

The Role of Fission Yeast Nuclear Actin-Related Protein in Mitosis

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Abstract

Nuclear actin-related proteins (Arps) have 20-30% identity to conventional actin and many are found to be in chromatin remodelling complexes. There are two families of chromatin remodelling complexes, one of which carries out covalent modification on histones such as acetylation. The other is an ATPase complex, which alters the nucleosomal spacing, and nuclear Arps are found in both complexes. These complexes are believed to be required for transcriptional activation by increasing the accessibility of the transcription machinery to the target DNA.

The *alp5-1134* mutant was isolated from a screen for temperature-sensitive (*ts*) mutants with altered polarity and shows severe mitotic defects. Cloning of *alp5*⁺ revealed that Alp5 is an essential actin-related protein, most similar to budding yeast Arp4 and human BAF53. Alp5 localises to the nucleus and immunoprecipitates with Mst1, a histone acetyltransferase. These results strongly indicate the role of Alp5 in chromatin remodelling process, as its homologues.

Given the interaction between Alp5 and Mst1, its function in histone acetylation was investigated both genetically and biochemically. It was found that Alp5 is required for acetylating the N-terminus tail of histone H4 lysine residues, and functionally counteracts with the histone deacetylases Clr6, Hst4 and Sir2.

At the restrictive temperature, the *alp5-1134* mutant shows a mitotic delay due to the activation of a spindle assembly checkpoint, which suggests a defect in the kinetochore-spindle interaction. This study also reveals that the function of Alp5 is required for the transcriptional repression at the core centromere region. Possible roles of Alp5 in mitosis are discussed.

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Abbreviations

5'-FOA	5'-fluorootic acid
APC/C	Anaphase promoting complex/cyclosome
ARP	Actin-related protein
CDK	Cyclin-dependent kinase
CFP	Cyan fluorescent protein
ChIP	Chromatin immunoprecipitation
CRC	Chromatin remodelling complex
DAPI	4', 6-diamidino-2-phenylindole dihydrochloride
EMM	minimal medium
GFP	Green fluorescent protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HU	Hydroxy-urea
IP	Immunoprecipitation
MAPs	Microtubule-associated proteins
MNase	Micrococcal Nuclease
PCR	Polymerase chain reaction
RFP	Red fluorescent protein
SAC	Spindle assembly checkpoint
SPB	Spindle pole body
TBZ	Thiabendazole
TSA	Trichostatin A
H4K16	H4 lysine 16
H3K9	H3 lysine 9
UV	Ultraviolet
YE5S	Yeast extract 5 supplements

Chapter 1

Introduction

Mitosis transmits an equal set of DNA to daughter cells in every cell division. Avoiding chromosome missegregation that would most likely lead to aneuploidy, a phenomena often observed in cancer cells (Cahill, Lengauer et al. 1998; Lee, Trainer et al. 1999), is imperative to maintaining genomic stability.

Alp5 protein of *Schizosaccharomyces pombe* (*S. pombe*) is an essential nuclear actin-related protein that is most homologous to budding yeast Arp4 and human BAF53, which are both present in chromatin modification complexes. Alp5 was identified from a morphology screen, the mutant of which displayed both morphology and mitotic phenotypes. This thesis will describe the work that has been carried out to determine the mitotic roles of Alp5 in fission yeast *S. pombe*.

1.1 Fission yeast cell cycle

Fission yeast cells are usually haploid and contain three chromosomes the size of 5.7, 4.7 and 3.5 Mb. During vegetative growth, where the conditions are optimal, fission yeast spend approximately 70% of the time in G2 phase of the cell cycle (2n DNA content), 10% in S phase (DNA replication), 10% in M phase (mitosis) and 10% in the G1 phase (1n DNA content) (Nasmyth, Nurse et al. 1979) (see Figure 1.1.1). A major advantage of studying fission yeast lies in the similarity of the mitotic cell cycle to higher eukaryotes.

Transitions of one phase of the cell cycle to the next are controlled by so-called checkpoints. Checkpoints ensure that the conditions are optimal to proceed with the cell cycle, which arrests the cell cycle otherwise. For example, a checkpoint would monitor environmental conditions (e.g. nutrients) and cell

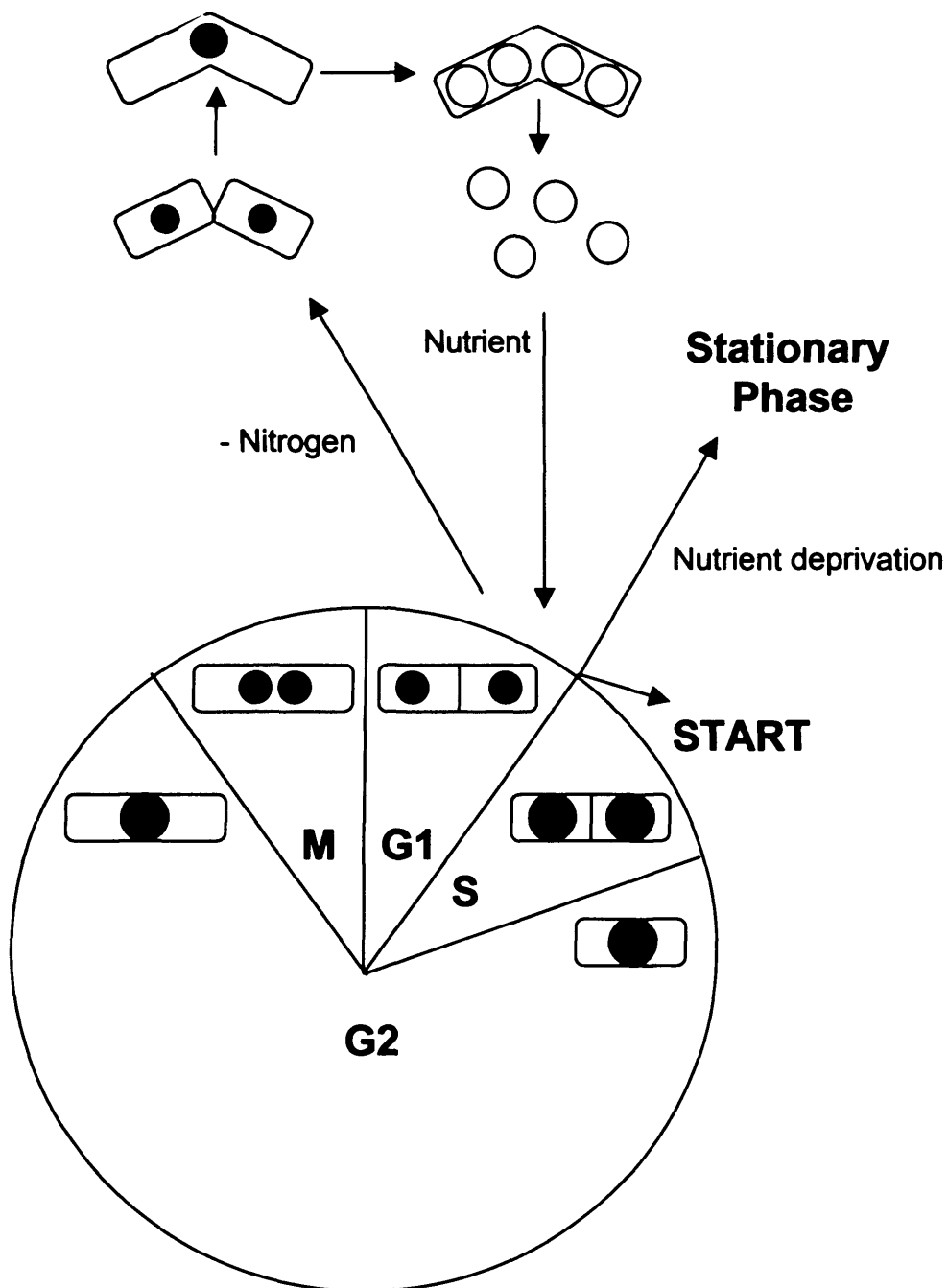


Figure 1.1.1 Life cycle of fission yeast. Haploid mitotic cell cycle (bottom) is divided into four phases of G1, S, G2 and M. Relevant duration of each phase is indicated by the area content. Haploid cells mate to form a diploid zygote followed by meiosis and sporulation in the absence of optimal nutrients (top).

size. If both are above the critical threshold, the cell enters S phase; this process is referred to as passing through Start and describes the commitment of the cell to mitosis. Under a poor nutrient condition, a cell will not pass Start but instead will go into a stationary phase or enter a sexual cycle and meiosis in the presence of a compatible mating type (see Figure 1.1.1). Once the cell passes through Start, it is committed to the mitotic cell cycle and is not able to go into the stationary phase or the sexual cycle until a round of cell cycle is completed (i.e. in the next G1).

1.2 **Mitosis**

Mitosis can be divided into four phases; prometaphase (chromosome condensation), metaphase (chromosome alignment), anaphase (chromosome segregation) and telophase (chromosome decondensation). During interphase, (not in mitosis) fission yeast contains three to four cytoplasmic microtubules parallel to the cell axis. These microtubules disappear rapidly upon mitotic entry, and a short intranuclear spindle is formed in prometaphase. The mitotic spindle consists of two sets of microtubules emanating from the duplicated spindle pole bodies (SPBs) that are embedded in the nuclear membrane. One set of microtubules are the polar microtubules that emanate from the opposite poles. They consist of both continuous pole-to-pole and non-continuous microtubules that form an overlapping mid-zone. The other set of microtubules are the pole-to-kinetochore microtubules, which are essential for chromosome separation in anaphase.

Metaphase

During metaphase, each condensed chromosomes are captured by the mitotic spindles and align in between the two SPBs. It is vital that all six kinetochores of sister chromatids are captured by the spindle to segregate chromosomes accurately to the opposite poles. If anaphase is initiated prior to the completion of this capturing process, this will lead to unequal chromosome segregation resulting in lagging chromosomes (uncaptured sister chromatids that are not

pulled by the spindle to the pole). This has been observed in some mitotic mutants such as those required for the kinetochore formation.

Anaphase

The phase in which sister chromatids are separated to the opposite poles is referred to as Anaphase. This period can be split into two sub-phases: Anaphase A and B. Anaphase A occurs when the sister chromatids move to the opposite poles whilst the length of the pole-to-pole microtubule is kept constant to that of the nuclear span. This movement is due to depolymerisation of the spindle at both ends, at the kinetochore and at the SPB. Anaphase B involves the movement of the SPBs with sister chromatids to the opposite ends of the cell.

There are two crucial factors for anaphase onset to occur. The first requirement is the destruction of cyclin B, called Cdc13 in fission yeast. Cdc13 is required for mitotic entry as a complex with Cdc2, which needs to be degraded by APC/C (anaphase promoting complex/cyclosome; an E3 ubiquitin ligase) in order to exit mitosis. Secondly, cohesion has to be resolved for anaphase onset. This requires poly-ubiquitination-dependent degradation of Securin (Cut2 in fission yeast) by APC/C (Funabiki, Yamano et al. 1996; Yamano, Gannon et al. 1996). Thus, the activity of APC/C is crucial for anaphase onset.

Telophase and Cytokinesis

Following anaphase, mitotic microtubules go through dramatic reorganisation to form cytoplasmic microtubules in telophase. At the beginning of telophase, an X-shaped microtubule is formed in the cytoplasm and a microtubule ring is formed at the division site. A septum is then formed across the centre of the cell and cell division occurs when this septum is cleaved by cell wall invagination. In rapidly growing cells, by the time the cells are septated, the two daughter cells would already have carried out DNA replication and are poised in G2 to enter mitosis. Another dynamic organisation of cytoplasmic microtubules is observed to form an array of longitudinal cytoplasmic microtubules.

1.3 Spindle assembly checkpoint

Anaphase segregates sister chromatids to transmit the same sets of information to each daughter cell for inheritance. If this step is not carried out in a faithful manner, it often leads to aneuploidy and eventual cell death, thus its accuracy is an absolute essential. It therefore becomes important to establish a stable spindle microtubule attachment to each and every sister kinetochore, and that each kinetochore is captured by the spindle emanating from the pole of destination, a state referred to as bi-oriented or amphitelic attachment. The metaphase to anaphase transition only occurs when such state is established at all the kinetochores, an event monitored by the spindle assembly checkpoint (SAC). Activation of the SAC leads to cell cycle arrest prior to anaphase through the inhibition of APC/C. In the presence of one or more unattached kinetochores, Mad2 is known to bind to the mitotic activator of APC/C, Cdc20 (fission yeast Slp1), which prevents its binding to APC/C and consequently the activation of APC/C (He, Patterson et al. 1997; Fang, Yu et al. 1998; Kim, Lin et al. 1998). In the absence of APC/C activity, Cdc13 and cohesin degradation, which are essential for anaphase onset and mitotic exit, are prevented, thus arresting cell cycle prior to anaphase (Yamano, Gannon et al. 1996).

Extensive studies have been carried out on the mechanism of SAC in both yeast and mammalian cells. The Mad1-3 (Mitotic arrest deficient, Mad3 is called BubR1 in vertebrate), Bub1 (budding uninhibited by benzimidazole), Bub3 and Mps1 (fission yeast Mph1 homologue) proteins were first identified in budding yeast and are conserved from yeast to humans (Hardwick 1998). The kinetochore functions as a site for signal transduction for the SAC, where the 'wait anaphase' signal is emitted in the absence of microtubule attachment, and most likely in the absence of tension at the kinetochore. Mad2 is targeted to such sites by Mad1, with which it forms a complex throughout the cell cycle (Chen, Shevchenko et al. 1998; Chen, Brady et al. 1999). Two additional complexes have also been characterised in budding yeast, which are formed transiently during the cell cycle at around the time chromosome bi-orientation is established. These are Mad1/Bub1/Bub3 complex (Brady and Hardwick 2000) and Mad2/Mad3/Bub3/Cdc20 complex (also called the MMC, mitotic checkpoint

complex, in vertebrates) (Fraschini, Beretta et al. 2001). Both complexes accumulate in cells treated with nocodazole, a microtubule-depolymerising drug, suggesting their stimulation by the presence of unattached kinetochores. Experiments measuring fluorescence recovery after photobleaching have shown that mammalian Mad2 turnover at the kinetochore is very fast and its half-life was determined to be ~24 s (Howell, Hoffman et al. 2000). Turnover rates for other mammalian checkpoint proteins (BubR1/Mad3, Bub3 and Cdc20) are also of similar magnitude (Kallio, Beardmore et al. 2002).

In fission yeast, about three microtubules are known to attach at each kinetochore (Ding, McDonald et al. 1993), compared with 10-45 in mammalian cells (Rieder 1982). Each budding yeast kinetochore, however, binds only one microtubule. Bi-oriented attachments are obtained through a 'trial and error' process, where dynamic microtubules 'search and capture' kinetochores. The chromosomes are observed to be very dynamic during prometaphase and metaphase. The rapid oscillatory movement of the chromosomes in between the two poles is known as directional instability (Rieder and Salmon 1998). This movement occurs during an error correction process of the kinetochore-to-spindle attachment, which corrects the wrongly attached spindle microtubules by initiating their detachment from the kinetochores, a process that requires the activity of Aurora kinase (Biggins, Severin et al. 1999; Tanaka, Rachidi et al. 2002). In theory, four different attachments can be obtained; amphitelic, monotelic, syntelic, and merotelic. Monotelic attachment occurs when one sister kinetochore is attached but not the other. Syntelic attachment is obtained when both of the sister kinetochores are attached to microtubules emanating from the same pole. If one sister kinetochore is captured by the spindles emanating from both poles, this is referred to as merotelic attachment (see Figure 1.3.1). It is of note that merotelic attachment can also be referred to the situation where although both of the sister kinetochores are attached to the microtubules, one of the kinetochores is captured by both poles. The correction process appears to be repeated until all the kinetochores form amphitelic attachments.

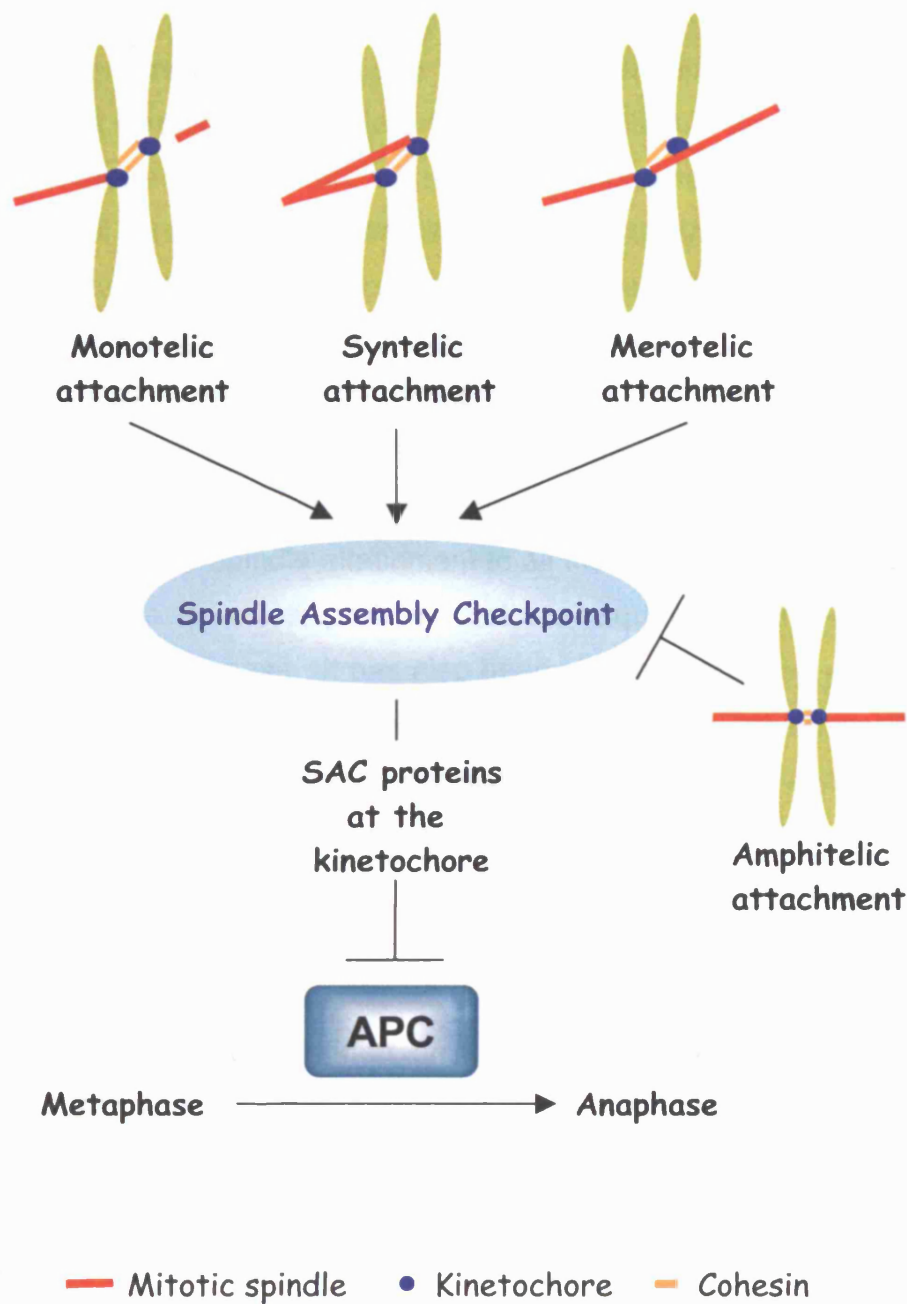


Figure 1.3.1 Spindle assembly checkpoint. Any attachment that is not amphitelic (bioriented and bipolar) is detected by the spindle assembly checkpoint (SAC), and the cell cycle is arrested prior anaphase through SAC protein localisation at such kinetochores and APC/C inhibition.

1.4 Centromeres

The centromere region was a site originally defined in 1880 by Walther Flemming as a cytologically visible primary constriction in the chromosome, as observed in the famous human X shaped chromosomes. The centromere is now defined as a region where the kinetochore is assembled - a large protein complex structure required for accurate chromosome segregation. There are at least three essential functions of a kinetochore. Firstly, it is a site of spindle microtubule attachment to the chromosome, required for the movement of the chromosomes during anaphase. Secondly, it is the site of the SAC machinery, which is activated to arrest the cell cycle prior to anaphase onset in the absence of correct microtubule spindle attachment to all the kinetochores. Lastly, it is the site of localisation for motor proteins that are required for the chromosome movement during anaphase. It has also been shown in budding yeast that the kinetochore has a role in establishing and maintaining a cohesion site at the outer centromere regions between the sister chromatids until anaphase onset (Weber, Gerton et al. 2004). Thus, a centromere is an essential structure for chromosome segregation as a platform to the kinetochore and its subsequent cohesion establishment.

How the centromeres are defined on the chromosomes is still unclear. In budding yeast, 125 bp DNA sequence that is conserved across all the chromosomes appears to be the primary determinant. In other eukaryotes, despite a huge effort in trying to determine the primary centromere sequence, a conserved sequence across the chromosomes does not seem to exist. However, the centromeric DNA sequences contain tandem repeats of 171 bp α -satellites (Koch 2000), which have been shown to be important for centromere function (Schueler, Higgins et al. 2001). The absence of a primary sequence is in fact consistent with phenomena such as the formation of neocentromeres, the ectopic centromeres that originate occasionally from normally non-centromeric chromosomal region (Voullaire, Slater et al. 1993). A repeat-rich region appears to be important for centromere function, since human α -satellite repeats are able to trigger de novo kinetochore assembly (Harrington, Van Bokkelen et al. 1997; Ikeno, Grimes et al. 1998). The question is how a single

centromere is identified and formed on each chromosome. One main determinant appears to be the specific localisation of the conserved histone H3 variant protein CENP-A to the centromere. It is a centromere-specific histone found in all eukaryotes examined to date, which replaces the major histone H3 subunits in a specialised octamer consisting of histones H4 and probably H2A and H2B (Westermann, Cheeseman et al. 2003), that has also been shown to be important for kinetochore protein recruitment (Van Hooser, Ouspenski et al. 2001). One of the original ideas regarding CENP-A incorporation was that centromeric DNA replication is coupled to CENP-A deposition, however, some of the recent studies do not seem to support this idea (Lo, Craig et al. 2001; Smith 2002), and is yet to be clarified.

Since there is no primary DNA sequence determining the centromere, the epigenetic status of the centromeric chromatin has proven to be essential for a functional centromere and kinetochore (see below), as well as neocentromere formation (Voullaire, Slater et al. 1993; Bjerling and Ekwall 2002). Epigenetic mechanisms include DNA methylation, histone acetylation and RNA interference, which result in heritable gene activation and inactivation without a change in the DNA sequence. Much of the repetitive centromeric DNA in metazoans has been shown to assemble into heterochromatin composed of hypoacetylated nucleosomes. These nucleosomes are found to contain the hallmark of the heterochromatin, methylated histone H3 at lysine 9 (H3K9) coated with the heterochromatin protein HP1 (Wreggett, Hill et al. 1994; Minc, Allory et al. 1999; Jacobs, Taverna et al. 2001; Maison, Bailly et al. 2002).

RNAi-mediated centromeric heterochromatin formation

Heterochromatin consists of DNA sequences with little or no coding potential, repeated thousands of times, and silenced by covalent modification of DNA and histones. From this, it was long thought that there is little or no transcription activity at these regions. Recent studies from *S. pombe*, however, have revealed one regulatory mechanism for heterochromatin formation and heterochromatic gene silencing that relies on the RNA interference (RNAi) pathway, and that it requires transcription from centromeric flanking repeats. Although RNAi pathway has been originally regarded as a post-transcriptional

process, responsible for post-transcriptional gene silencing through mRNA targeting and degradation, it has emerged that RNAi pathway can also be carried out to silence genes at a transcriptional level.

RNAi machinery includes Argonaute (Ago), which is a small RNA-binding protein, a double stranded RNA (dsRNA) ribonuclease called Dicer (DCR), and an RNA-dependent RNA polymerase (RdRP). The pathway involves processing of dsRNA into small interference RNA (siRNA) by Dicer, which is consequently loaded onto an effector complex called RISC (RNA-induced silencing complex) that contains an Argonaute and targets homologous mRNAs for degradation (Hannon 2002; Zamore 2002).

Fission yeast contains three regions where heterochromatic silencing is observed: at the centromeres, telomeres and mating type loci. Fission yeast *ago1*, *dcr1* and *rdp1* mutants are viable but were shown to be defective in silencing reporter genes inserted into centromeric repeats (*dg/dh*). They also show disrupted heterochromatin assembly, which correlates with the loss of Swi6 and H3K9 methylation (Volpe, Kidner et al. 2002; Hall, Noma et al. 2003). In contrast to the belief that heterochromatin is transcriptionally inactive, it was shown in *S. pombe* that both DNA strands of centromeric repeats are transcribed (Volpe, Kidner et al. 2002). In support of this notion, siRNA corresponding to centromeric repeats were also identified (Reinhart and Bartel 2002). In all three RNAi mutants mentioned above, Swi6 and methylation on H3K9 are lost from the centromeric reporter genes, indicating the requirement of RNAi for heterochromatic modifications (Reinhart and Bartel 2002).

Biochemical purification of chromodomain complexes in fission yeast recently identified RITS (RNAi-induced transcriptional gene silencing) complex, which includes Ago1, Chp1 (a heterochromatin-associated chromodomain protein) and Tas3 (Verdel, Jia et al. 2004). The complex was shown to directly bind to the outer centromere region and was shown to be required for the centromeric heterochromatin formation (Schramke and Allshire 2003; Verdel, Jia et al. 2004). In addition, the RITS complex also contains siRNA from centromeric repeats, the generation of which is dependent on Dicer. These siRNAs are required for the localisation of the RITS complex to the heterochromatic domains, suggesting their importance in complex targeting. In addition to the RNAi pathway, silencing at the outer centromere regions also require Clr3 and

Clf6 HDACs, histone H3K9 methyltransferase Clr4, and HP1 homologue Swi6 (Grewal 2000). Current model on RNAi-dependent heterochromatin formation is depicted in Figure 1.4.1.

Another study elegantly demonstrated a supporting notion for the RNAi pathway in heterochromatin formation. A hairpin RNA construct was generated, which was sufficient to silence the homologous locus, that is normally euchromatic, in trans (Schramke and Allshire 2003). This silencing was shown to be RNAi pathway and Clr4-dependent to form heterochromatin through Swi6 recruitment (Schramke and Allshire 2003). Thus, RNAi machinery appears to play an essential role in establishing heterochromatin structure at the centromeres.

Budding yeast centromere and kinetochore

A kinetochore consists of three layers; outer, middle and inner kinetochore, which are built on the core centromere. Without doubt, budding yeast centromere and kinetochore are the most extensively studied so far. Its centromere consists of a conserved 125 bp *cis*-DNA element among the different chromosomes that is both necessary and sufficient for kinetochore assembly (Carbon and Clarke 1990). The 125 bp are divided into three conserved regions; CDEI, CDEII and CDEIII (Fitzgerald-Hayes, Clarke et al. 1982) (Figure 1.4.2.A), where CDEI and CDEIII are conserved imperfect palindromes. CDEI is ~15 bp sequence bound by centromere-binding factor 1 Cbf1 as a homodimer, which has a similarity to CENP-B (Mellor, Jiang et al. 1990). Cbf1 has been shown to be required for both transcriptional regulation and chromosome segregation (Cai and Davis 1990). Although Cbf1 is not essential for the kinetochore function, it has been shown to induce DNA bending (Niedenthal, Sen-Gupta et al. 1993), which may contribute to the higher order structure of the kinetochore. Mammalian CENP-B also binds centromeric DNA and is able to induce bending (Tanaka, Nureki et al. 2001).

The non-conserved CDEII sequence varies from 78-86 bp, composed of AT rich DNA, and is the region where CENP-A homologue Cse4 binds, which is essential for chromosome segregation (Stoler, Keith et al. 1995; Ortiz, Stemmann et al. 1999; Cheeseman, Drubin et al. 2002).

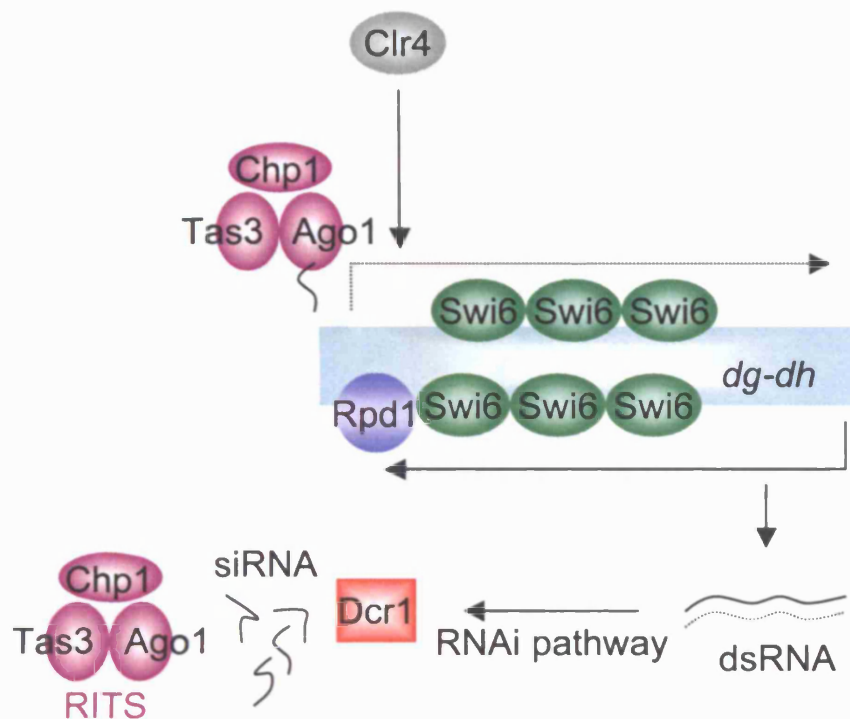


Figure 1.4.1 A proposed model for siRNA-dependent initiation of heterochromatin assembly by RITS. Dcr1-produced siRNA from *dg-dh* transcript is thought to target the RITS complex to the outer centromere. [Adapted from Ekwall 2004.]

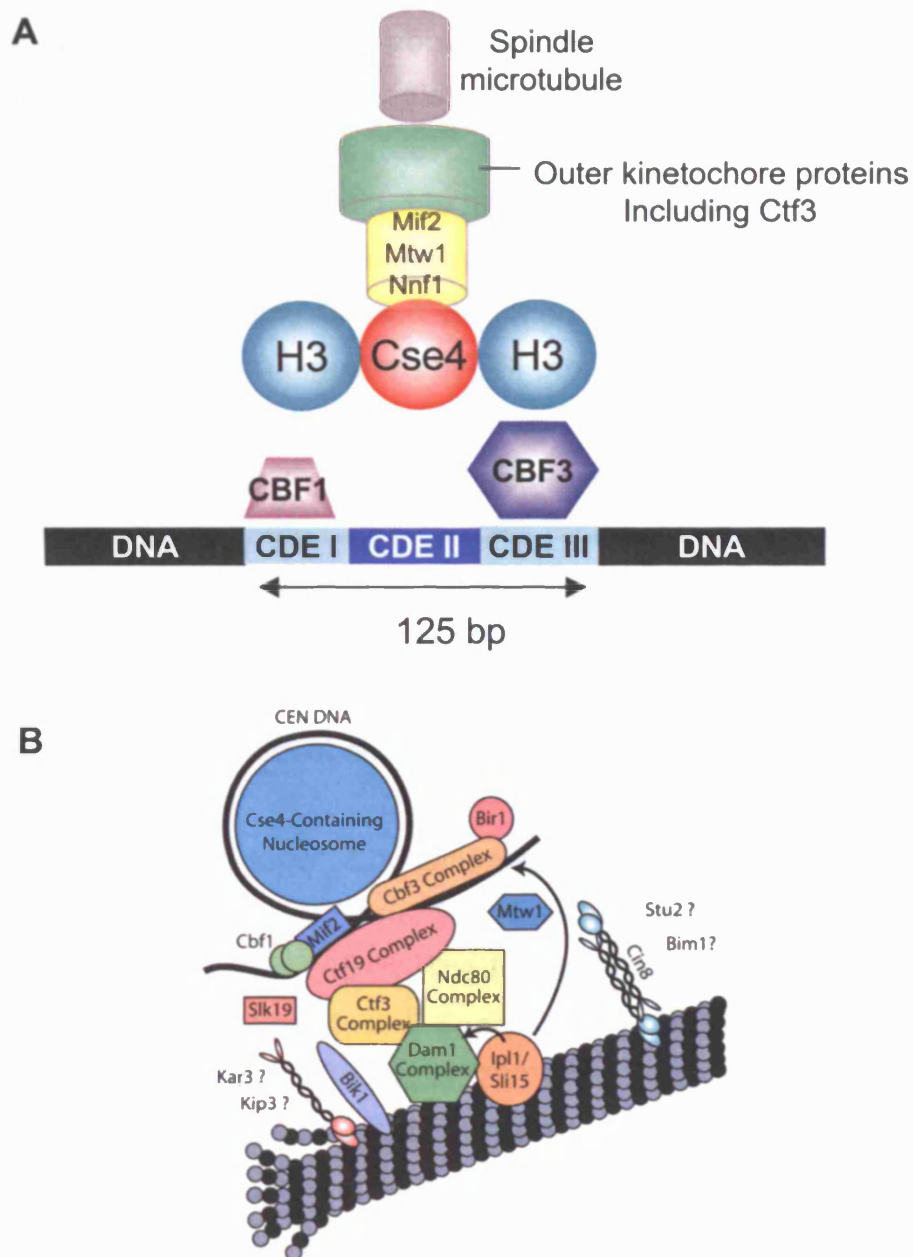


Figure 1.4.2 Budding yeast centromere **A** Budding yeast centromere is located on an essential 125 bp DNA, containing three conserved regions, CDE I, CDE II and CDEIII. CBF3 is essential for the localisation of all other kinetochore proteins, the binding of which to CDEIII also determines Cse4 localisation. [Adapted from Amor *et al.* 2004 Trends in Cell Biol. and Cheeseman *et al.* 2002 J. Cell Biol.]

CDEIII is the only essential element composed of ~25 bp, where a point mutation within this sequence abolishes the centromere function (Ortiz, Stemmann et al. 1999). CDEIII region acts as a binding site for the essential CBF3 complex (which contains Ndc10, Cep3, Ctf13 and Skp1) (Lechner and Carbon 1991; Goh and Kilmartin 1993; Connelly and Hieter 1996), which is required for the subsequent binding of all other kinetochore proteins to the centromeric DNA, including Cse4 (Ortiz, Stemmann et al. 1999) (see Figure 1.4.2.B). Loss of function mutations in CBF3 proteins lead to disruption in the microtubule-kinetochore attachments in vitro and in vivo (Goh and Kilmartin 1993; Sorger, Doheny et al. 1995; He, Rines et al. 2001). Thus in budding yeast, the recruitment of the CBF3 complex (and not Cse4) appears to be the primary determinant of centromere/kinetochore formation, which in turn is determined by the CDE III sequence. This is in contrast to other eukaryotes where CENP-A appears to be the primary determinant, and not the DNA sequence (Ortiz, Stemmann et al. 1999; Measday, Hailey et al. 2002).

The central kinetochore layer of budding yeast consists of Ctf19 complex, Ctf3 complex and Ndc80 complex (Ortiz, Stemmann et al. 1999; Janke, Ortiz et al. 2001; Wigge and Kilmartin 2001). The Ndc80 complex contains four protein subunits, Ndc80, Nuf2, Spc24, and Spc25, all of which are essential for the kinetochore function (Janke, Ortiz et al. 2001). Mutants of Ndc80 complex are completely defective in chromosome segregation and show complete detachment of the chromosomes from the microtubules, similar to CBF3 mutants (He, Rines et al. 2001; Janke, Ortiz et al. 2001; Wigge and Kilmartin 2001). The mutants of these proteins are also checkpoint defective (Wigge and Kilmartin 2001), suggesting their role in checkpoint proteins recruitment to the kinetochores. The Ndc80 complex is also required for the recruitment of the DASH complex (also known as Dam1 complex) to the kinetochore (Cheeseman, Brew et al. 2001; Janke, Ortiz et al. 2001), which is localised at the outer kinetochore amongst other proteins that include the motor proteins, and other regulators of microtubule dynamics (Cheeseman, Brew et al. 2001).

1.5 Cohesion

Throughout DNA replication in S phase and up to anaphase, the sister chromatids must remain together in order to achieve a faithful genetic transmission into two daughter cells. Evolution has developed a clever and efficient way to keep the two sister chromatids together by “gluing” them with a set of proteins called cohesin. The cohesin complex contains at least four proteins (Scc1/Rad21, Scc3/Psc3, Smc1/Psm1 and Smc3/Psm3 in budding and fission yeast, respectively) that have been postulated to form a ring around the sister chromatids (Lengronne, Katou et al. 2004; Nasmyth and Schleiffer 2004).

In fission and budding yeasts, cohesin dissociates from the chromosomes through cleavage of the cohesin subunit Rad21/Scc1 by the protease separase/Cut1/Esp1 (Uhlmann, Lottspeich et al. 1999). The timing of Rad21/Scc1 cleavage is crucial for faithful chromosome segregation. Thus, the activity of separase is controlled by securin/Cut2/Pds1 (Cohen-Fix, Peters et al. 1996; Funabiki, Kumada et al. 1996; Funabiki, Yamano et al. 1996; Ciosk, Zachariae et al. 1998; Kumada, Nakamura et al. 1998). When securin is degraded through proteolysis by APC/C^{Cdc20}, separase becomes free to cleave Rad21/Scc1 (Cohen-Fix, Peters et al. 1996; Funabiki, Yamano et al. 1996; Ciosk, Zachariae et al. 1998; Tomonaga, Nagao et al. 2000), which is both necessary and sufficient for anaphase onset (Uhlmann, Lottspeich et al. 1999).

Cohesion is established both along the chromosome arms and at the centromeres. However, the timing of cohesion degradation at these two regions differs in different organisms. In mammalian cells, most of the cohesin complex is dissociated from chromosomal arms before metaphase (Losada, Hirano et al. 1998; Schmiesing, Ball et al. 1998; Darwiche, Freeman et al. 1999). This results in the familiar X-shaped chromosome at metaphase, since the only factor keeping the two sister chromatids together at metaphase is cohesion at the centromeres. In budding yeast, cohesin is found both at the centromere region and along the chromosomal arms until anaphase, although cohesin concentration is much higher at the centromeres (Blat and Kleckner 1999). Fission yeast chromatids also appear to have more concentrated

cohesin at the centromere and pericentric region compared to the arm region, but the protein level of Rad21 associated with these regions does not seem to change from metaphase to anaphase (Tomonaga, Nagao et al. 2000). However, a small proportion (<5%) of Rad21 is cleaved at the onset of anaphase, which is in fact essential for proper anaphase progression, and an overproduction of Rad21 blocks sister chromatid separation (Tomonaga, Nagao et al. 2000).

Cohesion function in bipolar attachment of sister chromatids

Upon establishment of sister chromatids bi-orientation, tension is generated at the kinetochores. This is due to the stretching caused by microtubule depolymerisation in the presence of cohesin at the centromeres. Tension appears to be one of the factors that is sensed by the SAC, and in the absence of tension, for example, in the case of merotelic or syntelic attachments, the microtubules are proposed to be detached from the kinetochores in an aurora kinase-dependent manner to correct such attachments (Tanaka, Fuchs et al. 2000; Biggins and Murray 2001; Skoufias, Andreassen et al. 2001; Stern and Murray 2001; Tanaka, Rachidi et al. 2002; Zhou, Yao et al. 2002; Lens and Medema 2003; Dewar, Tanaka et al. 2004). In the absence of cohesin, bi-orientation is established at a much lower rate. Sister chromatids of cohesion mutant were observed to reside at the same pole frequently, and the pole swapping movement of the sister chromatids that normally occurs in the wild-type was not observed (Tanaka, Fuchs et al. 2000). These observations suggest the important role of cohesin in efficient establishment of bi-orientated kinetochore attachments.

In addition, cohesin is thought to play a more structural role of positioning or orienting the kinetochores properly so that they are facing the pole of destination, thereby increasing the chance of the kinetochores being captured by the spindle emanating from the pole of destination.

Chromatin

DNA is packaged into a higher ordered structure called the chromatin, the basic unit of which is called the nucleosomes. Each nucleosome is made of 147 bp of DNA wrapped around histone octamers, which consists of two copies of each histones H2A, H2B, H3 and H4 (Luger and Richmond 1998). Studies have revealed the importance of chromatin structure in transcription regulation and a picture of very dynamic chromatin is emerging. Chromatin dynamics is a consequence of a wide variety of complexes that modify histones, remove histone modifications, mobilise or stabilise nucleosomes, some of which are described below. Actin-related proteins have been identified as subunits of many chromatin modification complexes including histone acetyltransferase (HAT) and ATP-dependent chromatin remodelling complexes.

1.6 Actin-related proteins

Actin-related proteins (Arps) are defined as proteins with sequence similarity to conventional actin, which may range from 45% to 15% (Frankel and Mooseker 1996). The similarity resides mainly in the ATP-binding pocket at the core of the proteins, termed the 'actin fold', and thus, these proteins belong to the superfamily of ATPases. The actin fold is a flexible, hinged domain that is characterised by its ability to convert between conformational states in an ATP-dependent manner (Kabsch and Holmes 1995).

Studies have shown that nuclear Arps are found in many different chromatin modification complexes, including HAT and ATP-dependent chromatin remodelling complexes (see Figure 1.6.1) (Olave, Reck-Peterson et al. 2002). Various roles have been proposed for these proteins, including complex assembly, regulation of the catalytic activity of the chromatin modifying enzymes, and in the targeting of the complexes to the cytoskeletal-like nuclear architecture, called the nuclear matrix (Boyer and Peterson 2000; Olave, Reck-Peterson et al. 2002; Shumaker, Kuczmarski et al. 2003). The role of nuclear Arps in specific targeting of the complex is also intriguing. In vitro experiments have shown that budding yeast Arp4, for example, is able to bind to all four core

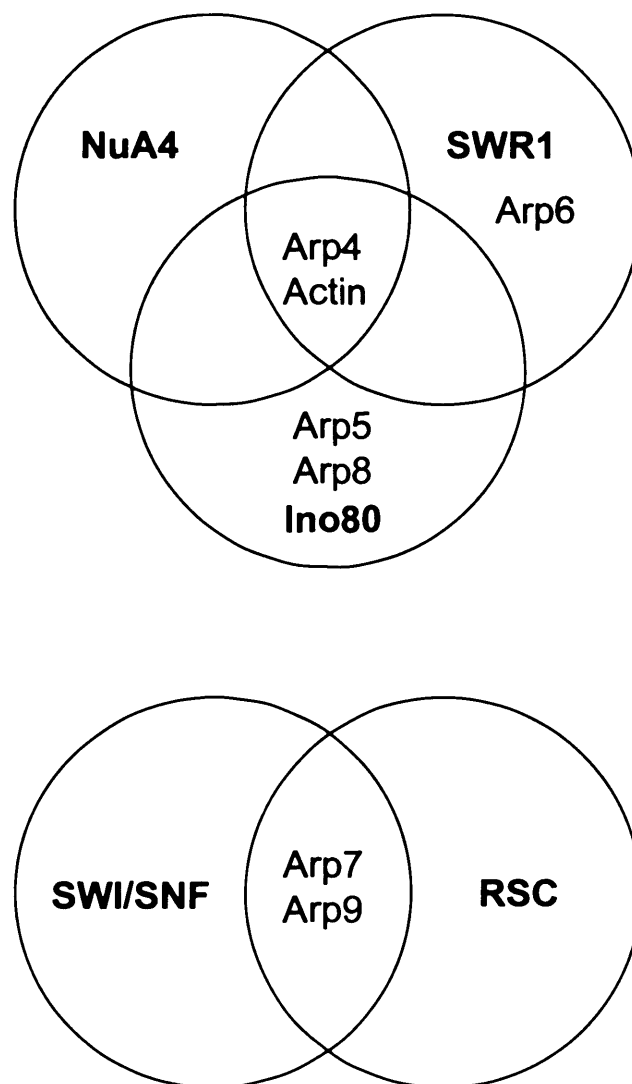


Figure 1.6.1 Chromatin modification complexes and actin-related proteins. A schematic diagram of the shared subunits of Arps amongst various chromatin modification complexes of both HAT and ATP-dependent chromatin remodelling complexes.

histones (Harata, Oma et al. 1999) and that many chromatin modifying complexes are known to be targeted to specific promoters (Hassan, Neely et al. 2001).

ARP family in budding yeast

There are ten Arps in *Saccharomyces cerevisiae* (*S. cerevisiae*) named Arp1 – 10 (Poch and Winsor 1997), where Arp1 is the most similar to conventional actin. Whereas the well-known proteins such as Arp2 and Arp3 are localised in the cytoplasm, most of the Arps characterised so far (Arp4 - Arp9) are nuclear proteins (Weber, Harata et al. 1995; Cairns, Erdjument-Bromage et al. 1998; Shen, Mizuguchi et al. 2000; Mizuguchi, Shen et al. 2004). Although it has been regarded as contamination for a long time, it is now generally accepted that actin itself is also found in the nucleus and comprise subunits of the chromatin modification complexes (Pederson and Aebi 2002), although its function is yet to be determined. Since different Arps have been found in various chromatin modification complexes, an interesting possibility is that different Arps are required to determine target specificity for each complex, but are also used as common subunits among different complexes at the same time.

Arp4

Arp4 is a nuclear protein throughout the cell cycle and is essential for cell viability (Harata, Karwan et al. 1994; Weber, Harata et al. 1995; Harata, Mochizuki et al. 1999). It was initially characterised as a *SPT* gene (suppressor of Ty). Ty1 (also called the δ -element) is a budding yeast retrovirus-like transposable element, which causes inactivation of adjacent genes upon its insertion (Winston and Carlson 1992) and *SPT* genes suppress this inactivation. Many of these genes have been shown to be involved in transcription regulation, for example, *SPT11* and *SPT12* encode histones (Clark-Adams, Norris et al. 1988) and *SPT3/7/20* encode components of Spt-Ada-Gcn5 HAT complex (Grant, Duggan et al. 1997). Certain mutations in the *arp4* gene suppress the transcription defect caused by δ -element insertion into *his4* promoter (*his4-912 δ*), which indicates a function for Arp4 in transcription regulation (Jiang and Stillman 1996). Consistent with this observation, Arp4

has been shown to be a subunit of the NuA4 (Nucleosome acetyltransferase of histone H4) HAT complex, and that it binds to all the core histones in vitro and to H2A in vivo, which requires a domain that is not conserved in other Arps (Harata, Karwan et al. 1994; Weber, Harata et al. 1995; Harata, Mochizuki et al. 1999; Galarneau, Nourani et al. 2000). It has also been shown in budding yeast that Arp4 is bound to the entire promoter region of *his4-912 δ* . In the conditional mutant of *arp4*, transcription from the *his4-912 δ* promoter is affected, which was shown to correlate with a decrease in the binding of Arp4 and a change in nuclease sensitivity of the chromatin (Harata, Zhang et al. 2002). *arp4* mutation also causes a slight alteration in chromatin structure (Harata, Karwan et al. 1994; Weber, Harata et al. 1995; Harata, Mochizuki et al. 1999). Its similarity to actin mainly resides in the actin-fold, where the ATP-binding pocket is found. However, this region does not seem to be essential for the chromatin remodelling function of Arp4, since mutations of this region does not affect its function (Stefanov 2000).

The role of Arp4 in DNA replication and repair has also been postulated, since some of the *arp4* mutants have been shown to be hypersensitive to HU (hydroxyurea, which inhibits ribonucleotide reductase and prevents dNTP incorporation for DNA synthesis) and UV irradiation under permissive temperature (Gorzer, Schuller et al. 2003). In support of this idea, Arp4 is also found as a subunit of an ATP-dependent chromatin remodelling complex called the Ino80 complex, that may play an important role for DNA damage repair (Shen, Mizuguchi et al. 2000). Moreover, a more direct evidence for the role of Arp4 in DNA damage repair comes from a study on the NuA4 complex, which showed the recruitment of Arp4 specifically to DNA double-strand breaks generated in vivo (Bird, Yu et al. 2002). Acetylation of histone H4 tail by the NuA4 complex was shown to be required for the repairing of DNA double-strand break.

In addition to NuA4 complex, Arp4 in yeast is also found in SWI/SNF-like ATP-dependent chromatin remodelling complexes (CRCs) of SWR1 and Ino80 (see later for complex functions, Figure 1.6.1) (Galarneau, Nourani et al. 2000; Ikura, Ogryzko et al. 2000; Shen, Mizuguchi et al. 2000; Krogan, Keogh et al. 2003;

Kobor, Venkatasubrahmanyam et al. 2004; Mizuguchi, Shen et al. 2004). The functions of Arp4 in these complexes are yet to be determined, although its presence in these complexes clearly suggests its importance in both histone acetylation through NuA4 complex and histone modification through ATP-dependent CRCs.

Human BAF53 is an Arp similar to yeast Arp4, and is also found in both ATP-dependent chromatin remodelling (BAF and PBAF complexes) and HAT (Tip60) complexes. BAF53 has been shown to be directly bound to both β -actin and Brg1 (the ATPase subunit of BAF and PBAF complexes), which is in fact required for the assembly and tight association of the PBAF complex to the nuclear matrix/chromatin in vivo (Zhao, Wang et al. 1998). β -actin and BAF53 are also required for the maximal ATPase activity of Brg1 (Zhao, Wang et al. 1998). Furthermore, BAF53 has been shown to interact with c-myc, which is required for oncogenic transformation (Park, Wood et al. 2002).

1.7 ATP-dependent chromatin remodelling

Chromatin remodelling refers to a change in histone-DNA interactions in a nucleosome. ATP-dependent chromatin remodelling complexes (CRCs) are able to utilise the energy from ATP hydrolysis to alter the chromatin structure, which are required for transcriptional regulation, DNA replication, repair, homologous recombination and chromatin assembly (Lusser and Kadonaga 2003). Activities of the chromatin remodelling factors have been observed mainly in vitro including catalysing the mobilisation and repositioning of the nucleosomes, transferring a histone octamer from nucleosomes to separate DNA template, facilitating access of nucleases to nucleosomal DNA and generation of superhelical torsion in DNA (Becker and Horz 2002). These complexes all share the catalytic core ATPase subunit, which belong to the SNF2 superfamily of proteins, first identified in the budding yeast SWI/SNF complex (Eisen, Sweder et al. 1995). The ATP-dependent CRCs can further be classified into several subclasses according to their sequence similarities and associated domains: SWI2/SNF2 (contains a bromodomain in addition to

ATPase), ISWI (imitation SWI) and CHD1/Mi-2. In budding yeast, the SWI2/SNF2 group includes SWI/SNF (contains Swi2/Snf2 ATPase), RSC (remodels structure of chromatin, contains Sth1 ATPase), Ino80 (contains Ino80 ATPase), and SWR1 (contains Swr1 ATPase) complexes (see Figure 1.7.1). These Swi2/Snf2-like proteins all contain DNA-dependent ATPase domain and C-terminal bromodomains, which are required for binding to the acetylated lysine residues on histones.

Yeast SWI/SNF and RSC complexes

SWI/SNF complex of budding yeast, which is around 2 MDa in size, was the first ATP-dependent CRC to be discovered and is highly conserved in all eukaryotes (Cote, Quinn et al. 1994; Imbalzano, Kwon et al. 1994). It was initially predicted to be involved in transcription activation of many genes, however, a systemic genome-wide analysis showed only a small fraction of genes required SWI/SNF for activation (~3%), and a similar number of genes were also shown to be repressed directly by SWI/SNF (Holstege, Jennings et al. 1998; Sudarsanam, Iyer et al. 2000). It is possible that a wider range of genes are regulated by the SWI/SNF complex, but were not detected from the mutational analysis due to functional redundancy that is known to exist between the ATP-dependent CRCs (Tsukiyama, Palmer et al. 1999; Tran, Steger et al. 2000) and between ATP-dependent CRCs and HATs (Pollard and Peterson 1998; Biggar and Crabtree 1999).

In contrast to SWI/SNF complex, RSC complex is essential for cell viability, is ten times more abundant than SWI/SNF complex, and appears to be required for a broad range of cellular processes (Cairns, Lorch et al. 1996). RSC complex is also required for cell cycle progression, as many *rsc* mutants arrest at G2/M transition, which is dependent on the spindle assembly checkpoint (Cao, Cairns et al. 1997; Du, Nasir et al. 1998; Tsuchiya, Hosotani et al. 1998; Angus-Hill, Schlichter et al. 2001). The *rsc* mutants also display sensitivity to tubulin-depolymerising agents (Tsuchiya, Hosotani et al. 1998), which is a common phenotype with the kinetochore mutants. In addition, *rsc* mutants exhibit increased frequency of non-disjunction of mini-chromosomes, which may be due to defective kinetochore-spindle interaction (Tsuchiya, Hosotani et al.

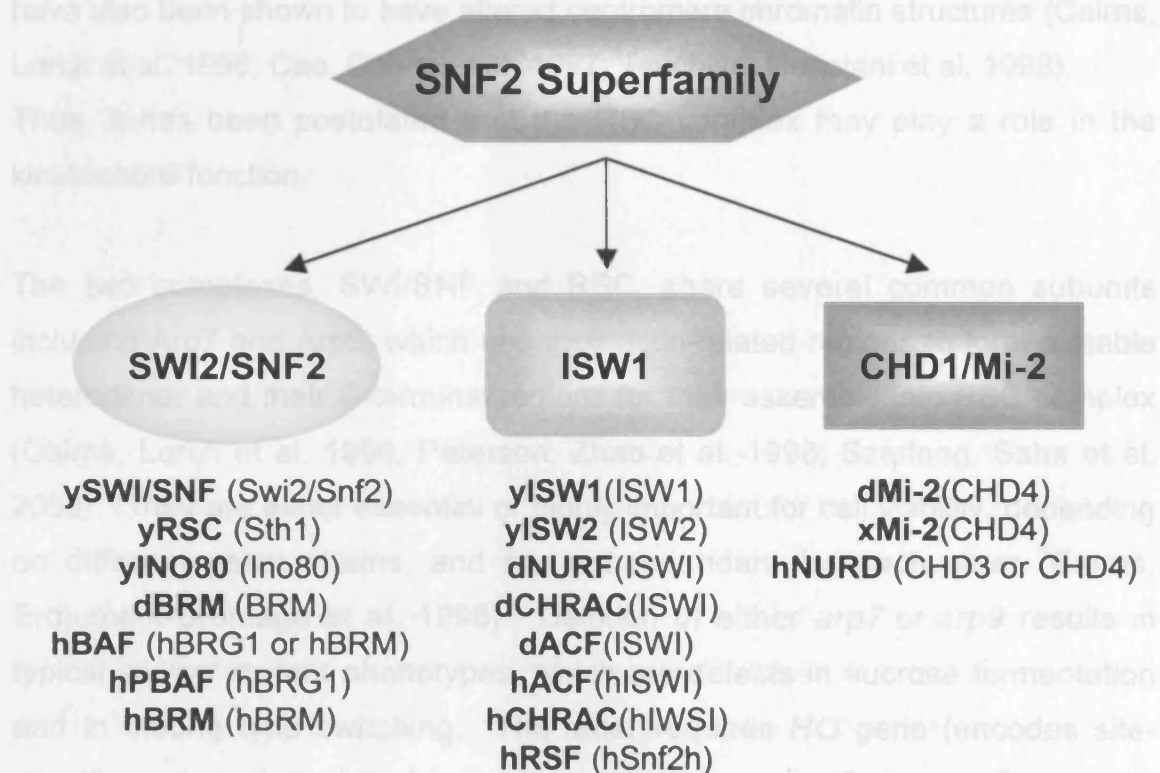


Figure 1.7.1 SNF2 superfamily. A list of known complexes in each subfamily of the Snf2 superfamily. The Snf2-like ATPase subunits of each complex are indicated in brackets. y-yeast (*S. cerevisiae*); d-*Drosophila*; x-*Xenopus*; h-Human.

1998). The *sth1* (the ATPase subunit of the RSC complex) and *rsc* mutant have also been shown to have altered centromere chromatin structures (Cairns, Lorch et al. 1996; Cao, Cairns et al. 1997; Tsuchiya, Hosotani et al. 1998).

Thus, it has been postulated that the RSC complex may play a role in the kinetochore function.

The two complexes, SWI/SNF and RSC, share several common subunits including Arp7 and Arp9, which use their actin-related regions to form a stable heterodimer and their C-terminal regions for their assembly into RSC complex (Cairns, Lorch et al. 1996; Peterson, Zhao et al. 1998; Szerlong, Saha et al. 2003). They are either essential or highly important for cell viability, depending on different yeast strains, and are not redundant for each other (Cairns, Erdjument-Bromage et al. 1998). Deletion of either *arp7* or *arp9* results in typical *swi/snf* mutant phenotypes, which are defects in sucrose fermentation and in mating type switching. The latter requires *HO* gene (encodes site-specific endonuclease) that initiates switching process (Peterson, Zhao et al. 1998). Thus, *swi/snf* mutants grow slowly on media containing glucose and cannot grow on media containing galactose, glycerol or sucrose as sole carbon sources (Stern, Jensen et al. 1984).

Deletant strains of *arp7* or *arp9* were also observed to cause an Spt⁻ phenotype, as the *arp4* mutant (see before), which is caused by mutations in genes involved in transcription and chromatin regulation such as TATA-binding protein, histones and certain members of the SAGA complex (Eisenmann, Dollard et al. 1989; Roberts and Winston 1997). *arp7* and *arp9* deletion mutants are such mutants, and since this phenotype is not observed with other *swi/snf* mutants (Happel, Swanson et al. 1991), RSC function may be required for an Spt⁺ phenotype. In support of this idea, it was shown that mutations that suppress the defects of *swi/snf* mutants were not able to suppress *arp7* and *arp9* null mutations, including histone mutation (Cairns, Erdjument-Bromage et al. 1998).

The functions of Arp7 and Arp9 appear to be structural rather than enzymatic, since the deletion of either protein does not have an effect on DNA-dependent ATPase activity, DNA translocation activity, mononucleosome binding or nucleosome remodelling activity of the RSC complex in vitro (Szerlong, Saha et

al. 2003). In addition, site-directed mutagenesis of residues predicted to mediate ATP-hydrolysis were shown to have little impact on the function of either Arp7 or Arp9 (Cairns, Erdjument-Bromage et al. 1998), indicating the importance of their actin fold structurally, for example for dimerization, rather than the ATPase activity.

The Ino80 complex

An Ino80 complex, another SWI/SNF-like CRC, is localised to the nucleus unevenly and is associated with the chromosomes of dividing cells (Shen, Mizuguchi et al. 2000). This complex has been shown to display DNA helicase activity, as well as nucleosome remodelling activity (Shen, Mizuguchi et al. 2000). The DNA helicase activity is most probably derived from the two proteins (Rvb1 and Rvb2) found in the complex, which are related to bacterial RuvB DNA helicase proteins that catalyse branch migration of Holliday junctions. Since *ino80* mutant shows hypersensitivity to DNA-damaging agents in addition to defects in transcription, the complex is thought to be required for both chromatin remodelling and DNA repair.

The Ino80 complex also contains three Arps (Arp4, Arp5 and Arp8) and actin (Shen, Ranallo et al. 2003). Arp5 and Arp8 are not essential for cell growth, as Ino80 ATPase, and are not essential for the structural integrity of the complex. However, they were shown to be required for Ino80 ATPase activity, DNA binding and nucleosome mobilisation (Shen, Ranallo et al. 2003). In addition, Arp8 was shown to be required for recruiting Arp4 and actin to the complex, and also binds to histones H3 and H4 in vitro itself.

The SWR1 complex

The SWR1 complex has recently been identified as a SWI/SNF-like ATPase CRC that specifically exchanges histone H2A for its variant H2A.Z (Htz1 in budding yeast) in nucleosomes (Krogan, Keogh et al. 2003; Kobor, Venkatasubrahmanyam et al. 2004; Mizuguchi, Shen et al. 2004). Prior to the discovery of this complex, a strong enrichment of H2A.Z in chromatin adjacent to transcriptionally silent regions was reported, which was shown to be important in preventing the ectopic spread of silent heterochromatin into adjacent euchromatic regions (Meneghini, Wu et al. 2003).

The SWR1 complex also contains Arps (Arp4 and Arp6) in addition to actin (Mizuguchi, Shen et al. 2004). Rvb1 and Rvb2 proteins found in the Ino80 complex are also found in the SWR1 complex, making actin, Arp4, Rvb1 and Rvb2 the common subunits of the two complexes. Recent papers on Yaf9, a common component of SWR1 and NuA4 HAT complexes, have also reported genetic interactions between SWR1 and NuA4 complexes. It has been suggested that NuA4/SWR1/Htz1 pathway may be regulating both transcription and centromere function in yeast, in addition to preventing the spread of silencing near telomeres (Krogan, Baetz et al. 2004; Zhang, Richardson et al. 2004). This may not be surprising given that many proteins are shared between the complexes, including actin, Arp4, Rvb1, Rvb2 and Yaf9.

Human SWI/SNF-like complexes

ATP-dependent CRCs that belong to the SWI/SNF subclass in human include BAF (Brg1- or hBrm-associated factor) and PBAF (polybromo BAF) complexes (Kwon, Imbalzano et al. 1994; Wang, Cote et al. 1996; Armstrong, Bieker et al. 1998). These complexes have been implicated in diverse cellular processes, including transcription regulation (Khavari, Peterson et al. 1993; Muchardt and Yaniv 1993), integration of viral genomes into host chromatin (Kalpana, Marmon et al. 1994), viral DNA replication (Lee, Sohn et al. 1999), cell cycle regulation (Reyes, Barra et al. 1998; Shanahan, Seghezzi et al. 1999), and tumorigenesis (Dunaief, Strober et al. 1994; Versteeg, Sevenet et al. 1998). Either Brg1 or hBrm is found with the BAF complex, whereas the PBAF complex contains Brg1 only. These two complexes share homologous subunits with both yeast SWI/SNF and RSC complexes, and it has not been clear which are the equivalent homologues of BAF and PBAF complexes. However, recent studies suggest that BAF complex is more similar to SWI/SNF, since BAF250 that is only found in the BAF complex has similarity to Swi1 of SWI/SNF complex, which is not found in the RSC complex (Nie, Xue et al. 2000). Similarly, PBAF complex specifically contains BAF180, a homologue of chicken polybromo (PB1), which contains 5 bromodomain and two bromo-adjacent homology (BAH) regions (Workman and Kingston 1998), which are most similar to Rsc1, Rsc2, and Rsc4 (Xue, Canman et al. 2000). Similarly to yeast, the PBAF complex may be essential for cell viability, since deletion of Brg1 abolishes a

subset of PBAF complex and leads to cell death (Xue, Canman et al. 2000). A parallel observation is obtained with mice where mBrg1 deletion is inviable (Sumi-Ichinose, Ichinose et al. 1997), but mBrm deletion is viable (Reyes, Barra et al. 1998).

One interesting aspect of the PBAF complex is its localisation to the kinetochores of chromosomes from prometaphase to metaphase (Xue, Canman et al. 2000). The complex is also found to be localised on the spindle poles during prometaphase and metaphase, which are consistent with the requirement of the yeast RSC complex in G2/M progression (see before).

The ISWI subfamily

The ISWI (imitation SWI) subclass is one of the most diverse groups of the chromatin remodelling complexes, both structurally and functionally. The ISWI-like ATPases contain homology to *Drosophila* ISWI, and contain SANT (Swi3, Ada2, N-CoR and TFII B) domain, which is speculated to be a histone-binding module (Boyer, Latek et al. 2004). This class includes yeast ISWI, ISW2, *Drosophila* NURF, ACF, CHRAC, human hCHRAC and hACF (Elfring, Deuring et al. 1994; Tsukiyama, Daniel et al. 1995; Ito, Bulger et al. 1997; Varga-Weisz, Wilm et al. 1997; LeRoy, Loyola et al. 2000; Poot, Dellaire et al. 2000). The functions of *S. cerevisiae* Isw1p and Isw2p are related to the effects on RNA levels and are associated with transcriptional repression control (Mellor and Morillon 2004).

The CHD/Mi-2 subfamily

The CHD/Mi-2 subclass ATPase contains a chromodomain (chromatin organisation modifier) and a DNA binding domain in addition to helicase-like motifs. This class contains complexes such as NuRD and Mi-2 complexes, which additionally possess a HDAC subunit (Wade, Jones et al. 1998; Zhang, Ng et al. 1999), which may suggest the function of these complexes in facilitating deacetylation of histones or other DNA-associated proteins.

1.8 Histone modification

Post-translational modification of histones such as acetylation, phosphorylation, ubiquitination, and methylation are crucial for the correct regulation of gene expression (Figure 1.8.1) (Berger 2002; He and Lehming 2003; Iizuka and Smith 2003). Mounting evidence for the important role of so-called epigenetic mechanism is emerging as a heritable transcriptional control.

Epigenetics, which includes modifications of DNA or histones and RNA interference, is a mechanism by which the transcription state of a gene, active or inactive (or silent state), is determined without changing the primary DNA sequence. Covalent modification on histones may be functioning as markers for the specific binding of various proteins that lead to chromatin structural changes. For example, fission yeast heterochromatin correlates with di- or trimethylated lysine 9 on histone H3 (H3K9), which are believed to be catalysed by Clr4 histone methyltransferase. Methylated H3K9 then acts as a binding site for Swi6 (Bannister, Zegerman et al. 2001; Nakayama, Rice et al. 2001), a homologue of *Drosophila* heterochromatic protein HP1 (also of humans). When this residue is unmethylated, Swi6 is no longer recruited to the region (Bannister, Zegerman et al. 2001; Nakayama, Rice et al. 2001). Thus, methylation on H3K9 can be considered to be a binding marker for Swi6. The exact mechanism for this specific recruitment is still unclear. One possibility is that by methylating a specific residue, the required structure or charge for Swi6 binding is provided.

Traditionally, it has been widely considered that the importance of histone acetylation lies in transcription activation, since a correlation between hyperacetylated histones and actively transcribed genes was first observed many years ago (Allfrey, Faulkner et al. 1964), and vice versa [see (Roth, Denu et al. 2001) for review]. However, recent studies have brought evidence that histone acetylation is not only important in transcription activation, but also in transcription repression (Reifsnnyder, Lowell et al. 1996; Ehrenhofer-Murray, Rivier et al. 1997), DNA replication (especially in determining the timing of firing of origins during S-phase) (Vogelauer, Rubbi et al. 2002) and DNA repair (Bird, Yu et al. 2002).



Current Biology

Figure 1.8.1 Post-translational histone modifications. Known modifications of the core histones are indicated in different colours.

[Taken from Bjerling *et al.* 2002 *Mol. Cell. Biol.*]

Histone acetylation

Most studies on histone acetylation, which is carried out by histone acetyltransferases (HATs), have been on lysine residues of histone amino-terminal tails, which are exposed on the surface of the core histone octamers. However, histone acetylation has also been shown to occur at the globular regions. There are four lysine residues on the histone H4 amino tail at residues 5, 8, 12 and 16, which are conserved from budding yeast to higher eukaryotes. On the amino tail of histone H3, there are lysine residues at 4, 9, 14, 18, 23 and 27 that can be acetylated. Acetylation on these residues is considered to be important for altering the chromatin structure, possibly by neutralising the positive charge of the histone tail, reducing the interaction between the DNA and the histones. Acetylated residues are also recognised by protein domains such as bromodomains that are commonly found in transcription machinery proteins (Zeng and Zhou 2002). Thus, acetylated histone residues may also be functioning as binding domains for the necessary proteins of transcriptional activity.

Budding yeast HATs include Gcn5, Hat1, Sas2, Sas3 and Esa1. Hat1 and Gcn5 belong to a superfamily of HATs called GNAT (Gcn5-related N-acetyltransferases), which show sequence and structural similarity to Gcn5 (Sterner and Berger 2000). The other three proteins are MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60)-type HATs (Utley and Cote 2003), which are classified based on the MYST domain that is homologous to the acetyl-CoA binding domain and zinc finger motif (Takechi and Nakayama 1999). Various studies have revealed a wide variety of cellular functions for these proteins including gene silencing in yeast (Reifsnyder, Lowell et al. 1996; Ehrenhofer-Murray, Rivier et al. 1997), dosage compensation in *Drosophila* (Hilfiker, Hilfiker-Kleiner et al. 1997) and oncogenic transformation in humans leading to specific diseases such as leukaemia (Park, Kunjibettu et al. 2001).

All the characterised HATs so far have been shown to exist in multiprotein complexes (Galarneau, Nourani et al. 2000; Shen, Mizuguchi et al. 2000), and substrate preferences of these enzymes have been shown to vary. For example, MYST HATs, Esa1 and its human homologue Tip60, acetylate free

histones H4, H2A and H3 but not nucleosomal histones. Only when present as components of native complex do they gain the ability to acetylate nucleosomal histones (Ikura, Ogryzko et al. 2000). Thus far, there has been no characterisation on fission yeast HATs.

Histone Deacetylation

Deacetylation of histones are carried out by HDAC (histone deacetylase) enzymes. Generally, histone deacetylation is associated with gene repression and silencing (Braunstein, Rose et al. 1993; Brownell and Allis 1996; Wolffe 1996; Hartzog and Winston 1997; Pazin and Kadonaga 1997). HDACs are classified into three classes according to sequence similarity to budding yeast HDACs (see below) (Leipe and Landsman 1997; Grozinger and Schreiber 2000), and there are six HDACs in fission yeast (Hos2/Phd1/Hda1, Clr6, Clr3, Sir2, Hst2 and Hst4) (see below and Figure 1.8.2 for Class I and II HDACs).

Class I HDACs

The non-essential Class I HDAC Hos2/Phd1/Hda1, which will be referred to as Hos2 from hereon, is a cytoplasmic protein. In its absence, a defect in sporulation is observed, which is also seen in budding yeast homologue *hos2* (Bilsland, Dahlen et al. 1998; Olsson, Ekwall et al. 1998). However, in vivo acetylation studies revealed that its deletion does not have any effect on histone acetylation level (Bjerling, Silverstein et al. 2002).

The second class I HDAC protein is Clr6, which is the only essential HDAC in fission yeast, unlike its homologue Rpd3 of budding yeast (Vidal and Gaber 1991). It has a very broad substrate specificity, as Rpd3, and its conditional mutant, *clr6-1*, shows increased acetylation level on all the lysine residues tested (Suka, Suka et al. 2001; Bjerling, Silverstein et al. 2002). Clr6 is localised in the nucleus on the chromatin, and *clr6-1* mutant shows a minor silencing defect at both the mating type loci and the centromere *imr* and *otr* regions, but not at the telomere (Grewal, Bonaduce et al. 1998). In contrast to these silencing defects, a marginal increase in repression is observed in the *clr6-1* mutant at the core centromere region of *cnt*. Thus, it appears that low acetylation level does not always result in transcriptional repression. Clr6 is also required for genomic stability, since the mutant shows a high chromosome

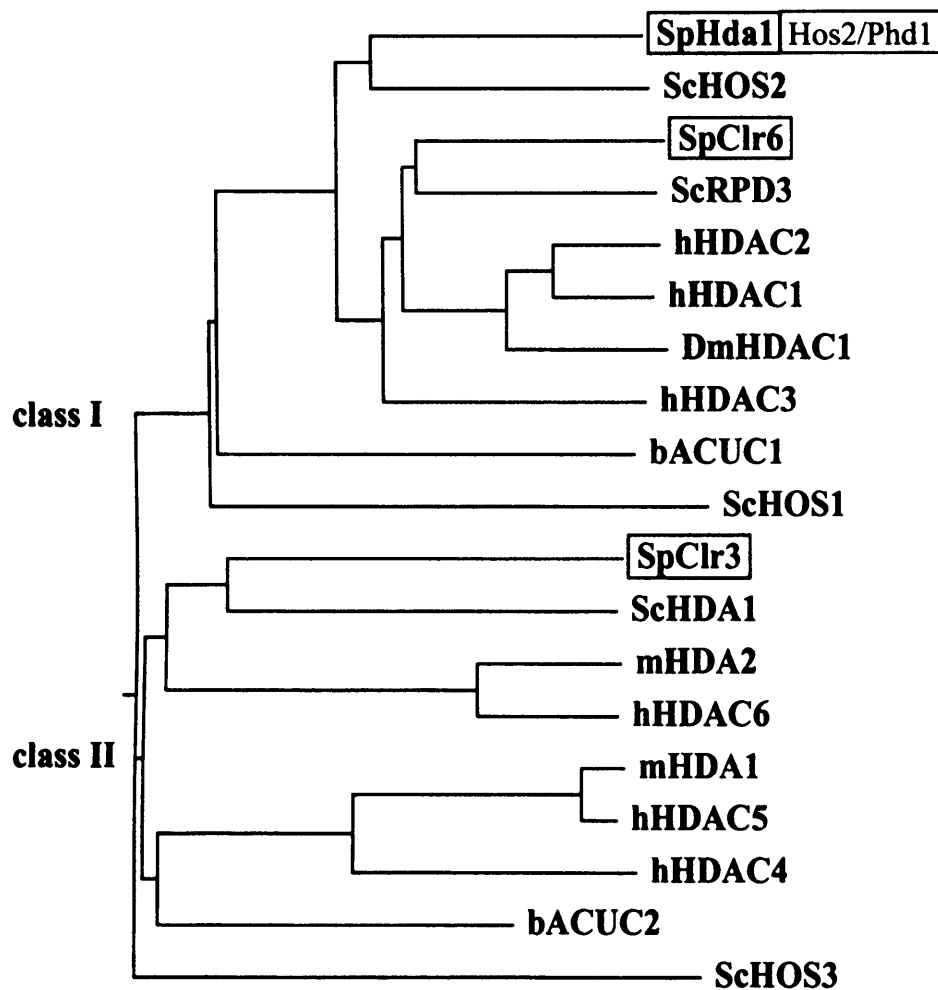


Figure 1.8.2 Class I and II HDAC proteins. Class I and II proteins are classified according to sequence similarity to *S. cerevisiae* Rpd3 and Hda1, respectively. *S. pombe* contains two Class I proteins, Hda1/Hos2/Phd1 and Clr6, and one Class II HDAC Clr3. [Taken from Bjerling *et al.* 2002 Mol. Cell. Biol.]

loss rate (Grewal, Bonaduce et al. 1998), suggesting its possible role in a centromere function.

Class II HDACs

Clr3 is required for global deacetylation of histone H3K14, and is localised in the nucleus, both on the chromatin and the nucleolus (Bjerling, Silverstein et al. 2002). Clr3 has been shown to be required for transcriptional repression at the centromere, the mating type locus, and the rDNA repeat regions. In addition, it is also required for the deacetylation of histone H3K9, H3K14, H4K8, and H4K12 within

rDNA repeats region (Thon, Cohen et al. 1994; Allshire, Nimmo et al. 1995; Grewal and Klar 1997; Bjerling, Silverstein et al. 2002).

Class III HDACs

Class III HDAC enzymes consist of those with sequence similarity to *S. cerevisiae* Sir2 protein, which are conserved from yeast to human to make up a family of HST (Homologues of Sir2) proteins (Brachmann, Sherman et al. 1995). The HSTs are divided into three subfamilies based on the overall sequence identities to *S. cerevisiae* proteins (Brachmann, Sherman et al. 1995). The first subfamily contains Sir2 and Hst1 homologues. The second contains Hst3 and Hst4 homologues (Freeman-Cook, Sherman et al. 1999), and the third subfamily consists of Hst2-like proteins (Brachmann, Sherman et al. 1995).

Budding yeast Sir2 is known to be required for silencing at all three loci that are transcriptionally silenced, at the mating type loci, telomere and rDNA repeats region (Shore, Squire et al. 1984; Ivy, Klar et al. 1986; Aparicio, Billington et al. 1991; Fritze, Verschueren et al. 1997). Defects in telomere silencing and chromosome segregation are observed in the double mutant of $\Delta hst3 \Delta hst4$, although single mutants display little phenotype (Chen and Clark-Walker 1994; Brachmann, Sherman et al. 1995).

Sir2 in fission yeast is required for histone H3K9 and H4K16 deacetylation in vitro and in vivo, and is required for the heterochromatin formation at the centromere and the mating type loci (Shankaranarayana, Motamedi et al. 2003). Sir2 requirement for the heterochromatin formation lies in the fact that Swi6, one of the hallmarks of heterochromatin, is recruited to methylated

histone H3K9, which must be deacetylated first for it to be methylated. Thus, *sir2* deletion leads to defects in silencing at these regions.

Hst4 of fission yeast is a nuclear protein, enriched in the nucleolus, and is also required for transcriptional silencing at the telomere and the centromere (Freeman-Cook, Sherman et al. 1999). Deletion of *hst4* leads to an increase in the frequency of chromosome loss and chromosome segregation, and also leads to both UV and TBZ sensitivity (Freeman-Cook, Sherman et al. 1999). No work has been reported on Hst2, and a functional relationship between three classes of enzymes remains elusive.

From these studies, it appears that different HDACs have different localisation with varying substrate specificity for different functions.

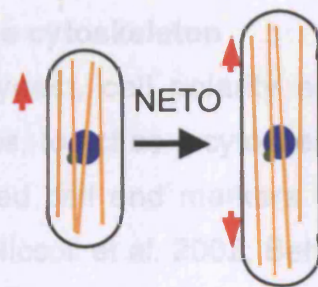
Chapter 2

Isolation and Characterisation of *alp5-1134*

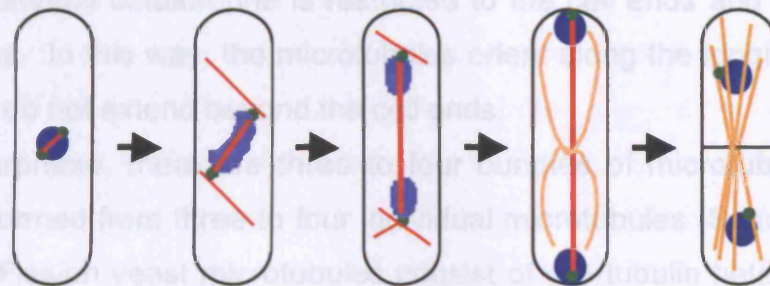
Fission yeast *S. pombe* has a unique rod cell shape, as a result of its highly polarised growth, which is restricted to the tips of the cells in a cell cycle-dependent manner (Hayles and Nurse 2001). After mitotic division, it grows in a monopolar fashion from its 'old' end, i.e. the end that existed before mitosis. After a process known as new end take off (NETO) during G2 phase, the growth switches to bipolar and cells grow from both ends (Mitchison and Nurse 1985) (Figure 2.1.1). This highly polarised growth makes fission yeast an ideal model organism to study cell shape and polarity as defects in this process regularly can be detected visibly, which often results in such morphologies as bent and branched shaped mutants (Snell and Nurse 1993).

A screen was undertaken prior to my joining to the lab, which isolated temperature-sensitive mutants with altered polarity (hence the isolated mutants were named *alp* mutants). The aim of this screen was to identify novel genes that are involved in the establishment of correct polarity. Fifteen *alp* mutants were isolated and phenotypic characterisations were undertaken to classify them according to their defects. Many of the genes were found to be genes encoding tubulin, tubulin cofactors and microtubule associated proteins (MAPs) (Radcliffe, Hirata et al. 1998).

Out of the uncharacterised mutants, I decided to study the *alp5-1134* mutant, which seemed to have defects with both cytoplasmic microtubules and mitosis.

AInterphase: Cytoplasmic
Microtubules**B**

Mitosis: Mitotic Spindle



— Microtubules

● DNA

— Spindle

● SPB

Figure 2.1.1 Microtubule reorganisation in the fission yeast cell cycle. Interphase microtubules (**A**) go through dynamic reorganisation to form a short nuclear mitotic spindle (**B**), which elongates in anaphase to reform interphase cytoplasmic microtubules upon mitotic exit.

2.1 Introduction

Microtubule cytoskeleton

In fission yeast, cell polarity establishment appears to require functional microtubules, to act as a cytoskeletal base, as well as their role in transporting the so-called cell end markers from the nuclear periphery to the cell ends (Arellano, Niccoli et al. 2002; Behrens and Nurse 2002). The cell end markers are localised at the plus-end (growing end) of the microtubules, and stabilise the microtubules by inhibiting microtubule catastrophe (a switch from growing to shrinking). Upon their deposition at the cell ends, microtubule catastrophe is initiated due to its uncapped state. Since their localisation is limited to the cell ends, microtubule catastrophe is restricted to the cell ends and not any other cortical sites. In this way, the microtubules orient along the longitudinal axis of the cell but do not extend beyond the cell ends.

During interphase, there are three to four bundles of microtubules, each of which are formed from three to four individual microtubules (Sagolla, Uzawa et al. 2003). Fission yeast microtubules consist of α/β tubulin heterodimers, like those of other eukaryotes, and possess a very dynamic structure, allowing them to repeatedly undergo growth and shrinkage (Waterman-Storer and Salmon 1997).

The screen and isolation of *alp* mutants

alp mutants were isolated in a large-scale screen for temperature sensitive mutants, which grow at 30°C but not at 36°C (Hirata *et al*, 1998). Fission yeast wild-type cultures were mutagenised with N-methyl-N'-nitrosoguanidine. 200,960 colonies were screened and 2,822 temperature sensitive alleles were isolated. After growth in rich medium at 36°C for 8 hours, calcofluor, which stains cell wall material and cell septum, was used to isolate mutants displaying abnormal cell morphology. Temperature sensitive mutants were further selected on the basis of displaying a bent or a branched cell shape at the restrictive temperature and fifteen genes were isolated from twenty-two *alp* mutants (revealed by complementation tests).

Most of these genes have now been cloned and many have been characterised extensively within the lab (Hirata, Masuda et al. 1998; Radcliffe, Hirata et al. 1998; Vardy and Toda 2000; Garcia, Vardy et al. 2001; Vardy, Fujita et al. 2002; Sato, Vardy et al. 2004). Nearly half of the gene products are either tubulin subunits, tubulin cofactors or microtubule-associated proteins (see Table 2.1).

Results

2.2 Microtubule integrity of *alp5-1134*

Cytoplasmic microtubule analysis

In comparison to the rod shaped wild-type cells, *alp5-1134* cells display a bent shape (see Figure 2.2.1.A). Since microtubules play an important role in generating cell shape, microtubule integrity was investigated by immunofluorescence in this mutant by staining tubulins with TAT-1 antibody, which recognises α -tubulin (see Materials and Methods). The procedure involved placing logarithmically growing *alp5-1134* cells in rich medium for 6 and 8 hours at 36°C, and fixing them with methanol. Two main microtubule characteristics became apparent with this mutant. One was that the microtubules were bundled together to form either one or two thick bundles (Figure 2.2.1.B). The second characteristic was that the bundles were longer than those of the wild-type cells, and curved at the tip of the cell, as indicated by the arrows in Figure 2.2.1B, which is never observed in wild-type cells. It is of note that this curved microtubule phenotype is also observed often with other bent mutants harbouring bundled microtubules (Vardy and Toda 2000).

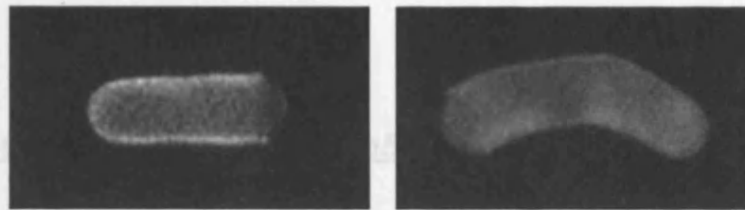
***alp5-1134* is resistant to a microtubule-depolymerising drug**

To investigate the possibility of stabilised microtubules in the *alp5-1134* mutant as a result of bundling, its sensitivity/resistance to the microtubule depolymerising drug TBZ (Thiobendazole) was investigated. A dilution assay (see Materials and Method) was carried out on rich media plates containing no drug, 10 μ g/ml or 20 μ g/ml TBZ and incubated at 26°C for five days for wild-type and *alp5-1134* mutant. The results showed that *alp5-1134* mutant is indeed

Loci	Morphology	Genes/product
<i>alp 1</i>	Bent/brancheds	<i>tsm1⁺</i> / Tubulin folding cofactor D
2	Bent/branched	<i>atb2⁺</i> /α2-tubulin
3	Curved/cut	<i>cut14⁺</i> /Condensin
4	Bent/cut	<i>SPC97</i> /GCP2
5	Bent	<i>Arp4</i> /BAF53
6	Bent/cut	<i>SPC98</i> /GCP3
7	Bent/cut	coiled coil MAP/TACC
8	Bent/branched	<i>tea1⁺</i> /cell end marker
9	Bent	
10	Curved	
11	Bent/branched	Tubulin folding cofactor B
12	Bent/branched	<i>nda3⁺</i> /β-tubulin
13	Bent/cut	Chromodomain/MRG15
14	Bent/branched	<i>Dis1</i> /TOG/XMAP215
15	Bent/cut	<i>top2⁺</i> /Topoisomerase II

Table 2.1. Isolated *alp* mutants and their genes (where known).

more resistant to TBZ than the wild-type (Fig. 2.2.1.C), which suggests that the microtubules in *alp5-1134* are more stabilised than that of the wild-type.

A

Defective chromosome segregation in *alp5-1134* mutant.

Another phenotype that was evident from the immunofluorescence picture was that the *alp5-1134* cells contained short spindles (< 2 µm length) (marked in bottom in Figure 2.2.1.A).

B

permissive temperature of 26°C and subsequently shifted up to 36°C for 6 and 8 hours. From the graph of Figure 2.2.1.A, it can be seen that the wild-type population consisted of around 7% of cells with short spindles, whereas it

contained roughly 20% in the *alp5-1134* mutant after 6 hours of shifting up.

C

When DAPI (chromosome stain, which binds specifically to DNA) staining was observed in a high degree of chromosome missegregation was observed (Figure 2.2.1.B). When the tangle of 8 hours shift up was observed, instead of condensed chromosomes, a high degree of chromosome missegregation was observed (Figure 2.2.1.B). This suggests that there may be a mitotic delay (rather than an arrest) in the *alp5-1134* mutant at its restrictive temperature.

Figure 2.2.1 Characterisation of *alp5-1134* **A** Calcofluor staining

of wild-type (left) and *alp5-1134* (right) after 6 hours at 36°C. **B**

Cytoplasmic microtubule structure from TAT1 staining shows

bundled microtubules in *alp5-1134*. **C** *alp5-1134* is resistant to

TBZ. 5×10^3 cells were spotted in the far-left spot and 10-fold

dilution in the subsequent spots onto rich plates in the absence

or the presence of TBZ, and incubated at 26°C for five days.

Scalebar indicates 10 µm.

more resistant to TBZ than the wild-type (Fig. 2.2.1.C), which suggests that the microtubules in *alp5-1134* are more stabilised than that of the wild-type.

2.3 Mitotic phenotypes of the *alp5-1134* mutant

Defective chromosome segregation in *alp5-1134* mutant

Another phenotype that was evident from the immunofluorescence pictures was that there were many cells containing short spindles ($< 2 \mu\text{m}$ length) (marked in Figure 2.3.1.A), which suggested that at the restrictive temperature, there may be a mitotic delay with the *alp5-1134* mutant cells. The number of cells containing short spindles was quantified by comparing asynchronous cultures of the wild-type and the *alp5-1134* mutant. The strains were cultured overnight at permissive temperature of 26°C and subsequently shifted up to 36°C for 6 and 8 hours. From the graph of Figure 2.3.1.A, it can be seen that the wild-type population consisted of around 7% of cells with short spindles, whereas it contained roughly 20% in the *alp5-1134* mutant after 6 hours of shifting up. When DAPI (diamidineophenylindole, which binds specifically to DNA) staining was observed, these cells with short spindles showed condensed chromosomes to a higher degree than normally observed in the wild-type cells (see Figure 2.3.1.B). When the sample of 8 hours shift up was observed, instead of condensed chromosomes, a high degree of chromosome missegregation was observed (Figure 2.3.1.B). This suggests that there may be a mitotic delay (rather than an arrest) in the *alp5-1134* mutant at its restrictive temperature.

Overall, the initial characterisation of the *alp5-1134* mutant revealed the presence of strong chromosome condensation with short mitotic spindles after 6 hours at its restrictive temperature of 36°C and severe chromosome missegregation after 8 hours.

Genomic instability of *alp5-1134*

Since the above observations suggested that genomic instability may arise in the *alp5-1134* mutant as a result of its mitotic chromosome segregation defect, the rate of mini-chromosome Ch16 (a 530 kb derivative of chromosome III) loss

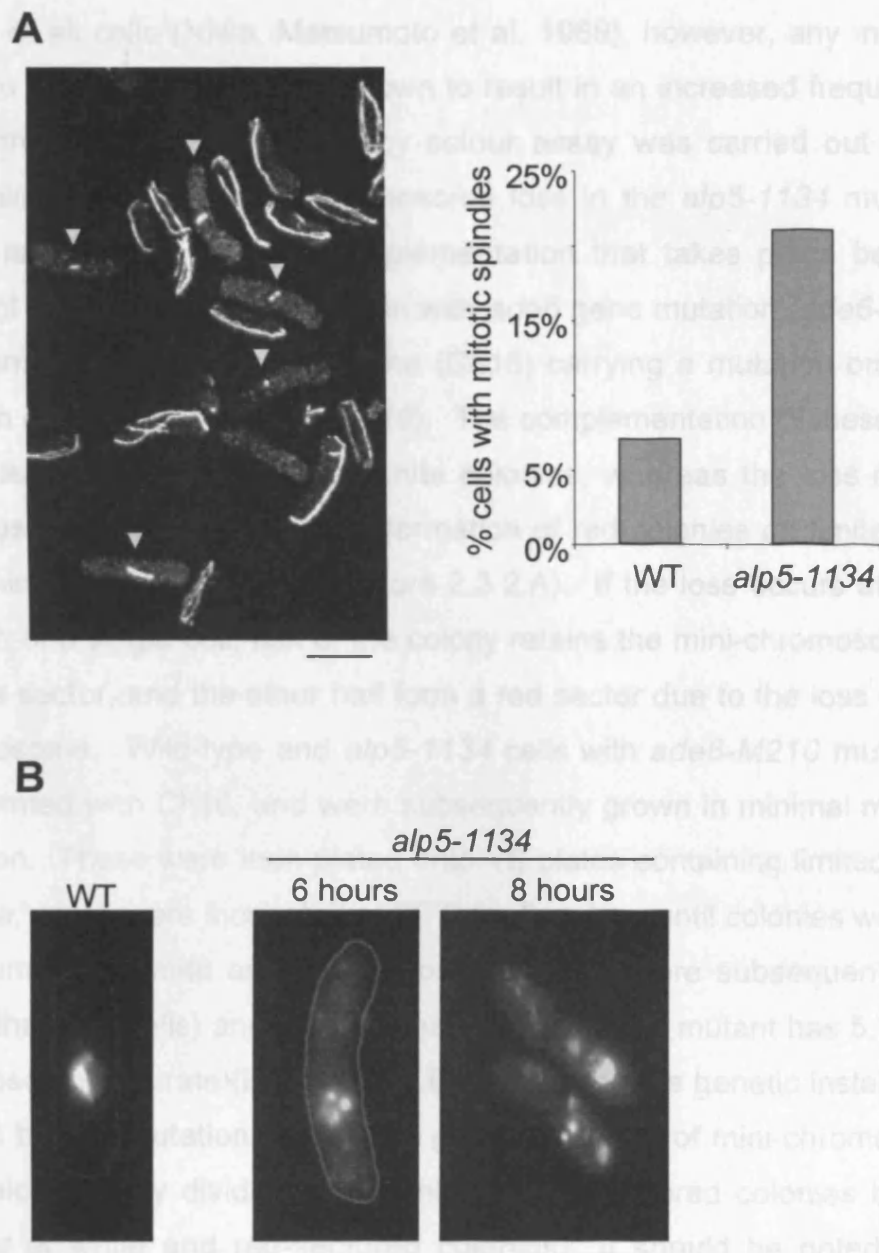
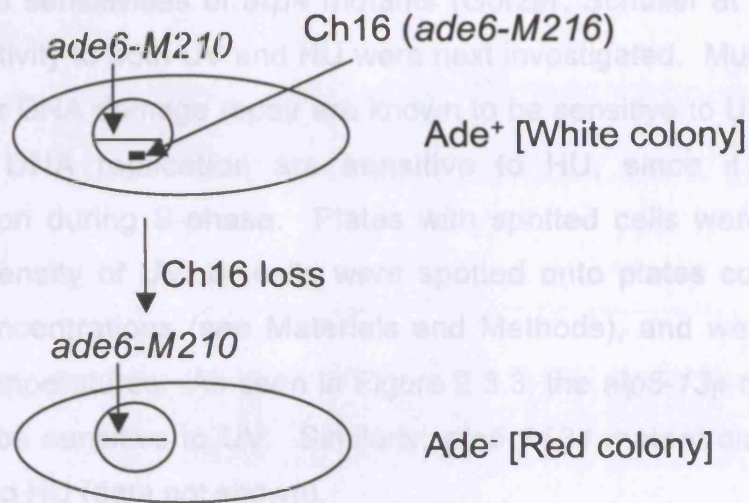


Figure 2.3.1 *alp5-1134* mutant shows mitotic phenotypes **A** *alp5-1134* cells with mitotic spindles after 6 hours at 36°C (left) and a graph showing the proportion of these cells with mitotic spindles in an asynchronous culture (right). **B** DAPI staining shows condensed chromosomes in *alp5-1134* after 6 hours of shift up at 36°C and chromosome missegregation at 8 hours. Scalebar indicates 10 μ m.

was then investigated. Under a wild-type situation, mini-chromosome is lost in ~0.2% of all cells (Niwa, Matsumoto et al. 1989), however, any mutation that leads to genomic instability is known to result in an increased frequency of the mini-chromosome loss. A colony colour assay was carried out in order to determine the rate of mini-chromosome loss in the *alp5-1134* mutant. This assay is based on genetic complementation that takes place between two different alleles of a gene. A strain with *ade6* gene mutation (*ade6-M210*) was transformed with mini-chromosome (Ch16) carrying a mutation on a different location on *ade6* gene (*ade6-M216*). The complementation of these mutations would lead to the formation of white colonies, whereas the loss of the mini-chromosome would result in the formation of red colonies on limited adenine-containing plates (depicted in Figure 2.3.2.A). If the loss occurs after the first division of a single cell, half of the colony retains the mini-chromosome to form a white sector, and the other half form a red sector due to the loss of the mini-chromosome. Wild-type and *alp5-1134* cells with *ade6-M210* mutation were transformed with Ch16, and were subsequently grown in minimal media for its selection. These were then plated onto YE plates containing limited amount of adenine, which were incubated at 27°C for five days until colonies were formed. The number of white and red-sectored colonies were subsequently counted (more than 10^4 cells) and showed that the *alp5-1134* mutant has 5.7% of mini-chromosome loss rate (Figure 2.3.2.B), confirming the genetic instability that is caused by this mutation in the *alp5* gene. The rate of mini-chromosome loss was calculated by dividing the number of red-sectored colonies by the total number of white and red-sectored colonies. It should be noted, however, although half-sectored colonies were the majority, those containing red sectors less than half of the colony were also counted. Thus, the resulting rate of mini-chromosome loss can be considered as an overestimate and does not reflect the chromosome loss rate per division. In order to obtain an accurate rate of chromosome loss per cell division, only those colonies with half red-sectors should be counted.

A**B**

Mini-chromosome loss
rate at 27°C

Wild Type	<i>alp5-1134</i>
< 0.01 %	5.7 %

n = 10,000

Figure 2.3.2 Mini-Chromosome Loss Assay **A** An *alp5-1134* strain with *ade6-M210* mutation was created containing Ch16. If the mini-chromosome is lost from the cell, it would no longer be able to form white, but red colonies. **B** *alp5-1134* with mini-chromosome was grown in minimal media, plated onto rich plates and incubated at 27°C for 4 days. Red-sectored colonies were visible with *alp5-1134* strain, which was quantitated.

Sensitivity of the *alp5-1134* mutant to UV

Since studies on budding yeast Arp4, which is homologous to Alp5, have shown UV and HU sensitivities of *arp4* mutants (Gorzer, Schuller et al. 2003), *alp5-1134* sensitivity to both UV and HU were next investigated. Mutations in genes required for DNA damage repair are known to be sensitive to UV radiation, and those for DNA replication are sensitive to HU, since it inhibits dNTP incorporation during S-phase. Plates with spotted cells were placed under varying intensity of UV, or cells were spotted onto plates containing HU at various concentrations (see Materials and Methods), and were incubated at different temperatures. As seen in Figure 2.3.3, the *alp5-134* mutant does not appear to be sensitive to UV. Similarly, *alp5-1134* mutant did not show any sensitivity to HU (data not shown).

2.4 Discussion

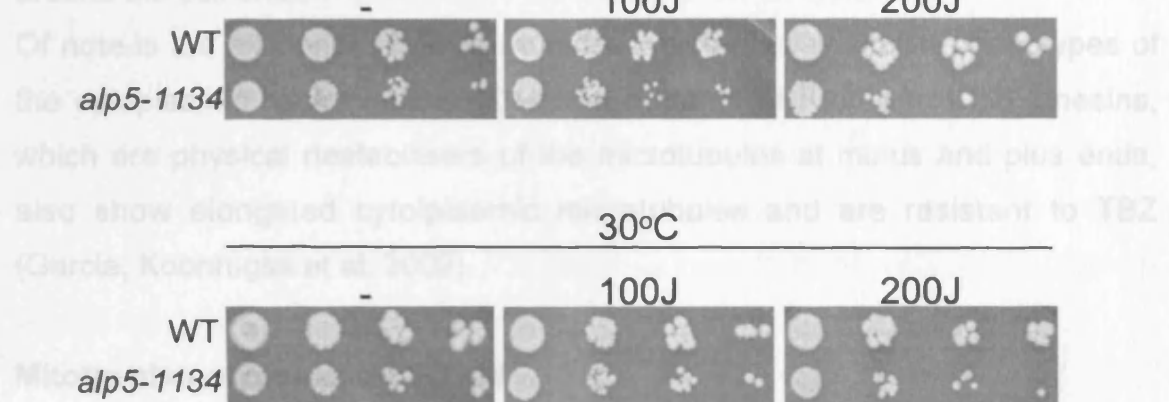
***alp* mutants**

By analysing the list of *alp* mutants that were isolated from the same screen, the list of proteins may be split into two main groups. The first group would consist of those required for microtubule polymerisation, including α -tubulin (Alp2), β -tubulin (Alp12), γ -tubulin complex (Alp4 and Alp6), tubulin cofactors (Alp1 and Alp11) and microtubule-associated proteins (Alp7 and Alp14). The other half would consist of nuclear proteins including condensin (Alp3), topoisomerase II (Alp15), Alp13 (Nakayama, Xiao et al. 2003) and Alp5 (see later).

Cytoplasmic phenotypes of *alp5-1134*

There are three main cytoplasmic microtubule phenotypes in the *alp5-1134* mutant. One is the stabilised microtubules, i.e. more resistant to the microtubule depolymerising drug TBZ than the wild-type. The second is the bundled phenotype, and the last is the curved microtubules at the ends of the cell. One interesting aspect of these phenotypes is which phenotype should be considered as the primary defect. There may be two possibilities; first is that microtubule bundling is the primary defect, which leads to TBZ resistance and curved microtubules at cell ends. The other possibility is that *alp5* mutation

causes stabilised microtubules, which may result in the bundling effect. The curving microtubulin phenotype at the tip may be due to the combination of both bundled and stabilised microtubules, which microtubules are pushed further around the cell and



At the restrictive temperature, *alp5-1134* mutant shows hyper-condensation after 6 hours, which is followed by gross chromosome missegregation. The *alp5-1134* mutant strain also shows a higher percentage of its population with mitotic spindles at 6 hours, suggesting a possible delay during mitosis, which may be due to the activation of a checkpoint (see Chapter 5). However, a complete arrest of the cell cycle at mitosis is not observed in this mutant, since chromosome missegregation is observed, which indicates the entry of the mutant cells into anaphase. These observations suggest that Alp5 function is required for faithful mitotic progression.

From the observation of DAPI-stained cells, it is not clear whether the hyper-condensation of the chromosomes that is observed after 6 hours of shifting up is a direct phenotype of the *alp5-1134* mutation, or whether it is simply due to the delay in metaphase that leads to increased chromosome condensation. However, since many mitotically delayed mutants show hyper-condensation, it may be more likely to be the latter case. In order to distinguish between these

Figure 2.3.3 UV sensitivity assay. 5x10³ wild-type (WT) or *alp5-1134* cells were spotted in the far-left spot, and 10-fold dilution in the subsequent spots, onto rich plates. They were then subjected to 100J or 200J UV and incubated at 26°C or 30°C for several days.

causes stabilised microtubules, which may result in the bundling effect. The curving microtubule phenotype at the tip may be due to the combination of both bundled and stabilised microtubules, where the microtubules are pushed further around the cell end.

Of note is the existence of other mutants, which display similar phenotypes of the cytoplasmic microtubules. Deletant mutants of Klp5 and Klp6 kinesins, which are physical destabilisers of the microtubules at minus and plus ends, also show elongated cytoplasmic microtubules and are resistant to TBZ (Garcia, Koonruga et al. 2002).

Mitotic phenotypes of *alp5-1134*

At the restrictive temperature, *alp5-1134* mutant shows hyper-condensation after 6 hours, which is followed by gross chromosome missegregation. The *alp5-1134* mutant strain also shows a higher percentage of its population with mitotic spindles at 6 hours, suggesting a possible delay during mitosis, which may be due to the activation of a checkpoint (see Chapter 5). However, a complete arrest of the cell cycle at mitosis is not observed in this mutant, since chromosome missegregation is observed, which indicates the entry of the mutant cells into anaphase. These observations suggest that Alp5 function is required for faithful mitotic progression.

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Chapter 3

Cloning and Sequencing of *alp5*⁺

This chapter describes the cloning and sequencing of the *alp5-1134* mutant and reveals that Alp5 contains high similarity in sequence to that of the conventional actin, which therefore classifies this protein as an actin-related protein (Frankel and Mooseker 1996).

The homologues of Alp5 in budding yeast (Arp4) and human (BAF53) reside in multi-protein chromatin modification complexes in the nucleus. Consistent with this, fission yeast Alp5 is also found to be a nuclear protein and is essential for cell viability.

3.1 Introduction

ARP family in fission yeast

A homology search was carried out on the fission yeast genome sequence database at the Sanger centre (<http://www.sanger.ac.uk>) and homologues were found for all ten budding yeast Arp proteins, which are shown in Figure 3.1.1. Fission yeast appears to have two proteins that are similar to budding yeast Arp4; Alp5 and SPAC23D3.09. The latter protein also contains some similarity to budding yeast Arp7.

A recent study showed that fission yeast Arp6 is a non-essential nuclear protein, which is required for telomere silencing, but not for centromere silencing, and that it binds to the telomere in a Swi6-independent manner (Sudo, Nitta et al. 2004). There has been no further characterisation on fission yeast Arps.

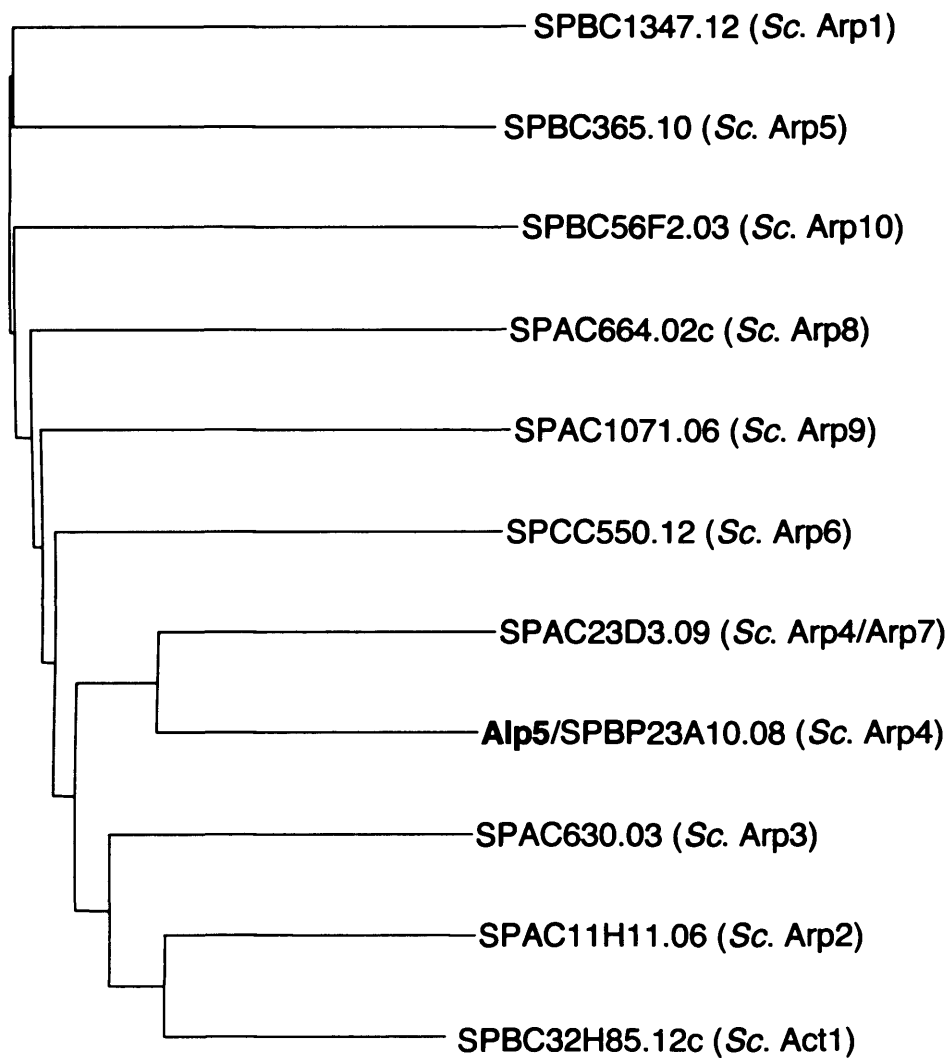


Figure 3.1.1 Actin-related proteins in fission yeast, showing the relevant budding yeast homologues in brackets.

Results

3.2 Identification of the *alp5*⁺ gene

Cloning of the *alp5*⁺ gene

The method of cloning *alp5*⁺ gene took advantage of the temperature sensitivity of *alp5-1134* mutant, and the wild-type gene was isolated by its ability to suppress its temperature lethal phenotype. A genomic library in a multi-copy vector pAL-KS (obtained from Taro Nakamura), which contains a leucine marker (*LEU2* of *S. cerevisiae*, complementing leucine auxotrophy of *leu1*⁻ strains), was transformed into *leu1*⁻ *alp5-1134* strain (see Materials and Methods) and transformants were selected for on minimal media (EMM) plates at the permissive temperature of 26°C. Colonies containing the plasmid were able to grow on these plates without the presence of leucine in the media due to the presence of the *ScLEU2* gene on the plasmid. These colonies were replica plated onto YE5S containing Phloxine B and incubated for 2 days at 36°C. Phloxine B is a dye, which stains dead cells red but stains the normally growing cells pink. The colonies that grew at 36°C and stained pink were selected as candidates for plasmid isolation. Transformation efficiency was about 1500 cells per plate with a total of 15,000 transformants being examined. Of these, 6 candidates were selected for the ability to grow at 36°C.

Plasmid dependency test

In order to determine if the rescue phenotype that was seen was due to the presence of a suppressing plasmid and not just a high frequency of reversion to wild-type, plasmid dependency tests were performed. Candidate colonies were grown overnight in rich medium (YE5S) at 26°C, which resulted in a high degree of plasmid loss. Approximately 200 cells were then plated onto YE5S at 26°C and replica plated onto Phloxine B containing plates at 36°C and to EMM-LEU plates at 26°C. To determine if the rescue phenotype was plasmid-dependent, candidates were checked for correlation between the ability to grow at 36°C and the presence of the plasmid (growth on EMM-LEU plates at 26°C). Growth at 36°C was determined to be plasmid dependent for all the candidates.

Plasmid isolation

Having established plasmid dependency with all the candidates, plasmids were then isolated from these strains. The candidate strains were streaked and grown on EMM-LEU at 26°C for several days, after which four colonies were picked for each candidate and were subsequently grown in EMM culture (selective condition) until late logarithmic phase. The plasmid DNA was then isolated using the Qiagen protocol (see Material and Methods) and amplified in *Escherichia coli* (*E. coli*). 2 µl of each purified plasmid were digested with either *Hind*III or *Eco*RI and run on a 1% TBE agarose gel. Banding patterns between different candidates were compared to determine if the plasmids contained the same gene (Figure 3.2.1.A). Of the six plasmids that suppressed *alp5-1134*, plasmid DNA were isolated from four, named pAL-3, pAL-6, pAL-9 and pAL-10, and two of these (pAL-6 and pAL-9) showed the same banding pattern after digestion. The plasmids were then retransformed into the *alp5-1134* mutant to check the degree of their suppression. Figure 3.2.1.B shows the suppression of *alp5-1134* temperature sensitivity by the plasmids on YE5S grown at 36°C. All the plasmids, except for pAL-3, suppressed temperature sensitivity of *alp5-1134*, although one or two in four isolated plasmids from different colonies failed to show full suppression (Figure 3.2.1.B underlined). This may be explained by the different plasmid copy numbers present amongst different cells. Together with the fact that the *LEU2* gene on the plasmid originates from *S. cerevisiae*, if the copy number is too low, it may not be sufficient to suppress the temperature sensitivity. Since both large and small colonies were selected for streaking, the small colonies may not have had enough plasmid copy numbers leading to failure in full suppression.

Gene identification by transposon mutagenesis

In order to identify the gene that rescued the mutant phenotype, the method of transposon mutagenesis was employed (Morgan 1996). By mixing a donor plasmid containing a transposon with the suppressing plasmid, if the transposon jumped into the suppressing gene, this gene would no longer be able to rescue the mutant phenotype at 36°C (see Figure 3.2.2). Primers designed against the flanking transposon sequence (-20 universal primer) could then be used to sequence the gene. The transposon employed here (Tn1000)

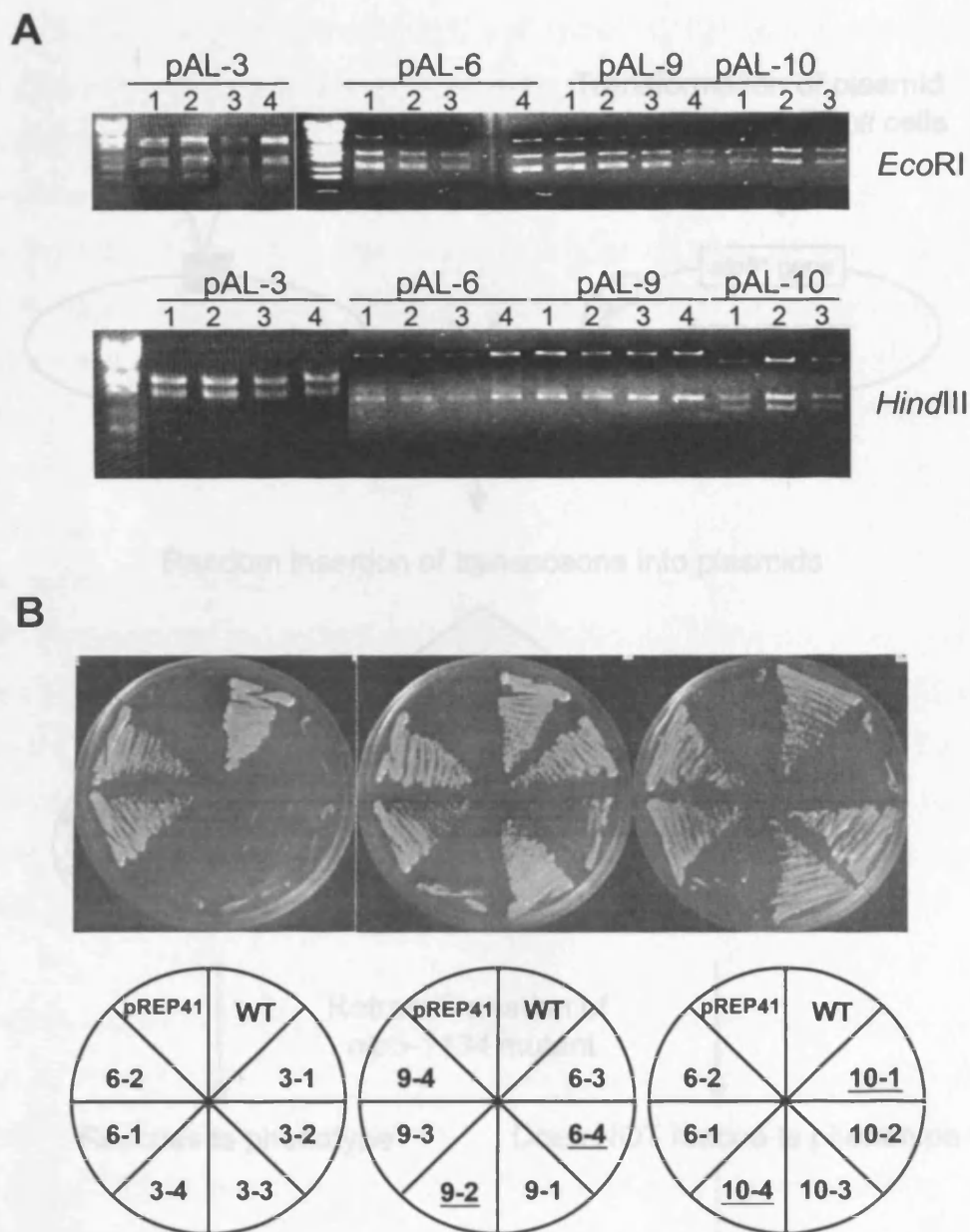


Figure 3.2.1 Cloning of *alp5*⁺ **A** *alp5-1134* cells were transformed with a genomic library. Obtained transformants were plated and incubated at 36°C. Plasmid DNA were then isolated from the strains that formed colonies at 36°C and were digested with *Eco*RI and *Hind*III. Digested fragments were separated on a 1% agarose gel. **B** Colonies containing candidate plasmids were re-streaked on YE5S and incubated at 36°C for two days.

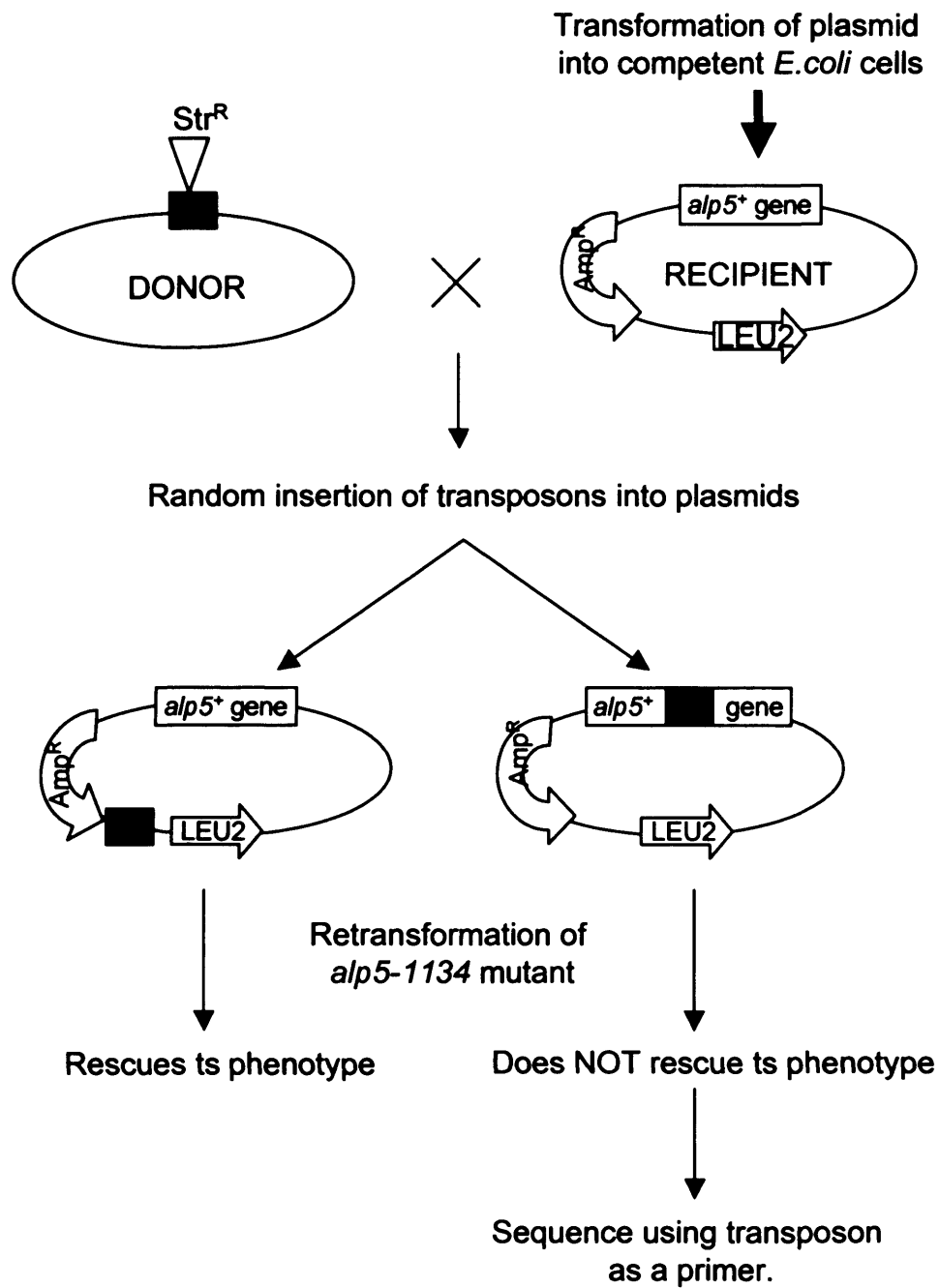


Figure 3.2.2 Transposon Mutagenesis: Sequencing of complementing plasmid, using transposon as a primer site.

contained the streptomycin resistance gene for selection (obtained from Jonathan Millar's lab). A competent *E. coli* strain HB101 was transformed with pAL-6 plasmid, which was then mated with the donor *E. coli* strain DH1 that contained the transposon in a plasmid. Streptomycin resistant colonies were then selected and the plasmids were isolated. On re-transformation back into the mutant, those plasmids that were no longer capable of suppressing the temperature lethality of *alp5-1134* at 36°C were selected for sequencing. Three plasmids were selected and sequenced by using the primers targeted against the two ends of transposon, thereby obtaining the sequence flanking the transposon.

Alp5 is an actin-related protein

The sequences obtained were then cross-referenced to the completed genome sequence database at Sanger Centre by using their BLAST server. This search identified the sequences as an actin-related protein (ARP), the gene of which is located on cosmid SPBP23A10.08, which encodes 433 amino acids with a predicted molecular weight of 49 KDa. *alp5*⁺ sequence has 30% identity and 50% similarity to conventional actin.

To determine if the correct gene, and not a multicopy suppressor, was cloned, a genetic cross was performed. The actin-related protein was tagged with the kanamycin resistance (*kan*^R) gene as a selectable marker, and was crossed to *alp5-1134*, which is kanamycin sensitive. No wild-type recombinant (*ts*⁺, *kan*^S) was obtained, suggesting that the mutant and the tagged gene were at the same locus.

Alp5 homologues are Arp4 in budding yeast and BAF53 in human

Having determined the Alp5 sequence, a homology search by BLAST search was carried out, which showed that the potential homologues are Arp4 in budding yeast (33% identity and 50% similarity) and BAF53 in human (31% identity and 51% similarity). Sequence comparison between Alp5, Alp5 homologues and actin is shown in Figure 3.2.3.

3.3 Structural and sequence analysis of Alp5

Alp5 has two insertions into actin

Figure 3.3.1.A shows a schematic structure comparison between Alp5 and the homologues. When Alp5 sequence is compared to conventional actin, it is clear that there are two insertions, insertion I (230-250) and insertion II (300-330), which are also present in budding yeast Arp4 (Harata, Karwan et al. 1994). It is of note that only insertion I appears to exist in BAF53 and not insertion II. These insertions are predicted to have roles in interacting with additional proteins.

***alp5-1134* contains a single point mutation**

The point mutation in *alp5-1134* was mapped by nucleotide sequencing to the C-terminus of the protein, as shown in Figure 3.3.1.B. It is a single missense mutation from G to A resulting in serine to asparagine change (S402N), which lies in a conserved region (although S402 itself is not conserved).

Structural analysis of Alp5

A predicted 3D structure of Alp5 was obtained based on the resolved crystal structure of actin (BioMolecular Modelling Lab, Cancer Research UK), which was superimposed onto the actin structure (Figure 3.3.2.A). From this, the two insertions discussed above can clearly be seen as two protruding loops. Position of the point mutation in the *alp5-1134* mutant is indicated by an arrow in Figure 3.3.2.B (left), and the precise mutation site in more detail is highlighted in green (right), which resides at the neck of the α -helix. Since asparagine contains a larger uncharged polar charge, this would most likely interfere with the internal folding of Alp5. These protruding loops are characteristic of Arp4-like proteins, which are not observed amongst other Arps. It would be of interest to determine the functions of these insertions, for example, they may be required for interacting with specific proteins, and/or required for specific localisation.

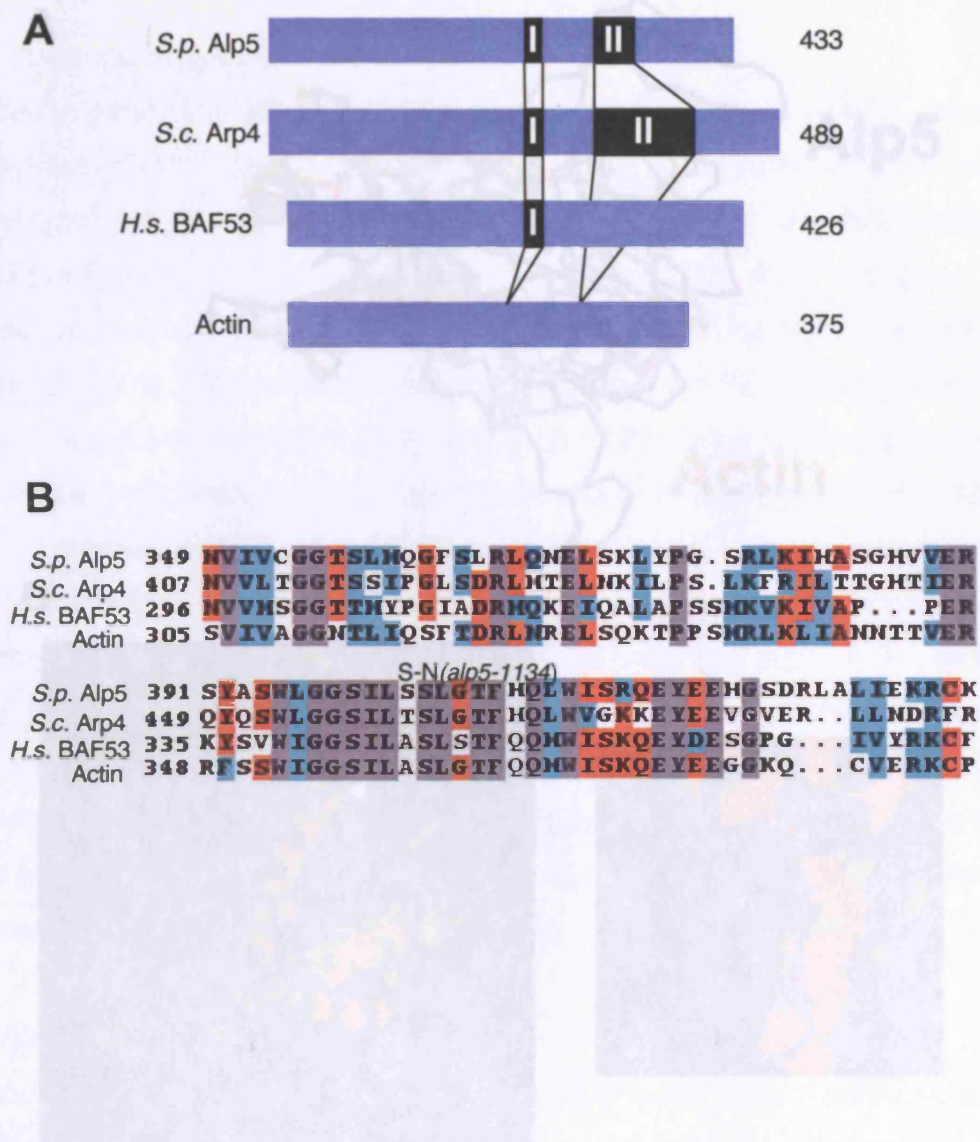
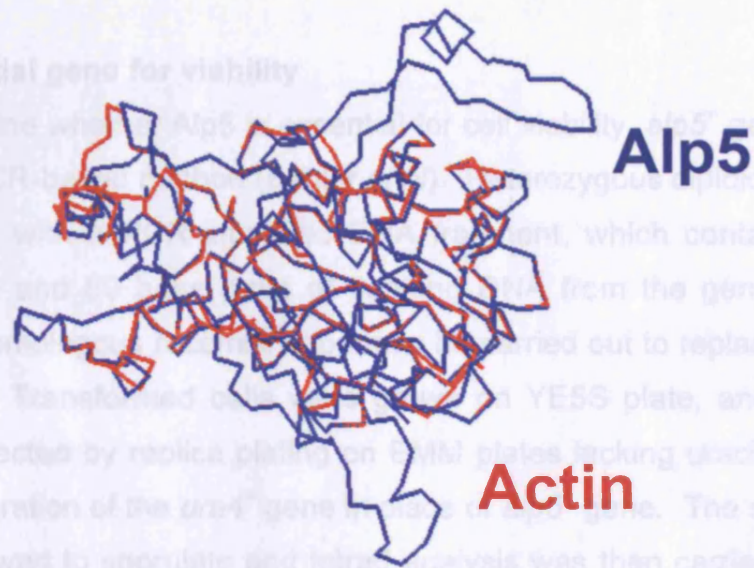


Figure 3.3.1 Comparison between Alp5 Homologues **A** Schematic structural comparison of Alp5, Arp4, BAF53 and actin. **B** Amino acid sequences of the region where point mutation occurs in *alp5-1134*. Amino acid sequences, which are identical in all the four members are shown in grey boxes, whilst residues identical between three members are shown in red boxes. The position of a point mutation (S402N) found in *alp5-1134* mutant is shown.

A



B

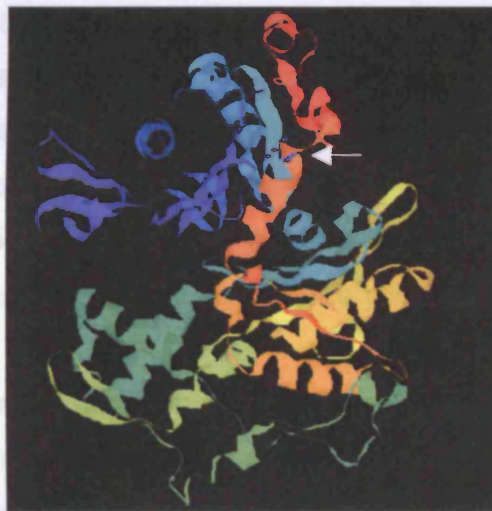


Figure 3.3.2 Predicted 3D structure of Alp5 A A superimposition of an actin (red) crystal structure to the predicted structure of Alp5 (blue). B A predicted structure of Alp5 based on the actin crystal structure. Blue and red regions correspond to N- and C-terminus, respectively. Arrow indicates the position of the single point mutation site of *alp5-1134*. (Right) A close up of the mutation site in *alp5-1134*, which resides at the top of α -helix, shown in green.

3.4 Analysis of *alp5*⁺ gene products

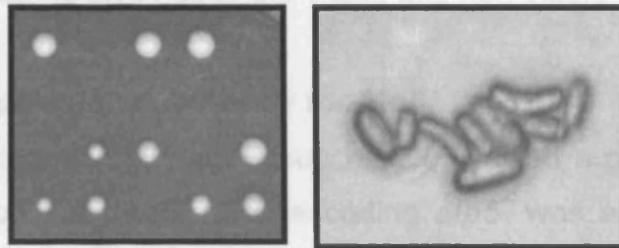
***alp5*⁺ is an essential gene for viability**

In order to determine whether Alp5 is essential for cell viability, *alp5*⁺ gene was deleted using a PCR-based method (Bahler *et al*). Heterozygous diploid strains were transformed with a PCR-amplified DNA fragment, which contains the whole *ura4*⁺ gene and 80 base pairs of flanking DNA from the gene to be deleted, so that homologous recombination may be carried out to replace *alp5*⁺ with *ura4*⁺ gene. Transformed cells were grown on YE5S plate, and *ura4*⁺ colonies were selected by replica plating on EMM plates lacking uracil, which indicated the integration of the *ura4*⁺ gene in place of *alp5*⁺ gene. The selected diploids were allowed to sporulate and tetrad analysis was then carried out to separate the four spores to determine the gene essentiality by their ability to germinate and form colonies. Figure 3.4.1.A shows that *alp5*⁺ is an essential gene since only two out of four spores were able to germinate and form colonies, which were verified to be Ura⁻. A closer examination revealed that the two spores, that did not form colonies, could in fact germinate and divide two or three times, but could not proceed into subsequent mitosis, revealing the terminal bent morphology phenotype.

Endogenous tagging of Alp5

In order to determine the localisation of Alp5 protein, both C-terminal and N-terminal tagging of Alp5 at its endogenous promoter was carried out using PCR-mediated long oligonucleotide methods (Bahler, Wu *et al*. 1998). A fragment was amplified by PCR, which contained an epitope (13-Myc and GFP; green fluorescent protein), a selectable marker of kanamycin resistance gene, and the flanking region of *alp5*⁺ gene for homologous recombination to take place. These fragments were then transformed into a wild-type strain and plated onto YE5S, incubated for 2 days and replica plated onto YE5S plates containing G418 to select those with kanamycin resistance gene. However, only one colony was obtained from myc-transformed cells, which turned out to be a diploid. Transformations were repeated several times with no success, thus, I reached the conclusion that tagging at either end of this protein is probably non-functional and thus lethal for cell viability. In order to show that

endogenous tagging of *Alp5* results in its loss of function, a heterozygous diploid of *alp5⁺/alp5-GFP-kan^r* could be constructed, after which a tetrad may be performed. Spores from 2:2 tetrads and non-viable colonies.



Raising a monoclonal antibody against *Alp5*. Since tagging of *Alp5* with GFP results in a loss of function, a monoclonal rabbit antibody against *Alp5* was raised. The coding *alp5⁺* was amplified and cloned into bacterial expression vector pET14b (Novagen, Madison, WI), which carries six copies of the histidine tag (6-His). The expressed 6-His tagged *Alp5* fusion protein was then tested for its solubility, and was found to be insoluble (see Figure 3.4.2.A). It was subsequently purified on Ni_2^{+} -NTA beads (QIAGEN, Valencia, CA), which was injected into two rabbits to generate two antisera. The resulting antisera were tested for immunoreaction against protein extract from *Basidi* yeast and against the original purified extract (Figure 3.4.3.A). Since the background was very high, the crude serum of rabbit 1 was affinity purified by immobilizing *Alp5* fusion protein on nitrocellulose filters. The resulting serum is very clean (see Figure 3.4.3.B). From Figure 3.4.3.B it is clear that *Alp5-myc* is a heterozygous diploid as mentioned before, since both the tagged and non-tagged *Alp5* bands are observed. Note that there are two bands detected in the wild-type protein extract with the affinity purified anti-*Alp5* serum. A possibility of immunoreaction of this antibody with another protein is investigated later in this chapter.

Alp5 abundance

In order to determine the abundance of the *Alp5* protein, 30 μg of whole cell extract of *Alp5-myc*, *Alp4-myc* and *Alp16-myc* were run on a 10% protein gel and blotted with anti-*myc* antibody. It is known that there are 2000 – 4000 molecules of *Alp5* per cell.

Figure 3.4.1 *alp5⁺* is essential for cell viability. Tetrad dissection of heterozygous diploid *alp5⁺/alp5⁺::ura4⁺* shows that *alp5⁺* is essential for viability (left). The spores are able to germinate and go through two to three rounds of division before showing the terminal bent phenotype. Scale-bar indicates 10 μm .

Abundance of *Alp5* was determined to be around 1000 molecules per cell. Although the *Alp5-myc* strain used for this analysis was a diploid (see Figure 3.4.3.B), this should not affect

endogenous tagging of Alp5 results in its loss of function, a heterozygous diploid of *alp5⁺/alp5-GFP-kan^R* could be constructed, after which a tetrad may be performed, to give rise to 2:2 viable and non-viable colonies.

Raising a polyclonal antibody for Alp5

Since tagging of Alp5 was unsuccessful, I raised a polyclonal rabbit antibody against Alp5. A whole ORF encoding *alp5⁺* was amplified and cloned into bacterial expression vector pET14-b (Novagen, Madison, WI), which carries six copies of the histidine tag (6-His). The expressed 6-His tagged Alp5 fusion protein was then tested for its solubility, and was found to be insoluble (see Figure 3.4.2.A). It was subsequently purified on Ni²⁺-NTA beads (QIAGEN, Valencia, CA), which was injected into two rabbits to generate two anti-sera.

The resulting antibodies were tested for immunoreaction against protein extract from fission yeast and against the original purified extract (Figure 3.4.3.A). Since the background was very high, the crude serum of rabbit 1 was affinity purified by immobilising Alp5 fusion protein on nitrocellulose filters. The resulting serum is very clean (see Figure 3.4.3.B). From Figure 3.4.3.B it is clear that Alp5-myc is a heterozygous diploid as mentioned before, since both the tagged and non-tagged Alp5 bands are observed. Note that there are two bands detected in the wild-type protein extract with the affinity purified anti-Alp5 serum. A possibility of immunoreaction of this antibody with another protein is investigated later in this chapter.

Alp5 abundance

In order to determine the abundance of the Alp5 protein, 30 µg of whole cell extract of Alp5-myc, Alp4-myc and Alp16-myc were run on a 10% protein gel and blotted with anti-myc antibody. It is known that there are 2000 – 4000 molecules of Alp4 and about 400 molecules of Alp16 in a fission yeast cell, which were determined on the basis of their protein level comparison with Cut2, the molecular number per cell of which is known as 20,000 (Funabiki, Yamano et al. 1996; Fujita, Vardy et al. 2002). As seen in Figure 3.4.4.A, Alp5 is more abundant than Alp16 but less than that of Alp4, thus Alp5 abundance was determined to be around 1000 molecules per cell. Although the Alp5-myc strain used for this analysis was a diploid (see Figure 3.4.3.B), this should not affect

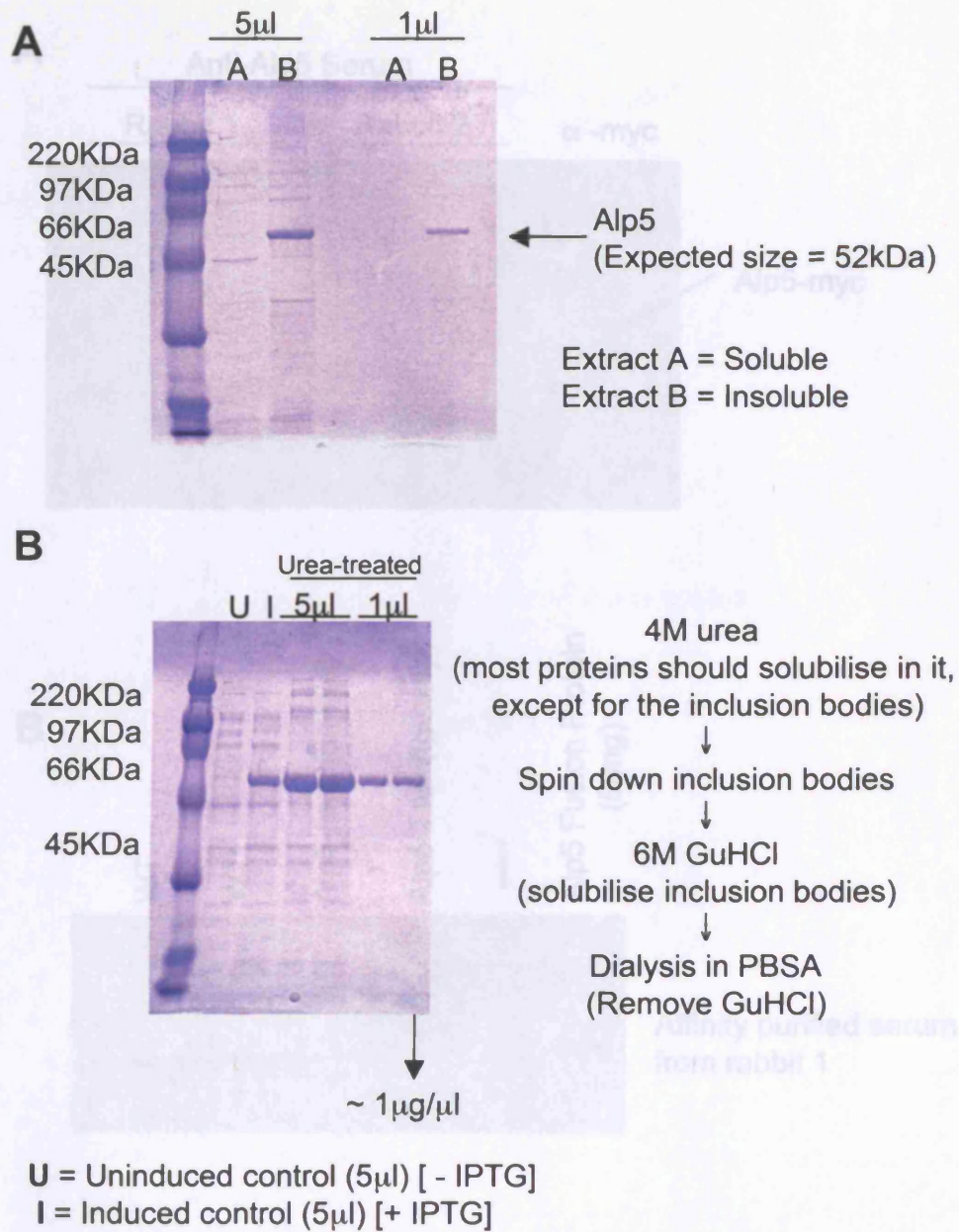


Figure 3.4.2 Production of polyclonal anti-Alp5 antibody **A** Alp5 was expressed from an *E.coli* pET vector, which was insoluble (i.e. formed inclusion bodies). **B** Expressed Alp5 from A was subsequently purified with 4 M urea, followed by an addition of guanidine hydrochloride (GuHCl) in order to solubilise the inclusion bodies. GuHCl was then removed through dialysis in PBSA to prepare for rabbit injection.

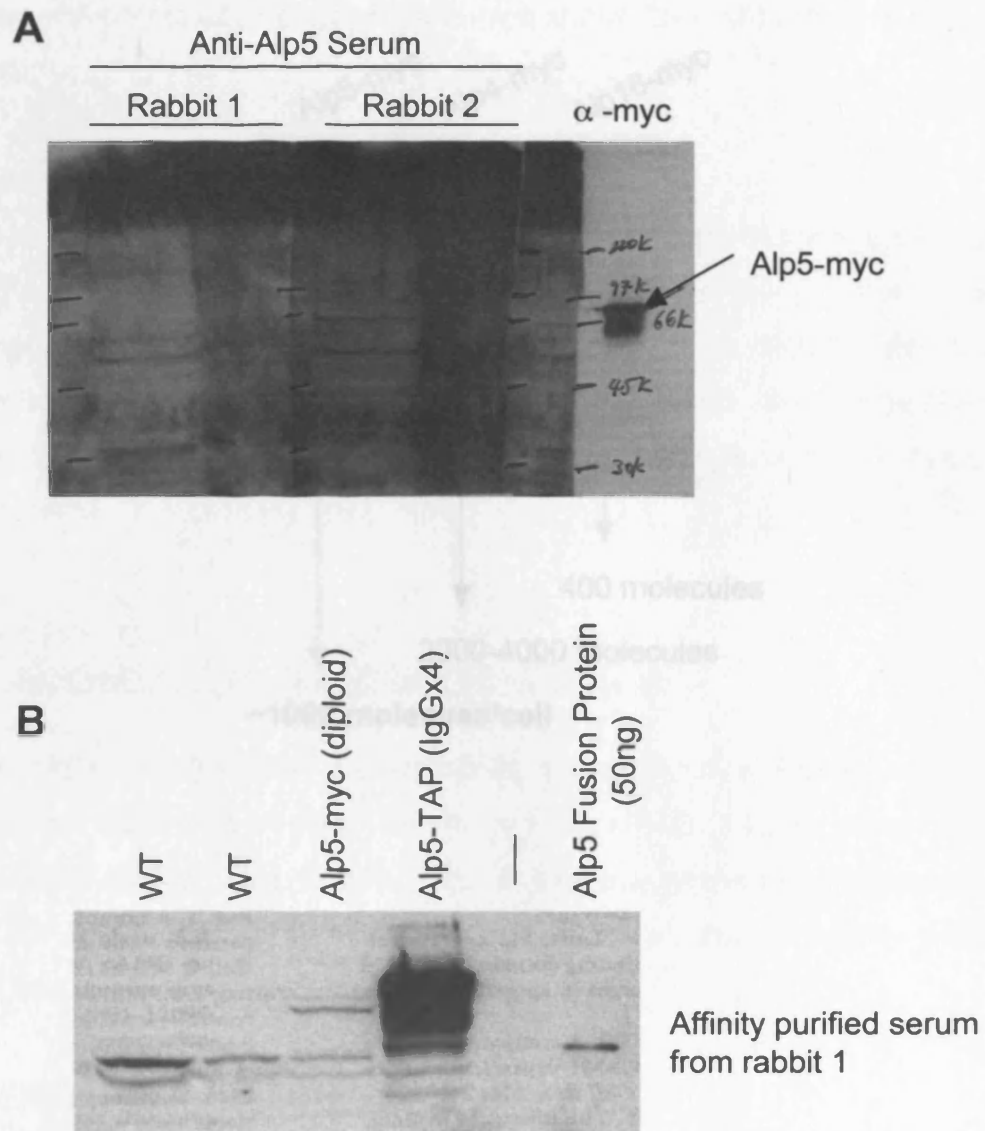
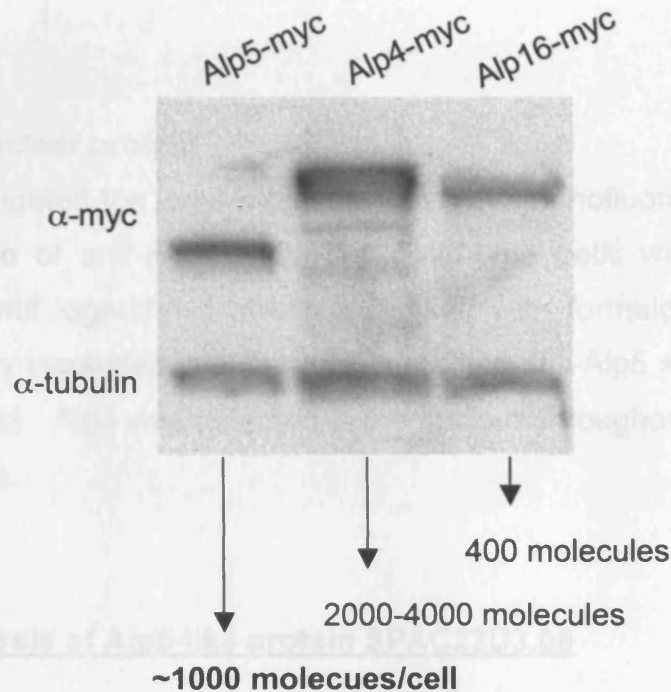


Figure 3.4.3 Polyclonal anti-Alp5 production **A** Polyclonal anti-Alp5 serum shows high background signal. Alp5 fusion protein was run on a protein gel and blotted with anti-Alp5 serum. Alp5-myc protein extract was also run and blotted with anti-myc antibody for comparison. **B** Serum from rabbit 1 was affinity purified and tested for its specificity with Alp5 strains of various tags.

the protein level of the myc-tagged Alp5, since it is a heterozygous diploid, the number of Alp5 molecules tagged with myc should be comparable to that of the untagged.

Alp5 is a nuclear protein

Next, to determine the localization of Alp5, cells were grown in YESS medium and then treated with formaldehyde, which were subsequently extracted with RNeasy lysis buffer. Alp5 protein (see Materials and Methods) was detected by Western blotting throughout the cell cycle (see Figure 3.4.3).



3.4.4 Analysis of Alp5 protein abundance

In an effort to characterize the acorn-related protein family in fission yeast, it was evident that there was a protein similar to Alp5, SPAC23D3.09, which contains 55% identity to Alp5 (see Figure 3.1). It also has similarity to budding yeast Alp7, and is encoded by 444 coding acids with predicted molecular weight of 47.8 KDa.

SPAC23D3.09 is localised on the chromatin

This protein was tagged with GFP to determine its localization and was also revealed to be a nuclear protein. The localization seems to be specifically on the chromatin, since it colocalises with the DAPI and the Hoechst staining which both stain the chromatin (see Figure 3.5.1.A).

SPAC23D3.09 is essential for cell viability

The wild-type was deleted in his⁻ background diploids by replacing with a his⁺ marker, thus showing that this gene is essential for cell viability.

No further characterisation was carried out with this protein since the localization is different to that of Alp5 and is nonessential.

Figure 3.4.4 Abundance of Alp5. 30 µg of protein extract from Alp5-myc, Alp4-myc or Alp16-myc expressing cells were run on SDS-PAGE gel and blotted with anti-myc antibody to compare protein amounts. 1000 molecules of Alp5 was estimated to be present in each cell.

the protein level of the myc-tagged Alp5, since it is a heterozygous diploid; the number of Alp5 molecules tagged with myc should be comparable to that of the haploid.

Alp5 is a nuclear protein

I next investigated the localisation of Alp5 by immunofluorescence microscopy with the use of anti-Alp5 antibody. Wild-type cells were grown in YE5S overnight until logarithmic phase and fixed with formaldehyde, which were subsequently incubated with the affinity purified anti-Alp5 serum (see Materials and methods). Alp5 was detected in the nucleus throughout the cell cycle (see Figure 3.4.5).

3.5 Analysis of Alp5-like protein SPAC23D3.09

In an effort to characterise the actin-related protein family in fission yeast, it was evident that there was a protein similar to Alp5, SPAC23D3.09, which contains 56% identity to Alp5 (see Figure 3.1). It also has similarity to budding yeast Arp7, and is encoded by 444 amino acids with predicted molecular weight of 47.8 KDa.

SPAC23D3.09 is localised on the chromatin

This protein was tagged with GFP to determine its localisation and was also revealed to be a nuclear protein. The localisation seems to be specifically on the chromatin, since it colocalises with the DAPI and the Hoechst staining, which both stain the chromatin (see Figure 3.5.1.A).

SPAC23D3.09 is not essential for cell viability

The whole ORF of this gene was deleted in heterozygous diploids by replacing with *ura4⁺* as before and was viable, thus is not an essential gene for cell viability, unlike *alp5⁺*.

No further characterisation was carried out with this protein since the localisation is different to that of Alp5 and is non-essential.

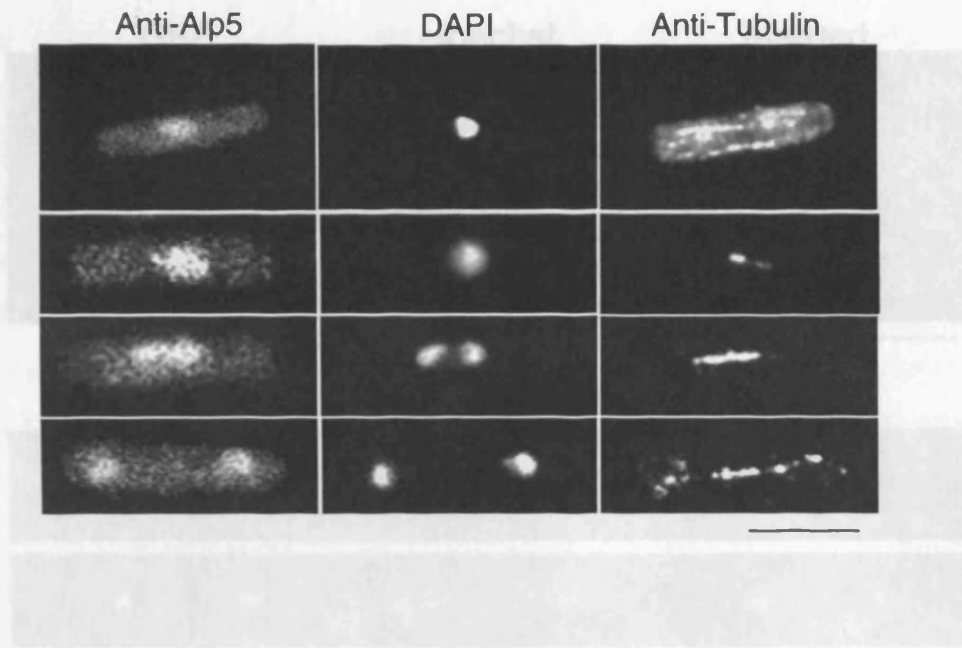
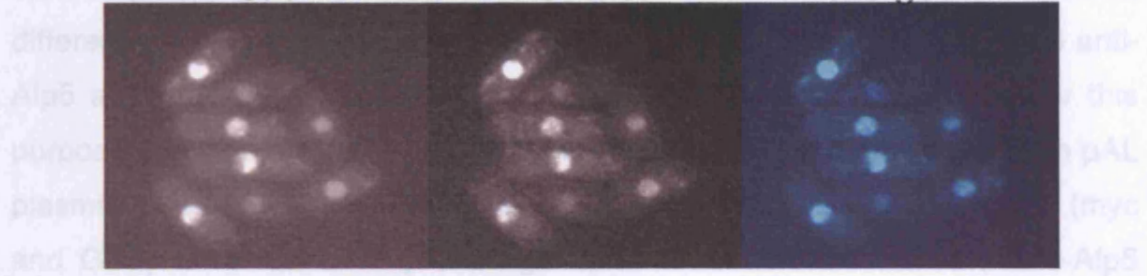


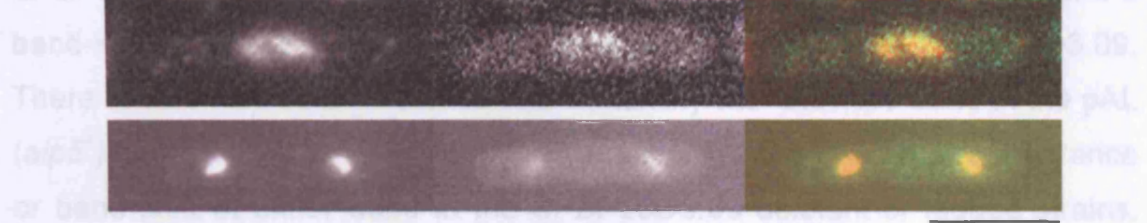
Figure 3.4.5 Alp5 is a nuclear protein. Exponentially grown wild-type cells were prepared for immunofluorescence and were stained with anti-Alp5 antibody, DAPI and anti-tubulin TAT1 antibody. Representative images during cell cycle are shown; interphase (top), early mitosis (second panel), and anaphase B (bottom panel). Scale-bar indicates 10 μm .

Anti-Alp5 antibody specificity

As described earlier, the anti-Alp5 antibody detects two bands in the wild-type extract (see Fig. 3.4.3.1), and there are no cross-reactions with



antibody. If either band is produced as a result of cross-reaction with SPBP23D3.09, then there should be a band in the SPBP23D3.09 strain and a



indicating neither band is produced as a result of cross-reaction with SPBP23D3.09. To further support that at least the top band is Alp5, an increase in its intensity was observed in the Alp5 plasmid-containing strain. It is yet unclear what the lower band is. It may be a by-product of Alp5 degradation or a cross-reaction band with another protein. Alternatively, it may be the unmodified Alp5, and the upper band may be that of the modified Alp5.

3.5 Discussion

Alp5 is an essential nuclear actin-related protein.

Through the isolation of a suppressor plasmid for *alp5-1134* temperature sensitivity, *alp5*⁺ was cloned and was subsequently sequenced to verify the gene as an actin-related protein on cosmid SPBP23A10.08. The homology

Figure 3.5.1 SPAC23D.09 is a nuclear protein. Exponentially-growing SPAC23D.09-GFP cells were fixed with formaldehyde and were stained with DAPI or Hoechst to observe DNA. Scale-bar indicates 10 μ m.

Anti-Alp5 antibody specificity

As described earlier, the anti-Alp5 antibody detects two bands in the wild-type extract (see Figure 3.4.3.B), and since there are only three amino acids difference in length between Alp5 and SPBP23D3.09, the possibility of the anti-Alp5 antibody cross-reacting with SPBP23D3.09 was investigated. For this purpose, the whole extracts of wild-type, *alp5* deletant containing *alp5⁺* on pAL plasmid (used for cloning), *SPBP23D3.09* deletant, and its tagged strains (myc and GFP) were run on a protein gel and immunoblotted with the anti-Alp5 antibody. If either band is produced as a result of cross-reaction with SPBP23D3.09, it should disappear in the *SPBP23D3.09* deletant strain, and a band-shift should be observed with the myc- or GFP-tagged SPBP23D3.09. There should also be an increase in the intensity with the Alp5 band in the pAL (*alp5⁺*)-containing strain. As seen in Figure 3.5.2, there was no disappearance or band-shift of either band in the *SPBP23D3.09* deletant or tagged strains, indicating neither bands are produced as a result of cross-reactivity with SPBP23D3.09. To further support that at least the top band is Alp5, an increase in its intensity was observed in the Alp5 plasmid-containing strain. It is yet unclear what the lower band is. It may be a by-product of Alp5 degradation, or a cross-reaction band with another protein. Alternatively, it may be the unmodified Alp5, and the upper band may be that of the modified Alp5.

3.6 Discussion

Alp5 is an essential nuclear actin-related protein

Through the isolation of a suppressor plasmid for *alp5-1134* temperature sensitivity, *alp5⁺* was cloned and was subsequently sequenced to verify the gene as an actin-related protein on cosmid SPBP23A10.08. The homology search using BLAST server led to the findings of its homologues as yeast Arp4 and human BAF53. Both of these proteins are known to reside in multi-subunit chromatin modification complexes (see Chapter 1). Arp4 in budding yeast is present in the NuA4 HAT complex, which acetylates histone H4, and also in the ATP-dependent CRCs of Ino80 and SWR1 (Galarneau, Nourani et al. 2000; Shen, Mizuguchi et al. 2000; Krogan, Keogh et al. 2003; Kobor,

Vankatasubramanian et al. 2004). Human BAF53 is found in both Tip60 HAT complex and also in ATP-dependent CRCs called the BAF and PBAF complexes (Zhao, Wang et al. 1998). In *S. cerevisiae*, Alp5 is a nuclear protein, as its homologues, the functions of BAF53 in humans and its orthologues in chromatin modification are predicted to be conserved.

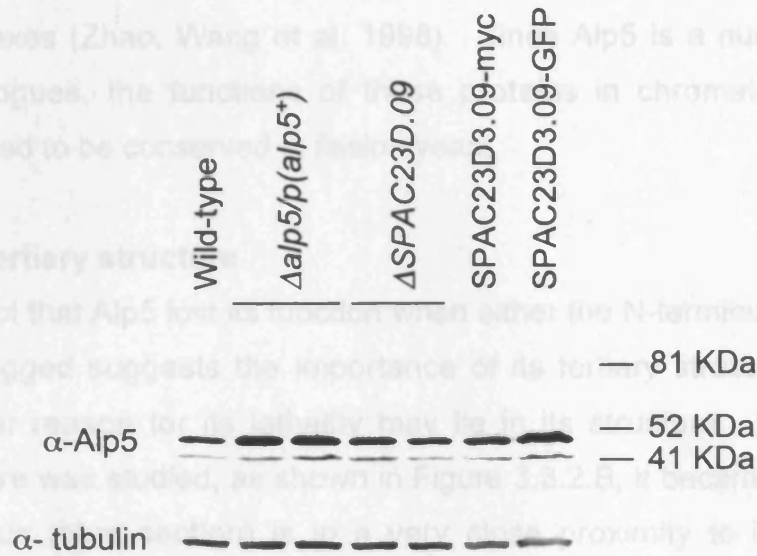
Alp5 tertiary structure

The fact that Alp5 lost its function when either the N-terminus or the C-terminus was tagged suggests the importance of its tertiary structure for its function. Another possibility for loss of function may be that its predicted structure was studied, as shown in Figure 3.3.2.B, it is quite evident that the N-terminus is in a very close proximity to its C-terminus (red section). Thus, tagging of Alp5 at either end may result in the disruption of its tertiary structure, which may be essential for its function.

An Alp5-like protein

SPAC23D3.09, a protein with high similarity to Alp5, is not essential for cell viability, and is localized to the chromatin region. The closest budding yeast protein is Arp4, followed by Arp7. Heterodimerisation of Arp4 has been observed in chromatin modification complexes, such as Arp7 and Arp9 in SWI/SNF and RSC complexes (Szerlong, Saha et al. 2005). Thus, Alp5 and SPAC23D3.09 may also be able to heterodimerise using their orthologous regions. Alternatively, the use of BAF53 in human may be conserved in *Saccharomyces*. The BAF and PBAF complexes contain either BAF53a or BAF53b as their subunit which are products of two distinct genes (Olave, Wang et al. 2002).

Figure 3.5.2 Specificity of anti-Alp5 antibody. The whole cell extracts of the indicated strains were run on a SDS-PAGE gel and immunoblotted with anti-Alp5 antibody. The possibility of anti-Alp5 antibody cross-reacting with SPAC23D3.09 was ruled out, since both of the bands remained in its deletion strain and no band shift was observed with the myc or GFP tagged strains. Anti-tubulin level was determined as a loading control. SPAC23D3.09 is more similar to BAF53 than Arp4. At this point, either possibility may be as likely as each



Venkatasubrahmanyam et al. 2004). Human BAF53 is found in both Tip60 HAT complex and also in ATP-dependent CRCs called the BAF and PBAF complexes (Zhao, Wang et al. 1998). Since Alp5 is a nuclear protein, as its homologues, the functions of these proteins in chromatin modification are predicted to be conserved in fission yeast.

Alp5 tertiary structure

The fact that Alp5 lost its function when either the N-terminus or the C-terminus was tagged suggests the importance of its tertiary structure for its function. Another reason for its lethality may lie in its structure. When its predicted structure was studied, as shown in Figure 3.3.2.B, it became evident that its N-terminus (blue section) is in a very close proximity to its C-terminus (red section). Thus, tagging of Alp5 at either end may result in the disruption of its tertiary structure, which may be essential for its function.

An Alp5-like protein

SPAC23D3.09, a protein with high similarity to Alp5, is not essential for cell viability, and is localised to the chromatin region. The closest budding yeast protein is Arp4, followed by Arp7. Heterodimerisation of Arps has been observed in chromatin modification complexes, such as Arp7 and Arp9 in SWI/SNF and RSC complexes (Szerlong, Saha et al. 2003). Thus, Alp5 and SPAC23D3.09 may also be able to heterodimerise using their actin-related regions. Alternatively, the use of BAF53 in human may be conserved in fission yeast. The BAF and PBAF complexes contain either BAF53 α or BAF53 β as their subunits, which are products of two distinct genes (Olave, Wang et al. 2002). There are only 3 amino acids difference in length between these two proteins (426 and 429 amino acids, respectively), which also is the case with Alp5 and SPAC23D3.09 (433 and 430 amino acids, respectively). In addition, BAF53 is almost equally similar to both Alp5 and SPAC23D3.09. Another possibility is that SPAC23D3.09 is a functional homologue of BAF53, and Alp5 of yeast Arp4; sequence comparison between protein sequences indicate that Alp5 is more similar to Arp4 than BAF53, and SPAC23D3.09 is more similar to BAF53 than Arp4. At this point, either possibility may be as likely as each

other, and it remains to be seen whether Alp5 carries out the functions of either Arp4 and BAF53, or both.

Chapter 4

The Role of Alp5 in Histone Acetylation

This chapter describes the role of Alp5 in histone H4 acetylation. Its acetylation role is most likely carried out through its interaction with Mst1 in a 2 MDa complex. Mst1 is a HAT homologous to budding yeast Esa1 and human Tip60. Here, the essential role of Alp5 in global histone H4 acetylation will be described. The counteracting HDAC enzymes to Alp5 are also investigated.

In addition, the results suggest that Alp5-dependent histone acetylation is importance for chromosome segregation and mitotic progression.

4.1 Introduction

The NuA4 HAT complex

Esa1, the HAT enzyme of the budding yeast NuA4 complex, is the only essential HAT in budding yeast. The complex acetylates all four lysine residues on the histone H4 amino tail (K5, K8, K12, and K16) (see Figure 4.1.1.A), and H2A amino tail to some extent (Allard, Utley et al. 1999). It is a multi-subunit complex with 13 subunits (~2 MDa), and contains an essential protein Arp4 (Allard, Utley et al. 1999; Galarneau, Nourani et al. 2000), the homologue of Alp5 (see Chapter 3). It has also been shown that the NuA4 complex is required for cell cycle progression, where a conditional *esa1* mutant arrests at G2/M, which is associated with a decrease in histone H4 acetylation at lysine 5 (Clarke, Lowell et al. 1999). The human homologue of Esa1 is Tip60 (HIV-Tat1-interacting protein), which is also found in a large complex, called the Tip60 complex. This complex contains TRRAP, the yeast homologue of Tra1, which interacts with c-Myc, E2F and E1A,

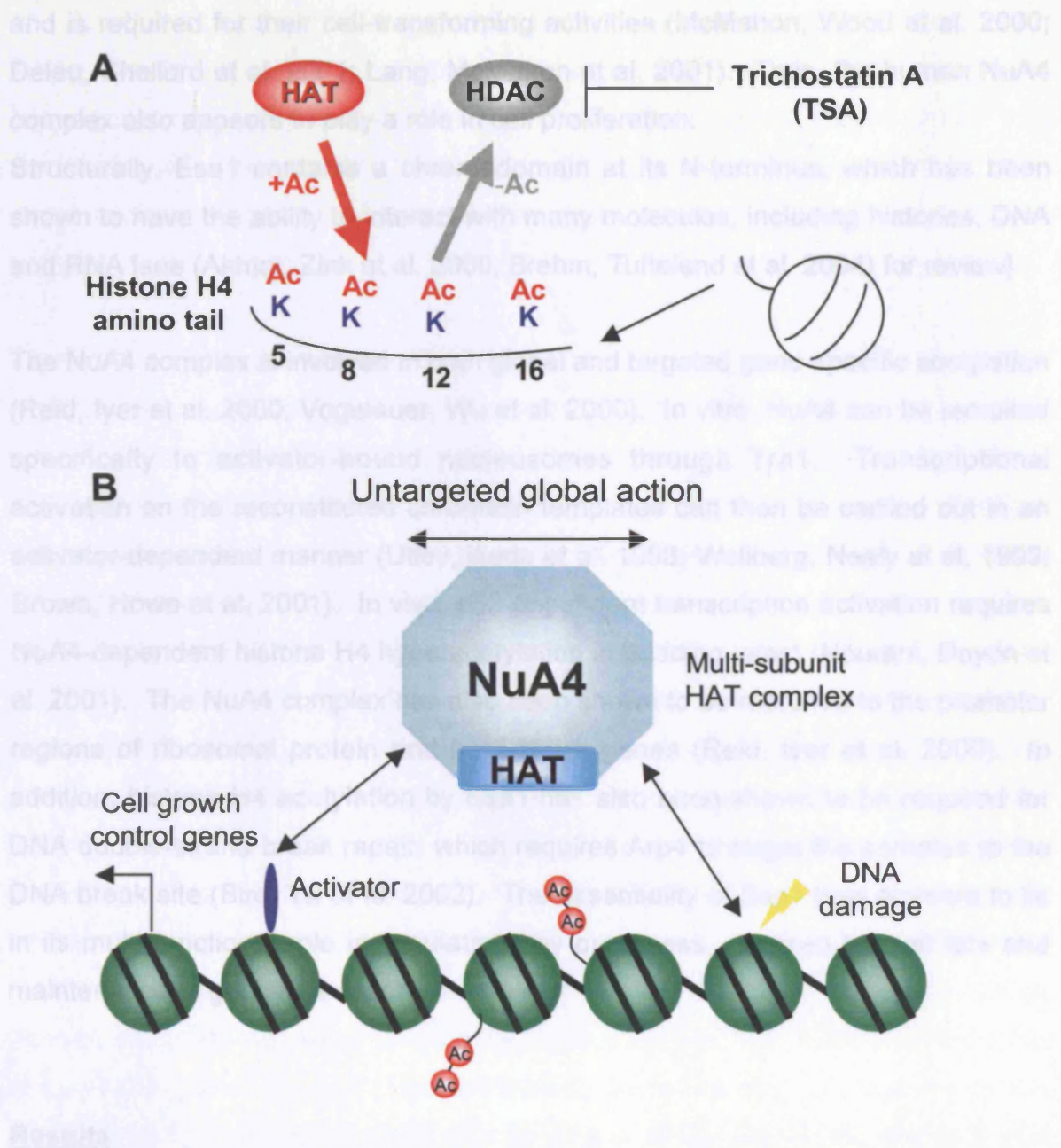


Figure 4.1.1 Histone H4 tail acetylation. **A** A schematic diagram of histone tail exposed out of the nucleosome. There are four lysine residues on histone H4 amino tail, at 5, 8, 12 and 16, which can be acetylated by HATs and deacetylated by HDACs. TSA is a Class I and II HDAC inhibitor drug. **B** NuA4 HAT complex affects transcription activation through global and targeted action and DNA repair. [Adapted from Carrozza *et al* 2003 Trends in Genetics 19: 321]

and is required for their cell-transforming activities (McMahon, Wood et al. 2000; Deleu, Shellard et al. 2001; Lang, McMahon et al. 2001). Thus, the human NuA4 complex also appears to play a role in cell proliferation.

Structurally, Esa1 contains a chromodomain at its N-terminus, which has been shown to have the ability to interact with many molecules, including histones, DNA and RNA [see (Akhtar, Zink et al. 2000; Brehm, Tufteland et al. 2004) for review].

The NuA4 complex is involved in both global and targeted gene-specific acetylation (Reid, Iyer et al. 2000; Vogelauer, Wu et al. 2000). In vitro, NuA4 can be recruited specifically to activator-bound nucleosomes through Tra1. Transcriptional activation on the reconstituted chromatin templates can then be carried out in an activator-dependent manner (Utley, Ikeda et al. 1998; Wallberg, Neely et al. 1999; Brown, Howe et al. 2001). In vivo, p53-dependent transcription activation requires NuA4-dependent histone H4 hyperacetylation in budding yeast (Nourani, Doyon et al. 2001). The NuA4 complex has also been shown to be recruited to the promoter regions of ribosomal protein and heat-shock genes (Reid, Iyer et al. 2000). In addition, histone H4 acetylation by Esa1 has also been shown to be required for DNA double-strand break repair, which requires Arp4 to target the complex to the DNA break site (Bird, Yu et al. 2002). The essentiality of Esa1 thus appears to lie in its multi-functional role in regulating key processes, required for cell fate and maintenance of genome integrity.

Results

4.2 Biochemical Analysis of Alp5

Alp5 interacts with Esa1 homologue Mst1

The budding yeast Arp4 is known to interact with Esa1 as part of the NuA4 complex, thus, I decided to find out whether this interaction is conserved in fission yeast by carrying out an immunoprecipitation assay (see Materials and Methods).

The Esa1 homologue in fission yeast is Mst1, and a myc-tagged strain (Mst1-myc) of this protein was constructed. When Mst1-myc was immunoprecipitated from the prepared whole cell extract with anti-myc beads, Alp5 was also pulled down. This interaction was not observed in the untagged strain (see Figure 4.2.1.A). This result shows that the interaction between Alp5 and Mst1 is conserved in fission yeast.

Alp5 is in a 2 MDa complex

In *S. cerevisiae*, Esa1 is known to exist in a NuA4 complex that has a size of around 2 MDa (Allard, Utley et al. 1999; Galarneau, Nourani et al. 2000). Since Alp5 was shown to interact with the homologue of Esa1, it was possible that Alp5 existed in a similarly large NuA4-type complex. This was investigated by a gel filtration assay using a Superose 6 column. Samples were prepared from the wild-type and the *alp5-1134* mutant, grown at 26°C and 36°C, which were then processed as necessary (see Materials and Methods). As shown in Figure 4.2.1.B, Alp5 exists in a complex with a molecular weight of roughly 2 MDa. It is of note that even at its restrictive temperature, the complex appears to be intact in the *alp5-1134* mutant, suggesting that the *alp5-1134* mutation does not affect the assembly and/or the maintenance of this large complex. Also of note is that Alp5 comes down in the fractions 23 and 25 in the wild-type and the *alp5-1134* mutant at permissive temperature. These are most likely to be Alp5 monomers and/or dimers, which do not seem to be present in the *alp5-1134* mutant at restrictive temperature. Since this point was not investigated any further, it is unknown if this observation has any significance. It is possible that the Alp5-1134 protein is less stable in its monomer or dimer form at its restrictive temperature.

Thus, Alp5 interacts with the homologue of a recognised NuA4 component, and is found in a complex of similar molecular weight to NuA4. Taken together, these results suggest that a NuA4-like complex is conserved in fission yeast, and that Alp5 may be required for histone acetylation through its interaction with Mst1.

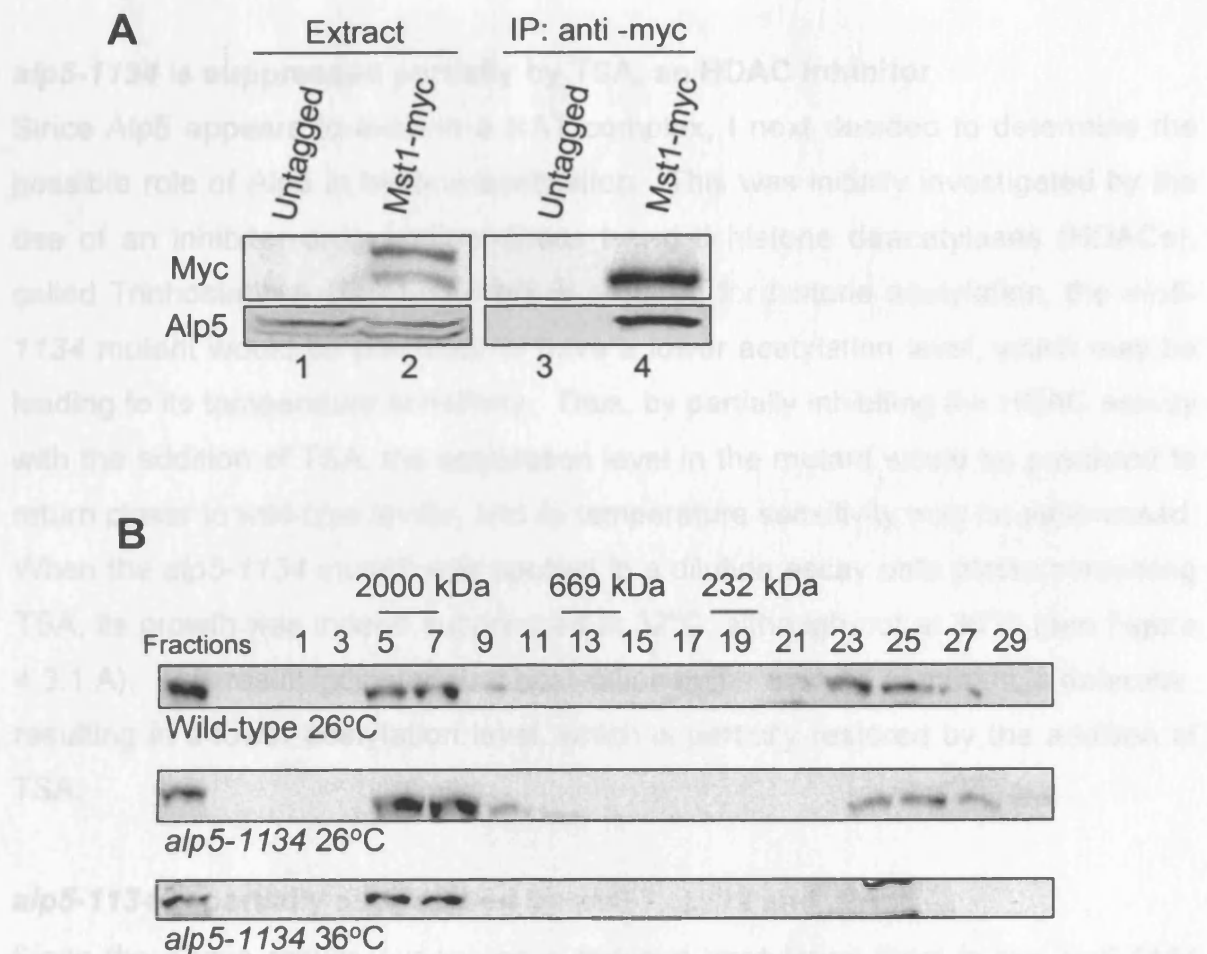
4.3 *Alp5 is essential for global histone H4 acetylation in vivo*

Figure 4.2.1 Alp5 interacts with Mst1 HAT and is in a large complex **A** Alp5 immunoprecipitates with Mst1. Immunoprecipitation was performed with anti-myc antibody with protein extract prepared from Mst1-myc cells, which was subsequently run on SDS-PAGE and immunoblotted with anti-myc or anti-Alp5 antibodies (lane 4). As a negative control, protein extracts were also prepared from an untagged strain (lane 3). **B** Gel filtration chromatography. Soluble cell extracts were prepared from wild-type cells grown at 26°C (upper panel) or *alp5-1134* cells at either 26°C (middle row) or 36°C (bottom panel) and loaded onto Superose 6 column. Each fraction was run on SDS-PAGE and immunoblotted with anti-Alp5 antibody.

4.3 Alp5 is essential for global histone H4 acetylation in vivo

***alp5-1134* is suppressed partially by TSA, an HDAC inhibitor**

Since Alp5 appears to exist in a HAT complex, I next decided to determine the possible role of Alp5 in histone acetylation. This was initially investigated by the use of an inhibitor drug against Class I and II histone deacetylases (HDACs), called Trichostatin A (TSA). If Alp5 is required for histone acetylation, the *alp5-1134* mutant would be predicted to have a lower acetylation level, which may be leading to its temperature sensitivity. Thus, by partially inhibiting the HDAC activity with the addition of TSA, the acetylation level in the mutant would be predicted to return closer to wild-type levels, and its temperature sensitivity may be suppressed. When the *alp5-1134* mutant was spotted in a dilution assay onto plates containing TSA, its growth was indeed suppressed at 32°C, although not at 36°C (see Figure 4.3.1.A). This result indicated that acetylation in the *alp5-1134* mutant is defective, resulting in a lower acetylation level, which is partially restored by the addition of TSA.

alp5-1134* is partially suppressed by *clr6-1*, Δ *sir2* and Δ *hst4

Since the above results suggested a reduced acetylation level in the *alp5-1134* mutant, I next sought to identify the HDAC that is genetically interacting with *alp5-1134* (i.e. the target of TSA, which is suppressing the *alp5-1134* mutant). Deletions or a mutation of each of the six known fission yeast HDACs were introduced into the *alp5-1134* background, and the temperature sensitivity were examined. Figure 4.3.1.B shows that out of the six double mutants, *clr6-1*, Δ *sir2* and Δ *hst4* mutants suppressed the slow growth of the *alp5-1134* mutant at 32°C (Figure 4.3.1.B, underlined). The suppression was nonetheless partial, since no growth was observed at 36°C. Since Clr6 is the only HDAC protein inhibited by TSA (TSA only inhibits Class I and II HDACs), this result suggested that the target of TSA for the *alp5-1134* suppression is Clr6. In contrast, the growth of *alp5-1134* Δ *hos2* was worse than that of the single mutants at 32°C. This may suggest that

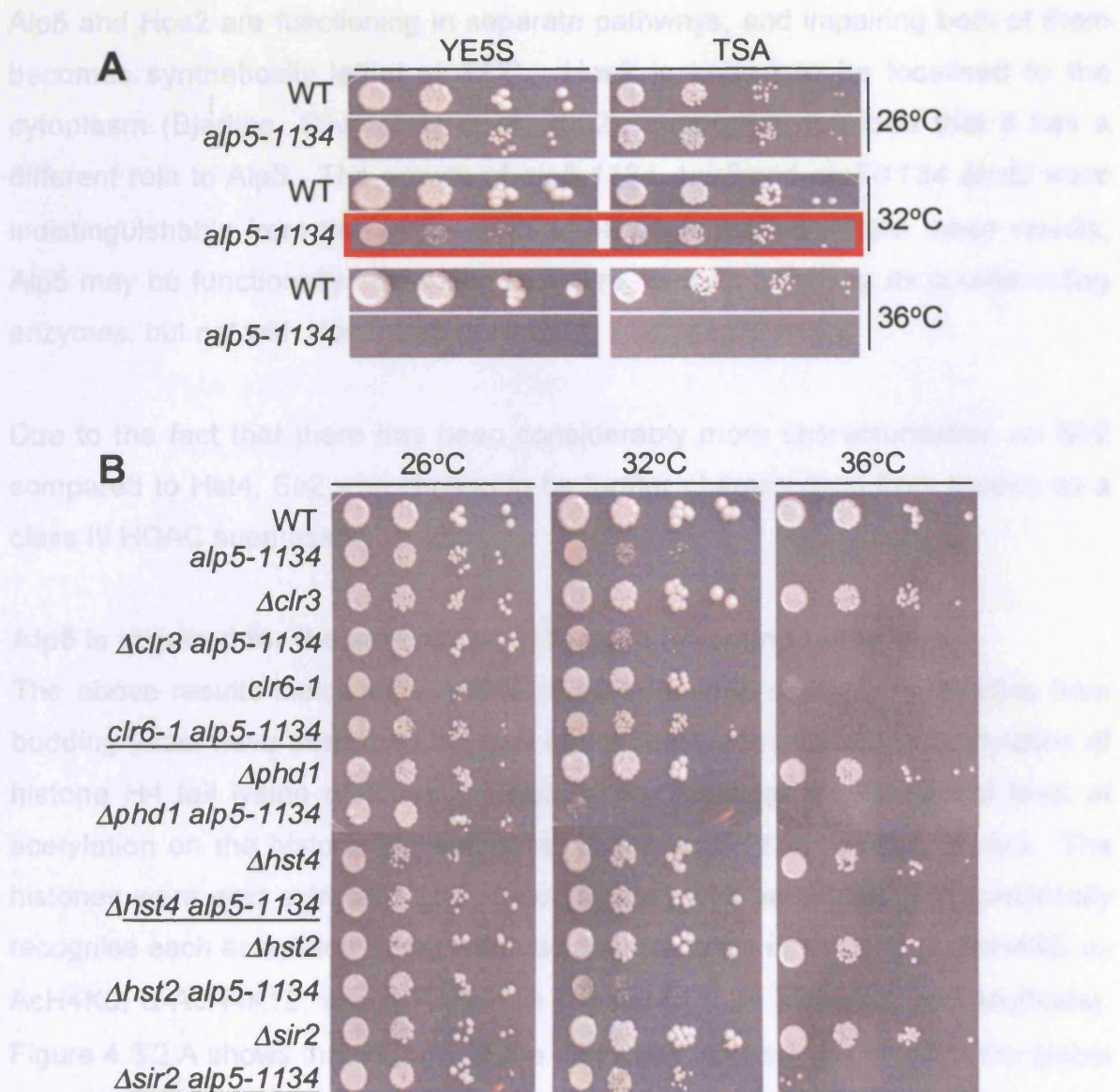


Figure 4.3.1 *alp5-1134* is partially suppressed by TSA and three of HDAC mutants **A** Wild-type (WT) or *alp5-1134* cells were spotted onto rich plates in the absence (left) or presence of TSA (50 μ g/ml), and incubated at indicated temperatures. Temperature lethality of *alp5-1134* is suppressed by TSA at 32°C (red box). **B** The indicated strains were spotted in a dilution assay onto rich plates and incubated at indicated temperatures. *clr6-1*, *Δhst4*, and *Δsir2* were able to suppress the temperature lethality of *alp5-1134* at 32°C (underlined).

Alp5 and Hos2 are functioning in separate pathways, and impairing both of them becomes synthetically lethal at 32°C. Hos2 is known to be localised to the cytoplasm (Bjerling, Silverstein et al. 2002), supporting the idea that it has a different role to Alp5. The growth of *alp5-1134 Δclr3* and *alp5-1134 Δhst2* were indistinguishable from that of the *alp5-1134* single mutant. From these results, Alp5 may be functionally interacting with Clr6, Sir2 and Hst4 as its counteracting enzymes, but not with Hos2, Clr3 or Hst2.

Due to the fact that there has been considerably more characterisation on Sir2 compared to Hst4, Sir2 was chosen to be further characterised from hereon as a class III HDAC suppressor.

Alp5 is required for the acetylation of histone H4 amino tail in vivo

The above results indicated a role for Alp5 in histone acetylation. Studies from budding yeast have described the role of the NuA4 complex in the acetylation of histone H4 tail lysine residues. Thus, I next investigated the global level of acetylation on the histone H4 amino tail in the *alp5-1134* mutant in vivo. The histones were acid extracted and immunoblotted with antibodies that specifically recognise each acetylated lysine residues on histone H4 amino tails [α -AcH4K5, α -AcH4K8, α -AcH4K12, and α -AcH4K16 (Upstate)] (see Materials and Methods). Figure 4.3.2.A shows that indeed, at the restrictive temperature of 36°C, the global acetylation level of all four lysine residues on histone H4 amino tail of the *alp5-1134* mutant are considerably lower compared to that of the wild-type. Also it is important to note that the levels of acetylation on H4K5, H4K8 and H4K12 in the double mutant of *alp5-1134 clr6-1* are restored to a similar level to that of the wild-type, which may relate to the *alp5-1134* growth suppression by *clr6-1* described earlier. The finding of Clr6 HDAC activity on H4K5, H4K8 and H4K12 is consistent with the previous report (Bjerling, Silverstein et al. 2002).

Thus, taken together, these results suggest that Alp5 is required for the global acetylation of histone H4 amino tail in vivo, and that its counteracting HDAC is Clr6.

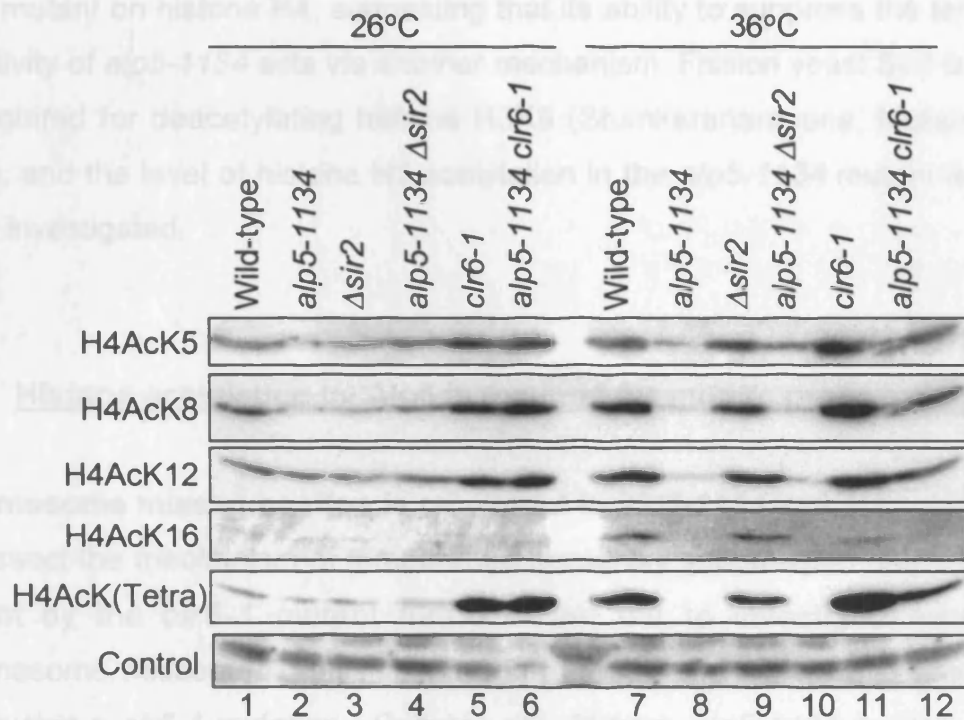


Figure 4.3.2 Alp5 is required for histone H4 tail acetylation in vivo.

A Acid-extracted histones from various strains as indicated were run on SDS-PAGE and immunoblotted with various antibodies. These antibodies recognise specific acetylated lysine residues on histone H4 amino tail. Signals from non-specific bands are shown as loading controls (bottom)

It is of note that the $\Delta sir2$ mutant did not restore the acetylation level of the *alp5-1134* mutant on histone H4, suggesting that its ability to suppress the temperature sensitivity of *alp5-1134* acts via another mechanism. Fission yeast Sir2 is known to be required for deacetylating histone H3K9 (Shankaranarayana, Motamedi et al. 2003), and the level of histone H3 acetylation in the *alp5-1134* mutant is currently being investigated.

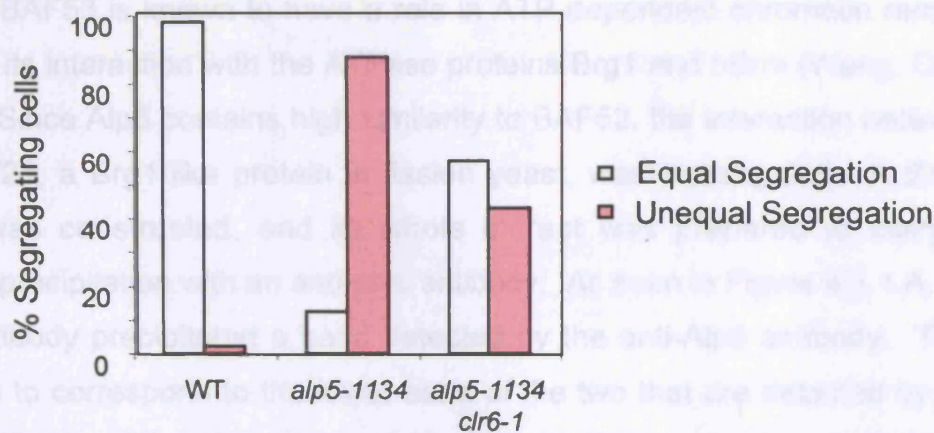
4.4 Histone acetylation by Alp5 is required for mitotic progression

Chromosome missegregation is recovered in *alp5-1134 clr6-1*

To dissect the mechanism of temperature sensitivity suppression of the *alp5-1134* mutant by the *clr6-1* mutant further, I set out to investigate whether the chromosome missegregation observed in the *alp5-1134* mutant is recovered by introducing a *clr6-1* mutation. Cultures of wild-type, *alp5-1134* mutant and *alp5-1134 clr6-1* double mutant were shifted to 36°C for six and eight hours, and fixed with formaldehyde. When DAPI staining was observed, chromosome missegregation in the double mutant appeared to be less severe than the single *alp5-1134* mutant. This was confirmed by quantification of missegregation rate in binucleate cells, as shown in Figure 4.4.1.A. Thus, the *clr6-1* mutation, in addition to being seemingly able to restore histone H4 acetylation level in the *alp5-1134* mutant, also appeared to partially restore the ability of chromosomes to segregate correctly in this background. Although this suppression was not a full recovery, it is consistent with the previous results, where the suppression of temperature sensitivity by *clr6-1* and TSA are also partial.

4.4 The role of Alp5 in ATP-dependent chromatin remodeling

Human BAF is a large protein complex involved in ATP-dependent chromatin remodeling, through interactions with the histone core proteins HsgF and HsgE (Yang, Gao et al. 1999). The BAF complex contains highly similar to BAF52, the interaction between Alp5 and Sp1. This protein interaction is also observed in the *alp5-1134* mutant. An immunoprecipitation assay with an anti-Alp5 antibody from the *alp5-1134* strain showed an immunoprecipitated band with an anti-Sp1 antibody. In the *alp5-1134* strain, the anti-Alp5 antibody appears to correspond to two bands that are detected by the anti-Alp5 antibody. This raises a possibility that Alp5 may also be involved in ATP-dependent chromatin remodeling process as well as histone acetylation. However, the immunoprecipitation assay could be verified as an Alp5 band from any further studies are carried out (see also Chapter 4.5 Discussion).



4.5 Discussion

The role of Alp5 in global histone H4 acetylation

These results show that Alp5 is required for global histone H4 acetylation in vivo, and that this function is likely to be important for accurate chromosome segregation. The enzymatic activity required for histone acetylation is most likely carried out by Mat1 HAT, which was found to interact with Alp5 (Figure 4.2.1). Mat1 is homologous to the catalytic HAT subunit of both budding yeast *Asp1* and *Tip60* complexes (Esa1 and Tip60, respectively), which have been shown to acetylate histone H4 (Galanteau, Hourani et al. 2000; Shen, Margolin et al. 2000). This study has also revealed that a H4-H3-HAT complex is most likely composed in

Figure 4.4.1 Partial suppression of chromosome missegregation in the *alp5-1134 clr6-1* mutant. Wild-type (WT), *alp5-1134* and *alp5-1134 clr6-1* cultures were incubated at 36°C for 8 hours and DAPI-stained cells were quantified for equal or unequal chromosome segregation among bi-nucleated cells.

4.5 The role of Alp5 in ATP-dependent chromatin remodelling

Human BAF53 is known to have a role in ATP-dependent chromatin remodelling, through its interaction with the ATPase proteins Brg1 and hBrm (Wang, Cote et al. 1996). Since Alp5 contains high similarity to BAF53, the interaction between Alp5 and Snf21, a Brg1-like protein in fission yeast, was investigated. A Snf21-myc strain was constructed, and its whole extract was prepared to carry out an immunoprecipitation with an anti-myc antibody. As seen in Figure 4.5.1.A, the anti-myc antibody precipitated a band detected by the anti-Alp5 antibody. This band appears to correspond to the lower band of the two that are detected by the anti-Alp5 antibody. This raises a possibility that Alp5 may also be involved in ATP-dependent chromatin remodelling process as well as histone acetylation. However, the immunoprecipitated band should be verified as an Alp5 band, prior any further studies are carried out (see also Chapter 4.6 Discussion).

4.6 Discussion

The role of Alp5 in global histone H4 acetylation

These results show that Alp5 is required for global histone H4 acetylation in vivo, and that this function is likely to be important for accurate chromosome segregation. The enzymatic activity required for histone acetylation is most likely carried out by Mst1 HAT, which was found to interact with Alp5 (Figure 4.2.1). Mst1 is homologous to the catalytic HAT subunit of both budding yeast and human NuA4 complexes (Esa1 and Tip60, respectively), which have been shown to acetylate histone H4 (Galarneau, Nourani et al. 2000; Shen, Mizuguchi et al. 2000). This study has also revealed that a NuA4-like HAT complex is most likely conserved in fission yeast; Alp5 interacts with Esa1-like protein and is found in a large complex around the size of 2 MDa, which is similar to that of budding yeast (Allard, Utley et al. 1999; Galarneau, Nourani et al. 2000). In order to verify that Alp5 and Mst1 exist in the same complex as predicted, a gel filtration assay should be carried out

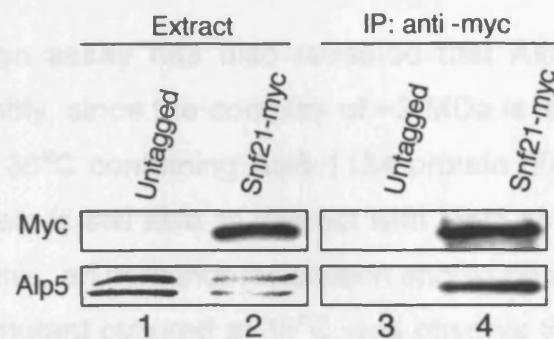


Figure 4.5.1 Alp5 interacts with Snf21 ATPase. Alp5 immunoprecipitates with Snf21. Immunoprecipitation was performed with anti-myc antibody in Snf21-myc cell extract, followed by immunoblotting with anti-myc or anti-Alp5 antibodies. As a negative control, immunoprecipitation was also performed in a protein extract prepared from an untagged strain (lane 3).

with Mst1-myc strain and observe whether Alp5 and Mst1 are present in the same fractions.

The gel filtration assay has also revealed that Alp5 may not be required for complex assembly, since the complex of ~2 MDa is still detected at the restrictive temperature of 36°C containing Alp5-1134 protein (Figure 4.2.1.B). Whether the Alp5-1134 protein is still able to interact with Mst1 at its restrictive temperature is unknown. For this, an immunoprecipitation should be carried out with the extract of the *alp5-1134* mutant cultured at 36°C, and observe the interaction between Alp5-1134 and Mst1 proteins. One possibility is that although Alp5-1134 is able to assemble into the complex, the interaction with Mst1, which may be required for histone acetylation, is lost at the restrictive temperature.

The role of Alp5 within the complex is yet to be determined. In budding yeast, Arp4 is able to bind to all core histones in vitro and is found on several promoters (Harata, Zhang et al. 2002), suggesting its role in specific targeting of the complex as well as its function in global histone acetylation. Arp4 is also required for targeting the NuA4 complex to the site of double stranded DNA breaks, by binding to the chromatin (Bird, Yu et al. 2002). Arp5 and Arp8 of yeast Ino80 complex were also shown to be required for Ino80 ATPase activity, DNA binding and nucleosome mobilisation (Shen, Ranallo et al. 2003). Thus, the exact role of Alp5 in fission yeast as a nuclear Arp in a HAT complex remains to be determined.

Alp5 and Clr6 are required for the regulation of histone H4 acetylation level

Suppression of both temperature sensitivity and chromosome segregation defects of the *alp5-1134* mutant by *clr6-1* mutant suggests that they may be functioning in the same pathway. Both mutants show an elevated level of mini-chromosome loss and both genes are essential for cell viability (this study, (Bjerling, Silverstein et al. 2002). Alp5 and Clr6 are required for acetylation and deacetylation of lysine residues on histone H4 amino tail, respectively. Regulation of acetylation at these residues appears to play an important role in accurate chromosome segregation.

However, it is not clear whether the function of Alp5 is important for accurate chromosome segregation through global acetylation, targeted acetylation, for example, at the centromere or gene promoters, or both. In either way, a regulation of the acetylation level at the centromere appears to be important, since a transient treatment of the cells with TSA was shown to result in chromosome missegregation, which is accompanied by an increase in the acetylation level at the centromere (Ekwall, Olsson et al. 1997).

Although the decreased global acetylation level of the *alp5-1134* mutant at restrictive temperature appears to be suppressed by introducing *clr6-1* mutation, the double mutant is still unable to form colonies on a plate at 36°C. This may suggest that the lethality of *alp5-1134* is not due to the decreased global acetylation level, but due to a defect in another function of Alp5. A possible candidate for another Alp5 function is its role in microtubule dynamics, most likely in its monomer/homodimer form (see Chapter 6 for Discussion). Alternatively, longer incubation of the double mutant at 36°C may gradually reduce the global acetylation level and thus, colonies cannot form on a plate after several days, but can be recovered after 8 hours.

Alp5, Sir2 and Hst4

The results also provided evidence of genetic interactions between Alp5 and Sir2-like proteins, Sir2 and Hst4 HDACs (Figure 4.2.2.B). Sir2 has been shown to be required for histone H3K9 deacetylation (Mellone, Ball et al. 2003), which is required for the heterochromatin formation and consequent cohesin recruitment to the outer centromere region (Partridge, Scott et al. 2002; Shankaranarayana, Motamedi et al. 2003). The possibility of Alp5-mediated acetylation on histone H3K9 is currently investigated. Although the target of Hst4 is unknown in fission yeast, suppression of the *alp5-1134* mutant by its deletion may suggest its involvement in deacetylating the target of Alp5-mediated acetylation. Hst4 is also important for genomic stability, the loss of which leads to an increased frequency of chromosome loss (Freeman-Cook, Sherman et al. 1999). The mechanism of *alp5-*

1134 mutant temperature sensitivity suppression by *sir2* and *hst4* deletions will be of interest.

A possible role of Alp5 in ATP-dependent chromatin remodelling

The immunoprecipitation assay suggests there may be an interaction between Alp5 and Snf21 (Figure 4.5.1). However, it appears that the lower band, of the two bands produced by the anti-Alp5 antibody, may be interacting with Snf-21-myc. As mentioned in Chapter 3, this lower band is yet to be verified as that of Alp5, and not that of a cross reaction, thus a firm conclusion is difficult to reach. Still, this result raises a possibility of a function of Alp5 in ATP-dependent chromatin remodelling. Since BAF53, an Alp5-like protein in human, is known to interact with Brg1 and hBrm of BAF and PBAF ATP-dependent CRCs, it would not be surprising if this interaction is also conserved in fission yeast.

Many studies have revealed the importance of co-operation between HAT and ATP-dependent CRCs, in facilitating efficient gene activation at specific gene promoters (Fry and Peterson 2001; Narlikar, Fan et al. 2002). Nuclear Arps are shared by many of these two types of chromatin modification complexes, and one possible reason for this may be that they are required for targeting of the complexes to specific sites, possibly through gene-specific activators. This would enable targeting both types of complexes to the same regions, where they are required to facilitate chromatin modification process for various purposes, including transcription and DNA damage repair.

Alp5 modification

When a whole cell extract is blotted with the anti-Alp5 antibody, two protein bands are observed. Immunoprecipitation experiments show that Mst1 interacts with the upper band, and Snf21 with the lower band (Figure 4.2.1.A and 4.5.1.A), although the lower band is yet to be clarified as Alp5. It remains possible that the lower band is a by-product of Alp5 degradation. At this point, however, the possibility of Alp5 modification cannot be ruled out, where the lower band may be that of the unmodified and the upper band being a modified Alp5. If Alp5 is in fact modified, it

raises an interesting possibility of different complexes recruiting different forms of Alp5, for example, the modified Alp5 might be assembled into the HAT complex, and the non-modified Alp5 could be recruited into the ATP-dependent CRC. Also of interest is whether the modification is carried out in the *alp5-1134* mutant at the restrictive temperature. It is possible that histone acetylation is compromised in the *alp5-1134* mutant at the restrictive temperature, due to its failure to be modified, which may be required for its interaction with Mst1 or complex function.

In conclusion, these results suggest that Alp5 is essential for the acetylation of histone H4 amino tail in vivo, which is important for accurate chromosome segregation and mitotic progression. The counteracting HDAC enzyme to Alp5 acetylation was found to be clr6.

Chapter 5

Centromere/Kinetochore Function of Alp5

This chapter describes a role of Alp5 in maintaining transcriptional repression at the core of the centromere. This was an unexpected finding, since histone acetylation is normally associated with transcriptional activation (Allfrey, Faulkner et al. 1964). This defect at the centromere is in line with the chromosome missegregation and an increase in the rate of mini-chromosome loss observed in the *alp5-1134* mutant. The desilencing phenotype at the *cnt* region of the centromere in the *alp5-1134* mutant can be suppressed by the *clr6-1* mutation, suggesting the importance of acetylation regulation in maintaining core centromere transcriptional repression. Interestingly, localisation of the kinetochore proteins do not appear to be affected in the *alp5-1134* mutant, indicating that the kinetochore is assembled. However, the SAC is activated in *alp5-1134* cells, indicating that Alp5 may play a role in establishing a stable kinetochore-spindle interaction that is required for accurate chromosome segregation. Possible roles of Alp5 in mitosis are discussed.

5.1 Introduction

Fission yeast centromere

The size of a fission yeast centromere varies between 40 to 120 kb, depending on the chromosome. Each centromere can be split into at least two different domains: the core and the outer centromere regions. The core centromere region is suggested to be the site of kinetochore formation, consisting of a non-repetitive *cnt* region, and a part of the inner repetitive region, called the *imr*. The *cnt* region has a unique chromatin structure that is not heterochromatic (see Figure 5.1.1.A), although weak transcriptional repression is observed. The *cnt* region is the site of centromere-specific histone H3 variant (SpCENP-A/Cnp1) binding, which requires the conserved kinetochore protein Mis6 for its

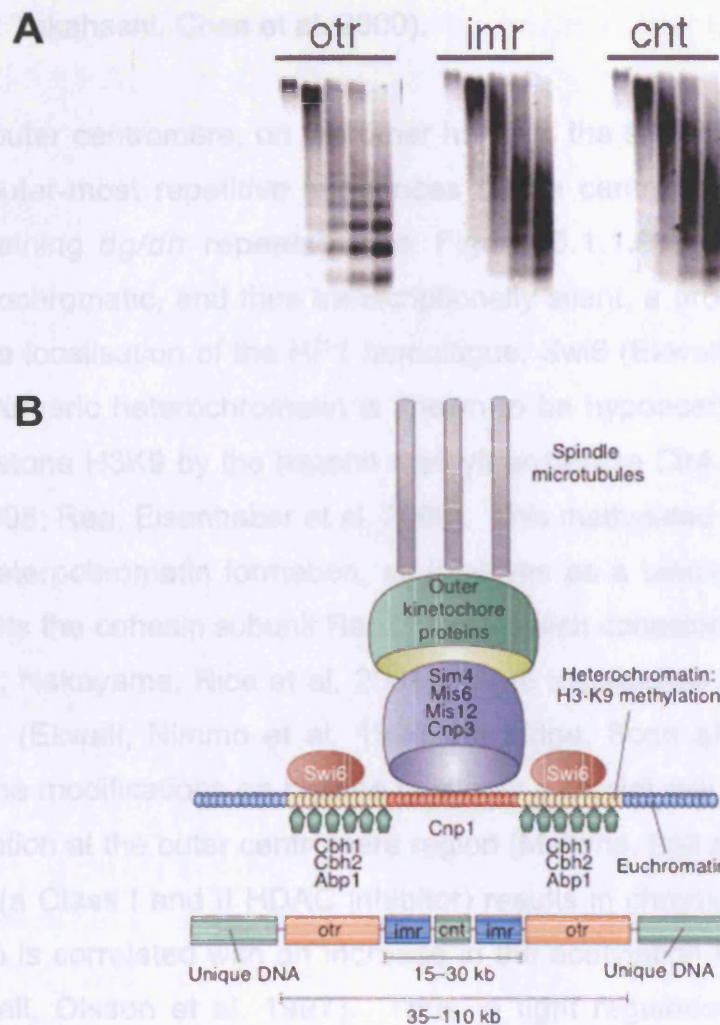


Figure 5.1.1 Fission yeast centromere. **A** Southern analysis of nuclear chromatin that was digested with MNase. The *otr* region shows a ladder pattern and a smear with the *cnt* region, indicating very different chromatin structures between the two centromere regions. [Adapted from Takahashi *et al.* 2000 Science] **B** Fission yeast centromere is divided into three regions, *otr* (inverted repeats, participates in heterochromatin formation and centromeric cohesion site), *imr* (inner most repeats) and a non-conserved 4.1 kb *cnt* (the centromere core, site of kinetochore formation). [Adapted from Amor *et al.* Trends in Cell Biology 2004].

loading during G1/S (Saitoh, Takahashi et al. 1997; Partridge, Borgstrom et al. 2000; Takahashi, Chen et al. 2000).

The outer centromere, on the other hand, is the site of cohesion. Situated on the outer-most repetitive sequences of the centromere are the *otr* repeats (containing *dg/dh* repeats) (see Figure 5.1.1.B). The *otr* regions are heterochromatic, and thus transcriptionally silent, a process that is dependent on the localisation of the HP1 homologue, Swi6 (Ekwall, Javerzat et al. 1995). Centromeric heterochromatin is known to be hypoacetylated, and methylated on histone H3K9 by the histone methyltransferase Clr4 (Ivanova, Bonaduce et al. 1998; Rea, Eisenhaber et al. 2000). This methylated histone H3K9 is crucial for heterochromatin formation, as it serves as a binding site for Swi6, which recruits the cohesin subunit Rad21 to establish cohesion (Bernard, Maure et al. 2001; Nakayama, Rice et al. 2001). Swi6 localisation also requires Rik1 and Chp1 (Ekwall, Nimmo et al. 1996; Partridge, Scott et al. 2002). Covalent histone modifications on histone tails play a crucial role in the heterochromatin formation at the outer centromere region (Mellone, Ball et al. 2003). Addition of TSA (a Class I and II HDAC inhibitor) results in chromosome missegregation, which is correlated with an increase in the acetylation level at the centromere (Ekwall, Olsson et al. 1997). Thus, a tight regulation of acetylation levels appears to be crucial for the formation of functional centromeres, which are required for proper mitotic progression and chromosome segregation.

Both chromosome missegregation and an increase in the rate of minichromosome loss are observed in the *alp5-1134* mutant (see Chapter 2). Thus, a role of Alp5 in the formation of functional centromeres was investigated. When a gene is inserted into a heterochromatic region, its transcription is repressed, i.e. transcriptionally silent. However, if the heterochromatin is altered, the inserted gene is desilenced (also referred to as derepressed), resulting in its expression (Allshire, Nimmo et al. 1995). In fission yeast, the *otr* region of the centromere is heterochromatic, and a reporter gene inserted into

this region is strongly repressed. However, the chromatin structures at the *imr* and the *cnt* regions are unique, with weaker transcriptional repression (see Figure 5.1.1.B).

Results

5.2 Centromeric chromatin in the *alp5-1134* mutant

***cnt* is desilenced in the *alp5-1134* mutant**

A standard assay for measuring transcriptional activity of a marker gene (Allshire, Nimmo et al. 1995) was carried out at centromeric sites of *alp5-1134*. Strains containing the *ura4⁺* marker gene in the *cnt* or the *otr* region were constructed in the *alp5-1134* background (see Figure 5.2.1.A), and a dilution assay was carried out to observe its silencing effect. Cells were spotted onto YE5S, EMM lacking uracil, and YE5S plates containing 5'FOA (5'-fluorootic acid, 100µg/ml), a drug that is toxic for cells expressing *ura4⁺*. By observing growth on plates containing 5'FOA, the extent of transcriptional repression at the centromeres in the *alp5-1134* mutant was determined. As seen in Figure 5.2.1.B/C, wild-type strains containing *ura4⁺* at the *cnt* or *otr* (*cnt::ura4⁺* or *otr::ura4⁺*) were able to grow on 5'FOA. Thus, the *ura4⁺* gene is transcriptionally repressed at these loci. It is of note that the wild-type strain containing *cnt::ura4⁺* showed some growth on plates lacking uracil; this weaker repression is thought to be due to the less heterochromatic structure as described before. In contrast to the wild-type growth, the *alp5-1134* mutant was sensitive to 5'FOA at the *cnt::ura4⁺* at the semi-permissive temperature of 32°C, and a substantial growth on plates lacking uracil was also observed. This result suggests some alteration in the *cnt* region in the *alp5-1134* mutant, which results in transcriptional desilencing. The desilencing of the *cnt* in the *alp5-1134* mutant was suppressed when the *clr6-1* mutation was introduced, which is an HDAC mutant, previously shown to suppress the temperature sensitivity of *alp5-1134* (Figure 5.2.1.B and Figure 4.3.1). These results suggest that the functions of Alp5 and Clr6 are required for maintaining transcriptional repression at the core centromere.

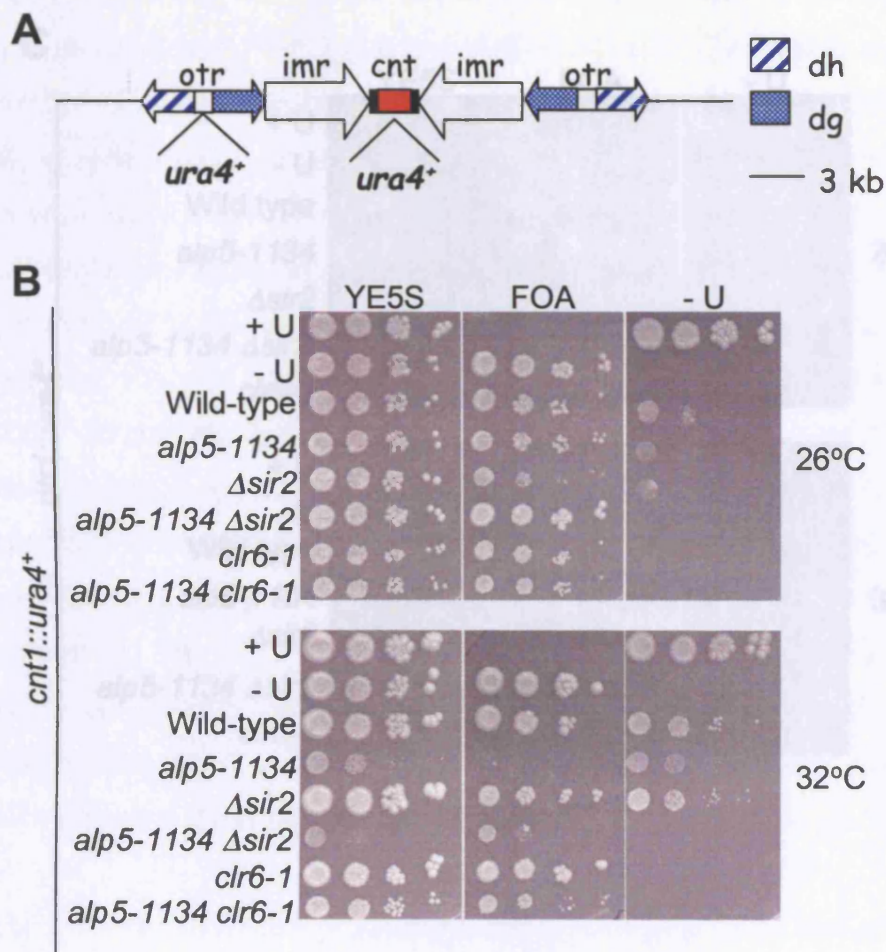


Figure 5.2.1 Alp5 is required for the core centromere silencing **A** A schematic diagram of the construct that was used in the desilencing assay. Strains containing *ura4⁺* marker gene inserted into *otr* or *cnt* were constructed in various mutation backgrounds. A spot test assay was carried out on rich media plates, FOA plates (toxic for uracil expressing cells) and plates lacking uracil, and incubated at indicated temperatures. Transcriptional repression ability of various mutants were observed at *cnt* (**B**) and *otr* (**C**) regions of the centromere.

C

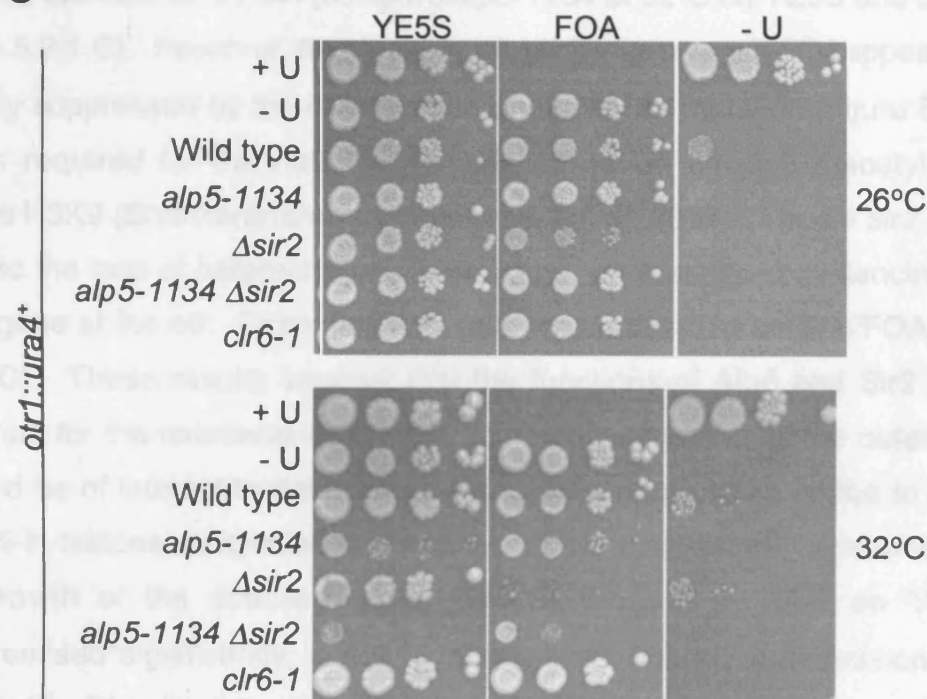


Figure 5.2.1 continued

In contrast to the *cnt*, the growth of the *alp5-1134* mutant containing *otr::ura4⁺* was less affected on 5'FOA (compare *alp5-1134* at 32°C on YE5S and 5'FOA in Figure 5.2.1.C). However, the inability of $\Delta sir2$ to grow on 5'FOA appears to be partially suppressed by the introduction of *alp5-1134* mutation (Figure 5.2.1.C). Sir2 is required for the heterochromatin formation through deacetylation of histone H3K9 (Shankaranarayana, Motamedi et al. 2003). Thus, a *sir2* deletion leads to the loss of heterochromatin structure, and results in desilencing of the *ura4⁺* gene at the *otr*. From this, the cells are unable to grow on 5'FOA (Figure 5.2.1.C). These results suggest that the functions of Alp5 and Sir2 may be important for the maintenance of heterochromatin structure at the outer region. It would be of interest to determine whether this suppression is due to the role of Alp5 in histone acetylation or through another mechanism. It is of note that the growth of the double mutant *alp5-1134* $\Delta sir2$ at 32°C on YE5S is compromised significantly, which is inconsistent with the suppression results obtained in Chapter 4.3. It is possible that *ura4⁺* insertion into the *otr* region results in a growth defect in this double mutant.

It should be noted that *alp5-1134 clr6-1 otr::ura4⁺* strain is not included in Figure 5.2.1.C, since an initial attempt to construct this strain failed. However, since *alp5-1134* appeared to have less of a defect in silencing at the *otr* region compared to the *cnt* region, the suppression effect to be tested with *clr6-1* would have been less significant, and thus, further attempt in obtaining the strain was not pursued.

Overall chromatin structure at the core centromere is not affected in *alp5-1134*

Mutations in proteins required for the establishment and/or maintenance of the core centromeres and the kinetochores are known to have altered MNase digestion patterns, such as those in Cnp1, Mal2, Mis6 and Sim4 (Takahashi, Yamada et al. 1994; Saitoh, Takahashi et al. 1997; Jin, Pidoux et al. 2002; Chen, Saitoh et al. 2003; Pidoux, Richardson et al. 2003). Since the *alp5-1134* mutant displayed a silencing defect at the *cnt* region, we investigated whether its *cnt* chromatin structure is altered. Nuclear chromatin fractions were prepared from wild-type or *alp5-1134* mutant cells grown at 36°C for 4 and 8

hours and digested with MNase for 0, 1, 2, 4, and 8 minutes. Analysis of the banding pattern was observed by agarose gel electrophoresis and Southern hybridisation using the core *cnt* probe (carried out by S. Saitoh and K. Takahashi) (Takahashi, Murakami et al. 1992). Figure 5.2.2.A shows an ethidium bromide stained gel (left), showing the digestion pattern of the nuclear chromatin. From this, there seems to be a slight difference between the wild-type and the *alp5-1134* mutant chromatin throughout the genome. The ladder pattern observed in the wild-type after 4 minutes is revealed in the *alp5-1134* mutant after one minute of MNase digestion, both at 4 and 8 hours after shift up. Thus, the *alp5-1134* mutant may be more sensitive to MNase digestion overall. However, it is also of note that there was a difference in the efficiency of nuclear chromatin extraction, where 60% of nucleosomal DNA was extracted from the *alp5-1134* mutant compared to that of the wild-type from the same number of cells. Conditions such as cell wall strength and growth ability are also known to have an effect on the nucleosomal DNA extraction and the MNase assay. Thus, further experiments need to be carried out in order to verify if the *alp5-1134* mutant has an overall altered chromatin structure.

When MNase digestion pattern was investigated, specifically at the *cnt* region by probing with the *cnt*, no drastic differences between the wild-type and the *alp5-1134* mutant were observed. From this, we conclude that the *cnt* desilencing effect observed in the *alp5-1134* mutant cannot be explained by a drastic change in the *cnt* chromatin structure. It is possible that Alp5 is required for maintaining transcriptional silencing at the *cnt* region of the centromere, although *alp5-1134* mutation is not sufficient to cause a structural change that is detectable by MNase assay.

Overall kinetochore assembly is maintained in *alp5-1134*

The localisation of centromere and kinetochore proteins were next investigated in order to determine the ability of the *alp5-1134* mutant to form kinetochores. Cnp1/SpCENPA, Mis6 and Nuf2 localisation to the centromere/kinetochore were determined. Cnp1 is a histone H3 variant protein that is localised specifically at the core centromere region, and Mis6 and Nuf2 are both core kinetochore proteins. These proteins are known to be delocalised in mutants

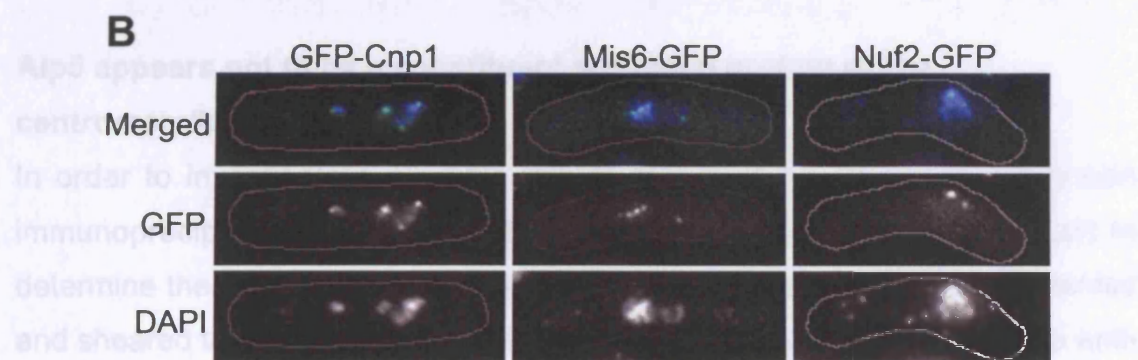
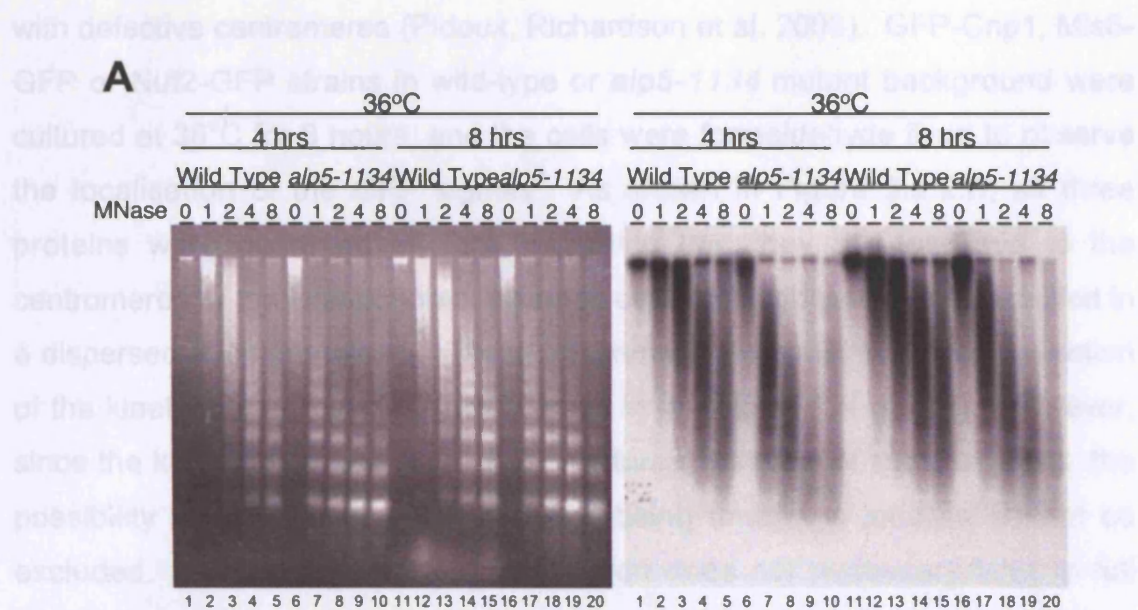


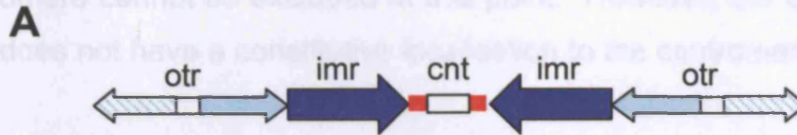
Figure 5.2.2 Overall kinetochore assembly is not affected in *alp5-1134* mutant **A** MNase digestion of nucleosomal DNA were performed with the nuclear extract prepared from wild-type or *alp5-1134* at 36°C for 4 or 8 hours [Performed by S. Saitoh and K. Takahashi] **B** Centromere protein GFP-Cnp1 and kinetochore proteins Mis6-GFP and Nuf2-GFP all localise as dots in *alp5-1134* mutant.

with defective centromeres (Pidoux, Richardson et al. 2003). GFP-Cnp1, Mis6-GFP or Nuf2-GFP strains in wild-type or *alp5-1134* mutant background were cultured at 36°C for 8 hours, and the cells were formaldehyde fixed to observe the localisation of the GFP signals. As shown in Figure 5.2.2.B, all three proteins were observed as foci, indicating that they are localised to the centromeres or the kinetochores, whereas delocalisation would have resulted in a dispersed nuclear staining. These observations suggest that the localisation of the kinetochore proteins is not affected in the *alp5-1134* mutant. However, since the kinetochore is a very large structure consisting of many proteins, the possibility of other kinetochore proteins being unable to localise cannot be excluded. Additionally, protein localisation does not necessary lead to full functionality, thus it is possible that these cells have defective kinetochores, even though these marker proteins are able to localise to these structures.

Alp5 appears not to be a constituent structural protein of the centromere/kinetochore

In order to investigate a possible role of Alp5 at the centromere, chromatin immunoprecipitation (ChIP) was carried out (see Materials and Methods) to determine the localisation of Alp5 at the centromere. Chromatin was extracted and sheared to 500-1000 bp by sonication, followed by incubation with the anti-Alp5 antibody. PCR was subsequently carried out with the immunoprecipitated chromatin with primers designed to detect each centromere region. As shown in Figure 5.2.3, under the condition where the positive control of Mis6 pulled down the *imr* (and weakly at *cnt*) region of the centromere, Alp5 was not bound at any of the three centromeric regions. Since Alp5 is not localised to the *cnt* or the *imr*, Alp5 is unlikely to be a structural component of the centromere or the kinetochore. Alternatively, the efficiency or nature of the crosslinking may have marked the ability to ChIP, especially when considering the fact that Alp5 is not a very abundant protein (see Figure 3.4.4). In addition, since an asynchronous culture was used to extract the chromatin, if Alp5 is localised at the centromere at a specific stage of the cell cycle, for example, only during prometaphase, this assessment will not be able to detect its localisation. For this purpose, the culture would have to be synchronised at the cell cycle stage of interest prior

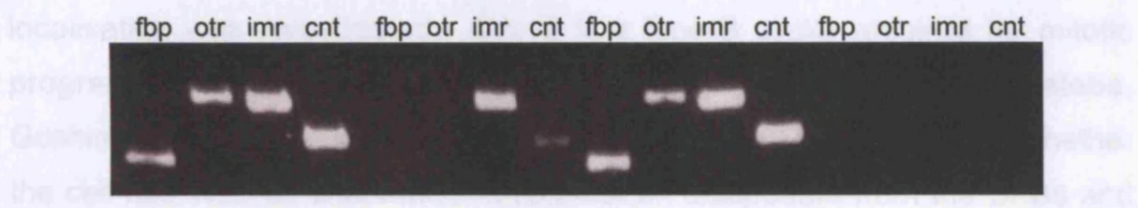
ChIP analysis. Thus, the possibility of a transient Alp5 localisation to the centromere cannot be excluded at this point. However, our data suggests that Alp5 does not have a constitutive localisation to the centromere.



5.3 Activation of spindle assembly and spindle function

Mitotic delay in *alp5-1134*

As described in Chapter 2.3, a *mis6-myc* strain was constructed in the *alp5-1134* mutant background.



incubated with spindles in anaphase due to its localisation by APCD (Yamano, Gannon et al. 1995; Decostre, Zaray et al. 2003). A *Cae13-GFP* strain in the *alp5-1134* background was constructed, grown at the permissive temperature of 36°C for 9 hours, and cells were fixed with formaldehyde, photographed for observation. The number of cells with *Cae13-GFP* localised to using the spindle and SPBs (Figure 5.3.1.A) were then quantified. As seen in Figure 5.3.1.B, over 30% of *alp5-1134* mutant cells from the asynchronous culture showed *Cae13-GFP* staining, whereas only 7% of the culture was observed to show positively before setting up, a number comparable to that of the wild type. This data suggests that there is 3-fold increase in mitotic cells in the *alp5-1134* mutant. Therefore, this data supports our conclusion that *alp5-1134* cells exhibit a mitotic delay before anaphase, as supported by *Cae13-GFP* staining.

Figure 5.2.3 Alp5 localisation at the centromere is not detected from an asynchronous culture **A** A schematic diagram of *S. pombe* centromere **B** ChIP assay was carried out using *Mis6-myc* strain. Total input or immunoprecipitated extracts, with the indicated antibodies, were amplified by PCR using primers of the indicated regions. *fbp*⁺ gene resides in the euchromatic region.

ChIP analysis. Thus, the possibility of a transient Alp5 localisation to the centromere cannot be excluded at this point. However, our data suggests that Alp5 does not have a constitutive localisation to the centromere.

5.3 Activation of spindle assembly checkpoint in *alp5-1134*

Mitotic delay in *alp5-1134*

As described in Chapter 2.3, a mitotic delay is observed in the *alp5-1134* mutant at the restrictive temperature. To confirm this mitotic delay, Cdc13-GFP localisation was investigated. Cdc13 is a type-B cyclin required for mitotic progression, and is localised at the mitotic SPBs and spindles (Tatebe, Goshima et al. 2001). Cdc13 has been used as a marker to determine whether the cell has entered anaphase; its localisation disappears from the SPBs and spindles in anaphase due to its destruction by APC/C (Yamano, Gannon et al. 1996; Decottignies, Zarzov et al. 2001). A Cdc13-GFP strain in the *alp5-1134* background was constructed, grown at the restrictive temperature of 36°C for 8 hours, and cells were fixed with formaldehyde or methanol for observation. The number of cells with Cdc13-GFP localisation along the spindle and SPBs (Figure 5.3.1.A) were then quantified. As seen in Figure 5.3.1.B, over 20% of *alp5-1134* mutant cells from the asynchronous culture showed Cdc13-GFP staining, whereas only 7% of the culture was observed to stain positively before shifting up, a number comparable to that of the wild-type. This data suggests that there is 3-fold increase in mitotic cells in the *alp5-1134* mutant. Therefore, this data supports our conclusion that *alp5-1134* cells exhibit a mitotic delay before anaphase, as supported by Cdc13-GFP staining.

Spindle assembly checkpoint (SAC) is activated in the *alp5-1134* mutant

The observation of a mitotic delay in the *alp5-1134* mutant suggested a possibility of SAC activation. Mad2 and Bub1 proteins are known to localise to unattached kinetochores upon SAC activation, and are often used as SAC activation markers. A Bub1-RFP Mad2-GFP strain was constructed in the *alp5-1134* background, grown at 26°C overnight and subsequently shifted to 36°C for 8 hours. Many cells contained Bub1 and Mad2 foci (Figure 5.3.2.A), and the

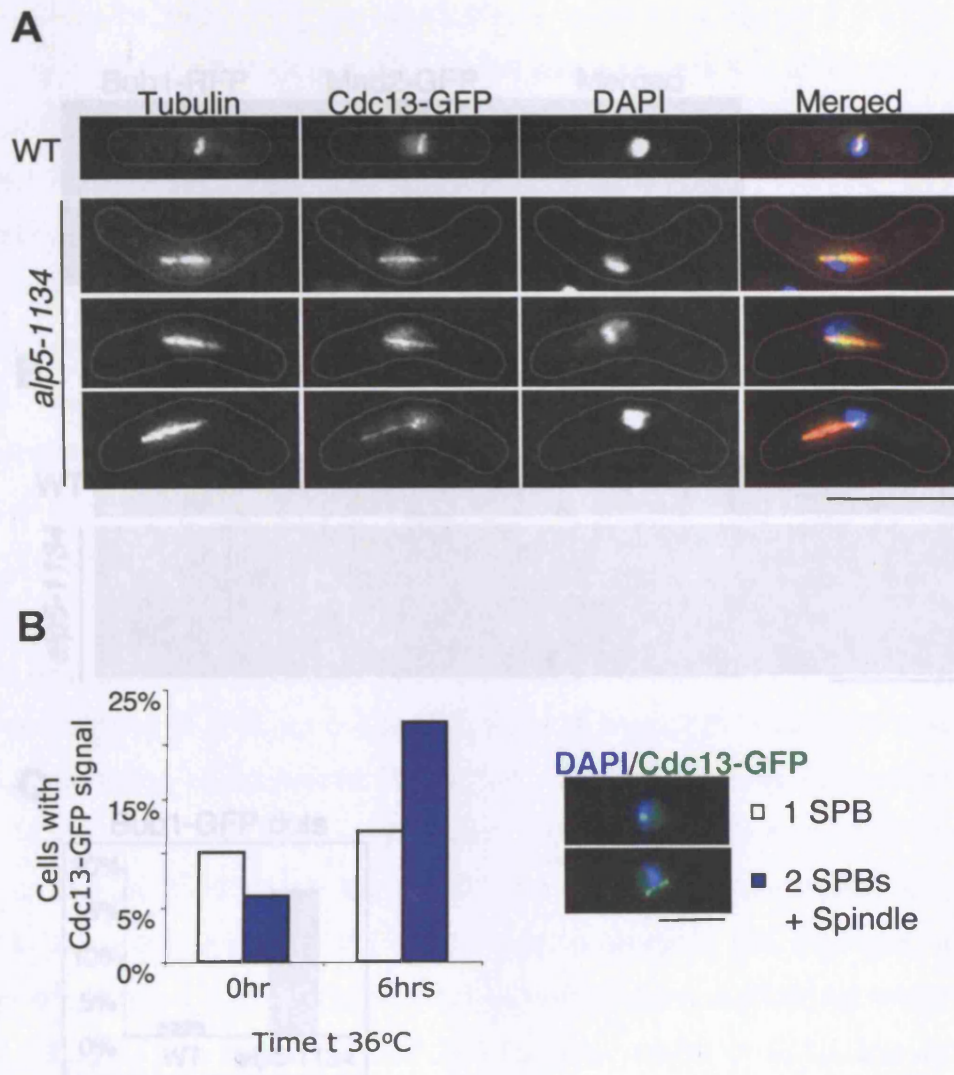


Figure 5.3.1 Spindle assembly checkpoint is activated in *alp5-1134*

mutant A Asynchronously cultured *alp5-1134* mutant cells at the

Figure 5.3.1 Metaphase delay in *alp5-1134* mutant **A** A wild-type cell in metaphase contains Cdc13-GFP signals on both SPBs and short mitotic spindle (top row). After 6 hours of incubation at 36°C, a 3-fold increase in the number of cells with Cdc13-GFP signals in *alp5-1134* mutant cells is observed compared to the wild-type population (**B**). Scalebar indicates 10 μ m.

localisation of Bub1 to the kinetochores was confirmed by the co-localisation of Bub1-GFP with Nuf2-CFP (a kinetochore protein) (Figure 5.3.2.B). The frequency of cells displaying kinetochore localisation was then determined, by quantifying the number of cells containing both Bub1-GFP and Nuf2-CFP signals, which was then compared to the total number of cells. Approximately 17% of cells displayed kinetochore localisation of Bub1-GFP, whereas approximately 17% of cells displayed kinetochore localisation of Nuf2-CFP (Figure 5.3.2.C).

This increase in Bub1, and most probably Mad2, localisation is consistent with the mitotic delay in the *alp5-1134* mutant. The results suggest that the delay observed in the *alp5-1134* mutant is dependent on the spindle assembly checkpoint (SAC). The dependency of SAC for the mitotic delay in the *alp5-1134* mutant was next tested by deleting components of the SAC. The mutants lacking either Bub1, Mad2, Mad1, or Bub3 were tested. Single or double mutant strains were grown overnight at 26°C and then shifted to 36°C for 8 hours and methanol fixed. The samples were stained with DAPI to observe microtubules, and the number of cells displaying kinetochore localisation were then quantified (summarised in Figure 5.3.2.D).

The dependency of SAC for the mitotic delay in the *alp5-1134* mutant was next tested by deleting components of the SAC. The mutants lacking either Bub1, Mad2, Mad1, or Bub3 were tested. Single or double mutant strains were grown overnight at 26°C and then shifted to 36°C for 8 hours and methanol fixed. The samples were stained with DAPI to observe microtubules, and the number of cells displaying kinetochore localisation were then quantified (summarised in Figure 5.3.2.D). The results confirmed that the mitotic delay in the *alp5-1134* mutant is dependent on the components of the SAC. The reason for Bub3-independency for the mitotic delay in the *alp5-1134* mutant is unclear.

Figure 5.3.2 Spindle assembly checkpoint is activated in *alp5-1134*

A Asynchronously cultured *alp5-1134* mutant cells at the restrictive temperature display a high number of cells containing Bub1-RFP and Mad2-GFP spots, which colocalise. **B** Localisation of Bub1 was confirmed to be at the kinetochores by the colocalisation of Bub1-GFP and Nuf2-CFP, the frequency of which was quantified from an asynchronous culture grown at 36°C for 6 hours in **C**. Scalebar indicates 10 μ m.

Under this condition, *alp5-1134* cells display increased chromosome condensation and an increase in the population of cells with short mitotic

localisation of Bub1 to the kinetochores was confirmed by the co-localisation of Bub1-GFP with Nuf2-CFP (a kinetochore protein) (Figure 5.3.2.B). The frequency of Bub1-GFP localisation to the kinetochores was then determined, by quantifying the occurrence of Bub1-GFP signals from asynchronously cultured wild-type or *alp5-1134* mutant cells at 36°C after 8 hours. Approximately 2% of the wild-type cells contained Bub1-GFP signals, whereas ~17% of the *alp5-1134* population showed Bub1-GFP dots (Figure 5.3.2.C). This increase in Bub1, and most probably Mad2, localisation is consistent with the mitotic delay in the *alp5-1134* mutant. The results suggest that the delay observed in the *alp5-1134* mutant is associated with SAC activation, and that the *alp5-1134* cells may contain defective kinetochore-spindle interaction, since Mad2 is known to localise to unattached kinetochores.

Mitotic delay in *alp5-1134* is SAC-dependent

The dependency of SAC for the mitotic delay in the *alp5-1134* mutant was next tested by deleting components of the SAC. The mutants lacking either Bub1, Mad2, Mad1, Bub3, or Mad3 were tested. Single or double mutant strains were grown overnight at 26°C, shifted to 36°C for 8 hours and methanol fixed. The samples were stained with TAT-1 antibody to observe microtubules, and the number of cells displaying mitotic spindles were then quantified (summarised in Figure 5.3.3.A). The absence of all the proteins, except for Bub3, alleviated the mitotic delay in the *alp5-1134* mutant. This result confirmed that the mitotic delay in the *alp5-1134* mutant is dependent on the components of the SAC. The reason for Bub3-independency for the mitotic delay in the *alp5-1134* mutant is unclear and unexpected, since Bub3 is known to be required for Bub1 localisation to the kinetochores in human cells (Sharp-Baker and Chen 2001). One possibility is that Bub3 function is compromised in the *alp5-1134* mutant, the exact mechanism of which is not yet fully established.

Requirement of the SAC for the viability of *alp5-1134*

Whether SAC activation is essential for the viability of *alp5-1134* was next investigated by looking at the growth of *alp5-1134 Δbub1* mutant after 6 hours at 36°C. Under this condition, *alp5-1134* cells display increased chromosome condensation and an increase in the population of cells with short mitotic

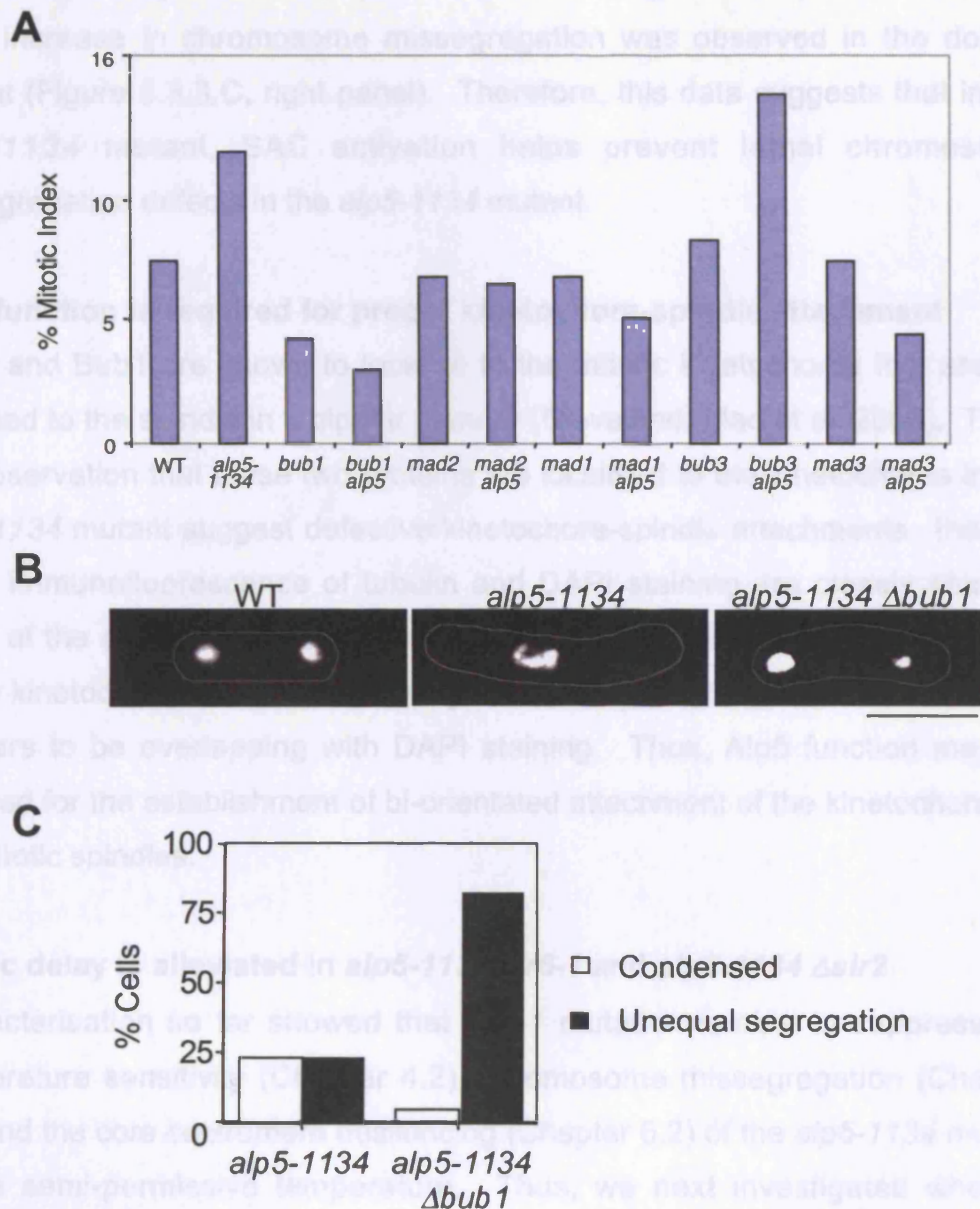


Figure 5.3.3 Requirement of SAC proteins for mitotic delay and viability of the *alp5-1134* mutant **A** Double mutants with SAC proteins were grown at 36°C for 8 hours, and a mitotic index was determined by quantifying the number of cells with mitotic spindles. **B** Wild-type (WT), *alp5-1134* and *alp5-1134 Δbub1* cultures were grown at 36°C for 8 hours and cells were stained with DAPI. Anaphase cells were then quantified for equal or unequal chromosome segregation (**C**). Scalebar indicates 10 μ m.

spindles (see Figure 5.3.3.B middle panel and Figure 2.3.1). In contrast, a large increase in chromosome missegregation was observed in the double mutant (Figure 5.3.3.C, right panel). Therefore, this data suggests that in the *alp5-1134* mutant, SAC activation helps prevent lethal chromosome missegregation defects in the *alp5-1134* mutant.

Alp5 function is required for proper kinetochore-spindle attachment

Mad2 and Bub1 are known to localise to the mitotic kinetochores that are not attached to the spindle in a bipolar manner (Cleveland, Mao et al. 2003). Thus, the observation that these two proteins are localised to the kinetochores in the *alp5-1134* mutant suggest defective kinetochore-spindle attachments. Indeed, when immunofluorescence of tubulin and DAPI staining are closely studied, many of the *alp5-1134* mitotic cells appear to have mono-oriented attachment of the kinetochores to the spindle (Figure 5.3.1.A); only one end of the spindle appears to be overlapping with DAPI staining. Thus, Alp5 function may be required for the establishment of bi-orientated attachment of the kinetochores to the mitotic spindles.

Mitotic delay is alleviated in *alp5-1134 clr6-1* and *alp5-1134 Δsir2*

Characterisation so far showed that *clr6-1* mutation is able to suppress the temperature sensitivity (Chapter 4.2), chromosome missegregation (Chapter 4.3) and the core centromere desilencing (Chapter 6.2) of the *alp5-1134* mutant at the semi-permissive temperature. Thus, we next investigated whether introducing *clr6-1* mutation also alleviates the mitotic delay. As shown in Figure 5.3.4.A, the double mutant of *alp5-1134 clr6-1* did not show any obvious mitotic delay (by quantifying the number of cells with mitotic spindles), which was also observed in the *alp5-1134 Δsir2* double mutant cells. These results may suggest that histone acetylation levels regulated by Alp5, Clr6 and Sir2 are important for preventing SAC activation. Alternatively, they may be required for a functional SAC.

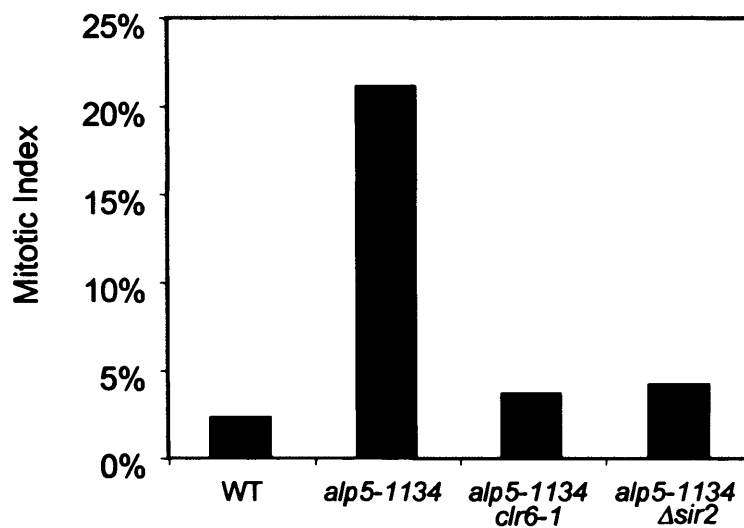


Figure 5.3.4 Regulation of histone tail acetylation is important for mitotic progression. Indicated strains were grown at 36°C for 8 hours and fixed to stain for microtubules. Mitotic index was determined by quantifying cells with mitotic spindles. Mitotic delay appears to be alleviated in the double mutants of *alp5-1134 clr6-1* and *alp5-1134 Δsir2*.

5.4 Discussion

The role of Alp5 in transcriptional repression at the core centromere

The results show that Alp5 is required for transcriptional repression at the core centromere *cnt* region. Transcriptional desilencing at the core centromere is also observed with the mutants of centromere and kinetochore structural proteins (Pidoux, Richardson et al. 2003). A change in the chromatin structure at the *cnt* region is displayed in these mutants, which can be detected by MNase digestion assays. Such obvious difference in the chromatin structure was not observed in the *alp5-1134* mutant. However, this may be consistent with the fact that Alp5 is not a constituent structural component of the centromere or the kinetochore (Figure 5.2.3), in contrast to the characterised mutants to date, which show *cnt* desilencing. Since MNase digestion assays detect gross nucleosomal spacing patterns, the result suggests that the change at the *cnt* region in the *alp5-1134* mutant does not alter the accessibility of MNase, which is consistent with the ability of *alp5-1134* cells to localise the kinetochore proteins.

Whether Alp5 plays a direct role in the establishment and/or the maintenance of transcriptional repression at the core centromere is not clear. Since both Alp5 and Ctr6 are required in regulating global histone H4 acetylation levels, they may be affecting transcription of genes required for such repression. Microarray analysis with the *alp5-1134* mutant may lead to some clue if this is the case, by determining the affected genes. If any of these genes are known to be involved in core centromere transcriptional repression, there would be a good possibility that Alp5 is required for this process through their regulation. Another possibility is that the decrease in global acetylation level in the *alp5-1134* mutant, as was shown in Chapter 4.3, also perturbs the acetylation level at the centromere, leading to transcriptional desilencing. Alternatively, Alp5 may play a more direct role at the centromere through targeted acetylation to maintain a unique acetylation level, which may be important for transcriptional repression. Although Alp5 was not detected at the centromere from an asynchronous culture, it remains possible that its association with the centromere is transient, and thus undetectable from an asynchronous culture.

Sgo2, for example, is a protein that localises to the outer centromere region during mitosis. Its localisation to the centromere is only detected in the synchronised populations of mitotic cells by ChIP (Kitajima, Kawashima et al. 2004). Since the NuA4 complex in *S. cerevisiae* is targeted specifically to some promoters, it is possible that Alp5 is also targeted to specific regions, which may include the centromere during mitosis.

The role of Alp5 in the outer centromere

It has been shown that hypoacetylation at the outer centromere by Sir2 is crucial for the heterochromatin formation (Ekwall, Olsson et al. 1997; Mellone, Ball et al. 2003). Deletion of *sir2* thus leads to the loss of heterochromatin at the *otr* region, as indicated by its inability to repress transcription of the marker gene (Figure 5.2.1.C). This desilencing phenotype was suppressed by the *alp5-1134* mutation to some extent (Figure 5.2.1.C). Since global acetylation levels in the *alp5-1134* mutant are decreased, an increase in silencing was to be expected in the outer *otr* region. However, in contrast to this prediction, *alp5-1134* cells showed some desilencing (Figure 5.2.1.C). One possibility to explain this result is that transcription of the genes required for the transcriptional repression at the outer centromere are Alp5-dependent. Alternatively, this unexpected finding may suggest that a basal level of acetylation at the outer centromere is in fact required for an eventual transcriptional repression at the outer centromere. Interestingly, recent studies have been describing a possible mechanism for the heterochromatin formation at the outer centromere that involves RNAi machinery and, possibly some transcriptional activity from the *otr* region (see Chapter 1). This may give rise to the possibility that Alp5 has a role in the heterochromatin formation at the outer centromere by mediating *dg/dh* transcription, which has been described to play an important role in heterochromatin establishment. It would be interesting to determine whether the level of *dg/dh* transcripts in the *alp5-1134* mutant is altered. If there is a reduction in its level, it may suggest that Alp5 plays a role in RNAi-mediated heterochromatin formation at the centromere.

A possible function of Alp5 at the outer centromere is raised from a study on Pst1, which is a SIN3-like protein. SIN3 is an HDAC corepressor that has the

ability to mediate HDAC targeting in the repression of promoters (Ahringer 2000). Pst1 was shown to interact physically with Clr6, and the *pst1-1* mutant displays TBZ sensitivity, anaphase defects, a decrease in the outer centromere silencing, an increase in histone acetylation on centromeric chromatin, and defective sister chromatid cohesion (Silverstein, Richardson et al. 2003). Since Pst1 colocalises with the centromere, particularly in binucleate cells, it has been postulated that Pst1 and Clr6 temporally associate with the centromeres to carry out the initial deacetylation necessary for the subsequent steps in heterochromatin formation. Given the genetic interaction between *alp5-1134* and *clr6-1* mutants, Alp5 may also be involved in this pathway by regulating histone acetylation levels.

Alternatively, it is also possible that the core centromere repression is important for the outer centromere repression. Since the desilencing effect observed at the *otr* region in the *alp5-1134* mutant is much weaker than that of the *cnt*, the *otr* region may in fact be affected by the *cnt* desilencing.

The kinetochore-spindle interaction in the *alp5-1134* mutant

The SAC activation in the *alp5-1134* mutant suggests that there may be a defect in kinetochore-spindle interactions. When DAPI and tubulin staining were analysed, ~50% of the mutant cells displayed chromosome bi-orientation defects, in which chromosomes seemed to attach to the spindles in a mono-oriented manner (Figure 5.3.1.A). This observation is also supported by Mad2 and Bub1 localisation to the kinetochores in *alp5-1134* at the restrictive temperature, suggesting the presence of unattached kinetochores. SAC activation is not sustained in the mutant however, and chromosome missegregation is eventually observed. One possible explanation for this is that although the *alp5-1134* cells are able to assemble the kinetochore proteins, the kinetochores are not stable, as suggested by desilencing phenotype at the *cnt* region. This may result in an unstable kinetochore-spindle interaction, which would be unable to retain kinetochore attachment to the spindle, resulting in SAC activation. Since the SAC is no longer activated in the *alp5-1134* mutant when the *clr6-1* mutation is introduced, regulation of histone acetylation level may play a role in either establishing a bi-oriented kinetochore-spindle

attachment, or the SAC function. Whether this regulation is important in a global acetylation manner or through targeted acetylation at the core centromere region remains to be determined.

Another possible explanation for SAC activation is stabilised microtubules in the *alp5-1134* mutant. As described in Chapter 2, the cytoplasmic microtubules appear to be stabilised in the *alp5-1134* mutant, displaying bundled and curved microtubules around the cell tips. This would suggest that the mitotic spindles may also be stabilised. Stabilised mitotic spindles would most likely lead to SAC activation, since it would lead to an unstable kinetochore-spindle attachment; a stable attachment is thought to be obtained in metaphase when two opposing but balanced forces are in operation. One force is derived from cohesion between sister chromatids, and the counteracting force is generated from microtubule depolymerisation at the kinetochores or the poles (Mitchison and Salmon 2001). If depolymerisation of spindle microtubules is prevented due to stabilised microtubules, the balance between the opposing forces would be lost, resulting in an unstable kinetochore-spindle attachment and/or its loss. This possibility may be investigated through a live analysis of the centromeres, since microtubule dynamics are thought to be required for their oscillation.

The role of Alp5 in spindle length regulation

The *alp5-1134* mutant appears to display elongated spindles during mitotic delay, when its lengths are compared to that of the wild-type (Figure 5.3.1.A). From the immunofluorescence pictures, it is not clear whether the spindles in the *alp5-1134* mutant are monopolar or bipolar, in other words, if the spindle nucleation is from one or both SPBs (which are embedded in the nuclear membrane). Upon entry into mitosis, one of the duplicated SPBs moves to the opposite side of the nuclear membrane to form a bipolar spindle. Monopolar spindles are observed in mutants such as those of γ -tubulin complexes, for example, *alp4* mutant, which also displays elongated spindles (Vardy, Fujita et al. 2002). If a bipolar spindle is established in the *alp5-1134* mutant (which may be determined by observing SPB signals in mitotic cells), it may suggest that the spindle is abnormally elongated. Thus, Alp5 may either be required for bipolar spindle formation or spindle dynamics/length regulation. It is intriguing

that *mis6* and *mis12*, two fission yeast kinetochore mutants, have also been described to have elongated spindles (Goshima, Saitoh et al. 1999). Since the distance between the two spindle poles determines the length of the mitotic spindle, the authors suggest that the elongated spindles may be due to the imbalance between the pulling forces of the kinetochore and kinetochore-independent pushing force on the centromere. Another possibility is that Alp5 is required for the regulation of nuclear morphology, since an elongated spindle could possibly arise from defective nuclear morphology, which dictates the positioning of the SPBs. SPB localisation in the *alp5-1134* mutant would need to be established in order to distinguish the difference between these possibilities. The final and most simple explanation would be that Alp5 is required to transcribe the necessary genes in determining the spindle length, including the genes mentioned above.

Chapter 6

Discussion

Alp5 is an essential nuclear ARP required for the global histone H4 acetylation in vivo

In this study, the *alp5*⁺ gene was cloned and sequenced to reveal it as a novel gene, encoding an essential nuclear actin-related protein. The most similar protein to fission yeast Alp5 are budding yeast Arp4 and human BAF53, which are also nuclear and are found in chromatin modification complexes. Alp5 was found to interact with Mst1, a HAT-domain containing protein, which is the homologue of Esa1 HAT in yeast NuA4 complex (~2 MDa) (Figure 4.2.1). Consistently, the temperature sensitivity of the *alp5-1134* mutant was partially suppressed by an HDAC inhibitor TSA (Figure 4.3.1), suggesting a decreased acetylation level in the mutant. The target of this inhibitor was later found to be Clr6 HDAC, a homologue of budding yeast Rpd3, which has a broad target specificity (Suka, Suka et al. 2001). Acetylation levels on all the lysine residues on histone H4 amino tail, except for H4K16, were shown to be drastically decreased in the *alp5-1134* mutant, which were recovered in the presence of *clr6-1* mutation (Figure 4.3.2). Thus, Alp5 is essential for global histone H4 amino tail acetylation in vivo, in an antagonistic manner to Clr6. Since Alp5 itself does not contain a HAT domain, the decrease in the global acetylation level in the *alp5-1134* mutant is most likely due to a functional defect in the Alp5-containing HAT complex. The responsible HAT protein is most likely to be Mst1, with which it interacts with Alp5 (Figure 4.2.1). These results suggest that a NuA4-like complex, which acetylates histone H4 in budding yeast and human (called Tip60 complex) (Eberharter, John et al. 1998; Kimura and Horikoshi 1998; Allard, Utley et al. 1999), is likely to be conserved in fission yeast.

Alp5 is required for mitotic progression

The requirement of Alp5 for proper mitotic progression is clear from the phenotypes the *alp5-1134* mutant displays. These include a mitotic delay,

which requires a functional SAC (Figure 5.3.3.A) where Mad2 and Bub1 are localized to the kinetochores (Figure 5.3.2), chromosome missegregation (Figure 2.3.1.B), and genomic instability (Figure 2.3.2.B). The observation that Mad2 is localised to the kinetochores indicate the presence of unattached kinetochores. There are three main possibilities to explain this, which may not be exclusive of each other. The first possibility is that Alp5 is required for transcribing one or more genes that are required for mitotic progression. This possibility may be tested by carrying out a microarray analysis. The second possibility is the formation of an unstable kinetochore due to whatever is causing transcriptional desilencing at the core centromere. This may result in weak kinetochore-spindle attachment, eventually leading to unattachment. A third possibility is that the mitotic spindles are stabilised in *alp5-1134* (see Chapter 5.4). This would lead to unstable kinetochore-spindle attachment due to lack of microtubule dynamics, since a stable attachment is thought to require the opposing forces of microtubule depolymerisation and sister chromatid cohesion. Microtubule dynamics are also thought to play an important role in establishing the bi-oriented attachment, through repeatedly detaching the wrongly connected spindles from the kinetochores (Lens and Medema 2003). Alternatively, a combination of two or all of these may be contributing to the phenotypes observed.

The important role of Alp5 in histone acetylation for mitotic progression is supported by the fact that mitotic delay is alleviated and chromosome missegregation is partially rescued in the double mutant of *alp5-1134 clr6-1* (Figure 5.3.4). To further support the importance of acetylation regulation in mitotic progression, a possible role of the NuA4 complex in centromere function was recently shown. A subunit of the budding yeast NuA4 complex was isolated from a genome-wide modifier screen, which identified genes required for chromosome transmission, and was shown to have genetic interactions with inner kinetochore proteins (Baetz, Krogan et al. 2004). Additional support for the role of the NuA4 complex at the centromere comes from observations that some of the NuA4 complex subunits have been described to be hypersensitive to the microtubule destabilising drug benomyl, which is one of the common

phenotypes of kinetochore and spindle checkpoint mutants (Le Masson, Yu et al. 2003; Kobor, Venkatasubrahmanyam et al. 2004).

Alp5 is required for maintaining transcriptional silencing at the core centromere

The finding that *alp5-1134* mutant displays an increased rate of mini-chromosome loss rate at the permissive temperature (Figure 2.3.2.B) suggested that there may be a defect with its centromere. In support of this idea, *alp5-1134* was found to be defective in transcriptionally silencing the core centromere (*cnt*) (Figure 5.2.1.A). When its chromatin structure was analysed at the *cnt* region by MNase digestion assay, however, no detectable defect was observed. MNase banding patterns would only detect gross changes in the chromatin structures, such as those caused by the loss of centromere or kinetochore structural proteins. Thus, *alp5-1134* mutation is sufficient to cause transcriptional desilencing at the core centromere without causing a gross change in the chromatin structure. This result is supported by the observation that the kinetochore proteins can be localised in the *alp5-1134* mutant (Figure 5.2.2), and that Alp5 is not a constituent structural protein of the centromere/kinetochore (Figure 5.2.3).

It is difficult to speculate what the change might be at the *cnt* region in the *alp5-1134* mutant that leads to *cnt* desilencing, since the core centromere region is far from fully understood. The chromatin structure of the *cnt* is distinct from that of both heterochromatin and euchromatin. Proteins found in the heterochromatin regions of the centromere are not present in the core centromere region, such as Swi6, and there are core centromere-specific proteins, such as the histone H3 variant protein Cnp1. Thus, whether transcriptional desilencing at the core centromere observed in the *alp5-1134* mutant is due to a loss of some known or unknown proteins is yet to be determined. A recent paper has also described a unique pattern of histone modification at the core centromere that is distinct from both euchromatin and heterochromatin, in human and *Drosophila melanogaster* (Sullivan and Karpen 2004). Thus, one possibility is that Alp5 is required for the maintenance of a

unique histone acetylation level at the core centromere, the disruption of which may result in transcriptional derepression.

Whatever the cause is for the core centromere desilencing in the *alp5-1134* mutant, there may be at least two possible roles of Alp5 in maintaining repression at the core centromere. The first is its function in regulating the level of global histone H4 acetylation. This may be important in transcribing the necessary genes for maintaining a transcriptionally silent state at the core centromere. Alternatively, the global histone acetylation level may affect the acetylation level at the centromere, which may lead to transcriptional derepression when disrupted. A second possibility is that Alp5 is targeted to the core centromere to regulate its unique acetylation level that may be important for a transcriptionally silent state. Measuring the histone acetylation levels at the centromere in *alp5-1134* may help clarify between the possibilities further. The second possibility is currently the least likely from the existing data, since the ChIP assay did not detect the presence of Alp5 at the core centromere (Figure 5.2.3.B). However, a transient Alp5 localisation at the centromere cannot be ruled out until the ChIP assay is also carried out with a synchronized culture in mitosis, and since Alp5 is not a very abundant protein (Figure 3.4.4), its detection may be difficult if it is transiently present at the centromeres.

The importance of histone acetylation in maintaining transcriptional silenced state at the core centromere is suggested from the observation that the silencing defect in the *alp5-1134* mutant is suppressed by the *clr6-1* mutant at the semi-permissive temperature (Figure 5.2.1.B). It is of note that *clr6-1* and budding yeast *rpd3* (Clr6 homologue) mutants have been shown to have an increased repression of marker genes at the core centromere and telomere, respectively (De Rubertis, Kadosh et al. 1996; Grewal, Bonaduce et al. 1998; Sun and Hampsey 1999), which is in line with *cnt* desilencing in the *alp5-1134* mutant. Consistent with this, *clr6-1* mutant shows genomic instability and chromosome missegregation (Ekwall, Olsson et al. 1997; Grewal, Bonaduce et al. 1998). Furthermore, as previously mentioned, a transient inhibition of HDACs with TSA also leads to chromosome missegregation, which correlates

with an increase in the acetylation level at the *cnt* region. Thus, a strict regulation of acetylation levels at the centromere appears to play an important role in centromere/kinetochore function.

A possible role of Alp5 in cohesion

The observation that the temperature sensitivity of the *alp5-1134* mutant was suppressed by *sir2* deletion is intriguing. In fission yeast, Sir2 is required for deacetylating histone H3K9 (Shankaranarayana, Motamedi et al. 2003). Deacetylated H3K9 at the outer region of the centromere is crucial for the establishment and maintenance of the heterochromatin structure, and subsequent cohesion establishment through Swi6 recruitment (Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002; Mellone, Ball et al. 2003; Shankaranarayana, Motamedi et al. 2003). When kinetochore protein localisation was investigated in the *alp5-1134* mutant, many cells contained more than three foci (Figure 5.2.2), which is not often observed in wild-type cells. This may indicate that there is a cohesion defect in the *alp5-1134* mutant, which could be along the chromosomal arms and/or at the centromeres. When Swi6 localisation at the centromeres are disrupted, centromeric cohesion between sister chromatids is lost, although it remains intact along chromosomal arms (Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002; Mellone, Ball et al. 2003; Shankaranarayana, Motamedi et al. 2003). Thus, it is possible that the multiple kinetochore dots in the *alp5-1134* mutant indicate a loss of centromeric cohesion. Recent studies suggest that the heterochromatin formation at the outer centromere requires functional RNAi machinery, and that *dg/dh* transcripts from the *otr* region plays an important role in this process (Volpe, Kidner et al. 2002; Volpe, Schramke et al. 2003). It would be interesting to see if the initial transcription at the *otr* region requires Alp5-mediated acetylation. Another possibility to explain a possible centromeric cohesion defect in the *alp5-1134* mutant is that cohesion establishment at the centromere is affected by the core centromeric chromatin structure. Since the desilencing phenotype displayed by the *alp5-1134* mutant is much stronger at the *cnt* region than at the *otr* region, it is possible that the original defect is at the *cnt* region, and the *otr* region is affected as a result. A recent study in budding yeast has shown the importance of the kinetochores in enhancing

cohesion establishment in the outer centromere region (Weber, Gerton et al. 2004), although it remains to be determined if this is a conserved mechanism. However, since there is no drastic change in the chromatin structure at the core centromere in the *alp5-1134*, this possibility may be unlikely. Alternatively, cohesion may be lost both at the centromeres and along chromosomal arms in the *alp5-1134* mutant, which is observed in the mutants of cohesin proteins, such as Rad21 (Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002; Mellone, Ball et al. 2003; Shankaranarayana, Motamedi et al. 2003). In such case, Alp5 may be required to transcribe cohesin genes, or those that are necessary to establish cohesion.

It remains possible that the mitotic phenotypes described in the *alp5-1134* mutant are partly originating from a cohesion defect. The loss of cohesion could possibly lead to a defective kinetochore-spindle attachment and establishment of chromosome bi-orientation, since cohesin is known to play an important role in these processes (Tanaka, Fuchs et al. 2000). An interdependency between the centromere/kinetochore structure and cohesion establishment also appears to exist (Meluh and Strunnikov 2002). Thus, the loss of cohesion could possibly be causing the core centromere desilencing in the *alp5-1134* mutant. However, since the silencing defect displayed in the *alp5-1134* at the *cnt* region is much greater than that of the *otr* region, the *otr* defects are unlikely to be the primary defect. Furthermore, it was shown that *sir2* deletion is able to suppress temperature sensitivity of the *alp5-1134* mutant (Figure 4.3.1). It is difficult to imagine how compromising the function of Sir2 would lead to its suppression if it is already defective in cohesion. Thus, the possibility of *otr* defects as the primary defects is less likely to explain the phenotypes and genetic interactions obtained for *alp5-1134*.

The role of Alp5 in ATP-dependent chromatin remodelling

In budding yeast, Arp4 has been shown to be present in two ATP-dependent CRCs, SWR1 and Ino80 complexes (see Chapter 1.7) (Galarneau, Nourani et al. 2000; Ikura, Ogryzko et al. 2000; Shen, Mizuguchi et al. 2000; Krogan, Keogh et al. 2003; Kobor, Venkatasubrahmanyam et al. 2004; Mizuguchi, Shen et al. 2004). Thus, it is likely that Alp5 would also be involved in the process of ATP-dependent chromatin remodelling. The SWR1 complex is required for

histone H2A variant (Htz1 in yeast) exchange with conventional histone H2A to buffer the spread of heterochromatin, and Ino80 complex appears to play a role in transcription and DNA damage repair (Shen, Mizuguchi et al. 2000; Krogan, Keogh et al. 2003; Kobor, Venkatasubrahmanyam et al. 2004; Mizuguchi, Shen et al. 2004). It was recently described that Htz1 is also deposited at the centromere in a Swr1-dependent manner, which raises the possibility of SWR1 and Htz1 regulating the centromere function, as well as transcription (Krogan, Baetz et al. 2004). Interestingly, this paper also describes a link between SWR1 and NuA4 complexes, which are both genetically linked to kinetochore components and genomic stability (Kobor, Venkatasubrahmanyam et al. 2004). The two complexes and Htz1 are suggested to operate in common, rather than in parallel, pathways. In addition, genetic interactions between SWR1/NuA4/Htz1 and known kinetochore components or SAC proteins were shown. These findings suggest that SWR1, NuA4 and Htz1 may be required for genomic stability in addition to transcription. Since Arp4 is a common subunit of the two complexes, it raises the possibility that Alp5 also functions at the centromere as part of the NuA4-like complex in fission yeast.

From the immunoprecipitation data, Alp5 may also interact with the ATPase protein Snf21, although the band that precipitated with Snf21 requires verification as an Alp5 band. In a human BAF complex, either Brg1 or hBrm is found as an ATPase subunit. In fission yeast, there are also two ATPase proteins that are similar to both Brg1 and hBrm, which are Snf21 and Snf22, although Snf21 may be slightly more similar to Brg1. Brg1 is also found in a PBAF complex, which is known to be required for diverse cellular processes including cell cycle progression (see Chapter 1) (Shanahan, Seghezzi et al. 1999), and has been shown to localise to the kinetochores of mitotic chromosomes, indicating a role in mitosis (Xue, Canman et al. 2000). Human Brg1 has also been shown to interact with HP1 α specifically, which is a centromeric protein (Nielsen, Sanchez et al. 2002). The interaction between Alp5 and Snf21 in fission yeast would not be surprising, since human BAF53 is known to bind Brg1, which is in fact required for the maximal ATPase activity of Brg1 (Zhao, Wang et al. 1998).

The budding yeast Brg1 homologue is Sth1 of the RSC complex, and there appear to be many parallels between Rsc and Alp5 proteins. These include being essential for viability (Cairns, Lorch et al. 1996), their function in mitotic progression (Cao, Cairns et al. 1997; Du, Nasir et al. 1998; Tsuchiya, Hosotani et al. 1998), and SAC activation in their absence (Tsuchiya, Hosotani et al. 1998). The *sth1* mutant displays an altered chromatin structure at the centromere and increased frequency of non-disjunction of mini-chromosomes (Tsuchiya, Hosotani et al. 1998), suggesting the role of RSC complex in the kinetochore-spindle interaction. Furthermore, mutations in genes encoding two core subunits of the RSC complex, Sth1 and Sfh1, interact genetically with mutations in genes encoding kinetochore proteins and with a mutation in centromeric DNA. The RSC complex also interacts genetically and physically with histones and histone variants of centromeric components, and localises to both centromeric and centromere-proximal chromosome regions (Hsu, Huang et al. 2003).

A possible defect of cohesion in the *alp5-1134* mutant was discussed earlier, and interestingly, recent evidence suggests the requirement of the RSC complex in cohesion along the chromosome arms, although it was not shown for the centromeres (Baetz, Krogan et al. 2004). The *rsc2* mutant was isolated from a screen, developed to identify genes required for faithful chromosome transmission using *ctf13-30* (a core kinetochore protein mutant) as a reference point, and was shown to interact genetically with kinetochore and cohesion mutants. Although cohesion and chromosome segregation defects are displayed in the mutant, the localisation of centromere and kinetochore proteins is not affected. Thus, the RSC complex may be required for both outer and core centromere functions.

The presence of Alp5 as a common subunit of a HAT complex and an ATPase CRC would be unsurprising given the mounting evidence of these two types of chromatin modification complexes cooperating in various processes, such as transcription regulation (Pollard and Peterson 1998). Although the main focus of this study was the role of Alp5 in histone acetylation, the ATP-dependent role of Alp5 should also be investigated. The phenotypes of the *alp5-1134* mutant,

which have been described here, may be due to the combination of defects in histone acetylation and ATP-dependent chromatin remodelling.

A possible role of Alp5 in microtubule dynamics

In addition to the mitotic phenotypes, *alp5-1134* mutant also displays defects in cytoplasmic microtubules. One possible explanation is that Alp5 is required for the transcription of genes that are necessary for normal cytoplasmic microtubule array formation. The bent morphology of the *alp5-1134* mutant is most likely due to microtubule defects.

However, the possibility of a separate, non-nuclear function of Alp5 cannot be excluded either. The localisation of Alp5 was determined in this study to be nuclear through immunofluorescence observation with an anti-Alp5 antibody. The possibility of its cytoplasmic localisation should not be ruled out at this stage, since some residual staining of Alp5 was detected in the cytoplasm (Figure 3.4.5). However, this signal could arise as a result of non-specific background staining, and remains to be determined if it is reflecting its true localisation. A recent study showed a very interesting finding that BAF53 has two alternatively spliced isoforms, one of which is a nuclear protein and the other cytoplasmic, although the function of this cytoplasmic protein remains to be determined (Ohfuchi, Nishimori et al. 2002). Another study has shown that BAF53 may be shuttling between the nucleus and the cytoplasm, and that the cytoplasmic staining appeared punctate. It remains to be determined whether the cytoplasmic localisation of BAF53 is conserved in fission yeast Alp5. In addition, as shown from the gel filtration studies, there appears to be a monomer/homodimer form of Alp5 present in the cell, in addition to a large complex (Figure 4.2.1). A cytoplasmic function of Alp5 in microtubule dynamic regulation in its monomer/homodimer form is an intriguing possibility. As described in Chapter 2, *alp5-1134* mutant was isolated from a screen which also isolated many proteins required for microtubule formation and/or dynamics, thus, it would not be a surprise if Alp5 also has a function in the regulation of cytoplasmic microtubules. In fact, many of the Alp proteins are involved in both cytoplasmic and mitotic microtubule regulation, and some are also shown to be important for the SAC function (Vardy and Toda 2000; Garcia, Vardy et al. 2001; Sato, Vardy et al. 2004).

The possibility of microtubule acetylation by Alp5 was also considered, by adding TSA to a culture and immunoblotting its extract with an antibody that detects acetylated proteins (obtained from Minoru Yoshida). However, the antibody did not detect any band at the size of tubulin (data not shown), and the acetylation site on tubulin does not appear to be conserved in fission yeast (personal communication with Minoru Yoshida). Thus, the microtubules do not appear to be acetylated in fission yeast (also from personal communication with Minoru Yoshida), although an absolute notion to the possibility of tubulin acetylation awaits future analysis. Taken together, Alp5 is unlikely to regulate microtubules through acetylation.

Concluding remarks

We have shown that Alp5 is an essential protein that is required for diverse cellular functions. The role of Alp5 in global histone H4 acetylation was described in this study, which is most probably carried out through its interaction with Mst1 in a ~2 MDa complex. These results suggest that the yeast NuA4-like complex is conserved in fission yeast. The temperature sensitive mutation *alp5-1134* displays a number of defects, namely mitotic delay, chromosome missegregation, genomic instability, and stabilised microtubules. Whether these functions are carried out through its role in chromatin modification complexes remains to be determined. However, its function in acetylation is clearly important for mitotic progression, since mitotic defects are suppressed by a *clr6-1* mutation. An unexpected finding of the role of Alp5 in maintaining a transcriptional silenced state at the core centromere may suggest the complexity of the centromere formation.

A recent study has reported that an Arp6 homologue in fission yeast is localised at the telomere in a Swi6-independent manner, and that it is required specifically for telomere silencing, but not for centromeric silencing (Ueno, Murase et al. 2004). This finding raises an intriguing possibility of different nuclear Arps playing positive roles towards silencing at specific regions. The exact mechanism of centromere formation is yet to be fully understood, and studies on nuclear Arps may assist in dissecting the complex workings of this fascinating and important phenomenon.

Chapter 7

Materials and Methods

This chapter details the experimental methods used throughout this thesis. It also gives details of the contents of buffers and derivation of strains used.

7.1 Laboratory stocks and solutions

All media, pipette tips, autoclaved glassware and other commonly used solutions were provided by the Cancer Research UK Central Services.

Solutions, buffers and media

The receipts of all the buffers and solutions described in this chapter are listed below.

5X loading buffer (PAGE)	60 mM Tris-HCl (pH 6.8), 25% Glycerol, 2% SDS, 14.4 mM 2-Mercaptoethanol, 1% Bromophenol blue.
10X loading buffer (DNA agarose gel)	60% (w/v) Sucrose, 0.1% (w/v) Bromophenol blue.
Elution buffer (QIAGEN)	1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% (v/v) Ethanol.
Equilibration Buffer (QIAGEN)	750mM NaCl ₂ , 50mM MOPS pH7.0, 15%(v/v) 50 mM MOPS pH 7.0, 15% (v/v) Ethanol.
Gel filtration buffer A	20 mM Tris-HCl pH 7.5, 20% Glycerol, 0.1mM EDTA, 1mM Mercaptoethanol, 5mM ATP, Cocktail of inhibitors (Sigma).
HB Buffer	25mM MOPS, pH 7.2, 60mM β -glycerophosphate, 15mM -p-Nitrophenyl-phosphate, 15mM MgCl ₂ , 15mM EGTA, 1 mM Dithiothreitol (DTT), 0.1mM Sodium vandate, 1% Triton X-100, 1 mM PMSF,

	20 µg/ml Leupeptin, 40 µg/ml Aprotinin.
L-Broth (LB)	170mM NaCl, 0.5% (w/v) Yeast extract, 1% (w/v) Bacto-tryptone.
Lysis buffer	50 mM Hepes-KOH (pH 7.5), 140 mM NaCl 1 mM EDTA, 1% (v/v) Sodium Deoxycholate.
EMM	14.7 mM KH phthalate, 15.5 mM Na ₂ HPO ₄ , 93.5 mM NH ₄ Cl, 111 mM Glucose, salt and vitamin stocks.
PBSA	170mM Sodium chloride, 3mM KCl, 10 mM Na ₂ HPO ₄ , 2 mM KH ₂ PO ₄ .
PM buffer	35 mM K-Phos pH 6.8, 0.5 mM MgSO ₄ .
PEM (pH 6.9)	100 mM PIPES, 1 mM EGTA, 1 mM MgSO ₄ .
PEMS	PEM + 1.3 M Sorbitol.
PEMBAL (pH 6.9)	PEM + 1% BSA, 1% NaN ₃ , 100 mM Lysine hydrochloride. Filter sterilised.
Plasmid solution 1 (P1)	50 mM Glucose, 25 mM Tris-pH 8.0, 10 mM EDTA.
Plasmid solution 2 (P2)	200 mM Sodium hydroxyde, 1% (w/v) SDS.
Plasmid solution 3 (P3)	3 M Potassium acetate.
SDS PAGE buffer	25 mM Tris, 250 mM Glycine pH 8.3, 0.1% SDS.
STOP Buffer	150mM NaCl, 50 mM NaF, 10 mM EDTA, NaN ₃ pH 8.
TBE	45 mM Tris base, 1 mM EDTA.
TE	10 mM Tris-HCl, pH 7.0, 0.1 M EDTA.
TES	50 mM Tris-HCl (pH 8), 10 mM EDTA, 1% SDS.
Transfer buffer	39 mM Glycine, 48 mM Tris base, 0.037% SDS, 20% Methanol.
YE5S	0.5% Difco yeast extract, 3% Dextrose + 250 mg/ml, Histidine, leucine, uracil, adenine, and lysine.
YPD	2% Dextrose, 2% Bacto-peptone, 1.1% Yeast extract.
Wash buffer (QIAGEN)	50 mM MOPS pH 7.0, 15% (v/v) Ethanol.
Wash buffer (ChIP)	10 mM Tris-HCl (pH 8), 0.25 M LiCl, 0.5% NP-40, 0.5% (w/v) Sodium Deoxycholate, 1 mM EDTA.

7.2 Yeast physiology

Nomenclature

A Fission yeast gene name consists of 3 italicised letters and a number e.g. *alp5*. A wild-type allele may be denoted by a plus sign e.g. *alp5*⁺. An allele number is also used to identify different mutant alleles of the same gene e.g. *alp5-1134*. Proteins are named using the same three letters indicative of the gene but are not written in italics with the first letter being an upper case e.g. Alp5.

Gene deletions by specific markers are shown by the gene name followed by two semicolons (::) and the replacement marker gene name. E.g. *alp5::ura4*⁺. A gene deletion can also be annotated by a delta (Δ) symbol in front of the gene name. The strains with resistance gene as a selectable marker are denoted by the superscript R resistance (and S for sensitive) e.g. *kan*^R.

Budding yeast nomenclature

The gene names of budding yeast are typically shown as 3 upper case letters and a number e.g. ACT1, a mutant allele is italicised and lower case e.g. *act1*. Protein names take the same format as fission yeast.

Other nomenclature

Mammalian gene names are usually written as upper case letters and gene products and take the same format as fission yeast. Drosophila proteins are denoted in upper case letters and the gene names are italicised and lowercase.

Strain Growth and maintenance

Techniques used to grow and maintain fission yeast strains and to store and revive cultures were performed as previously described (Ito, Fukuda et al. 1983). *S. pombe* cells were grown in either liquid media or on agar plates containing 1.6% agar. The contents of different media are shown above. Stocks of yeast strains were stored in YFM containing normal YE5S media with 15-20% glycerol and maintained at -70°C.

Transformation of Fission Yeast

Transformations of plasmid DNA into yeast cells were carried out using Lithium Acetate and Poly Ethylene Glycol (PEG) as previously described (Ito, Fukuda et al. 1983). Approximately 1 µg of plasmid DNA was used for these transformations.

PCR fragments for integration into the genome were transformed using Lithium acetate, PEG and DMSO previously described (Keeney and Boeke 1994; Bahler, Wu et al. 1998). Selective markers used in plasmid and integrant transformations were *LEU2*, *ura4⁺* and *kan^R*.

Cell Numbers

20 or 200µl of sample was added to 10 ml of Isoton (Beckman Coulter). Cells were sonicated briefly and counted on a Sysmex Microcell counter F-800 in the white blood cell channel.

Thiabendazole, Hydroxy Urea and Ultra Violet light dilution assays

Thiabendazole (TBZ) was added to plates at a concentration of 10µg/ml or 20µg/ml. Stock solutions were stored in DMSO at 1000x concentration, 4°C.

Hydroxy Urea (HU) was added to plates at a concentration of 5mM or 10mM. Fresh solution at 1M was made every time for use immediately.

Cells were serially diluted and spotted onto plates so that the first spot should contain 5×10^3 cells and the last spot should contain five cells. They were subsequently examined for growth at different temperatures.

For Ultra Violet light sensitivity, strains were spotted as above and the plates were subsequently exposed to UV light in a Stratalinker UV crosslinker 1800 at various doses for 30s and allowed to grow at various temperatures.

7.3 Strains

Strains	Genotypes	Derivations
972	<i>h⁻</i>	Our stock
513	<i>h⁻ leu1 ura4</i>	Our stock
AF6	<i>h⁻ leu1 ura4 alp16⁺-13myc-kan^R</i>	This study
AF7	<i>h⁻ leu1 ura4 alp4⁺-13myc-kan^R</i>	This study
AM11	<i>h⁻ leu1/leu1 ura4/ura4 his7/his7</i> <i>ade6-M210/ade6-M216 alp5⁺/alp5::ura4⁺</i>	This study
AM17	<i>h⁻ leu1 ura4 alp5⁺-GFP-kan^R</i>	This study
AM24	<i>h⁻ leu1 ura4 alp5⁺-13myc-kan^R</i>	This study
AM26	<i>h⁻ leu1 ura4 alp5⁺-TAP2120-kan^R</i>	This study
AM515	<i>h⁻ leu1/leu1 ura4/ura4 his7/his7</i> <i>ade6-M210/ade6-M216 alp5⁺/alp5⁺-GFP-kan</i>	This study
AM36	<i>h⁻ leu1 ura4 ade6-210 alp5-1134</i> containing <i>Ch16</i>	This study
AM44	<i>h⁻ leu1 alp5::ura4⁺</i> containing <i>p(AL-alp5⁺)</i>	This study
AM49	<i>h⁺ leu1 ura4 his2 mst1⁺-13myc-kan^R</i>	This study
AM50	<i>h⁺ leu1 ura4 his2 mad2::kan^R alp5-1134</i>	This study
AM53	<i>h⁻ leu1 phd1::LEU2 alp5-1134</i>	This study
AM57	<i>h⁻ leu1 ura4-DS/E otr1::ura4⁺ alp5-1134</i>	This study
AM60	<i>h⁻ leu1 ura4 snf21⁺-13myc-kan^R</i>	This study
AM68	<i>h⁺ leu1 ura4 his2 alp5-1134 ura4-DS/E cnt1::ura4⁺</i>	This study
AM115	<i>h⁺ leu1 ura4 clr3::kan^R alp5-1134</i>	This study
AM117	<i>h⁺ leu1 his2 cdc13⁺-GFP-LEU2 alp5-1134</i>	This study
AM121	<i>h⁻ leu1 ura4 mad2⁺-GFP-kan^R alp5-1134</i>	This study
AM123	<i>h⁺ leu1 ura4 his2 bub1⁺-GFP-kan^R alp5-1134</i>	This study
AM142	<i>h⁻ leu1 ura4 ade6-210 hst4⁺::his3⁺ alp5-1134</i>	This study
AM147	<i>h⁺ leu1 ura4 his2 mad3::ura4⁺ alp5-1134</i>	This study
AM149	<i>h⁺ leu1 ura4 his2 ade6 clr6-1 alp5-1134</i>	This study
AM152	<i>h⁺ leu1 ura4 his2 ade6-210 cnt1::ura4⁺ alp5-1134 clr6-1</i>	This study
AM156	<i>h⁻ leu1 ura4 sir2::kan^R</i>	This study
AM162	<i>h⁺ leu1 ura4 his2 otr1::ura4⁺ sir2::kan^R alp5-1134</i>	This study

AM163	<i>h⁻ leu1 ura4 otr1::ura4⁺ sir2::kan^R</i>	This study
AM167	<i>h⁻ leu1 ura4 ade6-210 otr1::ura4⁺ ura4⁺-DS/E sir2::kan^R</i>	This study
AM174	<i>h⁺ leu1 ura4 his2 ade6-210 cnt1::ura4⁺ ura4-DS/E alp5-1134 sir2::kan^R</i>	This study
AM170	<i>h⁺ leu1 ura4 his2 bub1::ura4⁺ alp5-1134</i>	This study
AM173	<i>h⁻ leu1 ura4 bub1⁺-RFP-kan^R mad2⁺-GFP-kan^R alp5-1134</i>	This study
AM181	<i>h⁺ leu1 ura4 his2 sir2::kan^R alp5-1134</i>	This study
AM184	<i>h⁺ leu1 ura4 his2 mad1::ura4⁺ alp5-1134</i>	This study
AM185	<i>h⁺ leu1 ura4 his2 bub3::ura4⁺ alp5-1134</i>	This study
AM196	<i>h⁹⁰ leu1 ura4 ade6-210 clr6-1 cen1R::ura4⁺</i>	This study
AM197	<i>h⁻ leu1 ura4 nuf2⁺-CFP-kan^R bub1⁺-GFP-kan^R</i>	This study
DH1134	<i>h⁻ leu1 alp5-1134</i> Hirata et al. 1998)	(Radcliffe,
FY648	<i>h⁺ leu1 ade6-210 ura4-DS/E otr1::ura4⁺</i>	Robin Allshire
FY336	<i>h⁻ leu1 ade6-210 ura4-DS/E cnt1::ura4⁺</i>	Robin Allshire
FY3223	<i>h⁺ leu1 ura4 ade6-210 clr3-735 cen1R::ura4⁺</i>	Robin Allshire
HU369	<i>h⁻ leu1 ade6-210 ura4 clr3::kan^R</i>	Karl Ekwall
LPY3277	<i>h⁻ ade6-210 arg3 hst4::his3⁺</i>	Lorraine Pillus
SPG1002	<i>h⁺ leu1 ura4 ade6-210 clr6-1 cnt1::ura4⁺</i>	Yota Murakami
SPS41	<i>mat msmt0 leu1 ade6-210 clr6-1</i>	Shiv Grewal

All the strains listed in this table contain *leu1-32*, *his2-245*, *arg3-D4*, and *his7-366*. The *ura4* used is *ura4-D18* unless otherwise stated.

7.4 Molecular Biological techniques

Molecular biological techniques were used as described (Fritsch, Maniatis et al. 1982) including growth and transformation of bacterial cells, gel electrophoresis of DNA in TBE and TAE buffer, restriction enzyme digests, minipreps and preparation of competent bacteria.

Nucleic Acid preparation and manipulation

Yeast genomic DNA was prepared based on the method described previously (Burke, Dawson et al. 2000).

Enzymatic digestion of DNA with restriction enzymes was carried out as recommended by the suppliers (New England Biolabs).

DNA fragments were examined on 1% and 1.5% (w/v) agarose in 1x TBE buffer. These gels were run at 100v in 1x TBE buffer in Embitech runone electrophoresis cells.

Polymerase Chain Reaction

Normal PCR reactions were carried out using Vent (New England Biolabs), Expand High Fidelity (Roche) or LA Taq (Takara) polymerases with buffers supplied by the manufacturers. dNTPs were supplied at 0.2mM in reaction volumes of 50 or 100 μ l.

For colony PCR, samples were prepared in a similar manner with a toothpick full of yeast colony suspended in the reaction mix.

All PCRs were performed in a Peiliter Thermal Cycler-200. All products were purified using wizard DNA clean up system (Promega) according to the manufacturers instructions and run on DNA agarose gels to check for product size.

Cloning of the *alp5*⁺ gene

An *S. pombe* pAL based genomic library (obtained from Taro Nakamura, Osaka City University) (Garcia, Vardy et al. 2001) was used for the isolation of plasmids suppressing temperature lethality of *alp5-1134* mutant. Suppressing plasmids were isolated and transformed into *E. coli* for the transposon method of gene isolation described previously (Morgan, Conlon et al. 1996). This involves a transposon jumping from a donor plasmid into the recipient suppressing plasmid that contains the gene of interest. If the plasmid can no longer suppress the mutant at 36°C it suggests that the transposon has jumped into the candidate gene. Primers designed against the transposon can then be used to sequence the gene. The transposon employed here (Tn1000) contains the Streptomycin resistance gene for selection.

Competent *E. coli* strain HB101 was transformed with pAL-6 plasmids and colonies were selected for on LB plates containing ampicillin and methicillin. 2ml of liquid donor culture DH1, containing the plasmid with the transposon tagged with a streptomycin marker, were pelleted and resuspended in 1ml of recipient culture from each transformation plate. After spreading onto LB plates for two hours, the mating mixture was washed and plated onto LB plates containing ampicillin (50 µg/ml) and streptomycin (100 µg/ml) in order to select for colonies that contained plasmids into which the transposon had jumped.

4 colonies were then selected from each plate, grown in selective LB media and minipreps were performed to re-isolate the plasmid. Purified plasmid DNA was then run on a DNA gel to check the presence of DNA. Digestion patterns were then compared between the original plasmid and the transposon tagged plasmid.

In order to check if the transposon had jumped into the suppressing gene the plasmids were transformed back into the relevant mutants. Three plasmids that no longer suppressed the mutant phenotype at 36°C were selected for sequencing. Primers designed against the two ends of the transposon were used to sequence the flanking sequences.

New primers were designed in order to sequence the entire gene of *alp5-1134*. Oligonucleotide primers of 20 base pairs were designated based on the obtained sequence. Sequencing was performed by the dideoxy method (Sanger, Nicklen et al. 1977). BLAST server at Sanger Centre was then used to cross-reference the obtained sequence to the *S. pombe* genomic database.

Recovering plasmids from yeast transformants

Transformant fission yeast colonies containing the plasmid were grown in liquid YE5S and the plasmid was isolated as described previously (MacNeill and Fantes, 1993). Plasmid DNA was purified using the wizard DNA clean up system (Promega) following the recommended protocol. Plasmid concentrations were determined by running 1 µl on a 1% DNA agarose gel.

Gene Disruption

All genes were disrupted using PCR generated fragments (Bahler, Wu et al. 1998). The 1.8kb *ura4⁺* gene was amplified with flanking sequences corresponding to the 5' and 3' ends of the relevant genes. These fragments were transformed into a *ura4⁻* diploid. *ura4⁺* colonies were selected on plates lacking uracil and then sporulated on plates lacking nitrogen. Asci from heterozygous diploids were dissected (Burke, Dawson et al. 2000) and germination of haploids monitored. Where a 2:2 ratio is seen with 2 growing spores lacking the ability to grow on ura- plates the disruption is deemed lethal to the cell. Genes were also disrupted in an identical manner but using the Kanamycin resistance gene.

C-terminal epitope tagging of genes in their chromosomal location

C terminal tagging of genes with HA, Myc GFP or RFP epitopes was carried out using PCR generated fragments as described (Bahler, Wu et al. 1998). All tagging was confirmed by PCR to look for increased gene size and western blotting with specific antibodies. For tagging with RFP, a fast folding RFP was used (provided by Drs. Elmar Schiebel and Michael Knop).

Transformation and isolation of plasmid DNA in *E. coli*

Competent *E. coli* stored at -70°C were thawed on ice and incubated for 20 minutes with 10 μg of plasmid DNA. Cells were then heat shocked at 42°C for 90s after which 1ml of LB media was added. The cells were allowed to recover at 37°C for 30 minutes to 1 hour before being plated onto LB plates containing ampicillin.

3-5ml of *E. coli* were grown overnight in LB + 100 $\mu\text{g}/\text{ml}$ ampicillin at 37°C . Cells were harvested by centrifugation at 4000rpm for 5 minutes and the pellet was resuspended in 150 μl of solution P1 (QIAGEN) containing 100 $\mu\text{g}/\text{ml}$ of RNase A.

150 μl of solution P2 (QIAGEN) was added and cells were left to incubate for 5minutes at room temperature to allow alkaline lysis of the bacterial cell membranes and denaturation of both plasmid and bacterial DNA.

150µl of chilled solution P3 (QIAGEN) was added to neutralise alkaline conditions and increase salt concentration. Cells were left to incubate on ice for 15 minutes to allow full precipitation of bacteria protein and chromosomal DNA. The plasmid DNA, which remains in solution, was separated from the cell debris by centrifugation 13,000rpm for 30 minutes. Plasmid DNA was then bound to a silica column (QIAGEN) previously equilibrated with equilibration buffer. The column was then washed twice with 1.5ml of wash buffer and the plasmid eluted from the column with 800µl of elution buffer. The Plasmid DNA was then recovered by isopropanol precipitation.

7.5 Protein biochemistry

Alp5 purification from bacterially expressed protein

Complementary DNA of *alp5+* was cloned into expression vector PET14b in frame with six copies of the histidine tag and under an IPTG inducible promoter. The plasmid was transformed into *E. coli* and selected by the ability to grow in the presence of the antibiotics, ampicillin and chloramphenicol. 100ml liquid cultures of *E. coli* were grown in LB with ampicillin and chloramphenicol overnight. 20 ml of this culture was diluted into 1 litre of fresh medium, grown for an hour until turbidity reached ~ 0.4 when measured with a wavelength of 600 nm. IPTG was added at a final concentration of 2 mM to induce the expression of Alp5. cells were grown for a further 4 hours. The cell culture was then spun at 6000 rpm for 10 minutes to pellet the cells. The pellet was then resuspended in 3 volumes of its wet weight with sonication buffer and frozen in dry ice and ethanol, followed by thawing in cold water. The solution was then kept on ice and sonicated at setting 15 (soniprep 150 sonicator MSE). The solution was given 5 pulses of 1 minute each with cooling intervals of 2 minutes between each pulse. The sonicated solution was spun for 20 minutes at 10,000 rpm and the supernatant was used in the nickel columns (QIAGEN) according to instructions supplied by the manufacture.

Western blot analysis

Fission yeast whole cell extracts (WCE) were prepared by lysing cells with glass beads in HB buffer (Moreno, Klar et al. 1991). Protein concentration was

determined using a Bradford assay (BioRad). Extracts were boiled in sample buffer for five minutes and 20 µg of extract was run on a 10% SDS-polyacrylamide gel (BioRad). For western blots the protein was blotted onto ImmobilonTM-P (Millipore) transfer membrane. Horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG (BioRad) and chemiluminescence system (ECL, Amersham) were used to detect bound antibodies.

Immunoprecipitation Assay

For immunoprecipitation analysis, 2 mg of soluble cell extract was prepared in the same way as for normal western blot analysis. 2.5 µg of monoclonal anti-myc antibody was incubated at 4°C for 1 hour. 30 µl of Protein A-Sepharose beads (Pharmacia Biotech. Co.), prewashed in HB buffer, were added and incubation continued for 1 hour. The protein A beads were then washed eight times in HB buffer followed by the addition of 10 µl sample buffer. Samples were run on a 10% SDS-PAGE gel and candidate proteins were detected as described above.

Chromatin Immunoprecipitation Assay

Standard methods (Pidoux, Mellone et al. 2004) were followed. Logarithmically grown cells that were cultured overnight were grown at 18°C for ~2 hrs, which were subsequently fixed with 16% formaldehyde with constant motion for 30 min. Cells were pelleted and washed with ice-cold PBS, followed by cell wall digestion with 0.4 mg/ml zymolyase in PEMS, incubated at 37°C for 20 min. The cells were then washed in 10 ml PEMS twice and resuspended in 1 ml PEMS, which were pelleted. 400 µl ice-cold Lysis buffer containing inhibitor cocktail and 2 mM PMSF were added to the pellet (on ice from hereon) and sonicated to shear chromatin to 500-1000 bp (17 µ for 20 sec, repeated 3 times). The samples were spun down and the lysates were transferred to a fresh tube with further centrifugation. A tenth of this lysates were kept as the whole cell extract control. Immunoprecipitations with the desired antibodies were carried out as above. The Sepharose beads were washed with 1 ml each of Lysis buffer, Lysis buffer with 0.5 M NaCl, Wash buffer and 1 x TE (pH 8).

250 μ l TES were added to the beads and 210 μ l TES to the stored WCE, which were incubated at 65°C for 6 hrs – overnight to reverse the cross-links. The samples were spun down and transferred to fresh tubes, to which 450 μ l TE and 30 μ l 10 mg/ml Proteinase K were added. Subsequent to a 2 hr incubation at 37°C, samples were extracted with phenol:chloroform and chloroform and precipitated with 0.3 M NaOAc (pH 5.5), glycogen, 2 volumes of 100 % ethanol and incubated on ice for 30 min. Following a 30 min centrifugation at 4°C, the supernatants were removed and the pellets were air-dried, which were suspended in 30 μ l TE (pH8). The extent of shearing was checked by treating the WCE sample with RNase and running it on a 1.5% agarose gel. The remaining WCE samples were diluted to a tenth. 2 μ l of ChIP or diluted WCE were used for the PCR reactions.

Antibodies used for Western and immunoprecipitation analysis

Alp5	This study	polyclonal	1/100
c-myc (9E10)	Babco (MMS-101R)	monoclonal	1/1,000
c-myc (PRB-150)	Babco (MMS-101R)	monoclonal	1/1,000
γ -tubulin	Sigma (T6557)	monoclonal	1/10,000
GFP	Clontech (8362-1)	monoclonal	1/200
α -tubulin	Sigma (T5168)	monoclonal	1/5,000
α -acetyl histone H4 (Lys5)	Serotec (AHP414)		1/600
α -acetyl histone H4 (Lys8)	Upstate (07-328)		1/1,000
α -acetyl histone H4 (Lys12)	Upstate (07-323)		1/5,000
α -acetyl histone H4 (Lys16)	Serotec (AHP417)		1/500
α -hyperacetylated histone H4 (Penta)	Upstate (06-946)		1/10,000
α -acetyl histone H3 (Lys9)	Upstate (07-352)		1/2,000
α -acetyl histone H3 (Lys14)	Upstate (07-353)		1/2,000

Gel filtration chromatography

Soluble protein extracts were prepared in Buffer A. Gel filtration chromatography was performed on a superose-6 column by FPLC (Pharmacia Biotech). The column was equilibrated with 2 column volumes of buffer A containing 100mM NaCl₂. A parallel column was run with the standard size markers dextran (2,000kDa), thyroglobin (669kDa) and α -amylase (232kDa).

50µl fractions were collected and separated on 10% SDS-PAGE gels and fractionated proteins were detected with specific antibodies.

Histone Acid Extraction

Standard methods (Pidoux, Mellone et al. 2004) were followed. Briefly cell pellets from exponentially growing culture (1×10^8) were disrupted with glass beads. The recovered lysate was centrifuged at $\sim 19,000 g$ for 10 min at 4°C . The pellet was resuspended in 0.5 ml of 0.4 M sulphuric acid and incubated for 1 h on ice. The extract was then centrifuged. The acid extraction was repeated once. The pooled supernatants (1 ml total) were precipitated overnight in glass at -20°C with 12 volumes of ice-cold acetone. The precipitate was collected by centrifugation. The pellet was air dried and resuspended in 100 µl of 4 M urea.

MNase digestion

The MNase digestion was performed using three centromeric probes corresponding to *cnt*, *imr* and *otr* regions described previously (Takahashi, Murakami et al. 1992). The 2.8 kb *HindIII/EcoRI* fragment derived from a pKT110 plasmid was used as a probe to detect the *cnt* region.

7.6 Microscopic analysis

Analysis of DNA and cell wall

Analysis of the DNA and septa were performed by fixing 1 ml of cell culture in 1/10 volume of formaldehyde for 10 minutes. After washing in PBSA, cells were visualised with the addition of DAPI or calcofluor and viewed under a fluorescence microscope.

Indirect immunofluorescence microscopy

Methods for indirect immunofluorescence procedures were adapted from Hagan and Hyams ((Hagan and Hyams 1988). 5×10^7 cells were filtered and fixed in methanol at -70°C for 12 hours. After 3 washes in PEM the cell wall was digested with 0.6 mg/ml zymolyase 20T in PEMS for 70 minutes. Cells were then pelleted and resuspended in 1% TritonX in PEMS for 1 minute. After 3

further washes in PEM, cells were incubated in PEMBAL for 30 minutes and primary antibody was added in 40 μ l of PEMBAL and left overnight with rotation. The primary antibody was washed out with PEMBAL, and the secondary antibody was added in 200 μ l PEMBAL and incubated in the dark for 2 hours. Finally, after washing the secondary antibody out with PEMBAL, cells were resuspended in PBSA + 0.2 μ g/ml NaN₃. Immunofluorescence images were viewed with a Zeiss Axioplan 2 imaging microscope attached to a chilled video CCD camera (ORCA-ER I C4742-95, Hamamatsu Photonics Ltd.). Images were processed with a PC containing Kinetic Imaging AQM Advance 6 software (Kinetic Imaging Ltd.) and Adobe Photoshop (Version 6 and 7).

Antibodies used for indirect immunofluorescence

Primary:

a-tubulin (TAT-1)	Dr.Keith Gull	monoclonal	1:40
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Secondary:

Cy3-conjugated sheep	anti-mouse IgG (Sigma C2128)	1:500
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Direct immunofluorescence microscopy

GFP-, RFP- and CFP-tagged strains were used for direct immunofluorescence. Cells were fixed with formaldehyde as DAPI staining or as described above for tubulin staining.

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BAF53/Arp4 Homolog Alp5 in Fission Yeast Is Required for Histone H4 Acetylation, Kinetochore-Spindle Attachment, and Gene Silencing at Centromere^D

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