Intrakinetochore localization and essential functional domains of *Drosophila* Spc105

Ralf B Schittenhelm, Romanas Chaleckis¹
and Christian F Lehner*

Institute of Zoology, University of Zurich, Zurich, Switzerland

The kinetochore is assembled during mitotic and meiotic divisions within the centromeric region of chromosomes. It is composed of more than eighty different proteins. Spc105 (also designated as Spc7, KNL-1 or Blinkin in different eukaryotes) is a comparatively large kinetochore protein, which can bind to the Mis12/MIND and Ndc80 complexes and to the spindle assembly checkpoint components Bub1 and BubR1. Our genetic characterization of *Drosophila* Spc105 shows that a truncated version lacking the rapidly evolving, repetitive central third still provides all essential functions. Moreover, in comparison with Cenp-C that has previously been observed to extend from the inner to the outer kinetochore region, full-length Spc105 is positioned further out and is not similarly extended along the spindle axis. Thus, our results indicate that Spc105 forms neither an extended link connecting inner Cenp-A chromatin with outer kinetochore regions nor a scaffold constraining kinetochore subcomplexes and spindle assembly checkpoint components together into a geometrically rigid supercomplex. Spc105 seems to provide a platform within the outer kinetochore allowing independent assembly of various kinetochore components.

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Introduction

Kinetochore are assembled within the centromeric region of eukaryotic chromosomes. They are crucial for accurate transmission of genetic information during mitotic and meiotic divisions (for reviews, see Westermann *et al*, 2007; Cheeseman and Desai, 2008; Welburn and Cheeseman, 2008). Kinetochore serve as attachment sites for spindle microtubules. In addition, they host a number of proteins functioning in surveillance mechanisms that ensure a correct orientation of chromosomes within the spindle before the start of anaphase. Among these are the proteins of the spindle assembly checkpoint (SAC), which delays anaphase onset

and exit from mitosis in the presence of unattached kinetochores (Musacchio and Salmon, 2007).

Surprisingly, despite the fundamental biological significance of kinetochores, its underlying centromeric DNA sequence organization has not been conserved during evolution (Allshire and Karpen, 2008). In contrast with the point centromeres of budding yeast, higher eukaryotes, including *Drosophila melanogaster* and humans have regional centromeres with megabase pairs of repetitive centromeric DNA embedded in pericentromeric heterochromatin. Nevertheless, eukaryotic kinetochores have recently been shown to be assembled from a shared set of homologous protein components (Meraldi *et al*, 2006; Westermann *et al*, 2007; Welburn and Cheeseman, 2008). More than 80 proteins contribute to functional kinetochores. The precise molecular functions and binding partners of individual kinetochore proteins are yet to be defined in many cases.

Findings from different organisms have emphasized the crucial role of a centromere-specific histone H3 variant (CenH3) at the top of the kinetochore assembly pathway. CenH3 (designated as Cenp-A in humans and Cid in *Drosophila*) is present in centromeres throughout the cell cycle and is thought to make an important contribution to the epigenetic specification of centromere identity in higher eukaryotes (Allshire and Karpen, 2008). Cenp-C is another conserved constitutive centromere component, which might provide a foundation for the assembly of the S/KMN network during mitosis. This protein network contains Spc105/KNL-1 as well as the Mis12/MIND and Ndc80 complexes (Welburn and Cheeseman, 2008). In yeast and humans, the Ndc80 complex is a heterotetramer with a central rod of about 50 nm coiled coil connecting globular end domains (Ciferri *et al*, 2005, 2008; Wei *et al*, 2005; Wang *et al*, 2008). The globular N-terminal domains of Ndc80/Hec1 and Nuf2 subunits on one end bind directly to microtubules (Wei *et al*, 2005; Cheeseman *et al*, 2006; Wilson-Kubalek *et al*, 2008). The unstructured N-terminal extension in Ndc80 is also important for microtubule binding (Guimaraes *et al*, 2008; Miller *et al*, 2008). The globular C-terminal domains of Spc24 and Spc25 on the other end of the complex are anchored within the kinetochore (Ciferri *et al*, 2005; Wei *et al*, 2005, 2006). This anchoring involves the Mis12/MIND complex and Spc105/KNL-1 (Cheeseman *et al*, 2006). High-resolution light microscopic analyses have clearly shown a polar orientation of the Ndc80 complex within the kinetochore with Spc24/Spc25 directed towards the inner centromere and Ndc80/Nuf2 towards the outer kinetochore microtubules (kMTs) (DeLuca *et al*, 2006; Schittenhelm *et al*, 2007). However, the molecular details of how the Mis12/MIND complex and Spc105/KNL-1 connect the Ndc80 complex with inner centromere components remain to be clarified. The co-purification behaviour and localization dependencies have suggested that the Mis12 complex associates with the Cenp-C homologue in yeast and *Caenorhabditis elegans* (Westermann *et al*, 2003; Cheeseman *et al*, 2004). *Drosophila*

*Corresponding author. Institute of Zoology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland.

Tel.: +41 44 635 48 71; Fax: +41 44 635 68 20;

E-mail: christian.lehner@zool.uzh.ch

¹Present address: UAB Biotechpharma, Akademijos g. 2, LT-08412 Vilnius, Lithuania

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Cenp-C has been shown to have an intrakinetochore localization that is entirely consistent with the suggestion that it might form a bridge between the inner Cenp-A chromatin and the outer S/KMN network (Schittenhelm *et al*, 2007). Although the C-terminal domain of Cenp-C is next to Cenp-A chromatin, its N-terminal domain is further out close to Mis12. However, direct molecular interactions between Cenp-C and Mis12 complex subunits or other S/KMN subunits have not yet been reported.

Spc105/KNL-1/Blinkin homologues are clearly crucial for kinetochore assembly in budding (Nekrasov *et al*, 2003) and fission yeast (Kerres *et al*, 2004), *C. elegans* (Desai *et al*, 2003), *D. melanogaster* (Przewloka *et al*, 2007) and humans (Kiyomitsu *et al*, 2007; Cheeseman *et al*, 2008). In principle, this comparatively large kinetochore protein might function as a linker connecting inner and outer kinetochore components. Initial analyses have shown co-immunoprecipitation with Cenp-C, and the Mis12 and Ndc80 complexes (Desai *et al*, 2003; Nekrasov *et al*, 2003; Cheeseman *et al*, 2004; Kerres *et al*, 2004; Obuse *et al*, 2004). The human Spc105/KNL-1 homologue Blinkin has recently been shown to interact with SAC components Zwint-1, Bub1 and BubR1 (Kiyomitsu *et al*, 2007). Moreover, the fission yeast microtubule plus end-binding protein Mal3/EB1 has been detected in Spc7/Spc105 immunoprecipitates (Kerres *et al*, 2004). Direct microtubule binding has been observed with *C. elegans* KNL-1 *in vitro* (Cheeseman *et al*, 2006). Therefore, it is also conceivable that Spc105/KNL-1 forms the kinetochore fibres that connect the outer kinetochore with kMT ends according to recent ultrastructural analyses (McIntosh *et al*, 2008).

The characterization of the *Drosophila* homologue described here constrains the models for Spc105 function. We show that drastically truncated versions can still provide all of the essential functions. These genetic analyses in combination with intrakinetochore mapping suggest that Spc105 does not function as an extended axial intrakinetochore linker, and is unlikely to act as a scaffold forcing all its interaction partners into a geometrically rigid supercomplex.

Results

Drosophila Spc105 is an essential kinetochore protein

In general, the sequences of known *Drosophila* centromere and kinetochore protein homologues are more strongly diverged than those of other eukaryotic lineages (Talbert *et al*, 2004; Heeger *et al*, 2005; Meraldi *et al*, 2006; Przewloka *et al*, 2007; Schittenhelm *et al*, 2007). Moreover, several genes for kinetochore components, which in a wide range of eukaryotes are clearly detectable by sequence-based searches, cannot be found in the *Drosophila* genome (Meraldi *et al*, 2006; CFL, unpublished observations). Cenp-C was therefore proposed to be absent initially, before it was revealed by a genetic modifier screen (Heeger *et al*, 2005). This genetic approach also led us to the highly diverged *Drosophila* Spc105 gene. We identified a deficiency, which is comparable to Cenp-C deficiencies (Heeger *et al*, 2005) in that it resulted in a strong dominant enhancement of a phenotype caused by Separase inhibition during eye development. Subsequent analyses revealed that the enhanced phenotype (Figure 1A) was caused by a second site mutation present on the chromosome carrying this particular deficiency. Mapping

placed the enhancer locus in a chromosomal region, which also contained the gene *CG11451*, a known E2F1 target (Dimova *et al*, 2003), important for normal proliferation and metaphase morphology in Schneider S2 cells (Bjorklund *et al*, 2006; Goshima *et al*, 2007). Therefore, it seemed conceivable that a mutation in *CG11451* further exacerbates progression through an already compromised mitosis. Indeed, sequence analysis revealed a DOC transposable element insertion in the *CG11451* gene isolated from the chromosome with the enhancer mutation (Figure 1B). This incomplete DOC retroposon disrupts the third exon. A transgene containing wild-type *CG11451* reverted the dominant enhancement of eye imaginal disk-specific Separase inhibition (Figure 1A and B) and prevented the recessive lethality associated with the DOC insertion in *CG11451*. Moreover, another chromosome with a P element insertion in *CG11451* (Figure 1B; Quinones-Coello *et al*, 2007) behaved like the DOC insertion. It also resulted in dominant enhancement of eye-specific Separase inhibition and recessive lethality, which were both suppressed by the wild-type *CG11451* transgene (data not shown). Therefore, we conclude that *CG11451* is essential and interacts genetically with Separase function, as previously reported for *Drosophila* Cenp-C.

To analyse the intracellular localization of the *CG11451* protein product, we expressed versions with N- or C-terminal EGFP extensions in S2R+ cells. EGFP fluorescence was observed at the kinetochore of mitotic chromosomes, as indicated by double labelling using an antibody against the centromere-specific Cenp-A/Cid protein (data not shown). During interphase, weak EGFP signals were detected mainly in the cytoplasm but not at centromeres. The mitosis-specific kinetochore localization was also observed in transgenic embryos expressing the *CG11451*-EGFP fusion (Figure 1C). Finally, we also obtained mitosis-specific kinetochore signals on staining S2R+ cells with specific antibodies raised against *CG11451* (Supplementary Figure 1).

A careful comparison of the *CG11451* sequence with that of kinetochore proteins described in other eukaryotes revealed very limited similarity to the Spc105/Spc7/KNL-1/Blinkin protein family (Supplementary Figure 2), as also described recently (Przewloka *et al*, 2007). The Spc105 homologues participate in the conserved S/KMN network of kinetochore complexes (Desai *et al*, 2003; Nekrasov *et al*, 2003; Cheeseman *et al*, 2004; Kerres *et al*, 2004; Obuse *et al*, 2004). Recent proteomic analyses have shown that the *CG11451* protein is a component of the *Drosophila* S/KMN network (Przewloka *et al*, 2007). Our independent proteomic analyses of EGFP-Nuf2 and EGFP-Mis12 immunoprecipitates from transgenic embryos confirmed this finding (Schittenhelm *et al*, 2007; data not shown). In addition, we carried out reciprocal Y2H experiments using *Drosophila* outer kinetochore components (Figure 1D and Supplementary Figure 3). The *CG11451* protein was found to interact with *Drosophila* Nsl1/Kmn1 (*CG1558*) and the N-terminal regions of Bub1 (*CG14030*, amino acids 1–182). Similar Y2H interactions have been observed with the human homologue Blinkin (Kiyomitsu *et al*, 2007). Our localization and interaction data therefore confirm and extend the evidence that *CG11451* encodes the *Drosophila* Spc105 homologue.

As an interaction between Spc105 and Bub1 is observed in both humans and *Drosophila*, we analysed whether the short, conserved RR[I/V]SF motif, which occurs in the N-terminal region of all Spc105 family members, might

Figure 1 Identification of *Drosophila* Spc105. (A) The *Spc105*¹ mutation was identified as a dominant enhancer of a rough eye phenotype caused by *GMR-thrΔC*, which results in eye-specific expression of a dominant-negative Separase regulatory subunit. *GMR-thrΔC* was expressed in either *Spc105*^{+/+} (*GMR-thrΔC*; +/+) or *Spc105*^{1/+} (*GMR-thrΔC*; *Spc105*^{1/+}) flies. The increased eye abnormalities observed in *Spc105*¹ heterozygous flies were reverted by the presence of the *gSpc105* transgene driving expression of wild-type Spc105 (*GMR-thrΔC*; *Spc105*^{1/+}; *gSpc105*). (B) The structure of wild-type and mutant *Spc105*/CG11451 alleles, as well as the *gSpc105* transgene is illustrated schematically. The triangles indicate the insertion sites of an incomplete DOC element in *Spc105*¹ and of a P element (*P01511LE*) in *Spc105*². The genomic fragment used for the construction of the *gSpc105* transgene is indicated by the thin black line below the gene model. Arrows above the gene models indicate the direction of transcription. Boxes represent exons with UTR regions (white) and coding regions (black). (C) The intracellular localization of *Drosophila* Spc105 was analysed in syncytial embryos expressing an Spc105-EGFP fusion (*Spc105-EGFP*) after double labelling with an antibody against Cenp-A/Cid (*Cenp-A*) and a DNA stain (DNA). EGFP signals were detected at centromeres only during mitosis (lower row) and not during interphase (upper row). Bar = 5 μm. (D) Y2H experiments were used for an analysis of protein-protein interactions of Spc105, Mis12/MIND complex subunits (Mis12, Nsl1, Nnf1a) and the N-terminal region of Bub1. Spc105 was divided into an N-terminal (N), middle (M) and C-terminal region (C). Interactions represented by single arrows were only observed when the protein next to the arrowhead was fused to the transcriptional activation domain, and the protein at the base of the arrow to the DNA-binding domain of *GAL4*, but not with the reciprocal fusion protein configuration. Interactions indicated by double arrows were observed with both configurations.

be required for Bub1 binding. However, on exchanging the RRISF sequence to AAGAA, we found this motif to be dispensable for Bub1 binding in Y2H assays. Moreover, an Spc105^{AAGAA}-EGFP version was observed to localize to the kinetochore and rescues the mitotic defects during mitosis 16 when expressed in *Spc105*¹ mutant embryos (see below, data not shown).

Drosophila Spc105 is required for normal kinetochore assembly and function

For an analysis of the Spc105 expression pattern during embryogenesis, we used a transgenic line expressing an Spc105-EGFP fusion under the control of the *Spc105* cis-regulatory region (*gSpc105-EGFP*). This transgene rescued the lethality of *Spc105*¹ and *Spc105*² hemizyosity. Moreover, the rescued flies were fertile. Therefore, we conclude that the *gSpc105-EGFP* transgene provides all essential Spc105 functions.

Immunoblotting indicated that Spc105-EGFP (data not shown) and Spc105 (Supplementary Figure 4) is present

during embryonic stages associated with mitotic divisions. The high Spc105 levels, which were observed during the initial syncytial stages, in which nuclei divide rapidly, presumably represent a maternal contribution. Cell-cycle progression after cellularization was accompanied by zygotic expression. Spc105 levels were found to be minimal during the final stages of embryogenesis, when only very few cells continue with mitotic cell-cycle progression. Unfortunately, the antibodies against Spc105 resulted in relatively high background signals in embryos, precluding reliable immunofluorescent detection of Spc105. However, analysis of EGFP fluorescence in *gSpc105-EGFP* embryos confirmed the correlation of Spc105 expression and mitotic proliferation. After germ band retraction, EGFP signals were no longer observed in the epidermis and other post-mitotic tissues, whereas they were still detected in mitotic cells of the nervous system (Supplementary Figure 4).

The maternal Spc105 contribution is expected to support normal development in *Spc105* mutant embryos initially. Indeed, phenotypic abnormalities in *Spc105* mutant embryos

were not detected before cells progress through the fifteenth round of mitosis. During mitosis 16, strong abnormalities were observed. In particular, abnormal anaphase and telophase figures were apparent (Figure 2A). Although centromeres are tightly clustered into two groups in over 90% of the anaphase/telophase figures observed in sibling control embryos, we detected lagging centromeres or far more severe perturbations in over 90% of the anaphase/telophase figures

of *Spc105* mutant embryos ($n > 100$ from > 10 embryos). *Spc105*¹ and *Spc105*² hemizygous and trans-heterozygous embryos showed comparable phenotypes as *Spc105*¹ homozygotes, suggesting that both alleles result in a complete loss of gene function (Figure 2A). Immunoblotting experiments confirmed that *Spc105* was severely reduced in *Spc105* mutant embryos at the stage of mitosis 16 to $< 5\%$ of the normal levels (Supplementary Figure 1).

Figure 2 Abnormal chromosome segregation during mitosis in *Spc105* mutants. (A) Embryos were collected from *Spc105*¹/*TM3*, *Ubx-lacZ* flies and fixed at the stage of the 16th round of mitosis. Embryos were labelled with a DNA stain (DNA), an antibody against Cenp-A/Cid (*Cenp-A*) and an antibody against β -galactosidase for genotype determination (data not shown). In *Spc105*⁺ sibling control embryos (*Spc105*⁺, left panel), centromeres form a tight cluster during anaphase/telophase on the poleward side of the chromosomes (see arrows). In *Spc105* mutants (*Spc105*⁻; right panel), centromeres are rarely clustered in anaphase/telophase and lagging chromosomes are frequent (see arrows). Moreover, more severe perturbations during anaphase/telophase were also apparent (dashed circles). The bar diagram indicates the percentage of abnormal anaphase/telophase figures in wild-type and *Spc105* mutant embryos. The total number of the observed anaphase/telophase figures ($n > 100$) in the analysed epidermal regions from at least six different embryos per genotype was set to 100%. Genotypes are indicated below the bars. Bar = 2 μ m. (B) Time-lapse *in vivo* imaging of mitoses in *Spc105*⁺ or *Spc105*⁻ embryos expressing Cenp-A/Cid-EGFP and histone H2AvD-mRFP1. The time (min:s) indicated in each frame is given relative to the first anaphase frame, which was set to zero in the top three rows, or relative to the start of chromosome condensation in the bottom row. The top two rows illustrate progression through mitosis 15, in which chromosome segregation during anaphase is already less synchronous in *Spc105* mutants (second row). The bottom two rows illustrate progression through mitosis 16 in *Spc105* mutants, in which chromosome congression was either no longer observed (class I, fourth row) or in which it was followed by an abnormally delayed anaphase (class II, third row). Bar = 3 μ m.

In vivo imaging was used for a further characterization of the apparent mitotic defects in *Spc105*¹ embryos. We analysed embryos expressing histone H2Av-mRFP and Cenp-A/Cid-EGFP. Time-lapse analysis of the fourteenth round of mitosis did not show obvious defects in chromosome congression and segregation, confirming that maternally contributed Spc105 is likely to be still sufficient at this early stage (data not shown). However, as also observed with fixed embryos, the following fifteenth round of mitosis was found to be slightly abnormal in *Spc105*¹ mutants (Figure 2B). Lagging centromeres were frequently observed during anaphase (27 lagging centromeres in seven mutant anaphases compared with no lagging centromeres in nine control anaphases), but the duration of mitosis 15 did not seem to be increased. During the subsequent mitosis 16, time-lapse analysis showed more striking abnormalities in *Spc105*¹ mutants. Two classes of mitotic abnormalities were evident (Figure 2B). Mitotic cells in the first class showed similar but more severe defects as those apparent in the previous mitosis. These cells were still able to align chromosomes in a metaphase plate. Moreover, after a prolonged metaphase (more than 10 min compared with 2.3 ± 0.3 min in sibling controls, $n = 10$) they entered an abnormal anaphase. Many chromatids were not moved efficiently towards the poles. Cells in the second class remained in prometaphase during the analysed time period, that is for more than 14 min (Figure 2B, $n = 5$). They were unable to align their chromosomes into a metaphase plate and did not enter anaphase. In contrast, the duration of prometaphase 16 in control embryos was only 4.5 ± 0.3 min ($n = 10$). These observations confirm that Spc105 is required for normal kinetochore function. In mutants, Spc105 levels remaining at the mitosis-16 stage are evidently no longer sufficient for efficient chromosome congression during prometaphase and chromosome segregation during anaphase.

The reduced Spc105 levels in the mutant embryos do not seem to abolish SAC function during mitosis 16. The unaligned kinetochores apparently still activate this checkpoint and thereby delay anaphase onset and exit from mitosis. To confirm SAC function in *Spc105*¹ mutants, we analysed their response to colchicine treatment. Colchicine depolymerises microtubules and therefore prevents mitotic spindle formation. The resulting unattached kinetochores activate SAC in wild-type embryos and thereby arrest cells in mitosis with phospho-histone H3 (PH3)-positive, condensed chromosomes. After a 20 min incubation in colchicine, we observed a similar increase in the number of PH3-positive cells in sibling control and *Spc105*¹ mutant embryos at the mitosis-15 and 16 stages (Supplementary Figure 5 and data not shown). In the absence of colchicine, the number of PH3-positive cells was not detectably elevated in *Spc105*¹ compared with sibling control embryos in both mitosis 15 and 16. These latter observations were not entirely consistent with the delayed mitoses observed in mutant embryos using time-lapse analyses at the mitosis-16 stage. However, class II behaviour was observed in a minority of cells ($\sim 20\%$). We assume therefore that more extensive quantitative analyses of fixed embryos would have shown the expected enrichment of PH3-positive cells in untreated *Spc105*¹ compared with sibling control embryos. However, the results obtained using colchicine-treated embryos clearly show that *Spc105*¹ embryos still have SAC function at the mitosis-16 stage. The fact that at

this stage residual amounts of EGFP-BubR1 (kinetochore signal intensity during prometaphase 68% of that in sibling control embryos; Figure 3A and Supplementary Figure 6) are detectable on kinetochores in *Spc105*¹ mutant embryos corroborates this conclusion.

In principle, the chromosome congression and segregation defects observed in *Spc105*¹ mutants could reflect a direct Spc105 involvement in microtubule binding (Cheeseman *et al*, 2006) or an indirect effect on other kinetochore components. Therefore, we analysed the role of Spc105 in kinetochore assembly. Our findings using mutant embryos confirmed and extended previous observations obtained using RNA interference (RNAi) in cultured *Drosophila* cells (Przewlaka *et al*, 2007). The mitotic localization of EGFP-tagged versions of the kinetochore components Mis12, Nsl1, Spc25, Nuf2 and Ndc80 was found to depend on Spc105. In contrast, EGFP-tagged Cenp-C could still be recruited to kinetochores in the absence of Spc105. Our results (Figure 3A and Supplementary Figure 6), which were observed in a highly reproducible manner in all embryos of a given genotype in multiple independently fixed embryo collections, agree with those of the RNAi analysis (Przewlaka *et al*, 2007) in case of Cenp-C and the Ndc80 complex components (Spc25, Nuf2 and Ndc80). They are at variance in case of Mis12 complex components, as both Mis12-EGFP and EGFP-Nsl1 failed to localize normally to the kinetochore during mitosis 16 in *Spc105*¹ mutant embryos (Figure 3A and Supplementary Figure 6). We also analysed the dependence of Spc105 kinetochore localization on various centromere and kinetochore components. Spc105 localization was clearly affected in *Cenp-A/cid*, *Cenp-C* and *Mis12* mutants (Figure 3B and data not shown). In summary, our findings in *Drosophila* largely agree with earlier analyses in *C. elegans* (Cheeseman *et al*, 2004) and human cells (Kiyomitsu *et al*, 2007; Cheeseman *et al*, 2008), although the relationship between Spc105 and the Ndc80 and Mis12 complexes might be different in human cells. In *Drosophila* embryos, Spc105 is clearly required for normal recruitment of the Mis12 and Ndc80 complexes and therefore crucial for kinetochore assembly.

Spatial organization of Spc105 within the kinetochore

The importance of Spc105 for kinetochore organization, as well as its size and overall organization are comparable to Cenp-C. Both proteins are comparatively large kinetochore components (> 150 kDa) with more strongly conserved N- and C-terminal domains connected with a rapidly evolving linker region (Supplementary Figure 2; Heeger *et al*, 2005). Cenp-C was recently shown to have an extended and polar orientation within the kinetochore with its N-terminal domain oriented towards the spindle pole and its C-terminal domain towards the inner centromere (Figure 4B; Schittenhelm *et al*, 2007). Cenp-C might therefore function as a linker protein connecting the inner centromere chromatin with microtubule binding complexes in the outer region of the kinetochore. To evaluate the possibility that Spc105 might also act as a linker protein, potentially in close association with Cenp-C, we analysed its spatial organization within the kinetochore using our previously described approach (Schittenhelm *et al*, 2007).

By imaging native chromosomes released from mitotic syncytial embryos expressing EGFP-Spc105 and red fluores-

Figure 3 Requirements for kinetochore localization of Spc105 and other kinetochore proteins. **(A)** Localization of EGFP fusions of Cenp-C, Mis12, Nsl1, Spc25, Ndc80, Nuf2 and BubR1 in *Spc105*⁺ (*Spc105*⁻) and *Spc105*⁺ sibling control embryos (*Spc105*⁺) during mitosis 16. Representative anaphase figures are shown (except for prometaphases in case of BubR1) with kinetochore proteins in green and DNA staining in red. Bar = 3 μm. **(B)** Localization of Spc105-EGFP (shown in green) in *Cenp-C*^{prl41} (*Cenp-C*⁻) and *Mis12*^{f03756} (*Mis12*⁻) mutant embryos in comparison with sibling control embryos (*Cenp-C*⁺ and *Mis12*⁺, respectively). Representative anaphases with DNA staining in red are shown at the stage in which mitotic abnormalities start in the mutant embryos, that is during mitosis 16 in *Cenp-C* mutants and during the later mitotic divisions in the CNS in *Mis12* mutants. Bar = 3 μm.

cent Cenp-A/Cid, we obtained an average separation of about 41.5 nm between the fluorescent tags (Figure 4A). This places the N-terminus of Spc105 in the region in which Mis12 and Spc25 are located according to our earlier measurements (Schittenhelm *et al*, 2007; Figure 4B). Measurements using Spc105-EGFP in combination with mRFP-Cenp-C showed a separation by 5 nm and placed the C-terminus of Spc105 close to its N-terminus (Figure 4B). An evaluation of EGFP-Spc105-mRFP indicated a wider separation of the N- and C-termini by 9 nm (Figure 4B). In summary, our analyses showed a smaller distance between the N- and C-termini of Spc105 than in the previously analysed Cenp-C (Schittenhelm *et al*, 2007), indicating that Spc105 does not have a similarly extended orientation within the kinetochore as Cenp-C.

As our Y2H data suggested a direct interaction between Spc105 and Nsl1, we also integrated the position of Nsl1 into our kinetochore map by measuring the average separation from the C-terminus of Cenp-C (Figure 4B). Consistent with a direct interaction, this placed Nsl1 at the same place as the

C-terminus of Spc105, and close to Mis12 and Spc25. As an additional control for the accuracy of our intrakinetochore mapping method, we measured the average separation between the N-terminal ends of the microtubule-binding proteins Ndc80 and Nuf2, which reside on the spindle proximal end of the Ndc80 complex. As expected from the known X-ray structure of the Ndc80 complex (Ciferri *et al*, 2008; Wilson-Kubalek *et al*, 2008), these N-termini were found to be close neighbours (Figure 4B).

The repetitive linker region of Spc105 is dispensable for protein function

To characterize the functional significance of the comparatively large size of Spc105 in further detail, we shortened its middle linker region. As in other Spc105 homologues, this central region is also repetitive in *D. melanogaster*. However, the motif which is repeated 16 times in *DmSpc105* forms a consensus that is different from the M[E/D][L/I][S/T] consensus observed in yeast, *C. elegans* and human homologues

Figure 4 High-resolution localization of Spc105 within the kinetochore. (A) The localization of Spc105 was compared with that of Cenp-A/Cid. Native chromosomes released from syncytial embryos co-expressing EGFP-Spc105 (*EGFP-Spc105*) and a red fluorescent Cenp-A/Cid (*Cenp-A-mRFP*) were labelled with a DNA stain (*DNA*). The green and red signal intensities were quantified along the inter-kinetochore axis (white line). The spatial separation between the red and green fluorescent proteins was determined as described by Schittenhelm *et al* (2007) by averaging data obtained from 100 chromosomes. Bar = 100 nm. (B) The average spatial separation observed in experiments with Spc105 and other kinetochore proteins is indicated in the context of a kinetochore map. The position of Cenp-A/Cid, N- and C-terminus of Cenp-C, Spc25, Mis12 and Nuf2 along the inner-outer kinetochore axis is given in nm as determined previously (Schittenhelm *et al*, 2007). Double arrows and numbers below indicate the spacing (nm) observed in additional experiments using Spc105, Nsl1 and Ndc80.

Figure 5 The rapidly evolving repeat region of Spc105 does not provide essential functions. (A) The repetitive middle region of Spc105 evolves rapidly by repeat loss and expansions. The repeat motifs in the middle region of *DmSpc105* (red boxes) and *DwSpc105* (blue boxes) are illustrated. Most of the *D. willistoni* (*Dw*) repeats seem to have arisen recently from an expansion of a period composed of four repeats. These 5.5 periods are indicated on the line below the blue boxes. Reflecting their recent expansion, the *D. willistoni* repeats are more similar to each other than the *D. melanogaster* (*Dm*) repeats, as illustrated by the logo representation (Crooks *et al*, 2004) of the repeat consensus sequences. The numbers (amino-acid positions) and dashed lines illustrate the repeat domain swap constructs (also see panel B). (B) For a functional characterization of the repetitive middle region, the transgenes *gSpc105^{wr30}* (*wr30*) *gSpc105^{wr18}* (*wr18*) and *gSpc105^{wr1}* (*wr1*) expressing *DmSpc105* with a variable number of *D. willistoni* repeats instead the *D. melanogaster* repeats were generated. These transgenes provide all essential *Spc105* functions in *D. melanogaster* according to complementation tests.

(Figure 5A and Supplementary Figure 2). The recent sequencing of 12 Drosophilid genomes (Clark *et al*, 2007) allowed evolutionary comparisons of *D. melanogaster* Spc105 with homologues in more closely related species. Interestingly, these comparisons showed that the repeats in the linker

region of *Drosophila willistoni* Spc105 are far less diverged than those in *D. melanogaster* and other Drosophilids at the amino acid (Figure 5A) and DNA (data not shown) sequence levels. Most of the 31 *D. willistoni* repeats are present in a tandem array with 5.5 copies of a higher order period each

composed of four repeats (Figure 5A). Thus, the *D. willistoni* repeats seem to have resulted from an evolutionarily recent repeat expansion possibly at the expense of older repeats. Apart from this recent repeat homogenization in the *D. willistoni* lineage, we detected an *Spc105* gene duplication in the lineage leading to the *obscura* group. The most extensive differences between the Spc105 duplicates in both *Drosophila pseudoobscura* and *Drosophila persimilis* represent repeat number changes in the linker region (Supplementary Figure 2). These observations show that the repetitive Spc105 linker region can evolve very rapidly.

To address the functional significance of the repetitive Spc105 linker region, we generated transgenes expressing *DmSpc105* with an altered central domain (Figure 5B). In a first transgene (*gSpc105^{wr30}*), the *D. willistoni* repeat region was used to replace the *D. melanogaster* repeat region. Therefore, the product of this transgene has more (30 instead of 16) and different repeats than *DmSpc105*. In a second transgene (*gSpc105^{wr18}*), the repeat region encompassed only 18 of the *D. willistoni* repeats. Finally, we deleted all but one of the *D. melanogaster* repeats in a third transgene (*gSpc105^{wr1}*). Strikingly, complementation tests showed that all three transgenes promoted the development of *Spc105¹* and *Spc105²* hemizygotes to fertile adults. Moreover, the *gSpc105^{wr1}* transgene almost completely prevented the abnormal anaphase/telophase figures at mitosis-16 stage in *Spc105¹/Spc105²* embryos (12% abnormal anaphases/telophases in *gSpc105^{wr1}*; *Spc105¹/Spc105²* embryos, *n* = 310 versus 5% abnormal anaphases/telophases in *gSpc105*; *Spc105¹/Spc105²* embryos, *n* = 118; compare with Figure 2A). Moreover, all the analysed kinetochore components (EGFP–Ndc80, EGFP–Nuf2, Spc25–EGFP, Mis12–EGFP and EGFP–BubR1) were found to have a normal localization in *Spc105* mutant embryos rescued by *gSpc105^{wr1}* (Supplementary Figure 6). We also failed to detect any defects during the progression through the syncytial cell cycles in embryos collected from *Spc105* mutant females rescued by *gSpc105^{wr1}* or *gSpc105^{wr30}*, and SAC was observed to be fully functional in these embryos (Supplementary Figure 7). We therefore conclude that the repeat region does not provide essential Spc105 functions.

The C-terminal region of Spc105 partially rescues the Spc105 mutant phenotype

As neither length nor exact sequence of the repeat region was found to be of functional importance, we analysed whether the N- or C-terminal regions of Spc105 can provide function when expressed individually. Therefore, we generated transgenic lines allowing expression of either the N- or C-terminal or the middle repetitive linker region fused to EGFP (Figure 6A). An expression of these constructs in embryos (Figure 6A) and S2R+ cells (data not shown) indicated that the C-terminal region is sufficient for kinetochore localization, as also described for human and fission yeast Spc105/Spc7/Blinkin (Kerres *et al*, 2004; Kiyomitsu *et al*, 2007). In contrast, the N-terminal and the linker domain were not detected on mitotic kinetochores (Figure 6A), whereas weak nuclear signals during interphase clearly confirmed their expression (data not shown).

To address whether isolated Spc105 subdomains can rescue the mitotic defects observed in *Spc105¹* mutants, we fixed embryos expressing different UAS transgenes at the mitosis-

16 stage and analysed the appearance of mitotic figures (Figure 6B and C). Although full-length Spc105–EGFP completely rescued the mitotic defects in *Spc105¹* mutants as expected, neither the N-terminal domain nor the linker region of Spc105 was able to prevent the mitotic defects (Figure 6B and C). Interestingly, however, mitotic defects were almost completely eliminated by the expression of the C-terminal region of Spc105 (Figure 6B and C). Moreover, all the analysed kinetochore proteins (EGFP–Ndc80, EGFP–Nuf2, Spc25–EGFP and Mis12–EGFP) were observed to be present at the kinetochore although slightly diminished in *Spc105¹* mutant embryos carrying *gSpc105(C)*, a transgene driving the expression of the C-terminal region of Spc105 under control of the normal *Spc105* cis-regulatory region (Supplementary Figure 6).

The N-terminal region of human Blinkin/Spc105 is required for kinetochore localization of Bub1 and BubR1, as well as for SAC function (Kiyomitsu *et al*, 2007). However, after incubation with the spindle inhibitor colchicine, *gSpc105(C)*; *Spc105¹* embryos showed an increase in mitotic cells comparable to that of control embryos and in contrast to *mad2* mutant embryos, which lack SAC function (Figure 6D). Moreover, EGFP–BubR1 was observed to be present at prometaphase kinetochores in *gSpc105(C)*; *Spc105¹* embryos at almost normal levels (85% of level in *Spc105⁺* sibling control embryos; Supplementary Figure 6).

As the extent of rescue observed in *Spc105¹* mutants expressing Spc105(C) was extensive, we determined whether *gSpc105(C)* is able to rescue all of the essential *Spc105* functions. However, *gSpc105(C)* transgenes were not able to rescue the recessive lethality associated with the *Spc105¹* mutations, in contrast to *gSpc105* or *gSpc105^{wr1}*. Moreover, after prolonged strong overexpression of UAS transgenes during wing or eye development, the C-terminal, but not the full-length Spc105 or the other domains (N-terminal and linker region), interfered with the normal development (Supplementary Figure 8). These observations indicate that the C-terminal region is not functionally equivalent to wild-type Spc105.

Discussion

Spc105 homologues have been shown to be important kinetochore components in budding and fission yeast, *C. elegans* and human cells. They are considerably larger than most other kinetochore proteins. An increasing number of interaction partners have been reported to associate with Spc105 homologues. Spc105 is therefore an attractive candidate scaffold or linker protein, promoting the assembly of additional kinetochore components and/or connecting between inner and outer kinetochore proteins or between outer kinetochore and kMTs. Our high-resolution mapping of Spc105 within the *Drosophila* kinetochore, in combination with our genetic data, provides new insights into the actual function of this kinetochore component.

In general, most kinetochore protein sequences evolve very rapidly, in particular in the *Drosophila* lineage (Meraldi *et al*, 2006). Sequence-based searches have therefore failed to identify the *Drosophila* Spc105 homologue. However, both proteomics (Przewlaka *et al*, 2007) as well as our genetic screen for potential regulators of Separase function have led to the identification of an Spc105 candidate

protein which we have shown here to be localized at the kinetochore. Sequence comparisons, including those of the recently published 12 *Drosophilid* genomes show a particu-

larly fast sequence evolution within the central repetitive part of Spc105, thereby dividing the protein into three parts, the N, M and C regions.

The N-terminal region of the human Spc105 homologue Blinkin/AF15q14 has recently been shown to bind the protein kinases Bub1 and BubR1 (Kiyomitsu *et al*, 2007), which are important for SAC and for kinetochore attachment to KMTs. Moreover, Blinkin has been shown to be required for the recruitment of Bub1 and BubR1 to the kinetochore and for SAC (Kiyomitsu *et al*, 2007). In *Drosophila*, we also detect an interaction between the N-terminal regions of Spc105 and Bub1 with Y2H assays. However, we have not observed an interaction with BubR1. Moreover, EGFP-BubR1 signals at kinetochores, in *Spc105* mutants at a stage at which Spc105 levels are clearly below 5% of the normal wild-type level, are only reduced by about 30%. Thus, *Drosophila* Spc105 might not be the only binding partner of *Drosophila* BubR1 at the kinetochore. The fact that *Drosophila* Bub1 also interacts with the Mis12 complex component Nsl1, according to our Y2H data, might hint at alternative pathways for Bub1 and BubR1 recruitment to the kinetochore. Consistent with the significant levels of EGFP-BubR1 remaining at kinetochores in *Spc105* mutants with or without the expression of the C-terminal Spc105 region, we find that SAC seems to be functional at the mitosis-16 stage.

The repetitive middle region of Spc105 evolves most rapidly. The significant size difference among eukaryotic Spc105 homologues (from 917 aa in *Saccharomyces cerevisiae* to 2342 aa in humans) largely reflects variability within the repetitive middle region. Our sequence comparisons have shown a concerted evolution of the repeat region of *Drosophilid* *Spc105* genes in a minisatellite-like manner. Repeat expansion and contractions seem to have caused rapid changes in repeat numbers. Moreover, occasional repeat homogenizations might explain why a repeat consensus different from that of other eukaryotes has been maintained in the *Drosophilid* lineage, even though this repeat region does not seem to be functionally important. Our study clearly shows that the repeat region, which amounts to 44% of the full-length *D. melanogaster* protein, does not provide essential functions. The *gSpc105^{wr1}* transgene, which lacks all but one of the repeats, complements the recessive embryonic lethality of *Spc105* null mutations.

We point out, however, that a slightly elevated number of abnormal mitotic figures were apparent in embryos that depend on the *gSpc105^{wr1}* transgene. In addition, the repeat

consensus in *Drosophila* contains several acidic as well as basic residues followed by a threonine, which seems to be phosphorylated *in vivo* (Bodenmiller *et al*, 2007), possibly by Aurora B, as it is within a corresponding kinase consensus site (Ohashi *et al*, 2006). Thus, it is tempting to speculate that the middle region could contribute to regulated electrostatic interactions with spindle microtubules, similar to the N-terminal tails of Ndc80 (Cheeseman *et al*, 2006; DeLuca *et al*, 2006; Guimaraes *et al*, 2008; Miller *et al*, 2008). As the *Drosophila* Spc105 repeat consensus seems to be different from that of other eukaryotes, the possibility of a distinct functional significance of the repeat region in *Drosophila* should be kept in mind.

The C-terminal region of Spc105 interacts with Mis12 complex components according to Y2H data from both human (Kiyomitsu *et al*, 2007) and *Drosophila* proteins (this study). Although heterodimerization of the Mis12 complex components Nsl1/hMis14 and Dsn1/hMis13 seems to be required for an association with Spc105-C in humans, *Drosophila* Nsl1 is alone capable of interacting with Spc105-C. This apparent discrepancy might reflect the possibility that *Dsn1* might have been lost during the evolution of *Drosophila*, in which extensive proteomic analyses have failed to show the corresponding homologue so far (Przewlaka *et al*, 2007; Schittenhelm *et al*, 2007). The suggestion that Nsl1 might have taken over the Dsn1 functions is also consistent with our additional Y2H data regarding interactions between Mis12 complex components. *Drosophila* Nsl1 can readily interact with the other components, Mis12 and Nnf1a, whereas an association of human Nsl1 with Mis12 and Nnf1 depends on Dsn1 (Kiyomitsu *et al*, 2007). Thus, although the S/KMN network, which includes Spc105/KNL-1, Mis12 and Ndc80 complexes, seems to be present in all eukaryotes, some of the molecular details of the interaction network seem to have evolved divergently in different eukaryotic lineages. The differences in Ndc80 complex organization further support this conclusion. Although the Ndc80 complexes of both humans and budding yeast are anchored at the kinetochore by an intimate Spc24/Spc25 heterodimer (DeLuca *et al*, 2006; Wei *et al*, 2006; Ciferri *et al*, 2008), *C. elegans* and *D. melanogaster* do not seem to express a canonical Spc24 homologue (Cheeseman *et al*, 2006; Przewlaka *et al*, 2007; Schittenhelm *et al*, 2007).

Figure 6 The C-terminal domain of Spc105 partially rescues the *Spc105* mutant phenotypes. (A) For an analysis of the different Spc105 domains, UAS transgenes, expressing either the N-terminal (N), middle (M) or the C-terminal (C) region fused to EGFP (green boxes), were generated with the indicated fragments. Numbers indicate amino-acid positions. The subcellular localization of these domains was analysed after zygotic expression of paternally inherited UAS transgenes driven by maternal $\alpha 4tub$ -GAL4-VP16. Embryos at the stage of mitosis 16 were double labelled with an antibody against Cenp-A/Cid (*Cenp-A*) and a DNA stain (DNA). Representative metaphase figures illustrate that the C-terminal domain localizes to the kinetochore, as full-length Spc105 (FL), whereas the middle and the N-terminal domain do not show kinetochore localization. Bar = 4 μ m. (B) *Spc105¹* embryos expressing the UAS transgenes (see panel A) were fixed at the stage of mitosis 16 and double labelled with an antibody against Cenp-A/Cid (*Cenp-A*) and a DNA stain (DNA). *Spc105¹* embryos without a UAS transgene served as controls (–). Expression of UAS-*Spc105(N)*-EGFP and UAS-EGFP-*Spc105(M)* in *Spc105¹* embryos does not prevent the mitotic defects during mitosis 16. Centromeres are rarely clustered in anaphase and lagging chromosomes are frequent (dashed circles). In contrast, in *Spc105¹* embryos expressing UAS-*Spc105(FL)*-EGFP or UAS-EGFP-*Spc105(C)*, centromeres form a tight cluster during anaphase on the poleward side of the chromosomes (arrows). Also see (C). Bar = 6 μ m. (C) The bar diagram indicates the percentage of abnormal anaphase/telophase figures in *Spc105¹* mutant embryos at the stage of mitosis 16 after $\alpha 4tub$ -GAL4-VP16-mediated expression of Spc105(FL)-EGFP (FL), Spc105(N)-EGFP (N), EGFP-Spc105(M) (M) or EGFP-Spc105(C) (C). *Spc105¹* embryos without a UAS transgene served as control (–). (D) To evaluate SAC function, 10–12 h embryos were incubated for 45 min in medium without (– colchicine) or with colchicine (+ colchicine) before fixation and labelling with a DNA stain (DNA) and antibodies against phospho-histone H3 (PH3) and against β -galactosidase for genotype identification (data not shown). High magnification views of CNS regions are shown from *Spc105⁺* sibling embryos (*Spc105⁺*), from *Spc105¹* mutant embryos with transgenes driving the expression of either full-length (*Spc105¹* + FL) or the C-terminal region of Spc105 (*Spc105¹* + C), as well as of *mad2* mutant embryos (*mad2^P*). Bar = 20 μ m. (E) Bar diagram represents quantification of the number of PH3-positive cells in the CNS of embryos treated as described and illustrated in (D). The average density of PH3 cells in mock-treated *Spc105⁺* sibling embryos was set to 1. Ten embryos were analysed for each of the genotypes indicated below the bars.

The variant S/KMN network details might also contribute to a striking difference in kinetochore localization dependencies. Although *Drosophila* Spc105 is clearly required for Ndc80 complex localization at the kinetochore (Przewloka *et al*, 2007; this study), the human and fission yeast Spc105 homologues are not (Kerres *et al*, 2007; Kiyomitsu *et al*, 2007; Cheeseman *et al*, 2008). The relationship between Spc105 and the Ndc80 complex in *Drosophila* is therefore the same as in *C. elegans* (Cheeseman *et al*, 2004). Interestingly, both these organisms seem to have lost the constitutive centromere-associated network (CCAN), which is present in fission yeast and humans. The CCAN has recently been shown to contribute to the Spc105-independent kinetochore localization of the human Ndc80 complex (Cheeseman *et al*, 2008). Apart from the variable interaction with the Ndc80 complex and BubR1, all other kinetochore localization dependencies observed for *Drosophila* Spc105 (Przewloka *et al*, 2007; this study) seem to correspond to those reported from yeast, *C. elegans* and humans. The localization of *Drosophila* Spc105 depends on Cenp-A/Cid and Cenp-C, but not vice versa. In contrast, the localization of Spc105 and the Mis12 complex seems to be inter-dependent, at least in embryos. Finally, Spc105 localization does not depend on the Ndc80 complex.

The C-terminal region of Spc105, which anchors this protein in the S/KMN complex, seems to provide the most important Spc105 functions in *Drosophila*. The expression of Spc105-C largely restores progression through mitosis 16 in *Spc105* mutant embryos. This observation might suggest that not only the Spc105-M but also the Spc105-N region might not be crucial. However, we emphasize that Spc105-C cannot support the development of *Spc105* mutants to the adult stage. Moreover, Spc105-C fails to restore kinetochore levels of EGFP-BubR1, Mis12 and Ndc80 complex components completely during mitosis 16, when low residual levels of maternally provided Spc105 might contribute to the extent of mitosis 16 rescue in *Spc105* mutants. In addition, prolonged Spc105-C expression during wing or eye development in an *Spc105*⁺ background results in defects that might reflect a dominant-negative effect. The N-terminal region therefore clearly provides important Spc105 functions.

Our finding that the Spc105-M region is not essential shows that the comparatively large size of *Drosophila* wild-type Spc105 is not required, for instance, to link kinetochore layers or kinetochore and kMTs. Our high-resolution mapping of Spc105 within the kinetochore argues in the same direction. In contrast to Cenp-C, a protein of comparable size with an extended polar orientation bridging between inner and outer kinetochore regions (Schittenhelm *et al*, 2007), Spc105 does not show a similarly extended layout along the inter-sister kinetochore axis. However, consistent with the known molecular interactions, it is close to the Mis12 complex components. Moreover, it is also close to the N-terminal region of Cenp-C and the Spc25 end of the Ndc80 complex, consistent with co-immunoprecipitation data (Westermann *et al*, 2003; Cheeseman *et al*, 2004; Przewloka *et al*, 2007; Schittenhelm *et al*, 2007). We emphasize that our kinetochore mapping data have been obtained using released native chromosomes that are not attached to the spindle. Our data thus leave open the possibility that the orientation of Spc105 might change on attachment of the kinetochore to kMTs. During revision of our study, high-resolution data regarding the intrakinetochore localization of Spc105 in yeast and human cells have

been reported (Joglekar *et al*, 2009; Wan *et al*, 2009). These studies have analysed kinetochore organization along the spindle axis in kinetochores that are attached to the spindle. A comparison with these studies suggests that the N-terminal region of Spc105 is localized further out towards the kMTs after attachment, whereas the C-terminal region remains close to the Mis12 complex and the Spc25 end of the Ndc80 complex.

In summary, rather than serving as an extended connecting linker or rigid scaffold protein, Spc105 is more likely to function as a platform which contributes to the recruitment of various kinetochore components, including SAC proteins without enforcing exact and rigid geometries between all the recruited entities.

Materials and methods

Fly genetics

The *Spc105*¹ allele was identified with the genetic modifier screen strategy described previously (Heeger *et al*, 2005) and the Exelixis deficiency set (Parks *et al*, 2004) (see Supplementary data for a detailed description of the screen). The *Df(3R)Exel9014* chromosome resulted in the strongest dominant enhancement of the adult rough eye phenotype caused by eye-specific Separase inhibition. Meiotic recombination resulted in a separation of the enhancer locus from the deficiency. The enhancer locus was mapped using meiotic recombination and complementation tests with deficiencies (see Supplementary data). The allele *Spc105*² (kindly provided by L Cooley, Yale University School of Medicine, New Haven, CT) was isolated using a GFP trap screen as line *P01511LE* (Quinones-Coello *et al*, 2007). It contains a recombinant P element with a GFP exon (PTT; Morin *et al*, 2001) in the first *Spc105* intron. The expressed GFP fluorescence is not localized to the kinetochore, indicating that the encoded product is not a functional Spc105 variant.

The mutations *mad2*^P, *Mis12*^{f03756}, *Cenp-C*^{prl41}, *cid*^{T12-1} and *cid*^{T22-4} have been described before (Heeger *et al*, 2005; Blower *et al*, 2006; Buffin *et al*, 2007; Schittenhelm *et al*, 2007), as well as the *GAL4* driver lines *ey-GAL4* (Hazelett *et al*, 1998), *MS1096* (Capdevila and Guerrero, 1994), *prd-GAL4* (Brand and Perrimon, 1993) and *α4tub-GAL4-VP16* (Hacker and Perrimon, 1998). In our experiments using *α4tub-GAL4-VP16*, maternally derived *GAL4-VP16* was used to drive zygotic expression from paternally inherited *UAS* transgenes, which starts during cellularization. The following transgenic lines have previously been described: *gHis2AvD-mRFP1* and *gcid-EGFP-cid* (Schuh *et al*, 2007); *gcid-mRFP1-cid*, *gCenp-C-EGFP*, *gEGFP-Ndc80*, *gEGFP-Nuf2*, *gSpc25-EGFP* and *gMis12-EGFP* (Schittenhelm *et al*, 2007); *gEYFP-Cenp-C* (Heeger *et al*, 2005) and *GFP-BubR1* (Buffin *et al*, 2005). Lines with the transgenes *gSpc105*, *gSpc105(C)*, *gEGFP-Spc105*, *gSpc105-EGFP*, *gEGFP-Spc105-mRFP1*, *gSpc105^{wr1}*, *gSpc105^{wr15}*, *gSpc105^{wr30}*, *UAS-Spc105-EGFP*, *UAS-Spc105(N)-EGFP*, *UAS-EGFP-Spc105(M)*, *UAS-EGFP-Spc105(C)*, *giEGFP-Cenp-C* and *gEGFP-Nsl1* were generated using standard P element-mediated germ line transformation. In contrast to *gSpc105* and *gSpc105-EGFP*, the *gEGFP-Spc105* and *gEGFP-Spc105-mRFP1* transgenes were not capable of rescuing *Spc105* mutants, although the products localized to kinetochores just like Spc105-EGFP. Lines expressing transgene combinations or transgenes in mutant backgrounds were established using standard crosses and/or meiotic recombination.

Plasmids, transfections and yeast two hybrid assays

Genomic DNA of *D. willistoni* was obtained from the *Drosophila* species stock center (University of Arizona, Tucson, USA). *pp{CaSpeR-4}* (Thummel and Pirrotta, 1991) was used for transgene constructs allowing the expression of fluorescently tagged or untagged kinetochore proteins under the control of their normal genomic regulatory region. *pPUAST* (Brand and Perrimon, 1993) was used for transgene constructs for *GAL4*-regulated expression. Moreover, these constructs were also used for transfection of S2R+ cells after which basal expression was analysed. The transfection of S2R+ cells was carried out using the FuGeneHD Transfection Reagent (Roche) essentially as described previously (Schittenhelm

et al, 2007). Protein–protein interactions were analysed using the Matchmaker Two-Hybrid system and the yeast strain AH109 (Clontech), after transformation with *pGADT7* and *pGBKT7* constructs essentially as described before (Jäger *et al*, 2004). A detailed description of all plasmids is provided in the Supplementary data.

In vivo imaging and immunofluorescence

Embryos collected from *gHis2AvD-mRFP1 II.2*, *gcid-EGFP-cid II.1*; *Spc105¹/TM3*, *Sb*, *twi-GAL4*, *UAS-GFP* parents were analysed by *in vivo* imaging essentially as described previously (Pandey *et al*, 2005). *Spc105* mutant embryos were identified because of the absence of *twi-GAL4*-directed *UAS-GFP* expression. As controls, we analysed *gHis2AvD-mRFP1 II.2*, *gcid-EGFP-cid II.1* embryos analogously.

Immunofluorescence and DNA labelling of fixed embryos and S2R+ cells was conducted as described before (Pandey *et al*, 2005; Schittenhelm *et al*, 2007). For SAC assays, embryo collections were split into two aliquots after dechorionization and incubated for either 20 min (during pre-cellularization stages) or 45 min (during post-cellularization stages) in a 1:1 mixture of octane and Schneider's tissue culture medium with or without demecolcine (10 µM, Sigma) before fixation. We used rabbit anti-Cenp-A/Cid (Jäger *et al*, 2005) at 1:1000, rabbit anti-Cenp-C (Heeger *et al*, 2005) at 1:5000, rabbit anti-phospho-histone H3 (Upstate) at 1:800 and mouse anti-β-galactosidase (Promega) at 1:1000. Two affinity-purified rabbit antibodies against the bacterially expressed and His-tagged N-terminus (amino acids 1–471) of *Drosophila* Spc105 were generated and used at 1:3000 with S2R+ cells. Details about the quantification and presentation of data acquired from light microscopy are given in the Supplementary data.

Embryo extracts, immunoblotting and immunoprecipitation

For the analysis of Spc105 levels during embryogenesis, extracts from *w* mutant embryos of defined stages were obtained as described by Edgar *et al* (1994). For the preparation of *Spc105¹* embryo extracts, we collected embryos from *Spc105¹/TM3*, *Sb*, *Ubx-lacZ* flies. After fixation with methanol and immunofluorescent staining with mouse anti-β-galactosidase (Promega; 1:300) and

Hoechst 33258, we sorted embryos with lacZ expression from those without lacZ expression using an inverted fluorescence microscope. The pooled embryos were solubilized in SDS–PAGE sample buffer. Hybond-ECL membranes and ECL-detection (Amersham Biosciences) were used for immunoblotting. The affinity-purified rabbit antibodies against Spc105 were used at 1:9000 and mouse anti-α-tubulin (Neomarkers) at 1:50 000.

Preparation and analysis of native chromosomes

We prepared and analysed native mitotic chromosomes from syncytial embryos expressing fluorescently tagged kinetochore proteins essentially as described previously (Schittenhelm *et al*, 2007). For the embryo collections, we used the following *Drosophila* strains: (1) *gEGFP-Spc105 II.1*, *gcid-mRFP1-cid II.1* (2) *gSpc105-EGFP II.1*, *gmRFP-Cenp-C II.2* (3) *gEGFP-Spc105-mRFP1 II.1* (4) *gEGFP-Nsl1 II.1*, *gCenp-C-mRFP1 II.1* and (5) *gEGFP-Nuf2 II.1*, *gmRFP1-Ndc80 II.1*. The distribution of the obtained *d_RGintra* values (see Supplementary Figure 9) was found to be similar to those described previously (Schittenhelm *et al*, 2007).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

References

- Allshire RC, Karpen GH (2008) Epigenetic regulation of centromeric chromatin: old dogs, new tricks? *Nat Rev Genet* **9**: 923–937
- Bjorklund M, Taipale M, Varjosalo M, Saharinen J, Lahdenpera J, Taipale J (2006) Identification of pathways regulating cell size and cell-cycle progression by RNAi. *Nature* **439**: 1009–1013
- Blower MD, Daigle T, Kaufman T, Karpen GH (2006) *Drosophila* CENP-A mutations cause a BubR1-dependent early mitotic delay without normal localization of kinetochore components. *PLoS Genet* **2**: e110
- Bodenmiller B, Malmstrom J, Gerrits B, Campbell D, Lam H, Schmidt A, Rinner O, Mueller LN, Shannon PT, Pedrioli PG, Panse C, Lee HK, Schlapbach R, Aebersold R (2007) PhosphoPep—a phosphoproteome resource for systems biology research in *Drosophila* Kc167 cells. *Mol Syst Biol* **3**: 139
- Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**: 401–415
- Buffin E, Emre D, Karess RE (2007) Flies without a spindle checkpoint. *Nat Cell Biol* **9**: 565–572
- Buffin E, Lefebvre C, Huang J, Gagou ME, Karess RE (2005) Recruitment of Mad2 to the kinetochore requires the Rod/Zw10 complex. *Curr Biol* **15**: 856–861
- Capdevila J, Guerrero I (1994) Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO J* **13**: 4459–4468
- Cheeseman IM, Chappie JS, Wilson-Kubalek EM, Desai A (2006) The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell* **127**: 983–997
- Cheeseman IM, Desai A (2008) Molecular architecture of the kinetochore–microtubule interface. *Nat Rev Mol Cell Biol* **9**: 33–46
- Cheeseman IM, Hori T, Fukagawa T, Desai A (2008) KNL1 and the CENP-H/I/K complex coordinately direct kinetochore assembly in vertebrates. *Mol Biol Cell* **19**: 587–594
- Cheeseman IM, Niessen S, Anderson S, Hyndman F, Yates III JR, Oegema K, Desai A (2004) A conserved protein network controls assembly of the outer kinetochore and its ability to sustain tension. *Genes Dev* **18**: 2255–2268
- Ciferri C, De Luca J, Monzani S, Ferrari KJ, Ristic D, Wyman C, Stark H, Kilmartin J, Salmon ED, Musacchio A (2005) Architecture of the human ndc80-hec1 complex, a critical constituent of the outer kinetochore. *J Biol Chem* **280**: 29088–29095
- Ciferri C, Pasqualato S, Screpanti E, Varetto G, Santaguida S, Dos Reis G, Maiolica A, Polka J, De Luca JG, De Wulf P, Salek M, Rappsilber J, Moores CA, Salmon ED, Musacchio A (2008) Implications for kinetochore–microtubule attachment from the structure of an engineered Ndc80 complex. *Cell* **133**: 427–439
- Clark AG, Eisen MB, Smith DR, Bergman CM, Oliver B, Markow TA, Kaufman TC, Kellis M, Gelbart W, Iyer VN, Pollard DA, Sackton TB, Larracuente AM, Singh ND, Abad JP, Abt DN, Adryan B, Aguade M, Akashi H, Anderson WW *et al* (2007) Evolution of genes and genomes on the *Drosophila* phylogeny. *Nature* **450**: 203–218
- Crooks GE, Hon G, Chandonia JM, Brenner SE (2004) WebLogo: a sequence logo generator. *Genome Res* **14**: 1188–1190
- DeLuca JG, Gall WE, Ciferri C, Cimini D, Musacchio A, Salmon ED (2006) Kinetochore microtubule dynamics and attachment stability are regulated by hec1. *Cell* **127**: 969–982
- Desai A, Rybina S, Muller-Reichert T, Shevchenko A, Shevchenko A, Hyman A, Oegema K (2003) KNL-1 directs assembly of the microtubule-binding interface of the kinetochore in *C. elegans*. *Genes Dev* **17**: 2421–2435
- Dimova DK, Stevaux O, Frolov MV, Dyson NJ (2003) Cell cycle-dependent and cell cycle-independent control of transcription by the *Drosophila* E2F/RB pathway. *Genes Dev* **17**: 2308–2320

- Edgar BA, Sprenger F, Duronio RJ, Leopold P, O'Farrell PH (1994) Distinct molecular mechanisms regulate cell cycle timing at successive stages of *Drosophila* embryogenesis. *Genes Dev* **8**: 440–452
- Goshima G, Wollman R, Goodwin SS, Zhang N, Scholey JM, Vale RD, Stuurman N (2007) Genes required for mitotic spindle assembly in *Drosophila* S2 cells. *Science* **316**: 417–421
- Guimaraes GJ, Dong Y, McEwen BF, Deluca JG (2008) Kinetochore-microtubule attachment relies on the disordered N-terminal tail domain of hec1. *Curr Biol* **18**: 1778–1784
- Hacker U, Perrimon N (1998) DRhoGEF2 encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in *Drosophila*. *Genes Dev* **12**: 274–284
- Hazelett DJ, Bourouis M, Walldorf U, Treisman JE (1998) decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc. *Development* **125**: 3741–3751
- Heeger S, Leismann O, Schittenhelm R, Schraidt O, Heidmann S, Lehner CF (2005) Genetic interactions of Separase regulatory subunits reveal the diverged *Drosophila* Cenp-C homolog. *Genes Dev* **19**: 2041–2053
- Jäger H, Herzig B, Herzig A, Sticht H, Lehner CF, Heidmann S (2004) Structure predictions and interaction studies indicate homology of separase N-terminal regulatory domains and *Drosophila* THR. *Cell Cycle* **3**: 182–188
- Jäger H, Rauch M, Heidmann S (2005) The *Drosophila melanogaster* condensin subunit Cap-G interacts with the centromere-specific histone H3 variant CID. *Chromosoma* **113**: 350–361
- Joglekar AP, Bloom K, Salmon ED (2009) *In vivo* protein architecture of the eukaryotic kinetochore with nanometer scale accuracy. *Curr Biol* **19**: 694–699
- Kerres A, Jakopc V, Fleig U (2007) The conserved Spc7 protein is required for spindle integrity and links kinetochore complexes in fission yeast. *Mol Biol Cell* **18**: 2441–2454
- Kerres A, Vietmeier-Decker C, Ortiz J, Karig I, Beuter C, Hegemann J, Lechner J, Fleig U (2004) The fission yeast kinetochore component Spc7 associates with the EB1 family member Mal3 and is required for kinetochore-spindle association. *Mol Biol Cell* **15**: 5255–5267
- Kiyomitsu T, Obuse C, Yanagida M (2007) Human Blinkin/AF15q14 is required for chromosome alignment and the mitotic checkpoint through direct interaction with Bub1 and BubR1. *Dev Cell* **13**: 663–676
- McIntosh JR, Grishchuk EL, Mophew MK, Efremov AK, Zhudnikov K, Volkov VA, Cheeseman IM, Desai A, Mastronarde DN, Ataullakhanov FI (2008) Fibrils connect microtubule tips with kinetochores: a mechanism to couple tubulin dynamics to chromosome motion. *Cell* **135**: 322–333
- Meraldi P, McAnish AD, Rheinbay E, Sorger PK (2006) Phylogenetic and structural analysis of centromeric DNA and kinetochore proteins. *Genome Biol* **7**: R23
- Miller SA, Johnson ML, Stukenberg PT (2008) Kinetochore attachments require an interaction between unstructured tails on microtubules and Ndc80(Hec1). *Curr Biol* **18**: 1785–1791
- Morin X, Daneman R, Zavortink M, Chia W (2001) A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. *Proc Natl Acad Sci USA* **98**: 15050–15055
- Musacchio A, Salmon ED (2007) The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* **8**: 379–393
- Nekrasov VS, Smith MA, Peak-Chew S, Kilmartin JV (2003) Interactions between centromere complexes in *Saccharomyces cerevisiae*. *Mol Biol Cell* **14**: 4931–4946
- Obuse C, Iwasaki O, Kiyomitsu T, Goshima G, Toyoda Y, Yanagida M (2004) A conserved Mis12 centromere complex is linked to heterochromatic HP1 and outer kinetochore protein Zwint-1. *Nat Cell Biol* **6**: 1135–1141
- Ohashi S, Sakashita G, Ban R, Nagasawa M, Matsuzaki H, Murata Y, Taniguchi H, Shima H, Furukawa K, Urano T (2006) Phosphoregulation of human protein kinase Aurora-A: analysis using anti-phospho-Thr288 monoclonal antibodies. *Oncogene* **25**: 7691–7702
- Pandey R, Heidmann S, Lehner CF (2005) Epithelial re-organization and dynamics of progression through mitosis in *Drosophila* separase complex mutants. *J Cell Sci* **118**: 733–742
- Parks AL, Cook KR, Belvin M, Dompe NA, Fawcett R, Huppert K, Tan LR, Winter CG, Bogart KP, Deal JE, Deal-Herr ME, Grant D, Marcinko M, Miyazaki WY, Robertson S, Shaw KJ, Tabios M, Vysotskaia V, Zhao L, Andrade RS *et al* (2004) Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome. *Nat Genet* **36**: 288–292
- Przewloka MR, Zhang W, Costa P, Archambault V, D'Avino PP, Lilley KS, Laue ED, McAnish AD, Glover DM (2007) Molecular analysis of core kinetochore composition and assembly in *Drosophila melanogaster*. *PLoS ONE* **2**: e478
- Quinones-Coello AT, Petrella LN, Ayers K, Melillo A, Mazzalupo S, Hudson AM, Wang S, Castiblanco C, Buszczak M, Hoskins RA, Cooley L (2007) Exploring strategies for protein trapping in *Drosophila*. *Genetics* **175**: 1089–1104
- Schittenhelm RB, Heeger S, Althoff F, Walter A, Heidmann S, Mechtler K, Lehner CF (2007) Spatial organization of a ubiquitous eukaryotic kinetochore protein network in *Drosophila* chromosomes. *Chromosoma* **116**: 385–402
- Schuh M, Lehner CF, Heidmann S (2007) Incorporation of *Drosophila* CID/CENP-A and CENP-C into centromeres during early embryonic anaphase. *Curr Biol* **17**: 237–243
- Talbert PB, Bryson TD, Henikoff S (2004) Adaptive evolution of centromere proteins in plants and animals. *J Biol* **3**: 18
- Thummel CS, Pirrotta V (1991) Technical Notes: new pCaSpeR P-element vectors. *Drosoph Inf Ser* **71**: 150
- Wan X, O'Quinn RP, Pierce HL, Joglekar AP, Gall WE, DeLuca JG, Carroll CW, Liu ST, Yen TJ, McEwen BF, Stukenberg PT, Desai A, Salmon ED (2009) Protein architecture of the human kinetochore microtubule attachment site. *Cell* **137**: 672–684
- Wang HW, Long S, Ciferri C, Westermann S, Drubin D, Barnes G, Nogales E (2008) Architecture and flexibility of the yeast Ndc80 kinetochore complex. *J Mol Biol* **383**: 894–903
- Wei RR, Schnell JR, Larsen NA, Sorger PK, Chou JJ, Harrison SC (2006) Structure of a central component of the yeast kinetochore: the Spc24p/Spc25p globular domain. *Structure* **14**: 1003–1009
- Wei RR, Sorger PK, Harrison SC (2005) Molecular organization of the Ndc80 complex, an essential kinetochore component. *Proc Natl Acad Sci USA* **102**: 5363–5367
- Welburn JP, Cheeseman IM (2008) Toward a molecular structure of the eukaryotic kinetochore. *Dev Cell* **15**: 645–655
- Westermann S, Cheeseman IM, Anderson S, Yates III JR, Drubin DG, Barnes G (2003) Architecture of the budding yeast kinetochore reveals a conserved molecular core. *J Cell Biol* **163**: 215–222
- Westermann S, Drubin DG, Barnes G (2007) Structures and functions of yeast kinetochore complexes. *Annu Rev Biochem* **76**: 563–591
- Wilson-Kubalek EM, Cheeseman IM, Yoshioka C, Desai A, Milligan RA (2008) Orientation and structure of the Ndc80 complex on the microtubule lattice. *J Cell Biol* **182**: 1055–1061