Mutations in the Drosophila rough deal gene affecting RZZ kinetochore function

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Background. The RZZ complex, composed of the proteins Rough-Deal (Rod), Zw10 and Zwilch, plays a central role in the spindle assembly checkpoint (SAC), which assures proper sister chromatid segregation during mitosis. RZZ contributes to the regulation of the spindle assembly checkpoint by helping to recruit Mad1–Mad2 and the microtubule motor dynein to unattached kinetochores. It is an important component of the outer kinetochore and specifically the fibrous corona whose expansion is believed to facilitate microtubule capture. How RZZ carries out its diverse activities is only poorly understood. The C-terminal region of the Rod subunit is relatively well-conserved across metazoan phylogeny, but no function has been attributed to it.

Results. To explore the importance of the Rod_C domain in RZZ function in Drosophila, we generated a series of point mutations in a stretch of 200 residues within this domain and we report here their phenotypes. Several of the mutations profoundly disrupt recruitment of RZZ to kinetochores, including one in a temperature-sensitive manner, while still retaining the capacity to assemble into a complex with Zw10 and Zwilch. Others affect aspects of dynein activity or recruitment at the kinetochore.

Conclusions and Significance. These results suggest that the Rod_C domain participates in the protein interactions necessary for RZZ recruitment and functionality at kinetochores.

Introduction

The spindle assembly checkpoint (SAC) assures proper sister chromatid segregation during mitosis [Musacchio, 2015]. The SAC surveys the attachment state of kinetochores and inhibits anaphase onset until all kinetochores are stably attached to the spindle. The central checkpoint components Mad1 and Mad2 are recruited onto unattached kinetochores at the start of mitosis, where together they catalyse the formation of an ‘anaphase inhibitor’ of the E3 ubiquitin ligase called the anaphase promoting complex, thus blocking anaphase onset. Once a kinetochore has properly attached to spindle fibres, Mad1–Mad2 is depleted from the kinetochore and transport along the microtubules in a dynein-dependent process called variously shedding, streaming or stripping. This mechanism is believed to be one of the major pathways by which the checkpoint activity is turned off.

The RZZ complex, composed of the proteins Rough-Deal (Rod), Zw10 and Zwilch, plays a central role in the SAC [Karess, 2005]. It is an important
Mutations of RZZ Rod_C domain

component of the outer kinetochore and specifically the fibrous corona [Basto et al., 2004; Sacristan et al., 2018], whose expansion is believed to facilitate microtubule capture. RZZ contributes to the regulation of the SAC by helping to recruit Mad1–Mad2 and dynein along with its associated regulatory proteins to unattached kinetochores [Buffin et al., 2005; Chan et al., 2009; Gama et al., 2017; Griffis et al., 2007; Kops et al., 2005; Starr et al., 1998]. Thus RZZ is involved not only in activating the checkpoint (by recruiting Mad1–Mad2) but also in inactivating it, via dynein-dependent streaming of RZZ and Mad1–Mad2. RZZ and its cofactor Spindly are also implicated in regulating kinetochore-microtubule (K-MT) attachments [Cheerambathur et al., 2013; Gassmann et al., 2008, 2010].

RZZ has an apparent molecular weight of about 700–800 kDa and contains two copies of each of the three subunits, Rod, Zw10 and Zwilch, with the 240-kDa Rod subunit serving as the scaffold [Civril et al., 2010; Mosalaganti et al., 2017; Scaerou et al., 2001; Williams et al., 2003]. Secondary structure predictions of Rod [Civril et al., 2010] reveal an N-terminal region folding into a β-propeller and a long α-solenoid region extending to the C-terminus. A model based on structural analysis and low-resolution cryo-EM of recombinant mammalian RZZ [Mosalaganti et al., 2017] shows an elongated axially symmetrical protein with the two Rod subunits assembling in a head-to-tail manner that juxtaposes the β-propeller of one subunit with the C-terminal domain of the other (Figures 1A and 2A). A Zwilch subunit associates with each head-tail domain, and the two Zw10 subunits are symmetrically bound in the middle, in association with a domain of Rod with homology to Sec39.

How RZZ carries out its diverse activities is only poorly understood. Even the pathways leading to its recruitment and accumulation at unattached kinetochores are not yet clear. Recent reports suggest that interactions with Bub1 and Knl1/Zwint complex [Caldas et al., 2015; Silio et al., 2015; Zhang et al., 2015] contribute but are not sufficient for RZZ recruitment at kinetochores. Regarding the domains of RZZ involved in its kinetochore recruitment, Gama et al. [2017] found that a sub-complex containing only tagged Rod and Zw10 is recruitment-competent in C. elegans. In human cells a Rod construct lacking the N-terminal β-propeller fold can still bind Zw10 (though not Zwilch) and still target kinetochores, though it could no longer generate expanded kinetochores [Gama et al., 2017].

The Rod_C domain (pfam10493) that covers approximately 550 residues towards the end of the Rod α-solenoid, is common to all metazoan members of the Rod family [Marchler-Bauer et al., 2017]. It is notably absent from the structurally related, but functionally distinct, Nag proteins, implicated in Golgi trafficking [Civril et al., 2010]. However it displays no obvious structural similarity to other conserved protein domains, and no specific function has been attributed to it. We previously described the phenotype of a Drosophila mutant called rodZ3 [Defachelles et al., 2015a], caused by a single amino acid change in the Rod_C domain. This mutation greatly reduces the recruitment of RZZ and Mad1–Mad2 to kinetochores, and in addition has a semi-dominant effect on dynein-mediated streaming of RZZ during metaphase (Defachelles et al., 2015a).

To further explore the contribution of the Rod_C domain in RZZ function in Drosophila, we generated a series of additional point mutations within this domain. Some of these interfere with RZZ kinetochore recruitment and others affect dynein activity, while maintaining the RZZ complex. Thus the Rod_C domain appears to have an important function in RZZ activity.

Results

Interdependencies of the three RZZ components for assembly and kinetochore recruitment in Drosophila

Each of the three RZZ components had been previously shown to be necessary for kinetochore localisation of the two others, based largely on immunostaining of fixed material [Scaerou et al., 2001; Williams and Goldberg, 1994; Williams et al., 1992; Williams et al., 2003]. To complete these studies, we (i) assayed fluorescently tagged RZZ recruitment to kinetochores during mitosis in live neuroblasts; (ii) employed co-immunoprecipitation of RZZ from larval brain extracts; and (iii) assayed the integrity of the RZZ complex by Blue Native polyacrylamide gel electrophoresis (BN-PAGE) [Fiala et al., 2011; Swamy et al., 2006].

We had previously reported that in wild-type Drosophila neuroblasts expressing both GFP-Zw10
and RFP-Rod, the two proteins were simultaneously recruited to and shed from kinetochores [Defachelles et al., 2015b], suggesting that they had indeed assembled into a functional RZZ complex. During interphase, both GFP-Zw10 and RFP-Rod were diffusely cytoplasmic but only GFP-Zw10 labelled cytoplasmic Golgi elements ([Defachelles et al., 2015b], confirmed in Figure 1B, lower panels).

In neuroblasts from rod− null mutant larvae, GFP-Zw10 remained detectable during the interphase in Golgi elements, while, as expected [Scaerou et al., 2001], it did not accumulate on the spindle during mitosis (Figure 1B, left). These results suggest that the Golgi localisation of Zw10 does not depend on an intact RZZ. In addition, GFP-Zw10 was no longer excluded from the interphase nucleus in rod− cells, but appeared freely diffusible throughout the cell. In zw10− mutant cells, the levels of RFP-Rod were greatly reduced relative to wild type (Figure 1B, right). Little or no RFP-Rod fluorescence was detectable at kinetochores or cytoplasm in either mitotic or interphase cells, suggesting that Rod is unstable in the absence of Zw10 (see below).

We next asked if Rod was required for the formation of a stable complex containing Zwilch and Zw10. We found that whereas immunoprecipitating GFP-Zw10 from wild-type extracts co-precipitated Zwilch robustly, Zwilch was completely absent from anti-GFP-Zw10 precipitates of rod− mutant extracts (Figure 1C, right). This result is consistent with the model that Rod serves as a scaffold binding the two other subunits. In the absence of Rod, Zwilch (but not GFP-Zw10) seemed to be slightly destabilised in whole extracts (Figure 1C, left). Blots of whole extracts from zw10 mutant cells (Figure 1D, left) confirmed that RFP-Rod was significantly reduced in the absence of Zw10, consistent with the fluorescence results above. Because the level of RFP-Rod is so low in zw10 mutant cell extracts, we could not determine by co-immunoprecipitation whether Rod-Zwilch interactions occur in the absence of Zw10 (Figure 1D, right).
Figure 2 | Mutations in the Rod_C domain examined in this study

(A) The model of RZZ based on the study of Mosalaganti et al. [2017] and the different domains of the Rod protein [Civril et al., 2010; Marchler-Bauer et al., 2017].

(B) Clustal-W alignment of the C-terminal half of the Rod_C domains from four vertebrates (human, mouse, chicken, zebrafish) and four dipterans (Aedes aegypti, Drosophila grimshawi, Drosophila willistoni, Drosophila melanogaster). Regions of predicted alpha helix across the alignment are indicated by green bars. The new D. melanogaster Rod mutations described here (numbered 1–17) corresponding to one or two altered residues are delimited by the red boxes, and the corresponding substitutions are indicated below in blue. The dotted box corresponds to the rod<sup>Z3</sup> mutation [Defachelles et al., 2015a] (see also Table 1).
The native RZZ complex (represented schematically in Figure 1A) has an apparent molecular mass of about 700–800 kDa [Mosalaganti et al., 2017; Williams et al., 2003] consistent with an assembly corresponding to two copies each of Rod, Zw10 and Zwilch [Mosalaganti et al., 2017; Williams et al., 2003]. By employing BN-PAGE, a technique capable of detecting and analysing high molecular weight multi-subunit complexes [Fiala et al., 2011; Swamy et al., 2006], we found that RFP-Rod, Zw10 and Zwilch were indeed enriched in a band migrating approximately at 700 kDa (Figure 1E, top lanes). (A weak Zw10 signal was displaced towards lower molecular weights in extracts of RFP-Rod expressing brains. In extracts of GFP-Zw10-expressing rod− brains however, the majority of the GFP-Zw10 and Zwilch signals were displaced towards lower molecular weights (Figure 1E, bottom lanes). (A weak Zw10 signal persisting around 400–700 kDa suggests that Zw10 may also be part of large complexes other than RZZ, perhaps related to its function in vesicle trafficking.) In the zw10− extracts (Figure 1E middle lanes), RFP-Rod is undetectable by BN-PAGE. Recently, it was shown in vitro that mammalian Rod is unstable in the absence of Zw10 and Zwilch [Mosalaganti et al., 2017] and it was reported in C. elegans that Rod is also destabilised in the absence of Zwilch [Gama et al., 2017]. All these data suggest that the assembly of the entire complex is required for Rod stability and to a lesser extent for Zwilch stability but not for Zw10.

Taken together with other published studies, these results confirm that the recruitment of Rod, Zw10 and Zwilch to kinetochores is mutually interdependent in Drosophila and suggests further that the RZZ complex is already preassembled in the cytoplasm, presumably during interphase and that this assembly is a pre-requisite for its recruitment to kinetochores. It also implies that, if RFP-Rod signal is detectable in the cytoplasm, it is most likely in a stable complex with Zw10 and Zwilch.

**New mutations in the Rod_C domain**

To further explore the contribution of the Rod_C domain to the RZZ function, we generated a series of 16 additional point mutations (named M1–M17; there is no M10) in a stretch of 200 residues covering the second half of this region, in proximity to the lesion of previously described rodM5 (Figure 2, Table 1). In most cases, we selected residues or residue pairs that had been conservatively substituted or remained identical either within diptera or across metazoans (Figure 2B) and replaced them by residues with different properties, such as acidic for basic, or charged for hydrophobic (summarized in Table 1). The majority of the most highly conserved residues in the Rod_C domain reside within the predicted short α-helical stretches comprising the α-solenoid.

These mutations were introduced in a previously validated RFP-Rod transgenic construct expressed from its natural promoter that fully rescued a rod null mutant [Buffin et al., 2005].

Hereafter, the mutant alleles carried by these transgenes are referred to as RFP-rodM1, RFP-rodM2 etc. and the corresponding proteins are RFP-RodM1 etc. Transgenic fly stocks bearing these RFP-rod mutations were established and examined by genetics, biochemistry and cell biology. Each line was characterised for its ability to complement a homozygous null rod mutant, and for the ability of the RFP-RodM variant protein to accumulate on kinetochores, both in the presence of an endogenous wild-type copy of Rod, and in homozygous rod− null cells.

Of the 16 RFP-rod transgenic alleles examined (Table 1), four (M8, M12, M13 and M16) produced too little stable mutant RFP-RodM protein, even in a rodM background, to conclude anything about their functionality. They were therefore excluded from further study. Of the remaining mutant lines, RFP-rodM5 and RFP-rodM6 failed to rescue the lethality of rod− null mutant animals; RFP-rodM11 showed only weak complementation, allowing rod− flies to eclose, but generally not to survive, and failed to repair the characteristic rough eye phenotype. Three alleles (mutants M4, M7, M9) restored viability, but the resulting flies were sterile: the females laying eggs that failed to develop (maternal effect lethal), even when the zygotes were genetically rod+. RFP-rodM15 proved to be temperature sensitive: lethal when raised at 29°C but viable and fertile when raised at 18°C (see below). The remaining five transgenic alleles (RFP-rodM mutants M1, M2, M3, M14, M17) were able to fully complement the null rod homozygote, producing viable fertile adults of normal appearance. Overall, these results indicate that mutations in this part of the protein domain can impact RZZ function and are consistent with an important role for the Rod_C domain.
Table 1 | Summary of the examined mutations and their phenotypes

| Mutant Transgene | rescue of rod null lethality | recruitment to kinetochore in rod<sup>−</sup> | RZZ streaming |
|------------------|-----------------------------|---------------------------------|---------------|
|                  | rod<sup>+</sup> | rod<sup>−</sup> | rod<sup>+</sup> | rod<sup>−</sup> |
| M1               | E1798, Q1799 | AA | + | + | + |
| M2               | R1817 | D | + | + | + |
| M3               | S1823, S1824 | RR | + | + | + |
| M4               | P1826, K1827 | VD | ++ | ++ | ++ |
| M5               | F1833, L1834 | DD | − | + | + |
| M6               | C1880, V1881 | DD | − | + | + |
| M7               | Q1912, R1973 | AA | + MEL/st | + | +(Q) |
| M8<sup>∗</sup>   | C1928, L1929 | DD | − | low | ND |
| M9               | P1936, Q1937 | DD | + MEL/st | + | low (Q) |
| M11              | N1940, G1941 | DD | +/− | low (Q) | low (Q) |
| M12<sup>∗</sup>  | K1944, R1945 | AA | −/− | low | ND |
| M13<sup>∗</sup>  | A1977, W1978 | DD | −/− | low | ND |
| M14              | P1985 | D | + | + | + |
| M15              | C2011, P2012 | AA | + {18°} − {29°} | greatly reduced (29°)(Q) | + (29°) |
| M16<sup>∗</sup>  | Q2066, I2067 | AR | − | low | ND |
| M17              | G2074 | R | + | + | + |

MEL: Maternal Effect Lethal
st: sterile
<sup>∗</sup>These mutant variants were poorly expressed or unstable even in rod<sup>+</sup> background.

Some mutations in the Rod_C domain profoundly inhibit RZZ recruitment to kinetochores without affecting RZZ complex assembly

While all 12 RFP-Rod<sup>M</sup> mutant proteins examined were recruited to kinetochores in a rod<sup>+</sup> context, four of them (mutants M5, M6, M11 and M15) displayed reduced kinetochore localisation in rod<sup>−</sup> neuroblasts (Table 1), consistent with their reduced ability to rescue rod<sup>−</sup> homozygotes. RFP-Rod<sup>M</sup> alleles M5, M6 and M11 all replaced pairs of highly conserved uncharged residues within predicted α-helices with negatively charged aspartates (Figure 2B, Table 1). In a rod<sup>−</sup> background, the kinetochore recruitment of these proteins was very low in the majority of cells of both RFP-Rod<sup>M5</sup> and RFP-Rod<sup>M6</sup> but somewhat higher in the case of RFP-Rod<sup>M11</sup> (Figure 3A, left). This was confirmed by quantification of the RFP signal normalised to the signal from outer kinetochore component Spc25-GFP (Figure 3B). A brief (15 min) colchicine treatment releases the kinetochores from K-MTs and leads to a significant increase in kinetochore recruitment of M5, M6 and M11 in the wild-type (rod<sup>+</sup>) context (Figure 3A right, Figure 3B); however, in rod<sup>−</sup> cells kinetochore signals of these three RFP-Rod<sup>M</sup> variants remained essentially unchanged.

We next asked if RFP-Rod<sup>M5</sup> and RFP-Rod<sup>M6</sup> were competent for assembly into a complex with Zw10 and Zwilch. The levels of RFP-Rod<sup>M5</sup> were similar in rod<sup>+</sup> and rod<sup>−</sup> brain extracts, showing that the stability of the mutant protein is not affected (Figure 3C). Similar results were found for RFP-Rod<sup>M6</sup> (data not shown). This is consistent with the strong cytoplasmic RFP signal visible in interphase RFP-Rod<sup>M5</sup> and RFP-Rod<sup>M6</sup>; rod<sup>−</sup> cells. Anti-RFP immunoprecipitates of RFP-Rod<sup>M5</sup> co-immunoprecipitated Zwilch and Zw10 equally well from rod<sup>+</sup> and rod<sup>−</sup> tissue extracts, suggesting that the assembly of the RZZ complex is not affected (Figure 3C). BN-PAGE analysis confirmed that RFP-Rod<sup>M5</sup>, Zw10 and Zwilch co-migrated as part of a ~700-kDa complex in both rod<sup>+</sup> and rod<sup>−</sup> extracts (Figure 3D). Together these results indicate that both RFP-Rod<sup>M5</sup> and RFP-Rod<sup>M6</sup> profoundly interfere with the recruitment of RZZ to kinetochores but do not disrupt RZZ complex assembly.
Mutations of RZZ Rod_C domain

Figure 3 | See Legend on next page
The mutation in \( \text{RFP-rod}^{\text{M15}} \) changes to alanines two residues (C2011, P2012) that are almost perfectly conserved in Rod proteins between arthropods and vertebrates (Figure 2B). Uniquely among the examined mutations, \( \text{RFP-rod}^{\text{M15}} \) is thermosensitive in the \( \text{rod}^- \) background. At 18°C, it rescues an otherwise \( \text{rod} \) null homozygote, generating fertile adults with normal appearance, at the expected 1:2 ratio with heterozygotes. At 29°C there are very few surviving \( \text{rod} \) adults (less than 5% of expected). In \( \text{rod}^- \) larvae raised at 18°C and 25°C, \( \text{RFP-rod}^{\text{M15}} \) is recruited robustly to kinetochores, but at the restrictive temperature (29°C), there is a significant reduction of \( \text{RFP-rod}^{\text{M15}} \) kinetochore signal in \( \text{rod}^- \) but not \( \text{rod}^+ \) cells (Figures 4A and 4B). In addition, streaming of \( \text{RFP-rod}^{\text{M15}} \) along the spindle appeared to be reduced at 25°C in the \( \text{rod}^- \) background.

To quantitate this temperature-sensitive phenotype, we compared \( \text{RFP-rod}^{\text{M15}} \) signal normalised to Spc25-GFP after a brief (15 min) colchicine treatment, in \( \text{rod}^+ \) and \( \text{rod}^- \) cells from larvae raised at 18°C or after a 1 h incubation at 29°C prior to dissection and treatment. As a control, cells expressing the wild-type \( \text{RFP-rod}^+ \) construct were similarly treated and examined (Figures 4C and 4D). \( \text{RFP-rod}^{\text{M15}} \) kinetochore signal was profoundly reduced by the prior incubation at 29°C, in the \( \text{rod}^- \) background, but not in \( \text{rod}^+ \). The \( \text{RFP-rod}^+ \) displayed no such thermolabile effect in either background.

Immunoprecipitation of \( \text{RFP-rod}^{\text{M15}} \) from extracts of either \( \text{rod}^+ \) or \( \text{rod}^- \) mutant animals raised at 29°C coprecipitated with Zw10 and Zwilch equally well (Figure 4E). Thus the \( \text{RFP-rod}^{\text{M15}} \) protein is thermosensitive for its recruitment to kinetochores but not thermosensitive for RZZ assembly.

**Mutations affecting streaming**

Three examined Rod mutant proteins, \( \text{RFP-rod}^{\text{M7}} \), \( \text{RFP-rod}^{\text{M9}} \) and \( \text{RFP-rod}^{\text{M11}} \) were competent for at least moderate recruitment to kinetochores but had profound effects on the dynein-mediated streaming that normally carries coronal components, including RZZ, along the spindle fibres. \( \text{RFP-rod}^{\text{M7}} \) and \( \text{RFP-rod}^{\text{M9}} \) are both capable of substantially rescuing \( \text{rod}^- \) homozygotes to normal appearing but sterile adults. Consistent with this, both mutant proteins appeared well recruited to kinetochores in a \( \text{rod}^- \) background (Figure 5A). As mentioned above, \( \text{RFP-rod}^{\text{M11}} \) protein accumulated to a limited extent on kinetochores of \( \text{rod}^- \) larval neuroblasts (Figures 3A, 3B, 5A). Strikingly, however, each of these three \( \text{RFP-rod} \) variants remained largely localised at kinetochores in \( \text{rod}^- \) cells, rather...
Mutations of RZZ Rod_C domain

Figure 4 | See Legend on next page

A

B

C

D

E

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than being distributed along the spindle, indicating reduced capacity to stream (Figure 5A and Supplemental Movie S1). To quantitate the degree of streaming, we classified cells into three categories: no streaming (RFP is found only on kinetochores), low streaming (most of the RFP signal is on kinetochores) and high streaming (RFP signal is present on kinetochores, along the spindle and at the poles). This analysis confirmed the significant reduction in streaming of these RFP-RodM proteins (Figure 5C). (Note that since streaming is a transient process, only occurring during part of metaphase, it is expected that a majority of mitotic cells will show no streaming). It also revealed a clear reduction in streaming of RFP-RodM9 (see Supplemental Movie S1) and RFP-RodM11 (but not RFP-RodM7) even in the rod+ background, that is, in the presence of endogenous wild-type Rod protein, suggesting that these mutant proteins exert a dominant negative effect on streaming.

Because streaming is a dynein-dependent process, and since RZZ is involved in recruiting dynein to kinetochores, we asked if the lack of streaming could be correlated with loss of dynein from these kinetochores, by monitoring the GFP-tagged dynein subunit DLIC, after a brief colchicine treatment (Figure 5B). GFP-DLIC was in fact still present on kinetochores in cells expressing RFP-RodM7 or RFP-RodM9 in both rod+ and rod− backgrounds, as well as in rod+ cells expressing RFP-RodM11, but was largely absent from RFP-RodM11; rod− kinetochores. Thus mutant Rod proteins can interfere with streaming even though dynein is still present at kinetochores, and in the case of RFP-RodM9 and RFP-RodM11 even in the presence of a wild-type copy of Rod. However, RFP-RodM11 appears to have a reduced capacity to recruit dynein on its own.

**Discussion**

We have generated mutations altering several conserved amino acids in the Rod_C domain and have found that some of these mutations profoundly impair the recruitment of RZZ to kinetochores and that others interfere with the dynein-mediated streaming of RZZ upon MT attachment.

The earliest observations of RZZ behaviour during mitosis led to the proposal that it was a major component of the fibrous corona [Basto et al., 2004; Williams et al., 1996], an expanded outer domain of unattached kinetochores that promotes microtubule capture. Features of the modelled structure of RZZ [Mosalaganti et al., 2017] display similarities to other proteins that can self-assemble into networks, such as COP1 and Clathrin, and recently several groups have reported direct cell biological and biochemical evidence for the participation of RZZ and its interacting partner Spindly in the assembly of the corona in vivo and filamentous structures in vitro ([Pereira et al., 2018; Rodriguez-Rodriguez et al., 2018; Sacristan et al., 2018], reviewed in [Suzuki and Varma, 2018]). These reports have focused particular attention on the N-terminal domain of Rod, which appears to be necessary for kinetochore expansion. The β-propeller is phosphorylated by
Mutations of RZZ Rod_C domain

Figure 5 | See Legend on next page

A

| Nomarski | RFP-Rod\textsuperscript{M7} | Nomarski | RFP-Rod\textsuperscript{M7} | GFP-DLIC |
|----------|-----------------|----------|-----------------|---------|
| \textit{rod\textsuperscript{+}} | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| \textit{rod\textsuperscript{-}} | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |

B

| Nomarski | RFP-Rod\textsuperscript{M9} | Nomarski | RFP-Rod\textsuperscript{M9} | GFP-DLIC |
|----------|-----------------|----------|-----------------|---------|
| \textit{rod\textsuperscript{+}} | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) |
| \textit{rod\textsuperscript{-}} | ![Image](image13) | ![Image](image14) | ![Image](image15) | ![Image](image16) |

C

![Bar chart](chart.png)

| Rod status | ns | ** | *** | **** |
|------------|----|----|-----|------|
| \textit{rod\textsuperscript{+}} | ![Bar](bar1) | ![Bar](bar2) | ![Bar](bar3) | ![Bar](bar4) |
| \textit{rod\textsuperscript{-}} | ![Bar](bar5) | ![Bar](bar6) | ![Bar](bar7) | ![Bar](bar8) |

Legend:
- No streaming
- Low
- High
sensitive RodM15, which seems to affect streaming recruitment levels. Together with the temperature-binding. physically close to the regions implicated in Spindly the RZZ subunits, the Rod_C domain is presumably cargo. Again, because of the head-to-tail assembly of the ondynein activity. Alternatively, it may be simply interfering with the ability of dynein to carry RZZ as cargo. Again, because of the head-to-tail assembly of the RZZ subunits, the Rod_C domain is presumably physically close to the regions implicated in Spindly binding.

Several of mutations described here (M7, M9 and M11) severely compromise dynein-mediated streaming, similar to the previously described classical rodZ3 point mutation of the Rod_C domain [Defachelles et al., 2015a]. And like RodZ3, RodM9 and RodM11 both display a certain ability to dominantly interfere with streaming even in the presence of wild-type Rod, presumably a consequence of the multi-subunit nature of RZZ at kinetochores, that would incorporate copies of both mutant and wild-type Rod proteins. Moreover, RodM11 seems to reduce dynein recruitment levels. Together with the temperature-sensitive RodM15, which seems to affect streaming at the semi-permissive temperature 25°C, these five mutations span about 100 amino acids of the Rod_C domain, suggesting that the integrity of this region at least is important for the proper maintenance of dynein-mediated transport of RZZ.

The interaction of dynein with RZZ is believed to be mediated by Spindly, which has been shown to bind the N-terminal domain of Rod. The fact that mutations in the Rod_C domain interfere with streaming may indicate an influence of this domain on dynein activity. Alternatively, it may be simply interfering with the ability of dynein to carry RZZ as cargo. Again, because of the head-to-tail assembly of the RZZ subunits, the Rod_C domain is presumably physically close to the regions implicated in Spindly binding.

In the RZZ structure proposed by Mosalaganti et al. [2017], the Rod_C domain would encompass a significant part of each end of the protein complex and is predicted to be in close proximity to and possibly interact with both the N-terminal β-propeller of the second Rod subunit and the Zwilch subunit. However, on the mutants where we performed co-IP tests, the altered Rod proteins co-precipitated with Zw10 and Zwilch even in the absence of wild-type Rod, and by BN-PAGE analysis, the RodM5 variant assembled into a complex of similar size as wild-type RZZ. Thus the failure of mutants M5, M6 and M15 (at the restrictive temperature) to accumulate on kinetochores in rod− cells is attributable directly to the mutation and not to failure to assemble into a stable complex. Although we did not look at every mutation in this way, it seems likely that all the RodM variants capable of recruitment to kinetochores when mixed with endogenous wild-type Rod (i.e. in the rod+ genetic background), necessarily retain the ability to assemble into an intact RZZ complex. We speculate that the mutant RFP-RodM proteins that were poorly expressed even in a rod+ background (alleles M8, M12, M13 and M16) may in fact be unstable precisely because they failed to assemble into RZZ complexes.

It is notable that the RFP-RodM variants presented in Figures 3 and 4 displayed higher kinetochore signal in the rod+ background than in rod−, whereas the wild-type RFP-Rod+ signal was reduced in the rod+ background. This suggests that the ability of the mutant RMZ to accumulate on kinetochores is enhanced by association with the unlabelled endogenous Rod+. In contrast, RFP-Rod+ would appear to be competing with the unlabelled Rod+ for kinetochore accumulation. These differences in behaviour support an interpretation that the lesions in the RodM
variants described here are affecting RZZ multimeric assembly into the corona.

The 16 mutations we generated were mostly designed to disrupt the charge or polarity at relatively conserved positions of the Rod_C domain. In some cases, the altered residues had been conserved since the divergence of arthropods and mammals (Figure 2B). Nevertheless, several of these mutations (M2, M3, M14, M17) functioned sufficiently well to substantially or fully rescue rod− null flies. The non-complementing mutations were nearly all within predicted α-helical sequences, which in general had conserved polarity features across phylogeny. Mutations M5 and M6, which eliminated nearly all RZZ recruitment, substituted highly conserved hydrophobic residues with glutamates. The cysteine–proline pair replaced by alanines in the mutant M15 is one of the few nearly invariant regions of the Rod_C domain that is predicted to fold into a loop between helices. The thermosensitivity of RMM15ZZ activity suggests that this loop may be intimately involved in the assembly of RZZ at kinetochores.

The mutations in this report argue that the Rod_C domain makes an important contribution to RZZ recruitment and functionality at the kinetochore. The RFP-rodM alleles failing to complement the rod null all show partial or near-total loss of RZZ from kinetochores, but were competent to assemble with Zw10 and Zwilch. The altered amino acids of these mutants are thus prime candidates for surfaces contributing to the initial recruitment or the higher order assembly of the coronal network. In particular, the fact that colchicine treatment did not detectably increase kinetochore levels of RFP-RodM5, M6 or M11 (Figures 3A and 3B) suggests that they are incapable of supporting kinetochore expansion.

Materials and methods

Fly stocks

The Drosophila strains were raised on standard medium composition and maintained at 18, 25 or 29°C depending on the experiment. Mutations (denoted M1–M17) were generated in vitro in conserved amino acids in the Rod_C domain. DNA fragments encoding the Rod mutations were cloned into a previously validated pCasper-4-RFP-Rod vector expressing RFP-Rod under the control of the natural endogenous rod promoter [Buffin et al. 2005]. P-mediated fly transformation was performed by BestGene (Chino Hills, CA). The P[RFP-RodM] transgenes were then crossed into a stock of rodAG1/TM6. rodAG1 is a null allele, bearing single nucleotide modification that changes the triplet CAA (Q) into the TAA stop codon at triplet 1004, truncating the open reading frame. In the text, ‘rod−’ refers to the heterozygous rodAG1/TM6 genotype. Except for Mutants 1 and 13 (for which only a single line was obtained), each mutant construct was represented by at least two independent transgenic inserts. When the transgene failed to complement rod−, we tested other inserts of the same construct to exclude possible position-specific expression effects as the source of the non-complementation. Other stocks used include flies expressing RFP-RodM [Buffin et al. 2005], GFP-DLIC (gift from Jordan Raff, Oxford, UK), Spc25-GFP [Schittenhelm et al., 2007] (gifts from Christian Lehner, University of Zurich, Zurich, Switzerland), GFP-Zw10 [Defachelles et al., 2015b].

Quantitation of ROD recruitment to kinetochores

For each mutant genotype, a stock containing P[RFP-RodM] rodAG1/TM6, Tb was crossed to P[Spc-25-GFP]Cy; rodAG1/TM6, Tb. Yellow non-Tb larvae carried one copy of RFP-RodM, one copy of the kinetochore marker Spc25-GFP and were homozygous for rodAG1. Sibling Tb flies were genotypically rod−.

Third instar larval brains were dissected in PBS and either mounted directly under a cover slip or after incubation in colchicine (10−4 M) for 15 min. The brief treatment was intended to detach the microtubules from kinetochores and thus promote RZZ accumulation. Fluorescence acquisition was performed with an Olympus IX71 inverted microscope equipped for video acquisition (CellR imaging system, Xenon lamp, Hammamatsu camera), using 60× objective, NA 1.3 (see Defachelles et al. [2015a] for other details).

Live larval brains were photographed in a single optical plane. A region encompassing all the in-plane kinetochores (visible by Spc25-RFP signal) was defined, and Red and Green pixel intensities were recorded. A corresponding background signal was also acquired. The quantitation of kinetochore signal (Figures 3B and 4B) compares the recruitment of RFP-RodM in the presence (rod+) and absence (rod−) of endogenous Rod, in cells treated, or not, with colchicine. This ‘internal’ control is preferable to a comparison with a wild-type RFP-Rod recruitment, in a separately derived transgenic fly line. It also demonstrates the capacity of the transgenic construct to be incorporated into a functional RZZ complex, even when it is not functional on its own. Recruitment of a control RFP-Rod+ in rod+ and rod− backgrounds was also measured.

Antibodies

For Western blot analysis, the following antibodies were used: rabbit anti-RFP (Abcam) and rabbit anti-Rod were diluted at 1/1000°, rabbit anti-Zw10 [Williams et al.; 1992] was used at 1/2000° and rabbit anti-Zwilch at 1/500°. The Rabbit anti-Rod used for western blot detects many unspecific bands and was only useful for immunoblotting semi-purified samples, such as following immunoprecipitation with RFP-Trap or GFP-Trap magnetic beads. Secondary antibodies, anti-rabbit horseradish peroxidase (HRP) (Sigma Chemical Co., Saint-Quentin Fallavier, France) and anti-mouse (HRP) (Promega, Charbonnière, France) were used as described by the manufacturers.
Immunoprecipitation
Third instar larval brains were dissected in PBS and lysed in lysis buffer (ChromoTek) supplemented with 0.5% NP40 and complete anti-protease inhibitors (Roche Diagnostics) using a Dounce homogenizer. The lysates were precleared by centrifugation (2 × 20 min at 13,000 × g). For co-immunoprecipitation, 200 µg of precleared protein extract (corresponding to 120–150 brains) was incubated with 5 µL of GFP-Trap or RFP-Trap magnetic agarose beads (ChromoTek) for 1 h at 4°C on a rotating wheel. Beads were separated magnetically, and supernatant was discarded. Beads were then resuspended in 30 µL 2× SDS sample buffer and boiled 10 min at 95°C to dissociate immunocomplexes from GFP-trap or RFP-trap beads. The supernatants were then analysed by PAGE.

Blue Native PAGE electrophoresis
The experiment was performed as described by Fiala et al. [2011] and Swamy et al. [2006] and according to the user guide ‘NativePAGENovexBis-Tris Gel System’ from Life Technologies. Samples were prepared as follows. Third instar larval brains (120–150 brains, about 200 µg) were dissected in PBS and lysed in native PAGE sample buffer 1× (Life Technologies) supplemented with 0.5% NP40 and anti-protease inhibitors (complete from Roche Diagnostics) using a Dounce homogenizer, and the lysates were precleared by centrifugation (2 × 20 min at 13,000 × g). Samples were loaded on precast native gels (native PAGE Novex 3–12% Bis-Tris Gels, Invitrogen). Samples migrated at 150 V during 120 min at room temperature using the buffers supplied. The lane of the native gel containing the sample of interest was excised and denatured in NuPAGE LDS Sample Buffer (1×) and NuPAGE Sample Reducing (1×) (Life Technologies). The strip was loaded onto a second dimension SDS-PAGE gel (6%), and electrophoresis was performed by standard protocols.

The Coomasie staining of the native gels was performed using the PAGE Blue Staining Solution (Thermo Fisher Scientific).

Author contribution
Concept and design of experiments: AM and RK. Experimental work: AM. Data analysis, interpretation, writing: AM and RK.

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Conflict of interest
The authors have declared no conflict of interest.

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