

REPORT

# Multimerization of a disordered kinetochore protein promotes accurate chromosome segregation by localizing a core dynein module

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Kinetochore connect chromosomes and spindle microtubules to maintain genomic integrity through cell division. Crosstalk between the minus-end directed motor dynein and kinetochore-microtubule attachment factors promotes accurate chromosome segregation by a poorly understood pathway. Here, we identify a linkage between the intrinsically disordered protein Spc105 (KNL1 orthologue) and dynein using an optogenetic oligomerization assay. Core pools of the checkpoint protein BubR1 and the adaptor complex RZZ contribute to the linkage. Furthermore, a minimal segment of Spc105 with a propensity to multimerize and which contains protein binding motifs is sufficient to link Spc105 to RZZ/dynein. Deletion of the minimal region from Spc105 compromises the recruitment of its binding partners to kinetochores and elevates chromosome missegregation due to merotelic attachments. Restoration of normal chromosome segregation and localization of BubR1 and RZZ requires both protein binding motifs and oligomerization of Spc105. Together, our results reveal that higher-order multimerization of Spc105 contributes to localizing a core pool of RZZ that promotes accurate chromosome segregation.

## Introduction

Kinetochores physically link the chromosomes and spindle microtubules (MTs) to mediate equal segregation of the genome into daughter cells. The affinity of kinetochores for MTs is carefully modulated to support accurate chromosome segregation. Misregulation of kinetochore-microtubule (kMT) attachment affinity compromises genomic integrity by causing chromosome missegregation and aneuploidies (Maiato and Silva, 2023). Modulation of attachment stability is governed by chemical and physical mechanisms, namely a phospho-regulation pathway and a mechanical or steric regulatory pathway. While phospho-regulation of kMT affinity by kinases has been extensively studied (Barbosa et al., 2022; Broad and DeLuca, 2020; Maresca and Salmon, 2010; Santaguida and Musacchio, 2009), a phospho-independent “crosstalk” pathway (Cheerambathur et al., 2013) involving mechano-regulation of kMT attachment factors by the motor protein dynein and its adaptor complex Rod-Zw10-Zwilch (RZZ) is far less defined. The core kMT attachment sites that are regulated by RZZ and dynein are located in the outer kinetochore in a milieu of the highly enriched intrinsically disordered protein KNL1 (Spc105 in *Drosophila*; Fig. 1 A).

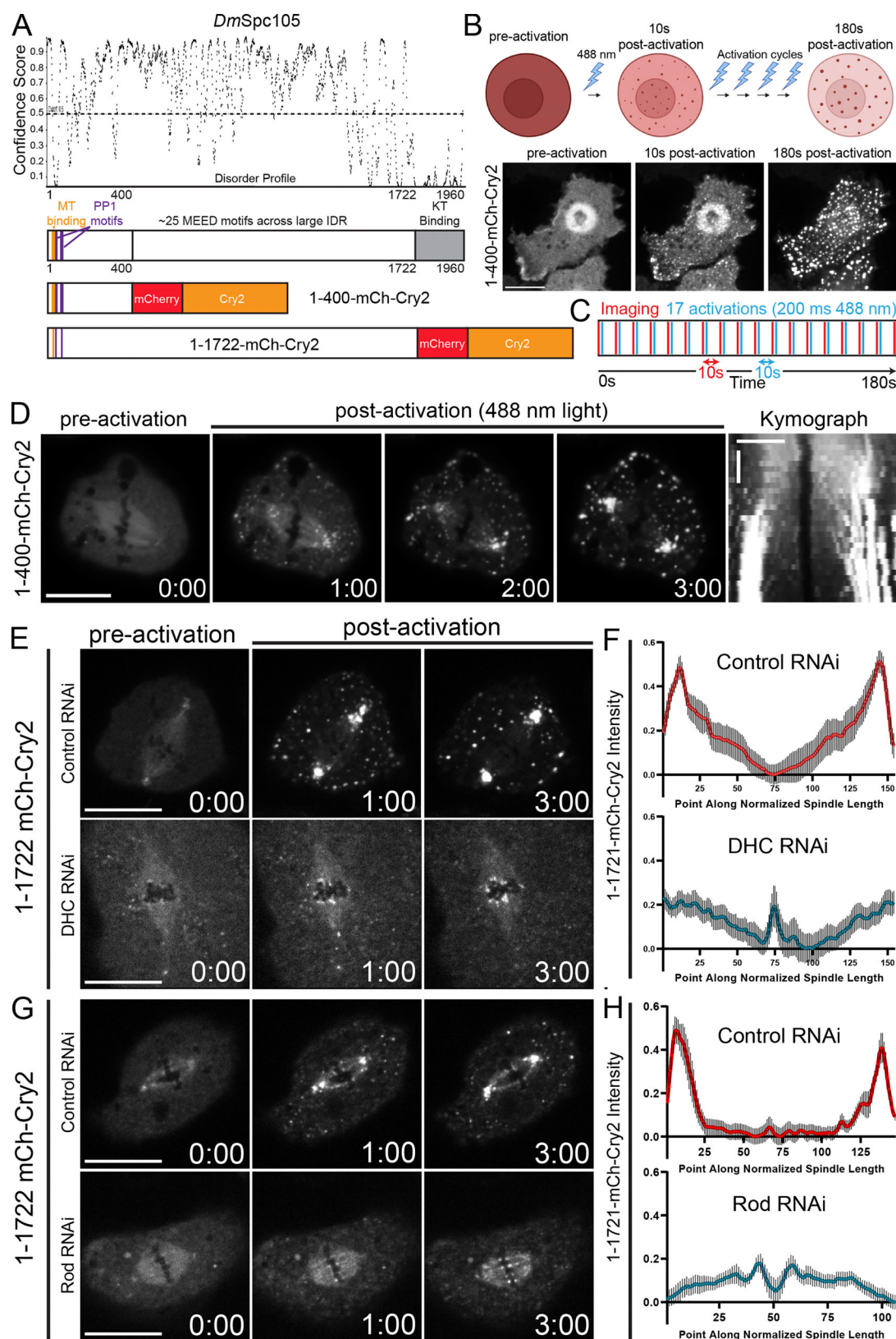
KNL1 (AF15q14/Blinkin/CASC5/D40/KNL1/Spc105; Desai et al., 2003; Genin et al., 2012; Hayette et al., 2000; Kiyomitsu

et al., 2007; Nekrasov et al., 2003; Przewloka et al., 2007; Wei et al., 1999) is the largest component of an outer kinetochore complex called the KMN network, which is made up of KNL1, the Mis12 complex, and the Ndc80 complex (Cheeseman et al., 2006). KNL1 orthologs are intrinsically disordered with the exception of a C-terminal kinetochore-targeting region comprised of RWD domains that bind to the Mis12 complex (Petrovic et al., 2014). The central intrinsically disordered regions (IDRs) of KNL1 orthologs contain a collection of phospho-regulated motifs that—when phosphorylated—bind directly to the checkpoint protein Bub3, which in turn recruits BubR1, Bub1, Mad1, and Mad2. Thus, KNL1 orthologs are conserved molecular hubs for spindle assembly checkpoint (SAC) signaling. Phosphorylation of “MELT” motifs by the checkpoint kinase monopolar spindle 1 (Mps1) promotes Bub3 binding to unattached kinetochores in many KNL1 orthologs (Krenn et al., 2012, 2014; London et al., 2012; Pagliuca et al., 2009; Primorac et al., 2013; Shepperd et al., 2012; Vleugel et al., 2013; Yamagishi et al., 2012; Zhang et al., 2014); however, the Bub3-binding motifs are phosphorylated by Polo-like kinase 1 (PLK1) in *Caenorhabditis elegans* (Espeut et al., 2015) and by Aurora B kinase in *Drosophila melanogaster* (Audett et al., 2022). Vertebrate KNL1 orthologs

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**Figure 1. Oligomerized Spc105 exhibits dynein- and RZZ-dependent poleward movement. (A)** Disorder plot of Spc105 and schematics of full-length Spc105 and truncations fused to mCherry-Cry2. **(B)** Schematic of the photo-oligomerization assay with a representative example of its application in living cells. **(C)** Schematic of the activation/imaging protocol applied to visualize oligomers in mitotic cells. **(D)** Representative example of photo-oligomerized 1-400-mCherry-Cry2 streaming poleward on a mitotic spindle with accompanying kymograph. **(E)** Representative control and DHC-depleted cells expressing 1-1722-mCherry-Cry2 and subjected to the activation/imaging protocol. **(F)** Quantification of the distribution of 1-1722 oligomers across mitotic spindles in control cells. **(G)** Representative control and Rod-depleted cells expressing 1-1722-mCherry-Cry2 and subjected to the activation/imaging protocol. **(H)** Quantification of the distribution of 1-1721 oligomers across mitotic spindles in control cells.

(red) and DHC-depleted (cyan) cells 3-min post activation (control,  $n = 10$  spindles; DHC RNAi,  $n = 12$  spindles). **(G)** Representative control and Rod-depleted cells expressing 1-1722-mCherry-Cry2 and subjected to the activation/imaging protocol. **(H)** Quantification of the distribution of 1-1722 oligomers across mitotic spindles in control (red) and Rod-depleted (cyan) cells 3-min post activation (control,  $n = 9$  spindles; Rod RNAi,  $n = 9$  spindles). Error bars are SEM. Scale bars, 10  $\mu\text{m}$ ; kymograph; 5  $\mu\text{m}$  (horizontal) and 1 min (vertical). Displayed times are min:s.

also bypass Bub3 and directly bind Bub1 and BubR1 via N-terminal (relative to most of the Bub3-binding motifs) KI motifs that have been implicated in regulating both checkpoint signaling and chromosome movement (Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2007, 2011; Krenn et al., 2012).

KNL1 orthologs also contain basic MT binding patches at their N-termini (Audett et al., 2022; Cheeseman et al., 2006; Espeut et al., 2012; Kerres et al., 2007; Pagliuca et al., 2009; Roy et al., 2019; Welburn et al., 2010). The MT binding activity of KNL1 orthologs contributes to SAC satisfaction rather than playing a direct physical role in establishing load-bearing kMT attachments (Audett et al., 2022; Espeut et al., 2012), which is instead mediated by the Ndc80 complex (Cheeseman et al., 2006; DeLuca et al., 2006). The MT binding region in KNL1 orthologs overlaps with two well-conserved protein phosphatase 1 (PPI) binding “SILK” and “RRVSF” motifs that are key contributors to SAC satisfaction (Audett et al., 2022; Bajaj et al., 2018; Espeut et al., 2012; Hendrickx et al., 2009; Liu et al., 2010; London et al., 2012; Meadows et al., 2011; Rosenberg et al., 2011; Shepperd et al., 2012). The N-terminus of KNL1 has also been implicated in Aurora B kinase-mediated phospho-regulation of kMT attachment stability in human cells (Caldas et al., 2013); however, we previously reported that deletion of the first 400 amino acids of Spc105 caused hyperstable kMT attachments without affecting Aurora B kinase activity in *D. melanogaster* cells (Audett et al., 2022). Interestingly, restoration of both the MT- and PPI-binding activities to the  $\Delta 1$ -400 Spc105 mutant did not restore normal kMT attachment stability, which led us to hypothesize that the first 400 aa of Spc105 recruits additional factors that weaken kMT interactions independent of Aurora B kinase.

## Results and discussion

In our prior work (Audett et al., 2022), the first 200 amino acids of Spc105 were fused to a minimal region of the *Arabidopsis thaliana* protein CRY2 that oligomerizes in response to blue light (Bugaj et al., 2013). The Spc105-Cry2 oligomerization assay recapitulates the complex multivalent nature of the kinetochore where there are >100 copies of Spc105 (Lawrimore et al., 2011; Schittenhelm et al., 2010) in cytosolic oligomers detached from the native kinetochore. Our previous study demonstrated the utility of multimerizing a kinetochore protein fragment lacking its targeting domain to assemble functional cytosolic oligomers, a point further reinforced by recent work characterizing cytosolic multimers of human CENP-T (Sissoko et al., 2023 Preprint). Since oligomerization of Spc105-1-200 allowed us to dissect functions that would have been challenging to uncover at intact kinetochores, the first 400 aa of Spc105 were optogenetically oligomerized to gain insights into possible kMT attachment-regulating activities contained therein (Fig. 1 A).

Spc105-1-400-mCherry-Cry2-expressing cells assembled cytoplasmic oligomers upon exposure to 488 nm laser light (Fig. 1 B). An activation/imaging protocol was applied to mitotic cells to examine the behavior of the 1-400 oligomers with relationship to the mitotic spindle (Fig. 1 C). Spc105-1-400 oligomers not only associated with the mitotic spindle but moved poleward and accumulated at spindle poles following photoactivation (Fig. 1 D and Video 1). The localization pattern and motile properties of the Spc105-1-400 fragment required oligomerization since Spc105-1-400-EGFP (without Cry2) typically localized diffusely throughout the cytoplasm and exhibited only a slight enrichment in the spindle region (Fig. S1 A) that was comparable to the localization of fluorescent protein tags alone (EGFP, mCherry, etc.) when expressed in S2 cells (unpublished observation). However, in some high-expressing cells cytosolic oligomers of Spc105-1-400-EGFP (without Cry2) spontaneously assembled, indicating that the 1-400 region has a tendency to self-associate at high concentrations (Fig. S1, B and C; and Video 2).

An mCherry-Cry2-tagged version of Spc105 containing the entire protein, with the exception of the C-terminal kinetochore targeting domain (1-1722-mCherry-Cry2), also photo-oligomerized and streamed poleward on the spindle (Fig. 1, A and E) substantiating the physiological relevance of the phenomenon observed using the shorter fragment of Spc105. The poleward movement of the Spc105-mCherry-Cry2 oligomers implicated the minus end-directed motor dynein, which is known to be targeted to the kinetochore by the Rod-Zw10-Zwilch (RZZ) complex (Gama et al., 2017; Mosalaganti et al., 2017). Upon depletion of the dynein heavy chain (DHC) by RNAi (Fig. S1, D-F), Spc105 oligomers did not accumulate at spindle poles but rather became enriched near the kinetochore region (Fig. 1, E and F; and Video 3). Since RZZ recruits the checkpoint proteins Mad1 and Mad2 (Caldas et al., 2015; Kops and Gassmann, 2020), which altogether are stripped from kinetochores in a dynein-mediated manner that looks identical to the movement of the Spc105 oligomers (Basto et al., 2004; Cane et al., 2013; Howell et al., 2000, 2001), we next assessed the motility of the 1-1722 oligomers in Rod-depleted cells (Fig. S1, G-I). Like the DHC RNAi condition, 1-1722 oligomers in Rod-depleted cells did not move poleward and accumulated near kinetochores rather than at spindle poles (Fig. 1, G and H; and Video 4). Thus, the minus-end directed movement and polar accumulation of Spc105 oligomers required both dynein and its adaptor—the RZZ complex.

We next examined the 1-400 region of Spc105 for features that could explain the molecular mechanism for the functional linkage of Spc105 to RZZ and dynein revealed by the oligomerization assays. T-COFFEE alignment (Notredame et al., 2000) of the Spc105 and human KNL1 aa sequences identified two putative KI motifs (KI1 and KI2) in the N-terminal region of Spc105 between aa 279-349 (Fig. 2 A). In human KNL1, KI motifs bind

directly to the N-terminal TPR motifs of the Bub checkpoint proteins with KI1 and KI2 exhibiting preferential binding for Bub1 and BubR1, respectively (Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2007, 2011; Krenn et al., 2012). The putative KI-motif-containing region was tagged with mCherry-Cry2 and subjected to photoactivation to further assess its functions. In support of the 266–384 region containing functional KI motifs, both Bub1-EGFP and BubR1-EGFP were enriched in the 266–384 oligomers with BubR1 exhibiting approximately twofold higher enrichment in the clusters than Bub1 (Fig. 2, C, D, G, and J–L). Importantly, the binding was specific since the EGFP tag alone was not enriched in the 266–384 oligomers over its entire expression range (Fig. 2, B and C). We noted that N-terminally tagged Bub1 was not recruited to the 266–384 oligomers (Fig. 2 C), possibly due to the N-terminal EGFP tag interfering with the binding of its TPR to the KI1 motif. We could rule out that enrichment of Bub1 and BubR1 in the 266–384 clusters was mediated by Bub3, which recruits subpopulations of Bub1 and BubR1 to intact kinetochores (Krenn et al., 2012; Larsen et al., 2007; Overlack et al., 2015; Primorac et al., 2013; Taylor et al., 1998) because there are no Bub3 binding motifs between aa 266 and 384, and Bub3 was not evidently enriched in the 1–1722 clusters (Fig. S1, J and K). Altogether, the data supported the conclusion that Spc105 contains functional KI motifs although we postulate that the KI2 motif may be more “active” because its sequence is better conserved than the KI1 motif and BubR1 was more robustly recruited to 266–384 oligomers than Bub1.

Since the N-terminus of KNL1 and Bub1 contribute to the localization of the RZZ complex at human kinetochores (Caldas et al., 2015; Varma et al., 2013; Zhang et al., 2015, 2019), we next investigated whether RZZ components were recruited to 266–384 oligomers. Consistent with prior findings, Zw10-EGFP and superfolder GFP (sfGFP)-Rod were similarly enriched in the 266–384 clusters (Fig. 2, C, E, F, H, I, and L) and some 266–384 clusters that recruited sfGFP-Rod moved poleward on mitotic spindles (Fig. 2 M and Video 5). Thus, oligomerization of a minimal KI motif-containing region (266–384) was sufficient to recruit the molecular machinery linking Spc105 to dynein and to support the minus-end directed motility observed for oligomerized 1–400 and 1–1722 Spc105 fragments.

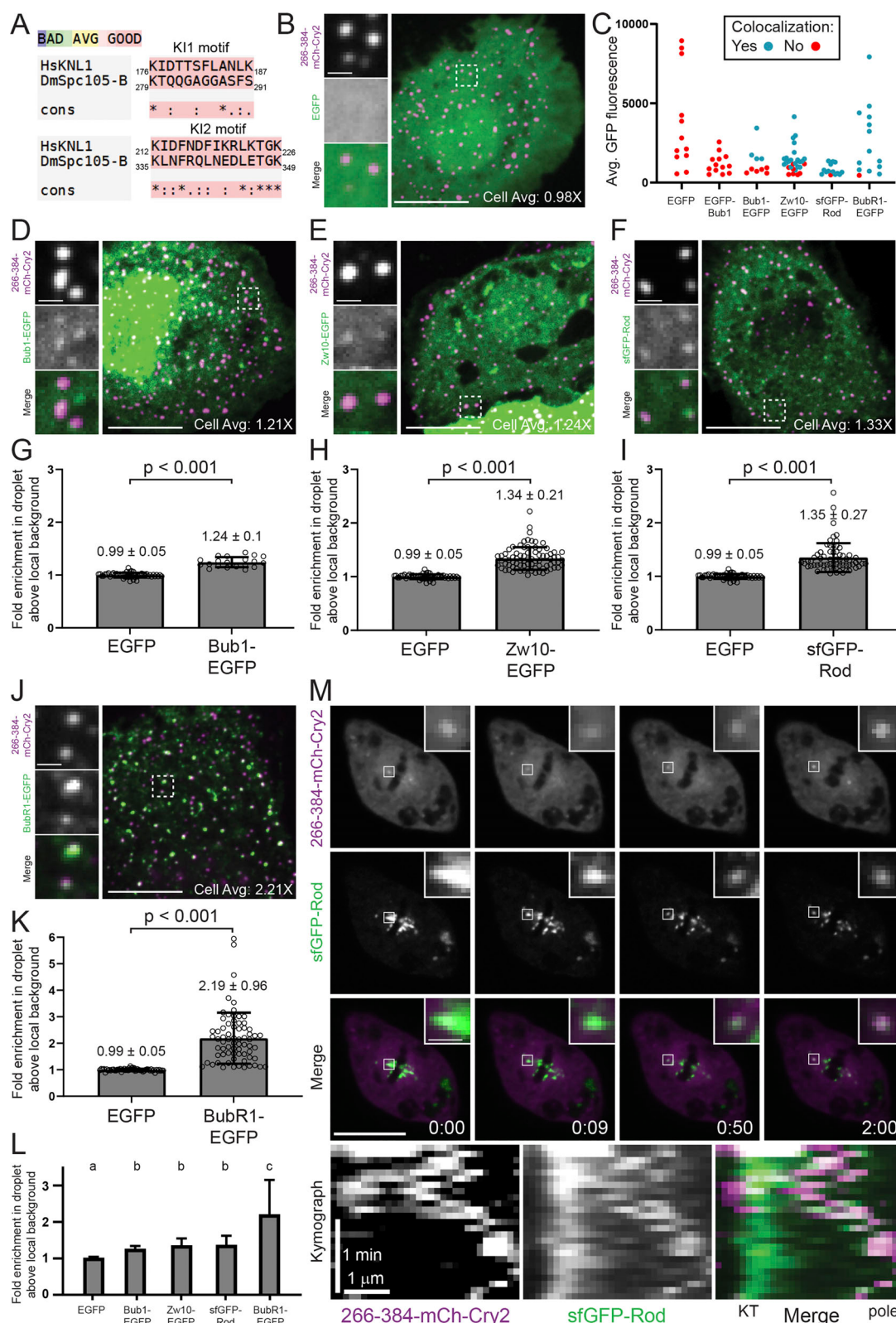
The KI-motif-containing region was next deleted from full-length Spc105 to determine if it was necessary for BubR1 and Rod recruitment to intact kinetochores. Bioriented kinetochores in the  $\Delta 267$ –383 deletion mutant exhibited ~45% and 60% reductions in BubR1 and Rod levels, respectively, compared with cells expressing wild-type (WT) Spc105 (Fig. 3, A–D). While the outer kinetochore pool of BubR1 was reduced, it was notable that an inner centromeric pool of BubR1 sometimes became enriched in cells expressing the  $\Delta 267$ –383 deletion mutant relative to the controls (personal observation). Since BubR1 likely binds directly to Spc105 via the KI2 motif, BubR1 was depleted by RNAi to determine if it affected the recruitment of RZZ to bioriented kinetochores (Fig. S1, L and M). Indeed, depletion of BubR1 from WT cells resulted in a comparable reduction in Rod levels at bioriented kinetochores, as was observed in the  $\Delta 267$ –383 deletion mutant (Fig. 3, D–F). Sequence alignments with fly and human Bub proteins identified an internal region of *DmBubR1*

with homology to a Bub1 motif that contributes to RZZ localization at human kinetochores (Fig. 3 G and Fig. S1 N; Zhang et al., 2015, 2019). Transient expression of a BubR1 deletion mutant ( $\Delta 734$ –872) lacking the human Bub1 homology region caused a significant reduction in Rod levels at bioriented kinetochores—comparable with that observed in BubR1-depleted cells (Fig. 3, F, H, and I). Thus, the KI motif region of Spc105 and BubR1 each contributed to the recruitment of a core pool of RZZ to bioriented kinetochores in fly cells.

Chromosome segregation was next evaluated via live- and fixed-cell analyses to determine if the KI motif region was necessary for normal kinetochore function. Consistent with prior work in human cells expressing a KNL1 mutant without KI motifs (Kiyomitsu et al., 2011), there was an increase in the frequency of lagging chromosomes during anaphase in cells expressing Spc105  $\Delta 267$ –383-EGFP compared with control cells (Fig. 3, J and K). Upon evaluation of the kMT attachment states of lagging chromosomes, there was a statistically significant increase in the frequency of merotelic attachments, an erroneous kMT attachment in which a single kinetochore is attached to MTs oriented toward opposite spindle poles (Fig. 3 L). Merotelic attachments with prototypical deformations into bilobed/kidney-shaped structures (Cimini et al., 2001) were evident in Spc105  $\Delta 267$ –383-EGFP-expressing anaphase cells visualized by spinning disk confocal microscopy (Fig. 3 M and Video 6). The abundance of persistently stabilized merotelic attachments in the deletion mutant supported the conclusion that loss of the KI motif-containing region contributed to the hyper-stable kMT attachments previously observed in the  $\Delta 1$ –400 Spc105 mutant (Audett et al., 2022).

While implementing the activation/imaging protocol, it was evident that oligomerization occurred more readily in cells expressing 1–400- and 1–1722-mCherry-Cry2 than in control cells with comparable levels of mCherry-Cry2 expression (Fig. 4 A and Video 7). Accordingly, the mean expression levels supporting cluster assembly were significantly different for each protein with mCherry-Cry2 requiring the highest expression, 266–384 being intermediate, and 1–1722 being the lowest (Fig. 4 B). The Cry2-based oligomerization assay was previously adapted to study the ability of “sticky” intrinsically disordered regions (IDRs) to form so-called optoDroplets in living cells (Shin et al., 2017). Interestingly, oligomerized 266–384 exhibited droplet-like properties as clusters could be observed to fuse in the cytosol similar to that observed for the Spc105-1–400-EGFP oligomers (Fig. 4 C, Fig. S1, B and C, Video 2, and Video 8). Furthermore, the 266–384 clusters rapidly disassembled upon introduction of the alcohol 1,6-hexanediol (1,6-HD; Fig. 4 D and Video 9).

Since the self-association of IDRs is implicated in a higher-order multimerization phenomenon known as liquid–liquid phase separation (LLPS) and the 266–384 oligomers exhibited LLPS-like properties, the propensity of Spc105 to undergo LLPS was computationally assessed. The catGRANULE algorithm (Bolognesi et al., 2016) identified seven stretches (between 8 and 40 aa) in Spc105 with LLPS propensity (Fig. 4 E). Consistent with the observed properties of 266–384 oligomers (Fig. 4, C and D), the two longest (40 and 35 aa) and highest scoring LLPS regions



**Figure 2. Oligomerization of the KI region of Spc105 recruits binding partners and is sufficient for poleward movement.** **(A)** T-COFFEE alignments of human (Hs) KNL1 and fly (Dm) Spc105 identify putative KI motifs. **(B, D–F, and J)** Representative confocal Z-planes of cells coexpressing the indicated EGFP-tagged protein (green) and oligomerized clusters of the KI motif-containing region (266–384) fused to mCherry-Cry2 (magenta). **(C)** Colocalization of the indicated EGFP-tagged proteins with 266–384-mCherry-Cry2 clusters in cells over a range of expression. Colocalization with clusters was never observed in cells over the full range of EGFP (control) or EGFP-Bub1 expression. With the exception of the EGFP control (to establish a baseline measurement for the assay), only cells exhibiting colocalization were used to measure the fold enrichment for each binding partner. **(G–I and K)** Quantification of the fold enrichment of the

signal of the indicated EGFP-tagged protein relative to the local background signal (EGFP;  $n = 53$  drops from 10 cells, Bub1-EGFP;  $n = 20$  drops from 4 cells, Zw10-EGFP;  $n = 80$  drops from 16 cells, sfGFP-Rod;  $n = 65$  drops from 13 cells, BubR1-EGFP;  $n = 70$  drops from 14 cells). **(L)** Measurements from G–I and K are shown on the same plot. In all plots, each binding partner is compared with the same control EGFP data set. **(M)** Representative confocal time-lapse of sfGFP-Rod (green) colocalized with a 266–384-mCherry-Cry2 cluster (magenta) moving poleward with accompanying kymograph. Boxed regions are highlighted in the insets. The average fold enrichment for the representative cell is indicated in each image. Bar plots report mean  $\pm$  SD. Different letters above each plot column indicate significant differences ( $P < 0.05$ ) determined by a randomization method (PlotsOfDifferences) for all pairwise combinations. Shared letters indicate that the difference is not significant ( $P > 0.05$ ). Scale bars, 10  $\mu\text{m}$ ; 1  $\mu\text{m}$  (insets), and 1  $\mu\text{m}$  (horizontal); 1 min (vertical) in kymograph. Displayed time is min:s.

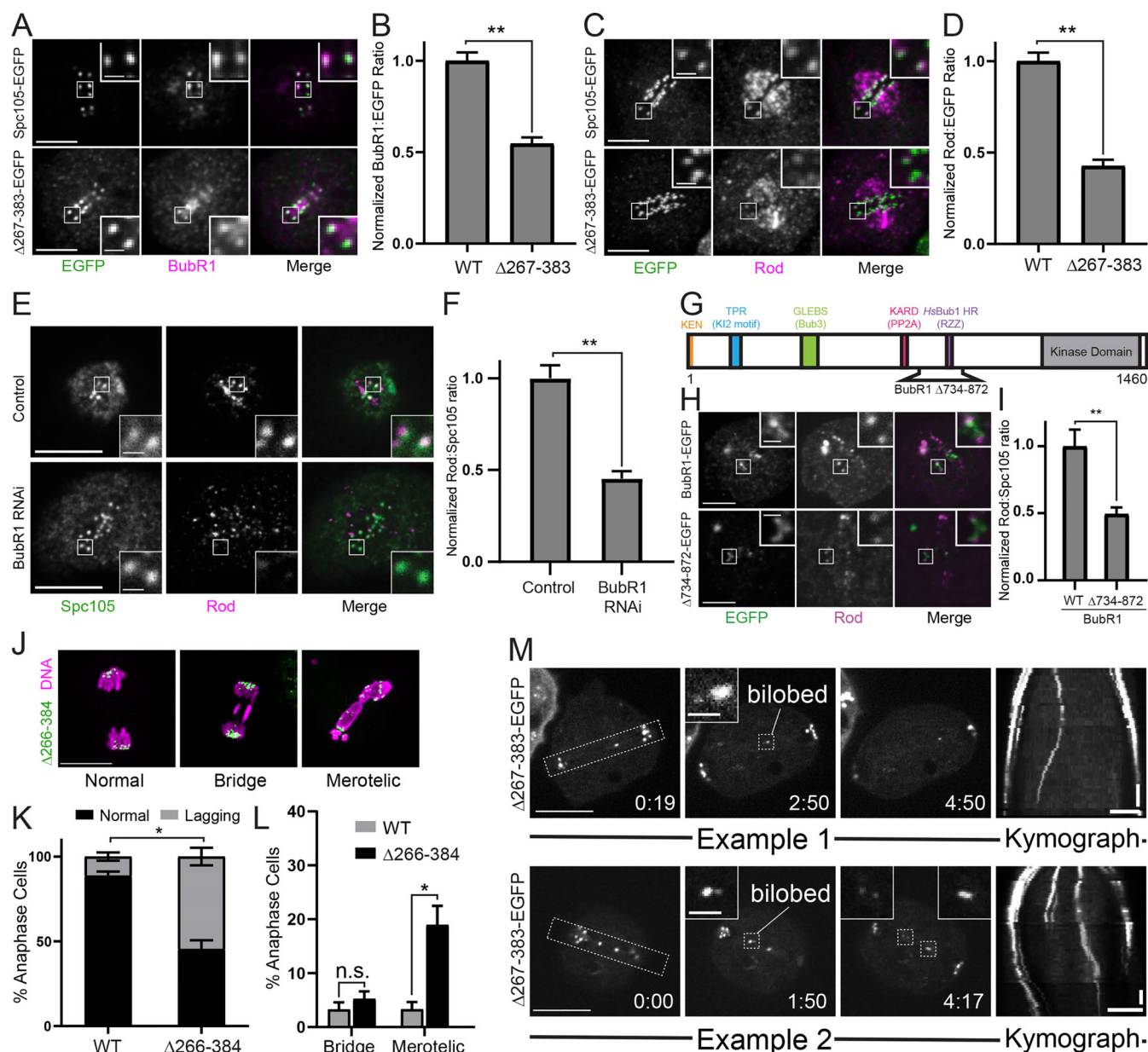
of Spc105 mapped between aa 266 and 384 (Fig. 4 E). Since the LLPS regions overlapped with the KI motifs, it was not possible to discern whether the  $\Delta 267$ –383 mutant phenotype was due to loss of protein binding motifs and/or reduced ability of the IDR to self-assemble into higher-order multimers. To separate these functions, we took advantage of the KI motifs in human KNL1, which do not overlap with predicted LLPS regions (Fig. S2 A), and the IDR of human FUS, which has no known functions at kinetochores but assembles higher-order multimers via a sticky IDR (Patel et al., 2015; Shin et al., 2017). Importantly, we assessed whether the human KNL1 KI2 motif was capable of binding fly BubR1 and observed that cytosolic droplets of the human KI motif-containing region (aa 196–276) fused to FUS-mCherry-Cry2 recruited similar levels of DmBubR1-EGFP as the 266–284-mCherry-Cry2 droplets. (Fig. 4, F and G).

Since cytosolic oligomers of the human KI motif region were capable of recruiting fly BubR1, a series of chimeras was next generated to scrutinize the contributions of KI motifs and higher-order multimerization at intact kinetochores. Introduction of the human KI motifs (HsKI) was insufficient to restore BubR1 and Rod localization at bioriented kinetochores to WT levels as they remained reduced to  $\sim 35\%$  and  $\sim 60\%$ , respectively, compared with control cells (Fig. 5, A–D; and Fig. S2, B and C). However, cells expressing a  $\Delta 267$ –383 chimera containing both the FUS IDR and the HsKI motifs recruited levels of BubR1 and Rod to their bioriented kinetochores that were not statistically different from WT Spc105-expressing cells (Fig. 5, E–H; and Fig. S2, B and C). The rescue of BubR1 localization was consistent with the observation that cytosolic oligomers of HsKI-FUS- and 266–384-mCherry-Cry2 recruited comparable levels of BubR1 (Fig. 4, F and G). The functionality of the chimeras was next evaluated by assessing the frequency of lagging chromosomes in anaphase. Cells expressing the  $\Delta 267$ –383 deletion and the HsKI chimera both exhibited a significant increase in lagging chromosomes compared with control cells, while possession of both the HsKI motifs and the FUS IDR rescued the chromosome missegregation defect (Fig. 5 I).

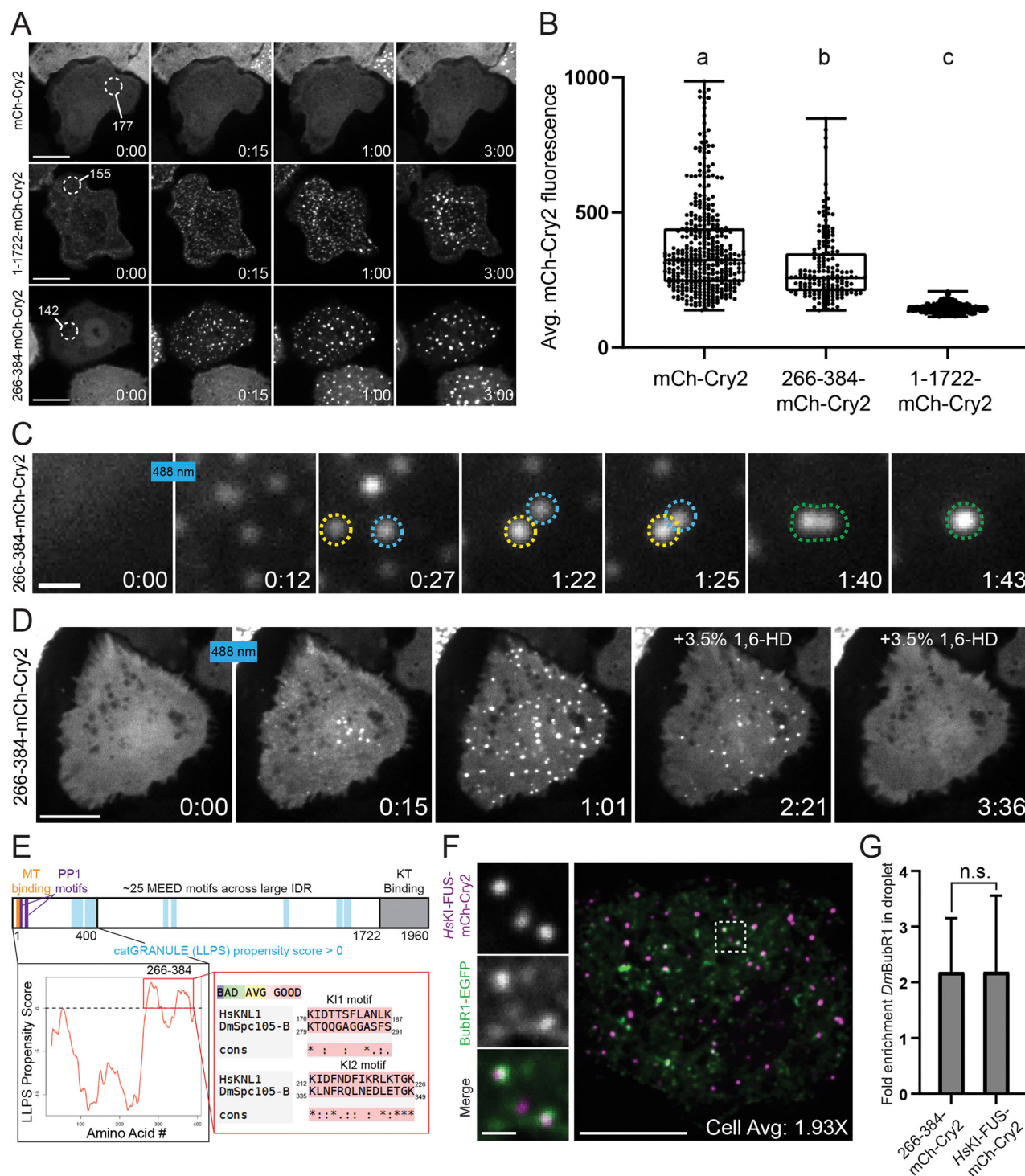
To further probe the extent of multimerization that was required for Spc105 function, two additional chimeras were generated containing the HsKI motifs and either the self-associating IDR from the human DEAD-box helicase DDX4 (Nott et al., 2015; Shin et al., 2017) or the leucine zipper of human GCN4, which assembles dimers and trimers (O'Shea et al., 1991; Oshaben et al., 2012; van der Horst et al., 2015). Introduction of the DDX4 IDR rescued chromosome missegregation as the percentage of anaphase cells with lagging chromosomes in the DDX4-HsKI chimera-expressing cells was indistinguishable from the WT Spc105-expressing control cells (Fig. 5 I). However, cells

expressing the GCN4-HsKI chimera had a statistically significant increase in chromosome missegregation compared with the WT controls (Fig. 5 I). The findings that chromosome missegregation could be rescued by introducing the self-associating IDR from DDX4 but not the structured leucine zipper of GCN4 indicates that multimerization of Spc105 above a dimer or trimer is required for its full functionality. While the GCN4 result does not exclude that assembly of self-associating Spc105 multimers via structured domains could support its kMT attachment-regulating function, it is unlikely to be the natural mechanism because—other than the C-terminal RWD domain—Spc105 is predicted to be intrinsically disordered and to contain multiple sticky IDRs.

Our findings are consistent with earlier models where RZZ modulates the stability of end-on kMT attachments by decreasing the affinity of the Ndc80 complex for MTs until it is inhibited by dynein via stripping or conformational changes (Amin et al., 2018; Cheerambathur et al., 2013; Gassmann et al., 2008). While earlier work envisioned that a coronal pool of RZZ functions early in mitosis to prevent premature end-on attachments, we propose that kMT attachment stability is actively modulated by a core pool of RZZ throughout mitosis—even at bioriented kinetochores. Our findings also further flesh out molecular details of the crosstalk pathway by identifying Spc105 as a potential receptor for the kMT attachment-regulating pool of RZZ. More specifically, we propose that recruitment of the core pool of RZZ is mediated by both Spc105 and BubR1, which potentially bridges Spc105 and RZZ (Fig. 5 J). The fact that dynein strips RZZ but not BubR1 in *D. melanogaster* (*Dm*) cells suggests that the affinity between BubR1 and Spc105 (via the KI2 motif) is stronger than RZZ's affinity for BubR1 and/or Spc105. We cannot rule out the existence of additional contacts beyond the KI motif region between Spc105 and RZZ since Rod was reduced but not absent from kinetochores in cells expressing Spc105  $\Delta 267$ –383, BubR1  $\Delta 734$ –872, and in BubR1-depleted cells. We also cannot exclude that Bub1 contributes to RZZ recruitment in flies as it was moderately enriched in the Spc105 clusters. Interestingly, the N-terminus of KNL1 and Bub1 are required for full recruitment of RZZ to kinetochores in human cells (Caldas et al., 2015; Zhang et al., 2019) and BUB-1 is required to localize a kMT-attachment-regulating pool of RZZ that also recruits the dynein-dynactin complex to kinetochores in *C. elegans* (Edwards et al., 2018). Thus, we hypothesize that the linkage to RZZ described here is likely mediated by KNL1 and Bub1 outside flies (Fig. S2 D). While our model posits direct recruitment of RZZ to Spc105 multimers, the mechanism by which the core pool of RZZ is localized deserves further inquiry since it is presently unclear if or how Spc105/BubR1 (or KNL1/Bub1) physically interacts with the RZZ complex.



**Figure 3. Dissecting the molecular linkage between Spc105 and dynein and its functional contribution to accurate chromosome segregation.** (A) Representative images of BubR1 (magenta) in cells expressing WT Spc105 or the Δ267-383-Spc105 deletion mutant (green). (B) Quantification of BubR1 intensities relative to EGFP signal in WT and Δ267-383-expressing cells (WT;  $n = 140$  kinetochores from 25 cells and 2 replicates, Δ267-383;  $n = 135$  kinetochores from 24 cells and 2 replicates). (C) Representative images of Rod (magenta) in cells expressing WT Spc105 or the deletion mutant (green). (D) Quantification of Rod intensity relative to EGFP signal in WT and Δ267-383-expressing cells (WT;  $n = 142$  kinetochores from 25 cells and 2 replicates, Δ267-383;  $n = 140$  kinetochores from 25 cells and 2 replicates). (E) Representative confocal Z-planes of control and BubR1 dsRNA-treated fixed cells stained for Spc105 (green) and Rod (magenta). (F) Quantification of Rod:Spc105 intensity ratios in control and BubR1-depleted cells (control;  $n = 105$  kinetochores from 21 cells and 2 replicates, BubR1 RNAi;  $n = 102$  kinetochores from 20 cells and 2 replicates). (G) Schematic of *DmBubR1* highlighting the KEN box (orange), TPR motif (cyan), GLEBS (green), and KARD (pink) domains, and homology region (HR) to *HsBub1* (magenta). The putative binding partner for each motif/domain is indicated in parentheses and the region (aa 734-872) deleted in the BubR1 mutant is shown. (H) Representative confocal Z-planes of metaphase cells transiently transfected with BubR1-EGFP or BubR1-Δ734-872-EGFP stained for EGFP (green) and Rod (magenta). (I) Quantification of Rod:EGFP intensity ratios in control (BubR1-EGFP) and BubR1-Δ734-872-EGFP-expressing cells (control;  $n = 60$  kinetochores from 12 cells, BubR1-Δ734-872;  $n = 55$  kinetochores from 11 cells). (J) Examples of the chromosome segregation categories quantified in K and L (Δ267-383 is green, DNA is magenta). (K) Percentage of anaphase/telophase cells with at least one lagging chromosome in WT- and Δ267-383-expressing cells ( $n = 150$  cells per condition from three replicates). (L) Percentage of WT- and Δ267-383-expressing cells with either bridge or merotelic lagging chromosomes. (M) Representative examples from confocal time-lapses of Δ267-383-expressing cells with persistent merotelic attachments in anaphase. Kymographs of the boxed regions are shown. Reported P values were generated by a randomization method (PlotsOfDifferences) in B, D, F, and I or two-tailed *t* tests in K and L: \* P value <0.05, \*\* P value <0.001. Boxed regions are shown in the insets. Plots report mean  $\pm$  SEM. Scale bars: 10  $\mu$ m (E, J, and M); 5  $\mu$ m (A, C, and H), 1  $\mu$ m (insets); 5  $\mu$ m (horizontal) and 1 min (vertical) in kymographs. Displayed times are min:s.



**Figure 4. Characterization of cytosolic Spc105 oligomers.** (A) Representative time-lapse confocal imaging of cells with comparable expression of the mCherry-Cry2 tag alone or tagged Spc105 truncations subjected to the same activation protocol. The average cytosolic mCherry fluorescence intensities are indicated for each circular region. (B) Box and whisker plots of the average cytosolic mCherry fluorescence prior to activation of all the cells that formed clusters in the activation/imaging protocol. (C) Representative confocal time-lapse of 266–384-mCherry-Cry2 clusters (yellow and blue circles) fusing (green circle). (D) Representative confocal time-lapse of optogenetic clustering in a 266–384-mCherry-Cry2-expressing cell being rapidly reversed upon the addition of 3.5% 1,6-HD. (E) Schematic of *D. melanogaster* Spc105-B highlighting the N-terminal MT (orange) and PP1-binding (magenta) motifs and the seven regions across Spc105 with a catGRANULE LLPS propensity score above 0 (blue) including the catGRANULE plot of the first 400 aa of Spc105-B with region 266–384 highlighted in the inset showing T-COFFEE alignments of the overlapping KI motifs (Hs: *Homo sapiens*; Dm: *D. melanogaster*). (F) Representative confocal Z-plane of a cell coexpressing BubR1-EGFP (green) and oligomerized clusters of the KI motif-containing region from human KNL1 (196–276) fused to FUS-mCherry-Cry2 (magenta). The boxed region is highlighted in the insets. The average fold enrichment for the cell (from five clusters) is indicated in the image. (G) Quantification of the fold enrichment of the signal of BubR1-EGFP relative to the local background signal (266–384-mCherry-Cry2;  $n = 70$  clusters from 14 cells [same data as plotted in Fig. 2 K], HsKI-FUS-mCherry-Cry2;  $n = 65$  clusters from 13 cells). Whiskers indicate min-max, box is 25th–75th percentile, and bar

is median in B. Bar plots report mean  $\pm$  SD in G. Different letters above each column in B indicate significant differences ( $P < 0.05$ ) determined by a randomization method (PlotsOfDifferences) for all pairwise combinations. The reported P value in G was generated by a two-tailed t test: n.s. is not significant ( $P > 0.05$ ). Scale bars, 10  $\mu$ m (A, D, and F); 1  $\mu$ m (C and insets). Displayed times are min:s.

Conventional protein binding motifs in Spc105 are insufficient to support RZZ/dynein recruitment since Spc105-mCherry-Cry2 fusions containing the KI motifs were not transported to spindle poles prior to photo-oligomerization. However, the functional linkage between Spc105 and RZZ/dynein could be established rapidly (seconds timescale) upon higher-order multimerization as evidenced by the immediate dynein-dependent motility of Spc105 clusters toward spindle poles following photoactivation. The variability of Cry2-based oligomers makes it difficult to know how many molecules of Spc105 must be present in the higher-order oligomers to support RZZ/dynein recruitment although this could be addressed in future studies using oligomerization tags of known stoichiometries. Regardless of how many Spc105 molecules are required to efficiently recruit RZZ, our findings suggest that the kinetochore localization of Spc105 may be insufficient. Rather, we hypothesize that enrichment of Spc105 upon kinetochore assembly stimulates its higher-order multimerization via sticky IDRs, which facilitates kMT attachment regulation by Spc105 through increasing its avidity for the RZZ complex (Fig. S2 E). Whether higher-order assembly via sticky IDRs is employed by other kinetochore proteins to achieve full functionality or impacts the SAC-regulating functions of Spc105 warrants future investigation. Relatedly, forces are likely transduced, albeit transiently, through Spc105 when the molecular linkage to dynein is established—especially at bioriented kinetochores. Force-dependent reorganization of the array of Bub3-binding motifs in the central IDRs of Spc105/KNL1 could influence SAC signaling (Audett and Maresca, 2020) and may contribute to the “unraveling” of human KNL1 that has been proposed to act as a tension-sensing mechanism (Roscioli et al., 2020).

In summary, we conclude that Spc105 multimerization supports the localization of sufficient levels of a core pool of RZZ that attenuates the affinity of the Ndc80 complex for MTs to reduce the prevalence of erroneous kMT attachments. The core pool of RZZ is likely dynamic in nature as its levels/activity must be defined by a balance of Spc105-dependent recruitment and dynein-mediated stripping or conformational inhibition. Finally, we propose that higher-order multimerization of Spc105—mediated by sticky IDRs—yields emergent kinetochore properties that contribute to accurate chromosome segregation and that intersect with force-dependent regulation by dynein.

## Materials and methods

### Cell culture and cell line production

*Drosophila* S2 cells were cultured at 24°C in Schneider’s medium supplemented with 10% heat-inactivated FBS (Invitrogen) and 0.5 $\times$  antibiotic-antimycotic cocktail (Invitrogen). All cell lines were made by transfecting WT S2 cells with 1–2  $\mu$ g of the appropriate plasmid DNA. The cells were transfected using the Effectene Transfection Reagent (Qiagen) according to the

manufacturer’s protocol. Stable cell lines were generated by selection with the appropriate antibiotic (0.025 mg/ml Blastidicin S HCl [Thermo Fisher Scientific] or 0.25–0.5 mg/ml Hygromycin B [Invitrogen]) until cell death ceased. Stable cells were occasionally split in the presence of an antibiotic to maintain expression levels.

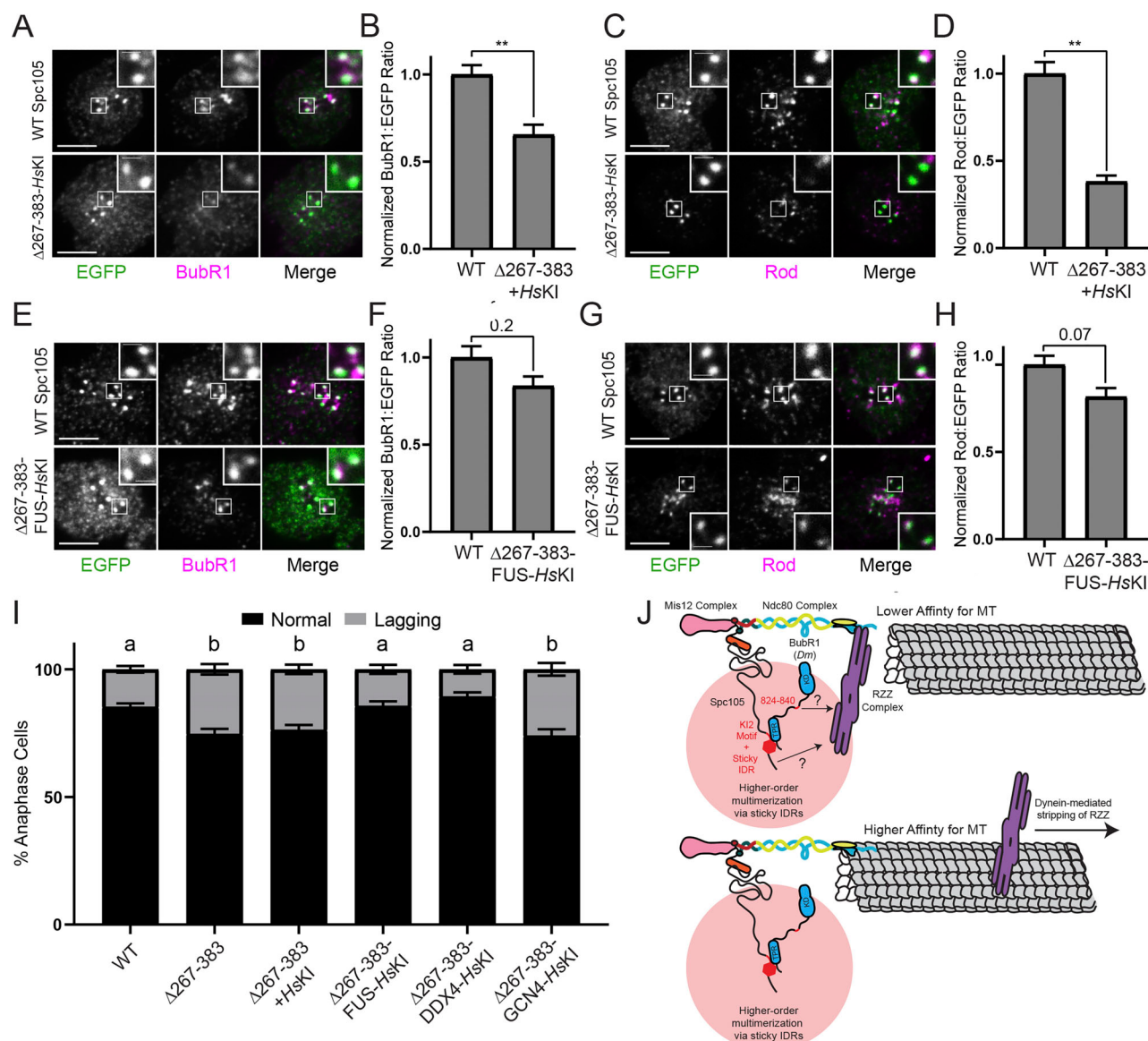
### Microscope image acquisition and live-cell imaging conditions

Cells were seeded onto Concanavalin A- (ConA; Sigma-Aldrich) treated 35 mm glass bottom petri dishes (Cellvis) and imaged on a TiE inverted microscope (Nikon) equipped with a Borealis (Andor) retrofitted CSU-10 (Yokogawa) spinning disk head and ORCA-Flash4.0 LT Digital CMOS camera (Hamamatsu) using a 100 $\times$  1.49 numerical aperture (NA) Apo differential interference contrast (DIC) TIRF objective (Nikon). Metamorph software (Molecular Devices) was used to control the imaging system and to analyze data. The cells were imaged at room temperature ( $\sim$ 22°C) in Schneider’s medium supplemented with 10% heat-inactivated FBS (Invitrogen) and 0.5 $\times$  antibiotic-antimycotic cocktail (Invitrogen). For live cell imaging, EGFP and mCherry fluorophores were imaged, and for fixations DAPI, alexa-488, Cy3, and Cy5, fluorochromes were visualized. To generate figures, microscopy images were processed in Metamorph, Photoshop (Adobe), and Illustrator (Adobe) with comparable images within a panel subjected to identical scaling and linear adjustments (no gamma adjustments were ever applied) to brightness and contrast.

### Optogenetic photoactivation experiments and analysis in mitotic cells

The same imaging protocol was used to analyze the behavior of 1–400-mCherry-Cry2, 266–384-mCherry-Cry2, and 1–1722-mCherry-Cry2 in mitotic cells. Cells were induced  $\sim$ 16 h overnight with 500  $\mu$ M CuSO<sub>4</sub> and seeded onto ConA-coated coverslips before subjecting them to the activation/imaging protocol outlined in Fig. 1 C, which consisted of a preactivation image with 561 nm light, followed by blue light activation at 488 nm and synchronous imaging of 561 nm at 10-s intervals for 3 min.

For phenotypic assessment of the DHC knockdown experiments, the above protocol was used to produce confocal time-lapses of which the final image was analyzed by linescan to quantify the distribution of 1–1722-mCherry-Cry2 clusters along the length of the spindle. The line width was set to  $\sim$ 30% of the spindle width and extended lengthwise along the long spindle axis terminating at each pole. Average fluorescence intensity values for each point along the line were transferred to a spreadsheet. Linescan data was pooled after normalizing for the variability in spindle length relative to the shortest spindle in a dataset. To do this, a running average of adjacent values was created for all spindles, and points were removed according to the extent to which a spindle needed to be normalized. For example, if a spindle needed to be corrected by 30%, every third



**Figure 5. Localization of dynein targeting factors and accurate chromosome segregation require sticky IDRs and protein binding motifs in Spc105.**

(A) Representative images of BubR1 (magenta) in cells expressing WT Spc105 or the HsKI chimera (green). (B) Quantification of BubR1 intensity relative to EGFP signal in WT and HsKI-expressing cells (WT;  $n = 150$  kinetochores from 30 cells and three replicates, HsKI;  $n = 145$  kinetochores from 29 cells and three replicates). (C) Representative images of Rod (magenta) in cells expressing WT Spc105 or the HsKI chimera (green). (D) Quantification of Rod intensity relative to EGFP signal in WT and HsKI-expressing cells (WT;  $n = 150$  kinetochores from 30 cells and three replicates, HsKI;  $n = 150$  kinetochores from 30 cells and three replicates). (E) Representative images of BubR1 (magenta) in cells expressing WT Spc105 or the FUS-HsKI chimera (green). (F) Quantification of BubR1 intensity relative to EGFP signal in WT and FUS-HsKI-expressing cells (WT;  $n = 150$  kinetochores from 30 cells and three replicates, FUS-HsKI;  $n = 150$  kinetochores from 30 cells and three replicates). (G) Representative images of Rod (magenta) in cells expressing WT Spc105 or the FUS-HsKI chimera (green). (H) Quantification of Rod intensity relative to EGFP signal in WT and FUS-HsKI-expressing cells (WT;  $n = 155$  kinetochores from 31 cells and three replicates, FUS-HsKI;  $n = 160$  kinetochores from 32 cells and three replicates). (I) Percentage of anaphase cells with at least one lagging chromosome in WT-, deletion mutant-, and chimera-expressing cells ( $n = 300$  cells per condition from three replicates). (J) Model for how Spc105 and BubR1 contribute to the recruitment of a core population of RZZ at bioriented kinetochores that reduces the Ndc80 complex's affinity for kMTs. A single Spc105 molecule is shown for clarity, but we propose that it is assembled into higher-order multimers via sticky IDRs (pink circle). Dynein relieves the weakening of kMT attachment affinity by the core pool of RZZ by stripping the complex in fly cells. Boxed regions are shown in the insets. All plots report mean  $\pm$  SEM. Reported P values were generated by a randomization method (PlotsOfDifferences) in B, D, F, and H and two-tailed t tests in I. \*\* $P < 0.001$ . Different letters above each column in I indicate significant differences ( $P < 0.05$ ) determined by two-tailed t tests for all pairwise combinations. Shared letters indicate that the difference is not significant ( $P > 0.05$ ). Scale bars: 5  $\mu$ m (A, C, E, and G), 1  $\mu$ m (all insets).

data point was removed but still accounted for by averaging adjacent data points along the linescan. For phenotypic assessment of the Rod knockdown experiments, linescans (drawn as

described previously) were conducted on maximum projections of confocal Z-stacks of the mCherry channel following the completion of the photoactivation protocol.

### Optogenetic clustering assays

*Drosophila* S2 cells stably expressing inducible mCherry-Cry2, 1-1722-mCherry-Cry2, or 266-384-mCherry-Cry2 were induced with 500  $\mu$ M CuSO<sub>4</sub> ~16 h prior to seeding them onto ConA-treated glass petri dishes. Since Spc105-B possesses MT binding activity, all chambers were treated with 25  $\mu$ M colchicine for 2 h prior to activation/imaging and then subjected to imaging for a maximum of 90 min at room temperature. A preactivation image (561 nm excitation) was taken prior to the first photoactivation event (488 nm excitation) while acquiring a 3-min time-lapse with mCherry images taken every 15 s. All replicates paired with a control (mCherry-Cry2) dish and an experimental dish (either 1-1722-mCherry-Cry2 or 266-384-mCherry-Cry2) each of which were subjected to identical activation/imaging protocols on the same day. To assess the expression levels necessary to support oligomerization, the average mCherry intensity of a 10  $\times$  10 pixel region prior to photoactivation was recorded and plotted for every cell that assembled discernible clusters during the activation/imaging protocol. To assess the effects of 1,6-HD on 266-384-mCherry-Cry2 drop assembly, cells were subjected to a 1-s photoactivation with 488 nm light, and shortly after drops had assembled, the media in the chamber was replaced with media supplemented with 3.5% 1,6-HD and time-lapse imaging of the mCherry channel continued.

### Fold enrichment assay

*Drosophila* S2 cells stably expressing inducible 266-384-mCherry-Cry2 were transiently transfected with plasmids encoding EGFP, EGFP-Bub1, Bub1-EGFP, Zw10-EGFP, sfGFP-Rod, or BubR1-EGFP each under low/endogenous-expression promoters using Effectene (Qiagen) according to the manufacturer's protocol. Within 1 wk of transfection, the cell lines were induced with 500  $\mu$ M CuSO<sub>4</sub> for ~16 h prior to seeding onto ConA-treated glass Petri dishes and subjecting them to the activation/imaging protocol outlined in Fig. 1 C. A confocal Z-section was then taken of each field at the conclusion of the 3-min photoactivation protocol. The time lapses were first assessed to determine if there was evident colocalization (obvious increase by eye of EGFP signal above the local background in the vicinity of 266-384 droplets), and the average EGFP intensity within a 10  $\times$  10 pixel region placed in a representative region of the cytoplasm was recorded from the preactivation image. This analysis was used to record the colocalization propensity as a function of expression levels of the cotransfected EGFP-tagged proteins shown in Figs. 2, 4, and S1. Only cells exhibiting evident colocalization were subjected to the fold-enrichment analysis with the exception of the control EGFP-expressing cells, which never exhibited colocalization but were analyzed as described below to establish a baseline for the assay and for comparison purposes. To measure the fold enrichment of 266-384-mCherry-Cry2 binding partners, color-combined planes from the confocal Z-sections were scanned to identify colocalized puncta. A 10  $\times$  10 pixel region was then centered on 266-384 droplets and transferred to the EGFP channel for analysis. The fold enrichment was defined as the integrated intensity of EGFP in the droplet divided by the integrated intensity of EGFP signal in the same

region positioned nearby in the cytoplasm lacking clusters. The fold enrichment of five droplets was measured per cell from different regions of the cell and from multiple planes.

### Immunofluorescence and quantification

For immunofluorescence, cells were seeded on ConA-coated coverslips, and after ~1 h, the coverslips were rinsed in 1 $\times$  BRB80 and fixed in either 100% methanol at -20°C (DHC and CID) or 10% paraformaldehyde in 1 $\times$  BRB80 (all other conditions) for 10 min. Cells were permeabilized in 1 $\times$  PBS + 1% Triton X-100 for 8 min, rinsed 3 $\times$  in 1 $\times$  PBS + 0.1% Triton X-100, and then blocked in 5% boiled donkey serum (Jackson ImmunoResearch) for 45 min. Cells were then incubated in the appropriate primary antibodies diluted in boiled donkey serum for 1 h. Next, the coverslips were washed 3 $\times$ , 5 min each, with PBS + 0.1% Triton X-100 and then incubated in the appropriate secondary antibodies (Jackson ImmunoResearch) diluted (1:200-1:500) in boiled donkey serum supplemented with 0.1 mg/ml DAPI for 45 min. The coverslips were washed 3 $\times$  (5 min each) in PBS + 0.1% Triton X-100 and then mounted in mounting media solution containing 90% glycerol, 20 mM Tris pH 8.0, and 0.5% N-propyl gallate. The following conditions and antibodies were used for immunofluorescence-based experiments. Cells were treated with 25  $\mu$ M colchicine for 1 h prior to fixation for DHC and Rod RNAi quantifications. Cells were treated for 1 h with 10  $\mu$ M MG132 for quantifying Rod and BubR1 at bioriented kinetochores. Primary antibodies were as follows: mouse anti-Dynein Heavy Chain (Cat #2C11-2; Developmental Studies Hybridoma Bank) at 1:200, rabbit anti-CID (Cat #ab10887; Abcam) at 1:200, rabbit anti-Rod serum (gift of M. Przewloka, University of Southampton, Southampton, UK) at 1:500, rabbit anti-BubR1 serum (gift of C. Sunkel and C. Conde, i3S, Porto, Portugal) at 1:500-1:1,000, sheep anti-Spc105 serum (gift of M. Przewloka) at 1:500-1:1,000, chicken anti-GFP (Cat #ab13970; Abcam) at 1:1,000, rabbit anti-Histone H3 phospho-S10 (Cat #ab5176; Abcam) at 1:5,000, and mouse anti-DM1 $\alpha$  (Cat #CP06; Sigma-Aldrich) at 1:1,000.

The region-in-region method (Ye and Maresca, 2018) was used to background correct and quantify integrated fluorescence intensities of the protein of interest ratioed to the indicated reference proteins at individual aligned/bioriented kinetochores from confocal Z-planes for the following experiments: RNAi knockdowns of DHC and Rod, BubR1 and Rod kinetochore levels in  $\Delta$ 267-383- and  $\Delta$ 267-383 chimera-expressing cells, and kinetochore levels in BubR1-depleted cells. In brief, region-in-a-region background correction was done by drawing concentric larger and smaller regions manually in MetaMorph around kinetochores in the protein of interest channel and subsequently transferred to the reference protein channel. The following equations were then used to calculate the total background corrected intensities of both channels: Background signal = (Integrated fluorescence intensity<sub>larger area</sub> - Integrated fluorescence intensity<sub>smaller area</sub>)/(Area<sub>larger</sub> - Area<sub>smaller</sub>). Total background corrected intensity = Integrated fluorescence intensity<sub>smaller area</sub> - (Background signal  $\times$  Smaller Area). To quantify the BubR1 RNAi depletion, the region-in-region method was used on maximum projections of confocal Z-sections. To

assess chromosome segregation phenotypes in Fig. 5 I, Spc105-EGFP (WT),  $\Delta$ 267–383-Spc105-EGFP,  $\Delta$ 267–383-Spc105-HsKI-GFP,  $\Delta$ 267–383-Spc105-FUS-HsKI-EGFP,  $\Delta$ 267–383-Spc105-DDX4-HsKI-EGFP, or  $\Delta$ 267–383-Spc105-GCN4-HsKI-EGFP were all transfected and selected to generate stable cell lines simultaneously. Once all the cell lines were stable ( $\sim$ 2 wk post-selection), as assessed by the percentage of EGFP-expressing cells in the population, all six cell lines were seeded on the same day onto different ConA-coated coverslips, fixed, and stained for phospho-H3, GFP, and  $\alpha$ -tubulin (DM1 $\alpha$ ). Cells in anaphase were scored as containing a merotelic when at least one evident (often stretched) GFP-positive kinetochore was observed in between the segregating chromosomes. A cell was defined as having a bridge when at least one chromosome was positioned between the segregating chromosomes but without any evident GFP-positive kinetochores.

### DNA constructs

All DNA constructs were made using isothermal (Gibson) cloning (Gibson et al., 2009) into a pMT-V5-His B vector using fragments amplified with the primers listed in Table S1. The mCherry-Cry2 constructs were made by amplifying mCherry-Cry2 DNA from Addgene plasmid #101221 (gift from Brangwynne, Princeton University, Princeton, NJ, USA). The PCR product was integrated between a 5' KpnI site and a 3' ApaI site downstream of the copper-inducible metallothionein promoter. The 1–400 Spc105-mCherry-Cry2 construct was made by amplifying Spc105-B corresponding to aa 1–400, which was previously amplified from cDNA (DGRC Clone IP22012). This fragment was flanked by KpnI sites and inserted into the mCherry-Cry2 vector described above, between the metallothionein promoter and the mCherry-Cry2 tag sequence. The 266–384-mCherry-Cry2 and 1–1722 Spc105-mCherry-Cry2 constructs were made by amplifying Spc105-B cDNA sequence corresponding to aa 266–384 (beginning with a start codon) and 1–1722, respectively, with flanking KpnI sites. The DNA was inserted into the pMT vector containing the mCherry-Cry2 tag. The Bub1-EGFP construct was made by amplifying Bub1 from Bub1 cDNA (LD22858). The PCR product was inserted into a pMT vector downstream of a Cenp-C promoter and upstream of EGFP via a 5' SpeI site and a 3' XbaI site. The N-terminal EGFP-Bub1 construct was made by amplifying Bub1 from cDNA (LD22858) and inserting the product, flanked by SacII sites, at the 3' end of GFP, downstream of a Cenp-C promoter. The BubR1-EGFP construct was made by amplifying the BubR1 promoter and gene from genomic DNA and inserting it into the pMT vector upstream of EGFP via a 5' XhoI site and a 3' NotI site. The ZW10-EGFP construct was made by amplifying ZW10 from cDNA CG9900-RB and inserting it downstream of the Spc105 promoter and upstream of EGFP using a 5' XhoI site and a 3' XbaI site in the pMT vector. sfGFP-Rod was a gift from Gohta Goshima (Nagoya University, Toba, Japan). The BubR1  $\Delta$ 734–872 construct was made by amplifying two fragments from the BubR1 cDNA (LD23835) corresponding to amino acids 1–733 and 873–1460 with a unique ApaI site engineered in between the two fragments. The fragments were designed to insert via a three-piece Gibson assembly reaction into a pMT vector downstream

of the CenpC promoter and upstream of the EGFP tag using a 5' SpeI and a 3' XbaI site. The Spc105  $\Delta$ 267–383 construct was made by amplifying the cDNA sequence corresponding to aa 1–266 and 384–1960 with overlapping primers engineered to position BamHI and BclI sites between the two fragments for the purpose of generating chimeras. The DNA fragments were inserted via a three-piece Gibson assembly reaction downstream of the Spc105 promoter and upstream of EGFP using a 5' XhoI site and a 3' XbaI site. The Spc105 HsKI chimera construct was made by inserting the human KI motif region amplified from the cDNA sequence corresponding to aa 201–249 into the BclI site between 1 and 266 Spc105 and 384 and 1960 Spc105. The Spc105 FUS- and DDX4-HsKI chimera constructs were made by inserting the sequences corresponding to the human FUS or DDX4 disordered regions in front of the KI motifs in the Spc105  $\Delta$ 267–383 HsKI construct into the BamHI site. The Spc105 GCN4-HsKI chimera construct was made by inserting a PCR fragment containing the sequence corresponding to the leucine zipper of GCN4 in front of the KI motifs in the Spc105  $\Delta$ 267–383 HsKI construct flanked by BamHI sites. The HsKI-FUS-mCherry-Cry2 construct was made by amplifying the DNA sequence of HsKI (DNA sequence corresponding to 196–276 amino acids)-FUS-mCherry-Cry2 from an existing plasmid in the Maresca lab. The resulting PCR product was ligated between 5' KpnI and 3' EcoRI sites of the pMT/V5-His vector (Invitrogen). All DNA constructs were confirmed by sequencing.

### RNA interference

Dynein template was made by amplifying  $\sim$ 600 base pairs from the dynein heavy chain cDNA (CG7507-RA). The T7 promoter sequence (5'-TAATACGACTCACTATAGGG-3') was followed by (5'-TGCCCAGGCGAATAGTTGGT-3') in the forward primer and (5'-CAAGTTTAAAGTATTTTCATT-3') in the reverse. Rod template was made by amplifying  $\sim$ 600 base pairs from an sfGFP-Rod construct (gift from Gohta Goshima). The T7 promoter sequence was followed by (5'-CGTCGCGAGGCATCTTCCAA-3') in the forward primer and (5'-GTGCTTTGATCTCCAGTGAT-3') in the reverse. The templates were then used to produce dsRNA using the T7 RiboMAX Express Large Scale RNA Production System (Promega) according to the manufacturer's protocol. Stable cells expressing inducible 1–1722-mCherry-Cry2 were treated with 20  $\mu$ g of dsRNA products (DHC or Rod) or control RNA in serum-free media for 1 h, followed by the addition of 1 ml of serum-containing media and 2- (DHC) or 4-d (Rod) incubations at 24°C before imaging.

### Statistical analyses

Statistical analyses reporting P values from two-tailed Student's *t* tests were done in Excel while P values generated with a randomization method were calculated using the PlotsofDifferences web tool at <https://huygens.science.uva.nl/PlotsOfDifferences> (Goedhart, 2019 Preprint). PlotsofDifferences does not rely on assumptions about the distribution of the data (normal versus non-normal) when calculating P values.

### Online supplemental material

Fig. S1 (related to Fig. 1, Fig. 2, and Fig. 3) shows the localization of Spc105-1–400 in mitotic cells and its ability to assemble

oligomers that undergo fusion when expressed at high concentrations. It also shows images and quantifications of the DHC, Rod, and BubR1 depletions as well as Bub3-EGFP localization and quantification relative to the 1–1722 oligomers, and an alignment of fly BubR1 versus human Bub1. Fig. S2 (related to Fig. 3, Fig. 4, and Fig. 5) shows a disorder plot of human KNL1 and a schematic of KNL1 highlighting important motifs and regions with a cat-GRANULE LLPS propensity score above 0. Fig. S2 also includes plots of BubR1 and Rod fluorescence quantifications from Fig. 3 and Fig. 5 shown together as well as a model for the possible linkage between KNL1 and RZZ in human cells and a model of how localization and enrichment of Spc105 at kinetochores could trigger its multimerization thereby increasing its avidity for client proteins including the RZZ complex. Table S1 lists the sequences of all the primers used in this study.

### Data availability

All the data underlying this study are available in the published article and its online supplemental material. A spreadsheet containing all the raw data for each plot is openly available in (McGory et al. underlyingdata\_repository) at <https://tinyurl.com/y6vumrhf>.

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Author contributions: T.J. Maresca: Conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, and writing (original draft, review, and editing). J.M. McGory: Formal analysis, investigation, methodology, validation, visualization, and writing (original draft, review and editing). V. Verma: Investigation, resources, and writing (review and editing). D.M. Barcelos: Investigation, resources, and writing (review and editing).

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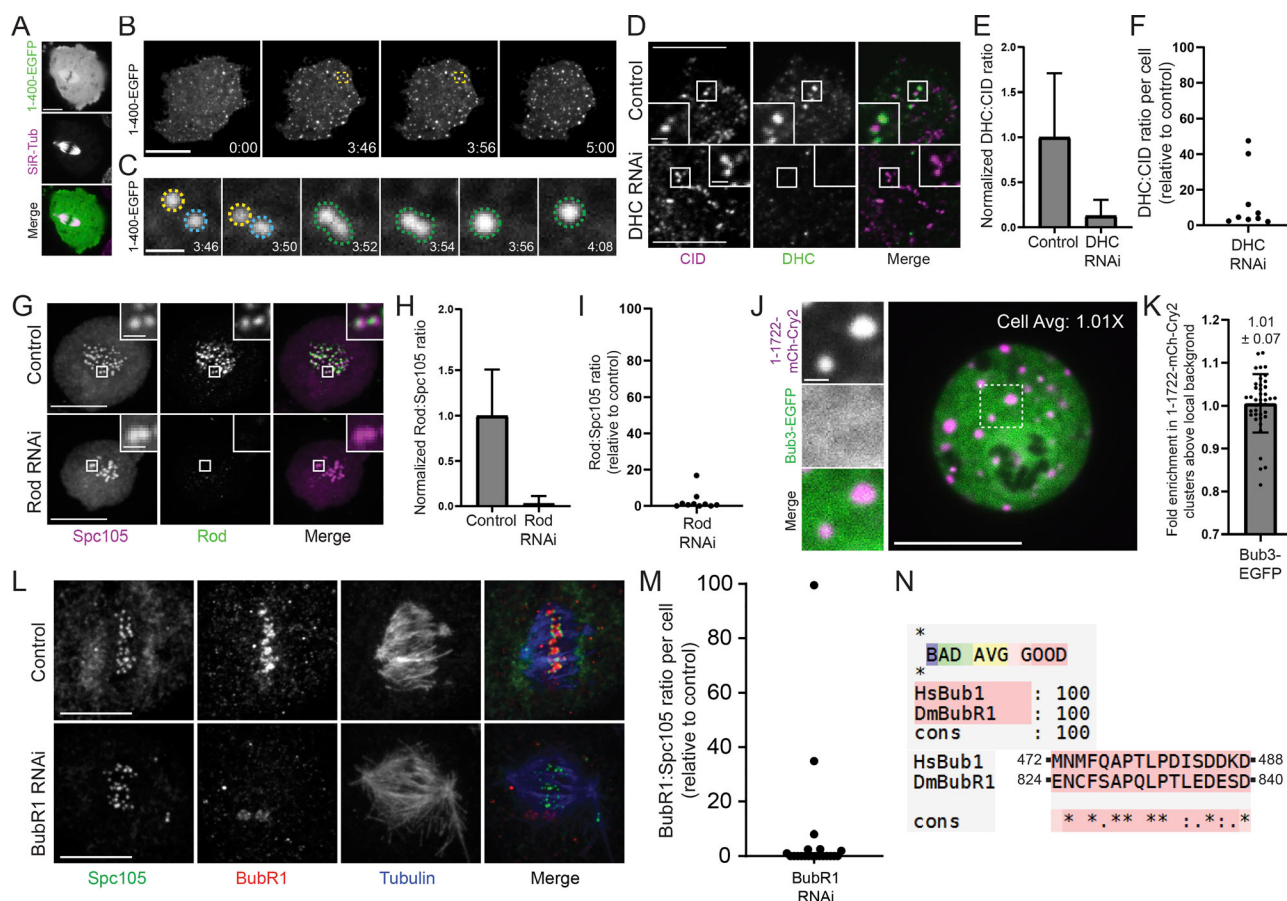
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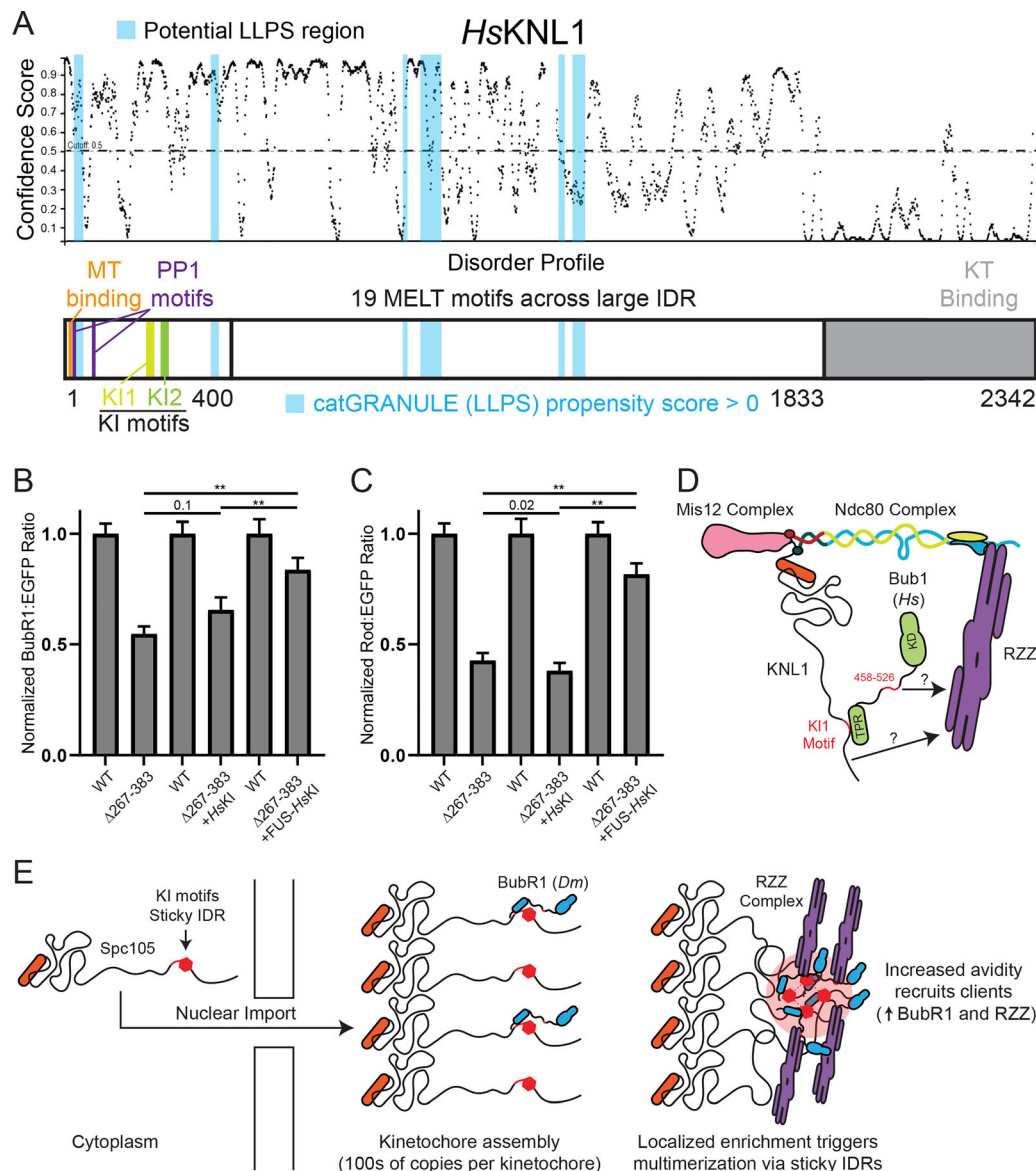
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# Supplemental material



**Figure S1. Characterization of Spc105-1-400-EGFP oligomerization, Bub3 exclusion from Spc105 clusters, and depletions of DHC, Rod, and BubR1.** **(A)** Representative maximum projection of confocal Z-sections of a mitotic cell expressing Spc105-1-400-EGFP. **(B and C)** Spinning-disc confocal time-lapse of cluster assembly in a cell with high expression of Spc105-1-400-EGFP. **(C)** Zoomed view of the boxed region (yellow dashed) in **B** showing a cluster fusion event. **(D)** Representative confocal Z-planes of control and DHC RNAi cells stained for CID (magenta) and DHC (green). **(E)** Quantification of DHC ratioed to CID fluorescence ( $n = 50$  kinetochores from 10 cells for each condition). **(F)** Cell averages (five kinetochores quantified per cell) of DHC to CID ratios for DHC dsRNA-treated cells relative to the mean DHC:CID ratio of control cells as a measure of the DHC RNAi penetrance. **(G)** Representative confocal Z-planes of control and Rod RNAi cells stained for Spc105 (magenta) and Rod (green). **(H)** Quantification of Rod ratioed to Spc105 fluorescence ( $n = 50$  kinetochores from 10 cells for each condition). **(I)** Cell averages (five kinetochores quantified per cell) of Rod to Spc105 ratios for Rod dsRNA-treated cells relative to the mean Rod:Spc105 ratio of control cells as a measure of the Rod RNAi penetrance. **(J)** Representative confocal Z-plane of a cell coexpressing Bub3-EGFP (green) and oligomerized clusters of Spc105-1-1722-mCherry-Cry2 (magenta). The boxed region is highlighted in the insets. The average fold enrichment for the cell (from five clusters) is indicated in the image. **(K)** Quantification of the fold enrichment of the signal of Bub3-EGFP in cluster regions relative to the local background signal ( $n = 40$  clusters from eight cells). **(L)** Representative maximum projections of confocal Z-sections of control and BubR1 dsRNA-treated fixed cells stained for Spc105 (green), BubR1 (red), and tubulin (blue). **(M)** Cell averages of BubR1:Spc105 ratios for BubR1 dsRNA-treated cells relative to the mean BubR1:Spc105 ratio of control cells as a measure of the BubR1 RNAi penetrance (control;  $n = 20$  cells from two replicates, BubR1 RNAi;  $n = 21$  cells from two replicates). **(N)** T-COFFEE alignment of human (Hs) Bub1 and *D. melanogaster* (Dm) BubR1 aa sequences identifying a motif in *Drosophila* BubR1 with homology to a region in human Bub1 implicated in RZZ recruitment to kinetochores. Boxed regions are highlighted in the insets. Bar plots report mean  $\pm$  SD. Scale bars, 10  $\mu$ m; 1  $\mu$ m (C and insets).



**Figure S2. The KI motifs and LLPS regions do not overlap in human KNL1; KNL1/Bub1 may be an RZZ receptor in human cells; and a model for how multimerization contributes to Spc105 function.** (A) Schematic of human (*Hs*) KNL1 highlighting the N-terminal MT-(orange) and PP1-binding (magenta) motifs, the KI motifs (shades of green), and the six regions across Spc105 with catGRANULE LLPS propensity scores above 0 (blue) overlaid onto a corresponding disorder plot of KNL1. (B) The BubR1 fluorescence quantifications from Figs. 3 and 5 shown together on the same plots (WT;  $n = 140$  kinetochores from 25 cells and two replicates,  $\Delta 267-383$ ;  $n = 135$  kinetochores from 24 cells and two replicates; WT;  $n = 150$  kinetochores from 30 cells and three replicates, HsKI;  $n = 145$  kinetochores from 29 cells and three replicates; WT;  $n = 150$  kinetochores from 30 cells and three replicates, FUS-HsKI;  $n = 150$  kinetochores from 30 cells and three replicates). (C) The Rod fluorescence quantifications from Figs. 3 and 5 shown together on the same plots (WT;  $n = 142$  kinetochores from 25 cells and two replicates,  $\Delta 267-383$ ;  $n = 140$  kinetochores from 25 cells and two replicates; WT;  $n = 150$  kinetochores from 30 cells and three replicates, HsKI;  $n = 150$  kinetochores from 30 cells and three replicates; WT;  $n = 155$  kinetochores from 31 cells and three replicates, FUS-HsKI;  $n = 160$  kinetochores from 32 cells and three replicates). Each replicate was done with a corresponding WT control and normalized to the control mean fluorescence intensity ratios for that experiment. (D) Model of how the pathway characterized using *D. melanogaster* cells in this study could function in human cells where Bub1, rather than BubR1, may be an RZZ receptor. (E) Model for how higher-order multimerization via sticky IDRs contributes to Spc105 function. Spc105 is too dilute in the cytoplasm to multimerize; however, the concentration of Spc105 upon kinetochores assembly stimulates multimerization thereby increasing its avidity for the RZZ complex. Bar plots report mean  $\pm$  SEM. The P values were generated by a randomization method (PlotsOfDifferences): \*\*\*P < 0.001.

**Video 1. Spinning disk confocal time-lapse of a mitotic cell expressing Spc105-1-400-mCherry-Cry2 during the photoactivation protocol.** Displayed times are min:s. Scale bar, 10  $\mu$ m. Relates to Fig. 1 D. Playback at 10 frames per second.

Video 2. **Spinning disk confocal time-lapse of cluster assembly (left) and fusion events (right) in a cell overexpressing Spc105-1-400-EGFP.** Boxed region indicates the area shown in the 4× zoomed video (right). Arrows mark fusion events in the movie on the right. Displayed times are min:s. Scale bars, 10 μm (left) and 1 μm (right). Relates to [Fig. S1, B and C](#). Playback at 15 frames per second.

Video 3. **Spinning disk confocal time-lapses of control (left) and DHC-depleted (right) mitotic cells expressing Spc105-1-1722-mCherry-Cry2 during the photoactivation protocol.** Displayed times are min:s. Scale bar, 10 μm. Relates to [Fig. 1, E and F](#). Playback at 10 frames per second.

Video 4. **Spinning disk confocal time-lapses of control (left) and Rod-depleted (right) mitotic cells expressing Spc105-1-1722-mCherry-Cry2 during the photoactivation protocol.** Displayed times are min:s. Scale bar, 10 μm. Relates to [Fig. 1, G and H](#). Playback at 15 frames per second.

Video 5. **Two-color spinning disk confocal time-lapse of Rod (green) colocalized with a 266–384-mCherry-Cry2 cluster (magenta) moving poleward.** Displayed times are min:s. Scale bar, 10 μm. Relates to [Fig. 2 M](#). Playback at 6 frames per second.

Video 6. **Spinning disk confocal time-lapses of representative merotelic attachments in anaphase cells expressing the Spc105 Δ267–383 deletion mutant.** Displayed times are min:s. Scale bars, 10 μm. Relates to [Fig. 3, L and M](#). Playback at 10 frames per second.

Video 7. **Spinning disk confocal time-lapses of cells expressing comparable levels of mCherry-Cry2 (upper left), 1–1722-mCherry-Cry2 (upper right), and 266–384-mCherry-Cry2 (lower left) during application of the same photoactivation protocol.** Displayed times are min:s. Scale bar, 10 μm. Relates to [Fig. 4, A and B](#). Playback at 6 frames per second.

Video 8. **Spinning disk confocal time-lapse of a representative 266–384-mCherry-Cry2 cluster fusion event.** Displayed times are min:s. Scale bar, 1 μm. Relates to [Fig. 4 C](#). Playback at 15 frames per second.

Video 9. **Spinning disk confocal time-lapse of 266–384-mCherry-Cry2 oligomerization followed by wash-in of 1,6-HD and rapid cluster disassembly.** Displayed times are min:s. Scale bar, 10 μm. Relates to [Fig. 4 D](#). Playback at 10 frames per second.

**Provided online is Table S1. Table S1 shows primers used in this study.**