

Self-assembled spindle formation relies on the kinetochores and the Ran-GTP gradient in fission yeast meiosis

Alberto Pineda-Santaella¹ and Alfonso Fernández-Álvarez¹

¹ Instituto de Biología Funcional y Genómica (IBFG). Consejo Superior de Investigaciones Científicas (CSIC). University of Salamanca (USAL). C/ Zacarías González, 2, 37007, Salamanca, Spain.

Introduction

Results

Generation of self-assembled spindles through alternative genotypes

The *bqt1Δ sad1.2* background has proven to be a very useful system for studying self-assembled spindle formation and behaviour, although it presents a margin of improvement in i) the residual formation of SPB-dependent spindles (from ~5% to 0%), ii) the incomplete rate of formation of self-assembled spindles (from ~70% to 100%) and iii) the requirement of mutations in 2 genes, in one of them involving thermosensitive and highly reversible point mutations (especially the T3S aminoacid change in *sad1.2* allele, from our experience).

These caveats prompt us to seek for genotypes alternative to *bqt1Δ sad1.2* that manage to more efficiently disrupt telomere/centromere contacts with Sad1 to prevent SPB-dependent spindle formation in favour of self-assembled spindle formation in a more penetrant manner. Also, these would need to involve a lower number mutations, less subject to reversions (or other type of suppression) and less vulnerable to environmental conditions (e.g. temperature fluctuations). With this purpose, we first explored within the nucleoplasmic portion of Sad1 protein, exposed to chromosomes, which specific regions are necessary to establish contacts with telomeres and centromeres by using a battery of truncated versions of *sad1* spanning aminoacids 10-80 in a 10-wise manner (10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80) together with data from region 1-10 from previous work in the lab (0).

Since centromere contacts are essential for eliciting SPB-mediated spindle formation in mitosis and thus to ensure the viability of the cell, we scanned for centromere contact regions by assessing spore viability after tetrad dissection of viable diploid strains containing one *wt* copy of *sad1* (*sad1⁺*)

and one copy of the respective truncated allele (*sad1ΔX-X*). In this manner, we identified aminoacids 10-30 as necessary for centromere contacts since dissections involving alleles *sad1Δ10-20* and *sad1Δ20-30* yield 2 very sick or inviable (*sad1ΔX-X*) and 2 viable (*sad1⁺*) haploid colonies per tetrad in 100% of the cases, in contrast with the rest of truncations, which yield 4 viable colonies per tetrad in 100% of the cases, as the control full-wildtype *sad1⁺* / *sad1⁺* dissection (Figure 1, A and B).

To scan for telomere contact regions, we monitored the rate of occurrence of such contacts by following a GFP-tagged Sad1 (Sad1-GFP) and a mCherry-tagged telomeric protein Taz1 (Taz1-mCherry) via fluorescent microscopy snapshots in meiocytes containing the viable *sad1* truncations (from 30-40 to 70-80). We classified the observed different modes of telomeres-Sad1 contacts into three categories: i) Normal, showing perfect co-localization of all telomeres (gathered at one single focus) and Sad1 focus; ii) Partial, where at least one telomere focus does not co-localize with Sad1 focus; iii) Abolished, where all telomere foci do not co-localize with Sad1 focus (Figure 1, C). According to these categories, we could identify aminoacids 30-50 as necessary for telomere-Sad1 contacts, since in genotypes *sad1Δ30-40* and *sad1Δ40-50* show predominantly abolished and partial contacts (quantification in Figure 1, D).

With this, we can determine that within the nucleoplasmic portion of Sad1, aminoacids 1-30 are essential for centromeric contacts (Figure 1, E, grey rectangle) and aminoacids 30-50 are essential for telomeric contacts (Figure 1, E, solid orange rectangle). However, since we could not assay telomeric contacts in non-viable truncated versions, comprising positions 1-30, due to impossibility of working with such strains, we cannot discard the existence of aminoacids necessary for telomeric contacts in such region (Figure 1, E, dashed orange rectangle).

At the light of these results, it can be derived that it may not be possible to obtain a *sad1* truncated version result of combination of centromeric and telomeric regions looking forward to affecting both centromeric and telomeric contacts

(as it happens in the *bqt1Δ sad1.2* setting), because of the high severity of viability loss when parts of the centromeric region are eliminated, rendering such hypothetical strain impossible to work with.

Despite of this outcome, we persisted in studying the effect of telomeric contact disruption produced by these truncations in the formation of the SPB-mediated and self-assembled spindles in a strain carrying a truncated version of *sad1* containing the whole identified telomeric region, *sad1Δ32–63*, with the *wt* and *bqt1Δ sad1.2* backgrounds as a reference. Strikingly, this *sad1Δ32–63* allele alone was sufficient to trigger the formation of self-assembled spindles in 44% of the meiocytes. Still, this rate of self-assembled spindle formation is lower than the average 76% rate in *bqt1Δ sad1.2* setting. Therefore, looking for the increase of self-assembled spindle formation rate in this new setting, we introduced mutations to further hamper telomeric or centromeric contacts.

On one hand, targeting telomeric contacts by introducing *bqt1* deletion into the *sad1Δ32–63* background did not increased but unexpectedly decreased the rate of self-assembled spindle formation, suggesting at least that disruption of telomeric contacts may not be aggravated by affecting structural telomeric proteins in such scenario. On the other hand, targeting both telomeric and centromeric contacts by deleting *csil*, a co-receptor of Sad1 that stabilises its interactions with telomeres and centromeres, increased the rate of self-assembled spindle formation up to 57%, much more similar to the rate in *bqt1Δ sad1.2* strain (quantifications in Figure 1, F).

Altogether, these results reveal that it is possible to elicit the formation of self-assembled spindles in fission yeast meiosis via mutations different from *bqt1Δ sad1.2* that likewise impair telomere/centromere contacts with Sad1 and with similar rates of self-assembled spindle formation.

Self-assembled spindle assembly is majorly independent of microtubule nucleation by γ-tubulin complex



In fission yeast mitosis and meiosis, after SPB insertion into the NE, the first step required for spindle formation is the nucleation, i.e., *de novo* formation, of the microtubules that will build up the body of the spindle inside the nucleoplasm. This microtubule nucleation is achieved by the activity of the γ-tubulin complex, a multiprotein complex composed of γ-tubulin and other subunits, that serve as a template for α/β-tubulin subunits polymerization into microtubule filaments.

To address whether this mode of microtubule nucleation driven by the γ-tubulin complex plays a role in the formation of the self-assembled spindle, we disrupted the activity

of this complex by depleting one of its essential components, Alp4 (conserved as GCP-2 in human), via the auxin-induced degron (AID) protein depletion system (see Methods).

Since the specific combination of AID components we chose had not been tested before, we first checked that our version of the AID system (AIDx) could achieve effective depletion of AID and GFP-tagged Alp4 (Alp4-AIDx-GFP). Thanks to the fact that the γ-tubulin complex activity is essential in vegetative (mitotically-growing) cells (for SPB-dependent spindle nucleation), we could readily assess such depletion via loss of growth in a spot assay (see Methods). Indeed, we could confirm lack of function of the γ-tubulin complex with the complete AIDx system (Alp4-AIDx-GFP and TIR1 receptor) and upon addition of the depletion activator, auxin, by a drastic loss of growth (Figure 2, YES + Auxin). On the contrary, growth was not affected in control backgrounds containing separate system components (Alp4-AIDx-GFP or TIR receptor) or in rich medium alone (Figure 2, YES) or with the carrier of the drug (Figure 2, YES + Ethanol). Therefore, depletion using our specific AIDx system is both effective and specific for the tagged protein.

Once we checked the functionality of our AID system, we caused the depletion of Alp4-AIDx-GFP in meiocytes in mid-late meiotic prophase by adding auxin, and immediately after, we monitored such depletion and its effect in spindle formation through fluorescence microscopy timelapse in SPB-dependent (*wt*) and self-assembled spindle (*bqt1Δ sad1.2*) contexts (Figure 3). Indeed, we confirmed Alp4-AIDx-GFP depletion in meiocytes during meiotic prophase for the *wt* (Figure 4, B, -75' to -45') and *bqt1Δ sad1.2* (Figure 4, D, -140' to -80') cases (quantification in Figure 5, A), while the Alp4-AIDx-GFP signal was maintained for controls (Figure 4, A and C). For *wt* meiocytes, disruption of the γ-tubulin complex resulted in an almost complete abolishment of SPB-mediated spindles (from 100% to 0%, 100% reduction), while in *bqt1Δ sad1.2* meiocytes, depletion caused an also significant although milder defect in self-assembled spindle formation, reducing the rate from 84% to 56% (37% reduction) (quantifications in Figure 5). Supplemental Figure 1 shows an example of a self-assembled spindle that persists to form after Alp4-AIDx-GFP depletion. Noteworthy, the residual formation of SPB-dependent spindles in the *bqt1Δ sad1.2* background (12%) is also abolished (to 0%) when the γ-tubulin complex is disrupted, resembling the abolishment observed in the *wt* background (quantifications in Figure 5).

Thus, these findings illustrate that SPB-dependent and self-assembled spindles present a differential degree of dependency on the microtubule nucleation driven by the γ-tubulin complex for its formation, suggesting that the latter may use alternative molecular mechanisms to assemble.

Self-assembled spindles formation relies on the kinetochores

In mammalian female meiosis, upon the absence of the major microtubule-organizing centre (MTOC) of the cell, the centrosome, oocytes opt for a diverse range of alternative mechanisms to nucleate and organize microtubules in order to assemble the meiotic acentrosomal spindle. One of the most studied alternative MTOC in oocytes are the kinetochores, described as points of microtubule nucleation and able to organize these newly synthesized microtubules to build up the body of the spindle.

In line with the idea that the self-assembled spindle occurring in meiosis in the *bqt1Δ sad1.2* fission yeast is at some extent equivalent to the acentrosomal spindle of mammalian female meiosis, we explored whether kinetochores contribute to the formation of the self-assembled spindle in the *bqt1Δ sad1.2* fission yeast system, as well as of the SPB-dependent spindle in a *wt* setting.

In fission yeast meiosis, the kinetochore undergoes a compositional and structural remodelling upon the activation of the meiotic program, transitioning from the mitotic version, which joins the two sister chromatids in each chromosome, to the meiotic version, which joins whole paired homologous chromosomes to ensure a correct reductional segregation in the first meiotic division. In detail, at the beginning of meiosis, components of the middle and outer kinetochore layers dismantle from centromeres and towards the end of meiotic prophase these reappear at centromeres and reassemble as the meiotic kinetochore, namely, the NMS (Ndc80-Mis12-Spc7) supercomplex and the DASH complex (in chronological order); contrarily, the inner kinetochore, the Mis6/Sim4 complex, remains assembled at the centromeres throughout meiosis.

According to this, to disrupt the kinetochore reassembly in meiosis, we targeted one component of the middle kinetochore, Mis12, for depletion by using the AIDx system as explained above, so as to prevent the reassembly of the outer layer, which builds upon the former, directly interacts with microtubules and presumably most likely harbours the microtubule-nucleating and organizing activities if it ressembles the mechanism from mammalian oocytes.

First, we confirmed the efficient and specific depletion of Mis12-AIDx-GFP in vegetative cells by spot assay (Figure 6) based on the fact that correct kinetochore assembly is essential for faithful chromosome segregation and thus for cell viability. After, we conveyed its depletion in mid prophase and afterwards assessed spindle formation. As described in the literature, in the *wt* control case, Mis12 is absent until it appears in late meiotic prophase before the onset of spindle formation as a single focus (Figure 7, -105' to -X') and later

as foci that segregate to spindle poles following the chromosomal masses along both meiotic divisions (Figure 7, A, 0' to 90' and quantification in Figure 8, A). Interestingly, depletion of Mis12-AIDx-GFP, noticed by the absence of Mis12-AIDx-GFP foci throughout the timelapse in the *wt* setting, does not affect SPB-dependent spindle formation in meiosis I (Figure 7, B, 0' to 40' and quantification in Figure 8, B), although it does abolish formation in meiosis II in all cases (Figure 7, B, 50' to 70'). Also, consistent with the defect in kinetochore reassembly, the spindle cannot attach chromosomes via disrupted kinetochores and pull these towards its poles, thus chromosomes remain unsegregated at the equator of the meiocyte (Figure 7, B, 15' to 30').

As for the *bqt1Δ sad1.2* setting, we likewise observe reappearance of Mis12-AIDx-GFP foci at late prophase, before self-assembled spindle formation (Figure 7, C, -80' to -15' and quantification in Figure 8, A), which later segregate along the spindle axis opposite poles upon its elongation (Figure 7, C, 10' to 30'). Strikingly, depletion of Mis12-AIDx-GFP in the *bqt1Δ sad1.2* setting, equally noticed by the absence of Mis12-AIDx-GFP foci, abolishes the formation of self-assembled spindles (Figure 7, D, 0' to 25') from rates 83% to 25% (70% reduction) (quantification in Figure 8, B). Also, of notice, all the minority cases where the self-assembled spindle persist to form upon depletion, chromosomes do not display polewards separation movements (Supp Figure 2, A, 20' to 30'), showing that these spindles are not able to attach and segregate them.

These results indicate that the kinetochores play a significant role in the formation of the self-assembled spindle, as described for mammalian oocytes, such as those from mouse and human, while they are more dispensable for the assembly of SPB-dependent spindles, at least for meiosis I.

Self-assembled spindle formation depends on the RanGTP gradient

Other important agent contributing to acentrosomal spindle formation in mammalian female meiosis is the RanGTP gradient, composed of the small GTPase Ran, conserved as Spil1 in fission yeast, and their regulators RanGAP (GTPase activating protein) and RanGEF/RCC1 (guanyl-nucleotide exchange factor), conserved as Rna1 and Pim1 in fission yeast, respectively, which modulate the balance between the active GTP-bound and inactive GDP-bound forms of Ran.

In oocytes, at the time of acentrosomal spindle assembly, the localization of RCC1 to chromatin concentrates the conversion of inactive Ran (RanGDP) to active Ran (RanGTP), promoting the activation of spindle assembly factors (SAFs) in the vicinity of chromosomes and thus eliciting nucleation, stabilization and organization of microtubules necessary for the assembly of the meiotic spindle.

Thus, given the importance of the RanGTP gradient in the chromatin-associated mechanism of spindle microtubule formation in acentrosomal meiosis, we explored whether this gradient could play a role in the formation of the self-assembled spindle in the *bqt1Δ sad1.2* system. For this task, we disrupted the RanGTP by targeting one of its essential components, Rna1, for depletion using the AIDx system.

First, we checked for a specific and efficient depletion of Rna1-AIDx-GFP in vegetative cells by spot assay (Figure 9), allowed by the fact that the RanGTP gradient activity is indispensable for spindle formation in mitotically-growing cells and thus, for cell viability. Following this, we performed the depletion of Rna1-AIDx-GFP in mid-late prophase in meiocytes, noticeable by a general fading of the GFP signal, and evaluated spindle formation afterwards in the *wt* and *bqt1Δ sad1.2* settings. In the *wt* control, Rna1-AIDx-GFP signal was maintained dispersed throughout the nucleus and cytoplasm of the meiocyte during late prophase and meiosis I (Figure 10, A, -85' to 30') until elongation of the SPB-mediated spindle in meiosis II, when it concentrated at the segregating chromosomes (Figure 10, A, 75' to 90'). Importantly, after depletion of Rna1-AIDx-GFP (Figure 10, B, -70' to -55' vs -20' to 25'), formation of SPB-dependent spindles was abolished from 100% to 47% rate (53% reduction), with a subtle accumulation of the signal at chromosomes (Figure 10, B, 0' to 25' and quantification in Figure 11). However, in the cases where the SPB-dependent persists to form despite the depletion of the general Rna1-AIDx-GFP signal (Supp Figure 3, A, -70' to -60' vs -5' to 125'), we observed meiosis I and meiosis II spindles and still localization of Rna1-AIDx-GFP at chromosomes in meiosis II (Supp Figure 3, A, 105' to 125').

Regarding the *bqt1Δ sad1.2* setting, the Rna1-AIDx-GFP signal was equally maintained throughout the whole meiocyte in the control case (Figure 10, C), although we did not observe an obvious concentration and localization of such signal at chromosomes at any stage, unlike the *wt* control case. Remarkably, after depletion of Rna1-AIDx-GFP (Figure 10, D, -115' to -85' vs -30' to 25'), formation of self-assembled spindles was abolished from 76% to 30% rate (60% reduction) (Figure 10, D, 0' to 25' and quantification in Figure 11). Also, after depletion of the general Rna1-AIDx-GFP signal, we still observe localization of Rna1-AIDx-GFP at the chromosomes in the cases where self-assembled spindle formation is abolished (Figure 10, D, -30' to 25') or not affected (Supp Figure 3, B, 0' to 40').

Together, these results uncover that the formation of the self-assembled spindle significantly depends on the activity of the RanGTP gradient, as it happens for the acentrosomal spindle in mammalian oocytes.

References