably reflects defects in the assembly of the kinetochore (Allshire *et al.*, 1995; Jin *et al.*, 2002; Pidoux *et al.*, 2003). The temperature sensitive *fta2* mutants show a very high number of aberrant mitosis with no or unequal separation of the condensed chromatin indicating the important role of Fta2 in kinetochore function. The high frequency of mitotic cells with non-separated chromatin on an elongating spindle (nearly 50 % in the *fta2-291* strain) has so far not been observed for other mutant components of the Sim4 complex and implies that kinetochore function is severely affected in *fta2* mutants.

Analysis of the segregation behaviour of tagged *cen1* sister centromeres indicated that Fta2 is required for bipolar chromosome orientation. In addition, 44 % of cen1-GFP signals did not appear to be spindle associated in anaphase *fta2* mutants, implying that Fta2 also plays a role in linking the kinetochore to microtubule-plus-ends.

The spindle checkpoint monitors spindle-kinetochore interaction and becomes activated when the mitotic chromosomes are not under tension or/and are not microtubule associated (Cleveland *et al*., 2003; Musacchio and Hardwick, 2002). Given the phenotype of the *fta2* mutants one would expect activation of the spindle checkpoint in these strains. Indeed, we observed that in the absence of the spindle checkpoint component Mph1, the temperature-sensitivity and chromosome-missegregation phenotype of a *fta2* mutant strain was increased significantly. However, double mutants between *fta2-291* and *mad2Δ* behaved like the *fta2-291* single mutant. Recently, it has been shown that Mis6 is required for the association of Mad2 with the kinetochore during mitosis (Saitoh *et al*., 2005). As the Mis6 protein is not localised correctly in *fta2* mutants, we presume that the Mad2-dependent part of the spindle checkpoint pathway is impaired in *fta2* mutants and thus unable to sense the aberrant microtubule-kinetochore interactions.

Fta2 is a component of the 13-component Sim4 kinetochore complex, that comprises the previously identified proteins Mal2, Mis6, Sim4, Mis15 and Mis17 and the DASH component

Dad1 and the seven new Fta1-7 proteins (Liu *et al.*, 2005). Apart from the non-essential Dad1 protein, all other Sim4-complex components analysed to date are essential for precise chromosome transmission. However the phenotypes caused by various mutant alleles coding for Sim4 complex components are not identical (Fleig *et al.*, 1996; Goshima *et al.*, 1999; Hayashi *et al.*, 2004; Jin *et al.*, 2002; Liu *et al.*, 2005; Pidoux *et al.*, 2003; Sanchez-Perez *et al.*, 2005). For example, *mal2-1* and *sim4-193* mutants are hypersensitive to microtubule destabilizing drugs while others, such as *mis6-302* or *fta2* mutants are not (Jin *et al.*, 2002; Pidoux *et al.*, 2003; our unpublished data). By analysing the interactions between Fta2 and other members of the Sim4 complex we have started to identify functional subgroups within this complex (Figure 6F). In particular, the tight functional interaction with Mal2 was not observed for other members of this complex. *mal2*$^{+}$ over expression strongly suppressed the ts phenotype of the *fta2* mutants in a dosage dependent manner. The same held true for the rescue of the *mal2-1* ts phenotype by extra *fta2*$^{+}$. Furthermore, kinetochore localisation of these proteins was absolutely dependent on each other. These data imply that Mal2 and Fta2 work together in a subgroup of the Sim4 complex. As *fta2 mal2-1* double mutants could not be obtained at any temperature, *fta2* and *mal2* mutants require the presence of the other wild-type partner protein for survival at the permissive temperature.

Such a close functional interaction has not been observed for any other members of the Sim4 complex. For example, all double mutants of essential Sim4 complex components generated to date were viable at the permissive temperature (22-26 °C) and showed growth impairment only at higher temperatures (Hayashi *et al.*, 2004; Pidoux *et al.*, 2003) (Figure 5B).

Furthermore, reciprocal suppression has not yet been observed for any other members of the Sim4 complex. Although Mis6, Mis17 and Mis15 proteins show strong co-immunoprecipitation and depend on each other for correct kinetochore localisation, they show no suppression of each others mutant phenotype (Hayashi *et al.*, 2004). Extra Sim4 protein is able to rescue the ts phenotype of a *mis6-302* mutant, however the converse is not true (Pidoux *et al.*, 2003). We have shown that *fta2*$^{+}$ or *mal2*$^{+}$ over expression did not rescue the ts phenotype of *sim4-193*, *mis15-68* and *mis17-362* mutant strains and that extra *fta2*$^{+}$ in the *mis6-302* mutant gave rise to a synthetic lethal phenotype. Interestingly, *gfp*-tagged *fta2*$^{+}$ in *mis17-365* and *mis15-68* but not other Sim4 component strains resulted in an increased ts sensitivity of these strains (our unpublished data), possibly implying that Fta2 and Mis17/Mis15 are in close proximity to each other.

Kinetochore localisation of Fta2 was absolutely dependent on Mal2, strongly dependent on functional Mis15, Mis17 and Mis6 proteins but unaffected in *sim4* mutant cells implying that the Sim4 complex is build up in a hierarchical manner (Figure 6F).

Interestingly, we found that wild-type like kinetochore localisation of Fta2 is dependent on the presence of a functional Spc7 protein. Spc7, which was isolated as a suppressor of the EB1 family member Mal3 and plays a role at the microtubule-kinetochore interface, is closely associated with the Ndc80 and MIND complexes (Kerres *et al.*, 2004; Liu *et al.*, 2005; Obuse *et al.*, 2004). Our data thus indicate an interaction between the Ndc80-MIND-Spc7 and Sim4 complexes, which is possibly mediated via Spc7 and Fta2. The functional significance of this interaction awaits further analysis; however given the finding that Spc7 associates with the microtubule-plus end associating protein Mal3 and the Sim4 complex Fta2 is required for kinetochore association of the DASH complex, it is possible that this interaction contributes to the dynamic microtubule-kinetochore interface.

Acknowledgements

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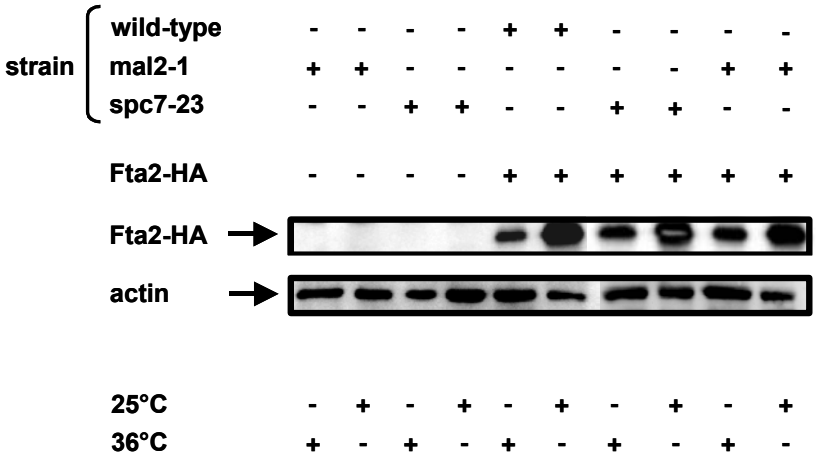
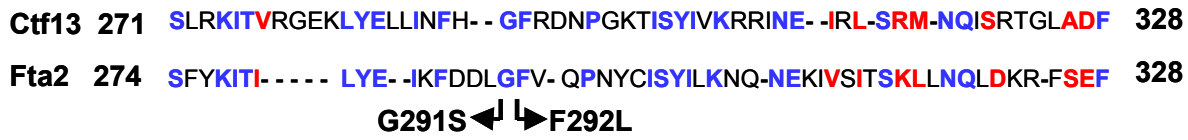
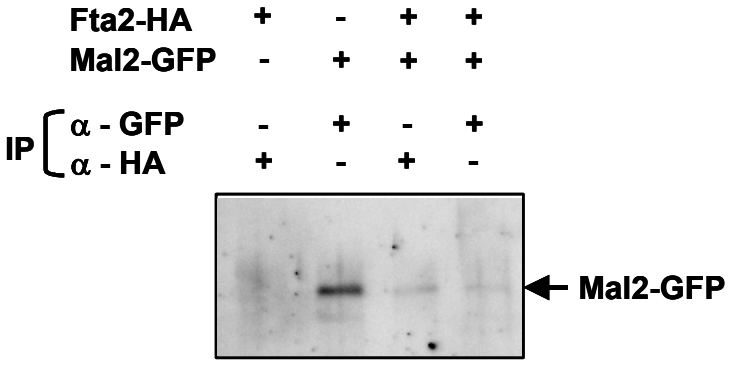
Supplementary Figures



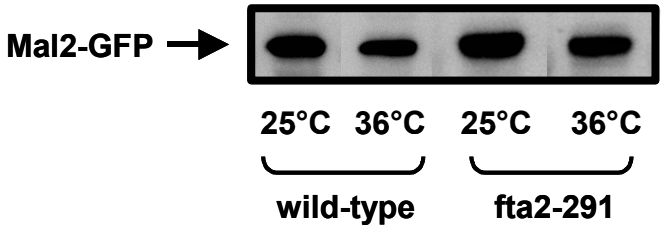
Supplementary Figure 1. Fta2 protein levels are unaltered in *spc7-23* and *mal2-1* mutant cells.
Protein extracts prepared from wild-type, *mal2-1* or *spc7-23* strains which expressed Fta2-HA endogenously were used for immunoprecipitations using an anti-HA-antibody. The immunoprecipitates were analysed by Western blot analysis using an anti-HA antibody. Strains were grown at 25 °C or shifted to 36 °C for 6 hours. Protein extracts used had very similar protein concentrations. Actin was used as a loading control.



Supplementary Figure 2. Sequence alignment of *S. pombe* Fta2 and *S. cerevisiae* Ctf13 proteins.
Blue letters denote identical residues, red letters indicate similar amino acids. The amino acid changes found in the Fta2-291 and Fta2-292 mutant proteins are indicated.



Supplementary Figure 3. Fta2-HA co-immunoprecipitates Mal2-GFP.
Co-immunoprecipitation of Fta2 and Mal2. Protein extracts from strains expressing Mal2-GFP, Fta2-HA or both were used for immunoprecipitation (IP) with an anti-HA or anti-GFP antibody. The immunoprecipitates were resolved by SDS-Page and probed with an anti-GFP antibody.



Supplementary Figure 4. Mal2-GFP protein levels are unaltered in *fta2* mutant cells.
Protein extracts prepared from wild-type or *fta2-291* strains which expressed Mal2-GFP were used for immunoprecipitations using an anti-GFP-antibody. The immunoprecipitates were analysed by Western blot analysis using an anti-GFP antibody. Strains were grown at 25 °C or shifted to 36 °C for 6 hours.

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8 Lebenslauf

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Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 30.08.06

(Anne Kerres)