Centromere localization and function of Mis18 requires Yippee-like domain-mediated oligomerization

Lakxmi Subramanian†, Bethan Medina-Pritchard†, Rachael Barton, Frances Spiller, Raghavendran Kulasegaran-Shylini, Guoda Radaviciute, Robin C Allshire & A Arockia Jeyaprakash*

Abstract

Mis18 is a key regulator responsible for the centromere localization of the CENP-A chaperone Scm3 in Schizosaccharomyces pombe and HJURP in humans, which establishes CENP-A chromatin that defines centromeres. The molecular and structural determinants of Mis18 centromere targeting remain elusive. Here, by combining structural, biochemical, and yeast genetic studies, we show that the oligomerization of S. pombe Mis18, mediated via its conserved N-terminal Yippee-like domain, is crucial for its centromere localization and function. The crystal structure of the N-terminal Yippee-like domain reveals a fold containing a cradle-shaped pocket that is implicated in protein/nucleic acid binding, which we show is required for Mis18 function. While the N-terminal Yippee-like domain forms a homodimer in vitro and in vivo, full-length Mis18, including the C-terminal α-helical domain, forms a homotetramer in vitro. We also show that the Yippee-like domains of human Mis18α/Mis18β interact to form a heterodimer, implying a conserved structural theme for Mis18 regulation.

Keywords  CENP-A; centromere; epigenetics; Mis18; Yippee
Subject Categories  Cell Cycle; DNA Replication, Repair & Recombination; Structural Biology

Introduction

The accurate distribution of genetic information to daughter cells during cell division relies on the physical attachment of chromosomes to spindle microtubules mediated by kinetochores. Kinetochores are large protein assemblies deposited at specific chromosomal loci known as centromeres [1–3]. Defective centromere function results in chromosome segregation errors that can contribute to genomic instability implicated in cancer [4]. Hence, understanding the molecular mechanisms that promote kinetochore establishment and maintenance at centromeres is of prime importance.

The location of most eukaryotic centromeres is determined by the assembly of specialized chromatin composed of nucleosomes in which canonical histone H3 is replaced by the centromere-specific H3 variant CENP-A in vertebrates and Cnp1 (CENP-ACnp1) in Schizosaccharomyces pombe [3,5]. Thus, the establishment and maintenance of kinetochores requires CENP-A to be recruited to and deposited at centromeres. In S. pombe, CENP-A Cnp1 is specifically incorporated into chromatin over the central domain of endogenous centromeres where it is flanked by heterochromatin formed on outer repeat elements [6,7].

During S phase, CENP-A Cnp1 levels at fission yeast centromeres are halved following DNA replication. Subsequently, CENP-A Cnp1 levels are replenished during G2 phase of the cell cycle [8]. In early mitosis, the Mis18 complex comprising Mis18, Mis16, Eic1/Mis19/Kis1, and Eic2/Mis20, along with the CENP-A Cnp1 chaperone Scm3 (counterpart of vertebrate HJURP; HJURP Scm3), dissociates from centromeres and re-associates in mid/late anaphase following chromosome segregation [9–15]. Exclusion of the Mis18 complex and HJURP Scm3 from centromeres during mitosis likely provides an opportunity for the CENP-A Cnp1 loading cycle to reset and thereby prevent continual CENP-A Cnp1 deposition [16]. HJURP Scm3 directly interacts with CENP-A Cnp1 and is essential for the deposition of new CENP-A Cnp1 at centromeres [14]. Genetic studies have shown that Mis16, Mis18, and Eic1/Mis19/Kis1 are essential genes that are required for the localization of HJURP Scm3 to centromeres and hence CENP-A Cnp1 maintenance at centromeres [10,11,13,14,17].

Most of the components of the CENP-A assembly pathway are conserved among eukaryotes, but with a few striking differences. Humans possess two isoforms of Mis18 (Mis18α and Mis18β) and Mis16 (RbAp46 and RbAp48) [10]. During telophase, the human Mis18 complex comprising Mis18α, Mis18β, and Mis18BP1/KNL2, along with RbAp46 and RbAp48, associates with centromeres [17]. Mis18BP1/KNL2 has no detectable S. pombe homolog, but Eic1/Mis19/Kis1 appears to perform an analogous function [11,13]. As in
S. pombe, the human Mis18 complex is required for HJURP recruitment to centromeres, where CENP-A is deposited during early G1 rather than G2 [9]. In addition, the stable incorporation of CENP-A at human centromeres requires the small GTPase activity of Cdc42 regulated by MgcRacGap/ECT2 [18].

Although key conserved players involved in the assembly and maintenance of CENP-A chromatin at centromeres have been identified, the molecular mechanisms through which they exert their function remain unclear. Mis18 is critical for the specification of centromeres from fission yeast to humans, however, what allows Mis18 to regulate centromere specification remains largely unknown. To gain insights into the structural features of S. pombe Mis18 that allow it to bind centromeres and promote HJURP recruitment and CENP-A-connection assembly, we determined the crystal structure of its highly conserved “Yippee-like” N-terminal globular domain. Our structural and biochemical analyses reveal that the Mis18 “Yippee-like” domain possesses a fold that is implicated in protein/nucleic acid binding and that this domain has an innate tendency to homodimerize both in vitro and in vivo. However, full-length Mis18 forms a homotetramer in vitro, highlighting a role for the C-terminal α-helical domain in influencing the overall oligomeric state of the protein. Genetic analyses using structure-guided mutants demonstrate that dimerization of the “Yippee-like” domain is essential for the centromere localization and hence the function of Mis18.

Results and Discussion

spMis18 possesses an N-terminal Yippee-like globular domain that is implicated in protein/nucleic acid binding

Amino acid sequence and the predicted secondary structure analysis of Schizosaccharomyces pombe Mis18 suggested the presence of a highly conserved N-terminal globular domain (residues 1–120; spMis181–120) mainly comprised of β-strands, followed by a moderately conserved C-terminal α-helical domain (residues 121–end; spMis181–term-α) (Fig 1A and B). Mis181–120 shares about 20% sequence similarity with a putative Zn2+-binding protein of unknown function named Yippee, originally identified in Drosophila, that is well conserved from yeast to humans [19]. spMis181–120 has two conserve C-X-C motifs, which are signature motifs present in metal ion-binding proteins. Previous analysis showed that mutations within the C-X-C motifs of human Mis18x perturb its centromere localization, highlighting the essential role of this domain [17]. To structurally characterize spMis181–120, we purified recombinant spMis181–120 as a mono-disperse sample and obtained crystals that diffracted X-rays to about 2.6 Å (Fig 1C). X-ray fluorescence scans of the crystals revealed the presence of bound Zn2+ ions. The structure was determined by single anomalous dispersion (SAD) exploiting the Zn anomalous signal from the bound Zn2+ ions in the crystals. The refined structure has an R factor of 22 and R-free factor of 26 with good stereochemistry (Table 1 and Fig EV1A). The final model includes amino acid residues 19–118 of spMis181–120 (Fig 1D). The N-terminal 18 and C-terminal 2 (119 and 120) residues are presumably disordered and hence could not be modeled.

The overall fold of spMis181–120 is formed by antiparallel β-sheets: a three-stranded (β1-β2-β9: β-sheet I) and a six-stranded (β3-β4-β8-β7-β6-β5: β-sheet II) sheet, arranged approximately perpendicular to each other (Fig 1D). The two β-sheets are held together by a Zn2+ ion coordinated via the C-X-C motifs from loops L1 and L5 (Fig 1D). Structural comparison of spMis181–120 with the available structures in the protein data bank (PDB) identified the thalidomide-binding domain of Cereblon (PDB: 4tzc; Q-score [20]: 0.47; RMSD: 2.12 Å), a component of an E3 ubiquitin ligase complex implicated in DNA repair, replication, and transcription [21], as the closest structural homolog (Fig EV1B). Other proteins that share a similar fold include RIG-I (PDB: 2qfd; Q-score: 0.38; RMSD: 2.19 Å), a nucleic acid-binding protein involved in innate anti-viral immunity [22]; Ms4 (PDB: 1fwq; Q-score: 0.34; RMSD: 2.03 Å), a guanine nucleotide exchange factor [23]; and MsB (methylamine sulfoxide reductase-B, PDB: 3eeo; Q-score: 0.32; RMSD: 1.81 Å), an oxidative reductase implicated in aging [24] (Fig EV1B). This is in agreement with a recent bioinformatics study suggesting an evolutionary relationship between Cereblon, Yippee, and Mis18 proteins [25]. Although these proteins recognize substrates as diverse as nucleic acids to proteins, they do so via a common cradle-shaped binding pocket formed by β-sheet II (Fig EV1C and D). This observation suggested that the putative substrate-binding site of spMis181–120 might play an important role in Mis18 function. To test whether the putative substrate-binding pocket was required for spMis18 function in vivo, we tested the ability of additional spMis18 expressed from a plasmid to complement the growth phenotype of mis18-262 (G117D) cells, which exhibit loss of function for spMis18 at the restrictive temperature (36°C) [10]. While expression of wild-type spMis18 restored growth at 36°C, expressing the pocket mutant (Y74A/Y90A/T105A/S107K, cell to varying degrees. Fivefold serial dilutions of mis18-262 cells transformed with plasmids harboring the indicated spMis18 constructs, spotted on PMG – uracil + phosphine B media supplemented with (repressed) or without (expressed) thiamine, and incubated at the indicated temperatures; dead cells stain dark pink.
Crystal structure of Mis18 "Yippee-like" domain  

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Figure 1.
Table 1. Data collection, phasing, and refinement statistics.

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Values in parentheses are for highest-resolution shell.

Figs 1D and EV1D) failed to complement the loss of spMis18 function, demonstrating the requirement of this pocket for Mis18 function (Fig 1E).

**spMis18₁₋₁₂₀ forms a homodimer**

The asymmetric unit of the spMis18₁₋₁₂₀ crystals contained three copies of spMis18₁₋₁₂₀ assembled in a linear arrangement via two different interfaces (interface I and interface II), resulting in two potential dimeric structures (dimer I and dimer II) (Fig 2A). While the interface stabilizing dimer I is formed by the stacking of loops L5 and L7 of one monomer over their dimeric counterpart, the dimer II interface is formed by the stacking of β-sheet I (Fig 2A). The oligomeric assembly observed in the crystals prompted us to characterize the oligomeric structure of spMis18₁₋₁₂₀ in solution. The molecular weight of spMis18₁₋₁₂₀, as measured using SEC-MALS (size exclusion combined with multi-angle light scattering), was 33,338 Da. Given that the calculated molecular weight of an spMis18₁₋₁₂₀ monomer is 16,160 Da whereas a dimer would be 32,320 Da, this analysis independently confirms that spMis18₁₋₁₂₀ forms a dimer in solution (Figs 2B and EV2A).

To identify the physiologically relevant dimer, we compared the extent of amino acid conservation of the interface residues and the buried surface area within the dimer I and dimer II interfaces. Dimer I is mainly stabilized by poorly conserved amino acid residues (F64, R66, L71, V73, S92, and I95 with the exception of R101) and involves 1,077 Å² buried surface area. In contrast, dimer II is stabilized by highly conserved residues (V22, I31, Y114, and L116) and has an extensive binding interface (as compared to dimer I) with a 1,431 Å² buried surface area. Based on this observation, we reasoned that dimer II is likely the physiologically relevant dimer. To test the basis of dimer formation, we generated several single point mutations at the dimer II interface (I31A, Y114A, Y114E, V22E) and determined whether they perturb the ability of spMis18₁₋₁₂₀ to dimerize. In size-exclusion chromatography (Fig 2B), recombinant spMis18₁₋₁₂₀I31A mutant protein eluted later (Ve = 12.4 ml) than wild-type spMis18₁₋₁₂₀ (Ve = 11.8 ml), suggesting that this mutant protein forms a smaller entity than wild-type. Further analysis of spMis18₁₋₁₂₀I31A using SEC-MALS confirmed that it is monomeric with a measured molecular weight of 15,955 Da (calculated molecular weight of a monomer is 16,160 Da) (Figs 2B and EV2A). Consistent with this, the other dimer II interface mutants Y114A, Y114E, and V22E also behaved as smaller entities compared to the wild-type protein when analyzed by native PAGE (Fig EV2B), thus demonstrating that the dimerization of spMis18₁₋₁₂₀ is mediated via the dimer II interface (Fig 2A).

**Yippee-like globular domains of Mis18 proteins possess an intrinsic ability to form dimers**

To test whether Yippee-like globular domains of other Mis18 orthologs also display an intrinsic ability to form dimers, we expressed and purified recombinant fragments of human Mis18α (residues 77–187; hsMis18α₇₇₋₁₈₇) and Mis18β (56–183; hsMis18β₅₆₋₁₈₃) containing their Yippee-like globular domains. SEC and SEC-MALS analyses were conducted to assess their ability to form oligomers. While hsMis18β₅₆₋₁₈₃ eluted as a dimer at 15.5 ml in SEC with a measured molecular weight of 31,254 Da in SEC-MALS (calculated MW of a dimer is 29,324 Da), the corresponding values for hsMis18β₅₆₋₁₈₃ were 17.3 ml and 14,993 Da (calculated MW of a monomer is 13,591 Da), respectively, indicating that hsMis18β₅₆₋₁₈₃ exists as a monomer in solution (Fig 2C). We next tested whether hsMis18β₇₇₋₁₈₇ and hsMis18β₅₆₋₁₈₃ could interact to form a heterodimer, by mixing equimolar quantities of recombinant hsMis18β₇₇₋₁₈₇ and hsMis18β₅₆₋₁₈₃. In SEC, hsMis18β₅₆₋₁₈₃ co-elutes with hsMis18β₇₇₋₁₈₇ at 15.4 ml suggesting that they form a heterodimer (Fig 2C). The molecular weight of this entity as measured by SEC-MALS was 27,476 Da, confirming that hsMis18α and hsMis18β can heterodimerize through their respective Yippee-like domains (Figs 2C and EV2C).

To test whether the mode of dimerization (dimer II mediated) is conserved from fission yeast to humans, we generated a homology model of the human hsMis18α₇₇₋₁₈₇–hsMis18β₅₆₋₁₈₃ heterodimer using the spMis18₁₋₁₂₀ crystal structure described above as a template (Phyre2 server: http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Fig 2D). Our analysis of the modeled dimer interface (containing Mis18α residues Val 82, Arg 89, Pro 91,
Figure 2.
Asp 94, Tyr 176, and Leu 178, and Mis18β residues Val 77, His 84, Val 86, His 92, Tyr 172, Leu 174, Lys 175, and Thr 176) did not show any steric clashes and involved 1,387 Å² buried surface area, similar to the spMis18α–120 dimer II interface. Moreover, mutations at the dimer interface (Mis18α V82E/Y172D and Mis18β V77E/Y172D) were sufficient to perturb the ability of hsMis18α–120 and hsMis18β–120 to form a heterodimer (Fig 2E and EV2D). This confirms that the Yippee-like domains within Mis18 proteins employ a conserved mode of dimerization.

Structural and biochemical analyses of Yippee-like domains from other proteins (Cerebion, RIG-I and MsrB) have so far yielded no direct evidence that they possess an innate tendency to oligomerize in vitro. We therefore refer to the Yippee-like domain of Mis18 orthologs as Mis eighteen Dimerization in Yippee (MeDiY) domain hereafter.

The C-terminal α-helical domain induces tetramerization of S. pombe Mis18

In addition to the N-terminal MeDiY domain, Mis18 orthologs possess a C-terminal α-helical domain spMis18C-term-α (aa residues 121–end). To test whether spMis18C-term-α can influence the overall oligomeric state of the protein, we purified recombinant full-length spMis18 (spMis18α). Obtaining intact samples of spMis18α proved difficult either with or without His/His-GFP tag, as it was sensitive to degradation from the C-terminus (Fig EV3A). The stable partially degraded His-GFP-spMis18α when analyzed by SEC-MALS appeared to form a tetramer (Fig 3A). Mass spectrometric peptide sequence coverage analysis of this sample revealed the loss of approximately 20 amino acids at the C-terminus. Close examination of the amino acid sequence revealed the presence of a low-complexity region at the extreme C-terminus (amino acid residues 171–end, 15 out of 20 amino acid residues being Lys/Arg) that is unique to spMis18 (Fig EV3B). We therefore expressed and purified recombinant spMis18 with a C-terminal truncation (spMis18ΔC; amino acids 1–168; Fig EV3C). SEC-MALS analysis of spMis18ΔC revealed that it predominantly formed a tetramer (Fig 3B) and demonstrated a role for the α-helical region downstream of the MeDiY domain (residues 121–168; Fig 1B) in spMis18 tetramerization. Since this α-helical region is structurally conserved (based on secondary structure prediction; Fig 1B) between Mis18 proteins from evolutionarily distant eukaryotes, we propose that Mis18 oligomerization (either homo or hetero) mediated through the C-terminus is also likely to be highly conserved.

MeDiY and the C-terminal α-helical domain are independent structural modules

To obtain further insights into the overall architecture of the spMis18 oligomer, we tested whether spMis18ΔMeDiY and spMis18C-term-α could interact with each other, or whether they existed as structurally independent modules. His-spMis18ΔMeDiY and His-GFP-spMis18C-term-α were purified individually and analyzed for complex formation using size-exclusion chromatography. His-GFP-spMis18C-term-α and His-spMis18ΔMeDiY eluted separately at distinct elution volumes (12.3 and 15.3 ml, respectively). This demonstrates that the spMis18ΔMeDiY domain alone is unable to associate with spMis18C-term-α (Figs 3C and EV3D).

We next tested whether spMis18C-term-α can self-associate to form oligomers. SEC-MALS analysis of His-GFP-spMis18C-term-α (untagged spMis18C-term-α (8,775 Da) is too small for accurate mass detection) revealed that it is a homotrimer with a measured molecular weight of 119,886 Da (theoretical molecular weight of a monomer is 38,337 Da while that of a homotrimer would be 115,011 Da) (Fig 3D). We therefore conclude that spMis18 has two structurally independent domains, and they each possess the ability to homo-oligomerize.

Oligomerization of spMis18 via the MeDiY domain is required for its function

We next tested whether spMis18 dimerization is functionally important in vivo. Co-immunoprecipitation assays were performed on S. pombe cells expressing endogenous spMis18α with a C-terminal 8xMyc tag, and spMis18α or spMis18αΔ31A expressed ectopically as a C-terminally GFP-tagged fusion protein. Endogenous wild-type spMis18α-Myc associated efficiently with ectopic wild-type spMis18α-GFP, confirming the interaction of spMis18α with itself in vivo (Fig 4A). Such self-association of wild-type spMis18α has also been demonstrated previously through yeast two-hybrid assays [13,15]. In contrast, the spMis18αΔ31A dimerization mutant showed significantly reduced association with endogenous spMis18α-Myc. Additionally, spMis18ΔMeDiY alone was sufficient to associate with spMis18α-Myc (Fig 4A). This highlights the role of spMis18ΔMeDiY in the overall oligomeric state of spMis18 in vivo.
spMis18-262 function at 36 °C [10]. Dimer-disrupting mutants spMis18flI31A and Y114A (in the context of both
expression assays in which we evaluated the ability of dimerization
essential for Mis18fl and spMis18MeDiY-mediated dimerization through
spMis18 via the MeDiY domain-mediated dimerization ensures optimal spMis18 association with centromeres.

Centromeric localization of spMis18 depends on MeDiY-mediated
dimerization
spMis18 is known to associate with centromeres, where it is required for the incorporation of CENP-A^{Cnp1} [10]. Chromatin
immunoprecipitation analyses were performed to determine
whether dimerization mediated by the MeDiY domain is required
for the association of spMis18fl with centromeres. Disruption of
spMis18 MeDiY-mediated dimerization through spMis18flI31A or
spMis18flY114A mutations resulted in reduced association of ectopically expressed spMis18fl-GFP with centromeres in wild-type cells
(Fig 4D), although no significant defects in subcellular localization
of spMis18fl-GFP were observed (Fig EV4A). Additionally, cell
growth and CENP-A^{Cnp1} association with centromeres were essentially unaffected in these cells (Fig EV4B and C). Thus, MeDiY
domain-mediated dimerization ensures optimal spMis18 association with centromeres.

To test whether spMis18MeDiY-mediated oligomerization is essential for spMis18 function, we performed genetic complementation
assays in which we evaluated the ability of dimerization mutants I31A and Y114A (in the context of both spMis18fl and
spMis18MeDiY), to rescue the temperature-sensitive growth phenotype of mis18-818 (T49A) and mis18-262 (G117D) cells in vivo
(Fig 4B and C) [10]. Dimer-disrupting mutants spMis18flI31A and
spMis18flY114A failed to rescue the viability defect at restrictive temperature in both mis18-262 and mis18-818 cells. Expression of
spMis18MeDiY alone, while failing to complement the loss of spMis18 function at 36°C, reproducibly conferred a dominant-negative effect
on growth in both mis18-262 and mis18-818 cells at 32°C. This inhibitory effect on growth of mis18-262 and mis18-818 cells depended on
the ability of spMis18MeDiY to dimerize, as the spMis18MeDiYI31A and Y114A mutations caused no such negative influence on growth
(Fig 4B and C). We conclude that dimerization of spMis18 via the MeDiY domain is crucial for spMis18 function in vivo.
Centromeric association of the Mis18 complex is one of the early key steps involved in the establishment of CENP-A nucleosomes at centromeres [17]. Although some of the components of the Mis18 complex show variations among different species (particularly the absence of Mis18BP1/KNL2 in fission yeast), the function of the Mis18 complex in targeting HJURP to centromeres is well conserved [14,15,26–28]. The molecular mechanism by which the Mis18 complex is targeted to centromeres, however, remains to be determined, and any structural insights into the molecular architecture of the Mis18 complex and/or its components have the potential to advance our understanding of centromere establishment.

The Mis18 complex is critical for CENP-A deposition at centromeres; however, Mis18 is poorly characterized in terms of its structure and function. Here, we show that fission yeast Mis18 and its human isoforms, Mis18α and Mis18β, possess two structurally distinct domains: an N-terminal “Yippee-like” globular domain and a C-terminal α-helical domain. Structural alignments revealed unambiguous structural homology between the N-terminal “Yippee-like” globular domain and proteins of diverse function such as Cereblon [21], RIG-I [22], MSS4 [23], and MsrB [24], as well as identified a conserved substrate-binding pocket in Mis18 required for its function. However, predicting the
identity and nature of Mis18 binding partner(s) seems non-trivial, as the amino acids that make up the binding pocket and their spatial orientation are markedly different among the various homologous proteins.

A key finding of our analyses is the ability of the “Yippee-like” globular domain (MeDiY) of S. pombe and human Mis18 proteins to dimerize. To date, none of the other related domains are known to form dimers. Interestingly, S. pombe Mis18 and also human Mis18 [29], in the presence of Mis18 C-terminal domains known to form dimers. Notably, ectopic expression of the MeDiY domain alone, while failing to complement the loss of spMis18 function conferred a dominant-negative effect on the growth of mis18 mutants, implicating both MeDiY and C-terminal α-helical domains in ensuring the required oligomeric structure for function. Specifically, perturbing the dimeric interface of the MeDiY domain affects the centromere localization of spMis18. Identifying the binding partners of MeDiY and determining how (and if) Mis18 oligomerization can influence substrate recognition are important questions to be addressed in the future to unravel the molecular mechanisms that mediate centromere localization and function of the Mis18 complex. A previous study has shown that HJURP dimerization is required for stable deposition of CENP-A nucleosomes at centromeres and implicated a direct role for HJURP dimerization in forming octameric CENP-A nucleosomes [30]. These observations highlight protein oligomerization-mediated regulation as an emerging regulatory theme for the inherently complex process of centromere establishment and maintenance.

Materials and Methods

Expression and purification of recombinant S. pombe proteins

Schizosaccharomyces pombe Mis18p, Mis18MeDiY, Mis18AC, and Mis18C-termα codon-optimized sequences (GeneArt) were cloned into pEC-K-3C-His or pGFP (Addgene) LIC vectors with N-terminal His or His-GFP tags cleavable with either a 3C or TEV site, respectively. Mutations were introduced using QuikChange site-directed mutagenesis protocol (Stratagene). All proteins were expressed using E. coli BL21 Gold; His-spMis18MeDiY was grown in Super Broth and induced for 6 h at 25°C using 0.3 mM IPTG. His-spMis18AC was grown in Super Broth, while His-spMis18C-termα and His-GFP-spMis18α were grown in 2× TY before inducing for 16 h at 18°C by adding 0.3 mM IPTG.

All proteins were lysed via sonication in a lysis buffer containing 20 mM Tris pH 8 (or 8.5 for His-spMis18MeDiY), 35 mM imidazole and 2 mM BME and 2 mM BME supplemented with 10 μg/ml DNase, 1 mM PMSF, and complete EDTA-free (Roche). The following NaCl concentrations were used in the lysis buffer: 500 mM for His-spMis18MeDiY, 50 mM for His-spMis18AC, and 100 mM for His-GFP-spMis18C-termα or His-GFP-spMis18α. After clarification, His-GFP-spMis18C-termα, His-GFP-spMis18α, and His-spMis18MeDiY proteins were purified using a 5-ml HisTrap HP column (GE Healthcare), while His-spMis18AC was purified by batch mode using HisPur Ni-NTA resin (Thermo Scientific). Resin was washed with lysis buffer, and then, His-spMis181–120, His-GFP-spMis18C-termα or His-GFP-spMis18α received additional washes with 20 mM Tris pH 8/8.5, 35 mM imidazole, 50 mM KCl, 10 mM MgCl2, 2 mM ATP, and 2 mM BME. The following NaCl concentrations were used: 500 mM for His-spMis18MeDiY or 1 M for His-GFP-spMis18C-termα and His-GFP-spMis18α. Proteins were finally washed in lysis buffer and then eluted with 20 mM Tris pH 8/8.5, 500 mM imidazole, and 2 mM BME. The following NaCl concentrations were used in the elution buffer: 500 mM for His-spMis18MeDiY, His-spMis18AC, and His-GFP-spMis18C-termα and 100 mM NaCl for His-GFP-spMis18α. Eluted His-spMis181–120 was used directly for crystallization trial, while all other proteins were subjected to size-exclusion chromatography. His-spMis18AC was loaded onto Superdex 200 Hi-load 16/600 (GE Healthcare) equilibrated with 20 mM Tris pH 8, 400 mM NaCl, and 1 mM TCEP. Appropriate fractions were pooled and injected onto a Superdex 200 increase 10/300 column (GE Healthcare) equilibrated with 20 mM Tris pH 8, 500 mM NaCl, and 4 mM DTT. His-GFP-spMis18α was applied to a Superdex 200 increase 10/300 column equilibrated with 20 mM Tris pH 8, 150 mM NaCl, and 2 mM DTT. His-GFP-spMis18α was applied to a Superose 6 10/300 column (GE Healthcare) equilibrated with 20 mM Tris pH 8, 300 mM NaCl, and 2 mM DTT. Fractions were analyzed on SDS-PAGE and stained with Coomassie blue.

Expression and purification of recombinant human proteins

Human Mis18α, Mis18β, Mis18γ, and Mis18δ codon-optimized sequences (GeneArt) were cloned into pEC-K-3C-His LIC vector or pGEX-6P-1 (GE Healthcare). Human proteins were expressed separately or co-expressed using pGEX-6P-1 and pEC-K-3C-His in BL21 Gold in a similar manner to spMis18AC.

His-hsMis18187–189 was lysed via sonication in 20 mM Tris pH 8, 500 mM NaCl, 35 mM imidazole, and 4 mM BME with 10 μg/ml DNase, 1 mM PMSF, and complete EDTA-free. Clarified lysate was loaded onto a 5-ml HisTrap HP column and washed with lysis buffer and then with 20 mM Tris pH 8, 1 M NaCl, 35 mM imidazole, 50 mM KCl, 10 mM MgCl2, 2 mM ATP, and 4 mM BME and re-equivibrated in lysis buffer before eluting with 20 mM Tris pH 8, 500 mM NaCl, 500 mM imidazole, and 4 mM BME. GST-hsMis18β56–183 was sonicated in 20 mM Tris pH 8, 500 mM NaCl, and 4 mM DTT with 10 μg/ml DNase, 1 mM PMSF, and complete EDTA-free. Cleared lysate was loaded on 12 ml glutathione-Sepharose (GE Healthcare) in batch mode and washed with lysis buffer and then with 20 mM Tris pH 8, 1 M NaCl, 50 mM imidazole, 50 mM KCl, 10 mM MgCl2, 2 mM ATP, and 4 mM BME and re-equivibrated in lysis buffer before eluting with 20 mM Tris pH 8, 500 mM NaCl, 500 mM imidazole, and 4 mM BME. GST-hsMis18β56–183 was applied to a Superose 6 10/300 column (GE Healthcare) equilibrated with 20 mM Tris pH 8, 200 mM NaCl, and 5 mM DTT.

His/GST pull-down assays

To test for protein-protein interactions, purified His-tagged spMis18MeDiY and His-GFP-tagged spMis18C-termα proteins were mixed and applied to a Superdex 200 increase 10/300 column equilibrated with 20 mM Tris pH 8, 150 mM NaCl, and 2 mM DTT and fractions analyzed via SDS-PAGE. To test for interaction of human hsMis18α and β, 10 μl of culture of co-expressed proteins was
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Crystallization and data collection

Crystallization trials were performed using a nanoliter crystallization robot at the Edinburgh crystallization facility. Crystals were grown by vapor diffusion method. Diffraction quality crystals were obtained using well buffer containing 0.2 M ammonium chloride/formate/acetate/phosphate and 20% PEG 3350 (with the measured pH of the solution in the range of 6.2–8). 15–20 mg/ml protein sample was mixed with the well buffer in a 1:1 ratio. Crystals were briefly transferred to cryoprotectant solution (crystallization solution supplemented with glycerol or ethylene glycol to a final concentration 25%) before flash cooling in liquid nitrogen. The crystals diffracted to 2.6 Å resolution at the MX beamlines of the Diamond Light Source (Table 1).

Crystal structure solution and refinement

The structure of spMis18MeDiY was determined by the single anomalous dispersion (SAD) method using the anomalous signal of intrinsically bound Zn²⁺ ion. Data were processed using XIA2 and scaled with SCALa of CCP4 [31]. SAD phasing and the calculation of the initial map were performed using phenix.autosol from the PHENIX suite of programs [32]. The model was built by iterative rounds of manual building with COOT [33], and refinement was done using Refmac5 of CCP4. Data collection, phasing, and refinement statistics are shown in Table 1.

SEC-MALS

Size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) was performed at the Edinburgh Protein Production Facility at room temperature using a ÄKTA FPLC. 100 µl of protein at 1 mg/ml was loaded onto either Superose 6 10/300, Superdex 75 10/300, or Superdex 200 10/300 columns (GE Healthcare) pre-equilibrated with 50 mM HEPES pH 8, 150 mM NaCl, and 5 mM DTT for His-spMis18MeDiY, His-GFP-spMis18Cterm, and hsMis1857,187, hsMis1899Glu–183, or 50 mM HEPES pH 8, 50 mM NaCl, and 5 mM DTT for His-spMis18AC or 50 mM HEPES pH 8, 300 mM NaCl, and 1 mM TCEP for His-GFP spMis18B. A MiniDAWN in-line detector (Wyatt Technology) was used to measure MALS, while a Viscotek RI Detector (Wyatt Technology) was used to detect refractive index. Data were analyzed using ASTRA™ software (Wyatt Technology).

Plasmids and S. pombe strains

For in vivo assays in S. pombe, spMis18 cDNA (wt, I31A, Y114A, Y90A or Y74A Y90A T105A S107K in the context of mis18Δ or mis18MeDiY) was PCR-amplified and cloned into the pDUAL-GFH41 vector, which allows for expression of C-terminally GFP-tagged spMis18 (wt or mutants) under the control of the medium-strength nmt41 promoter that is induced in the absence of thiamine in culture media [34]. spMis18 constructs cloned into the pDUAL-GFH41 vector were then either transformed as such and selected in the absence of uracil in growth media, or integrated into the genome at the leu1 locus, in wt, mis18-818, or mis18-262 cells. Genotypes of S. pombe strains used in this study are listed in Table EV1.

Genetic complementation assays

Fivefold serial dilutions of mis18-262, mis18-818, or wt cells expressing GFP-tagged spMis18 or spMis18MeDiY (wt or mutants) either from an ectopic pDUAL-GFH41 plasmid (selected for in media lacking uracil; Fig 1E) or from the leu1 locus (integrated in the genome; Figs 4B and C and EV4B), were spotted onto PMG media containing phloxine B supplemented with or without thiamine and incubated at the indicated temperatures for 3–5 days.

Co-immunoprecipitation and Western analyses

For co-immunoprecipitation experiments, cells expressing endogenous spMis18-Myc and ectopic spMis18-GFP (wt or mutants; from the leu1 locus) were cultured in complete PMG media lacking thiamine for 21 h and processed as previously described [11]. Immunoprecipitation was performed using rabbit anti-myc antibody A14 (Santa Cruz Biotech) and Western analyses using monoclonal anti-GFP (Roche) or anti-myc 9B11 (Cell Signaling).

Quantitative chromatin immunoprecipitation

Anti-GFP ChIP, anti-CENP-A<sup>Cop1</sup> ChIP, and real-time PCR analyses were performed as previously described [11], on wild-type S. pombe cells expressing ectopic GFP-tagged spMis18<sub>WT</sub> (wt or mutants; from the leu1 locus) cultured in complete PMG media lacking thiamine for 21 h.

Cytology

Immunolocalization and microscopy were performed as previously described [11] on wild-type S. pombe cells expressing ectopic GFP-tagged spMis18<sub>WT</sub> (wt or mutants; from the leu1 locus) cultured in complete PMG media lacking thiamine for 21 h.

Data deposition

Atomic coordinates of the structure and structure factors are deposited in the RCSB protein data bank (www.rcsb.org) with accession code 5HJ0.

Expanded View for this article is available online.

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Author contributions

LS, BM-P, RB, FS, RK-S, GR, and AAJ performed experiments and analyzed data. LS, BM-P, RCA, and AAJ designed experiments, analyzed data, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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