A maternal effect rough deal mutation suggesting multiple pathways regulating Drosophila RZZ kinetochore recruitment.

Lénaïg Défachelles¹*, Sarah G. Hainline²*, Alexandra Menant¹, Laura A. Lee², and Roger E. Karess¹†

¹Equipe Labellisée Ligue Contre le Cancer, CNRS, Institut Jacques Monod, UMR7592, Université Paris Diderot, Sorbonne Paris Cité, Paris Cedex 13 75205 FRANCE

²Department of Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN 37232-8240, USA

*Equal Contributions

†Corresponding author karess.roger@ijm.univ-paris-diderot.fr

RUNNING TITLE: An RZZ maternal-effect mutation

KEY WORDS: RZZ, Polar Body, Spindle Assembly Checkpoint, Mad1, Syncytial Embryo, Kinetochore
**ABSTRACT**

Proper kinetochore recruitment and regulation of Dynein and the Mad1-Mad2 complex requires the Rod-Zw10-Zwilch (RZZ) complex. We describe rod<sup>z3</sup>, a maternal-effect *Drosophila* mutation changing a single residue in the Rough Deal (Rod) subunit of RZZ. Although R<sup>z3</sup>ZZ complex is present in early syncytial stage embryos laid by homozygous rod<sup>z3</sup> mothers, it is not recruited to kinetochores. Consequently, the embryos have no spindle assembly checkpoint (SAC), and syncytial mitoses are profoundly perturbed. The polar body (residual meiotic products) cannot remain in its SAC-dependent metaphase-like state, and decondenses into chromatin. In neuroblasts of homozygous rod<sup>z3</sup> larvae, R<sup>z3</sup>ZZ recruitment is only partially reduced, the SAC is functional and mitosis is relatively normal. R<sup>z3</sup>ZZ nevertheless behaves abnormally: it does not further accumulate on kinetochores when microtubules are depolymerized; it reduces the rate of Mad1 recruitment; and it dominantly interferes with the dynein-mediated streaming of RZZ from attached kinetochores. These results suggest that the mutated residue of rod<sup>z3</sup> is required for normal RZZ kinetochore recruitment and function and moreover that the RZZ recruitment pathway may differ in syncytial stage embryos and post-embryonic somatic cells.
INTRODUCTION

Cell division is driven by oscillations in the activity of Cdks and their associations with regulatory cyclin subunits. Mitotic entry is driven by high Cdk1-Cyclin B activity, and mitotic exit is dependent on mechanisms that inactivate this complex and dephosphorylate its targets (reviewed in O’Farrell, 2001; Lindqvist et al., 2009; Mochida and Hunt, 2012). The initiation of mitotic exit at the metaphase-anaphase transition is under the control of the Spindle Assembly Checkpoint (SAC), which indirectly regulates the proteolytic degradation of Cyclin B by controlling its ubiquitylation by the E3 ubiquitin ligase known as the Anaphase Promoting Complex (APC/C) (reviewed in Musacchio, 2011; Lara-Gonzalez et al., 2012; Jia et al., 2013). Among the key SAC proteins in this process are Mad1 and Mad2, which form a complex at unattached kinetochores and help generate an inhibitor of the APC/C. Upon proper kinetochore attachment to the spindle, several SAC components, including Mad1, Mad2 and the Rough Deal-Zw10-Zwilch (RZZ) complex are removed from the kinetochore by a dynein-dependent process (Howell et al., 2000; Hoffman et al., 2001; Howell et al., 2001; Wojcik et al., 2001; Basto et al., 2004), thus dismantling the SAC apparatus and inactivating the source of the APC/C inhibitor.

RZZ is an important component of the outer kinetochore (Karess, 2005). It is a determinant of Mad1-Mad2 kinetochore levels, as it promotes both Mad1-Mad2 recruitment (Buffin et al., 2005; Kops et al., 2005) and recruitment of dynein and its associated regulatory proteins (Starr et al., 1998; Griffis et al., 2007; Chan et al., 2009). RZZ is also involved in regulating kinetochore-microtubule (K-MT) attachments (Gassmann et al., 2008; Gassmann et al., 2010; Cheerambathur et al., 2013), although how it performs these functions, and how it is itself recruited to kinetochores is poorly understood.

In the early division cycles of *Drosophila* embryogenesis, nuclei divide synchronously in a syncytium, using a streamlined cell cycle in which S and M-phase oscillate, driven by stockpiles of maternal mRNAs and proteins (reviewed in (Foe et al., 1993; Lee and Orr-Weaver, 2003)). During these syncytial cycles, the unfertilized nuclear products of female meiosis remain in M-phase at the periphery of the embryo. These nuclear products, referred to as polar bodies (PBs), coalesce to form a “starburst” of condensed chromosomes and maintain this state until they are culled from the embryo during cellularization.

Defective PB M-phase maintenance has been reported in mutants with either reduced Cdk1 activity or misregulated levels of Cyclin B. For example, the Pan Gu (PNG) kinase
complex promotes the translation of Cyclin B during female meiosis and syncytial embryogenesis. Mutant females of the \textit{png} kinase or its regulatory subunits lay eggs with abnormally low Cyclin B levels. As a result, PBs do not remain in M-phase but instead reenter interphase and undergo unregulated rounds of DNA replication, resulting in polyploid interphase-like PBs (Fenger et al., 2000; Lee et al., 2001). The SAC, which also regulates levels of Cyclin B, is another proposed mechanism by which PB M-phase is maintained. Indeed, eggs laid by females homozygous for certain viable mutations of the SAC genes \textit{mps1} and \textit{bubR1} present similar PB defects (Fischer et al., 2004; Perez-Mongiovi et al., 2005).

In a screen for new cell-cycle regulators specifically required during the syncytial stages of embryogenesis, we identified a novel, maternal-effect allele of the \textit{rough deal (rod)} gene, called here \textit{rod}\textsuperscript{Z3}. This mutation only slightly perturbs RZZ activity in homozygotes, which are viable and have relatively normal mitoses. However, in eggs laid by \textit{rod}\textsuperscript{Z3} mothers, RZZ is incapable of kinetochore recruitment. As a result, such embryos present decondensed PBs, profoundly perturbed syncytial mitoses, and a non-functional SAC. The phenotype of this mutation suggests that aspects of kinetochore assembly may differ in maternal and zygotic \textit{Drosophila} mitosis.
MATERIALS AND METHODS

Drosophila Stocks

Flies were maintained at 25°C using standard techniques. A y w stock was used as wild type for embryo experiments. The stock bw; rod^{23} st / TM6B was a gift from Charles Zuker (Columbia University). The rod^{23} allele had designation Z3-0733 in the Zuker stock collection. We shortened and superscripted it to indicate that it is an allele of rod. The following mutations and transgenes have been previously described: rod null alleles rod^{AG1}, rod^{X2} and transgene rod^*[C3L9] (Scaerou et al., 1999), GFP-Rod (Basto et al., 2004), RFP-Rod (Buffin et al., 2005), the null allele mad1^{b}, transgene Mad1-GFP, and Cherry-Mad1 (Emre et al., 2011), the null allele mad2^{p} (Buffin et al., 2007), the 2XCyclin B stocks, and transgenes Spc25-GFP and Spc25-RFP (Schittenhelm et al., 2007) (gifts from Christian Lehner, University of Zurich), the shtd^{b} stock (from the Bloomington stock center), transgene GFP-DLIC2 (gift from Jordan Raff, Oxford). The GFP-Zw10 construct will be described elsewhere. Transgenic lines Cherry-Rod, and GFP-Rod^{Z3} were obtained by P-element transposition. The transgenes correspond to genomic sequence (including the promoter region) originally described in (Basto et al., 2004). Fly transformation was performed by BestGene (Chino Hills, CA).

Embryo Immunostaining and Colchicine Treatment

0-2 hour embryos (unless otherwise noted) were collected as described (Rothwell and Sullivan, 2000). For Tubulin, Centrosomin, and DNA staining, embryos were dechorionated in 50% bleach, and fixed/devitellinized by shaking in a 1:1 mixture of methanol/heptane. For visualizing RFP/GFP fluorescence during cortical divisions, 0-30 minute embryos were collected, aged for 1 h 15 min, dechorionated and fixed/devitellinized in methanol/heptane. For PH3 immunostaining, embryos were dechorionated, fixed in a 1:1 mixture of 4% formaldehyde (in PBS) and heptane, and devitellinized as described above. For colchicine treatment, 0-1 hour embryos were collected, dechorionated, and incubated in 1:1 mixture of 250 μM colchicine (in PBS) and heptane for 30 minutes. Treated embryos were then formaldehyde fixed and devitellinized as above. SAC response in embryos was determined by comparing the percentage of mitotic embryos following a 30 min incubation with or without colchicine. “Mitotic” embryos were defined as those having at least 50% PH3-positive nuclei. For immunostaining, fixed embryos were rehydrated in PBS and incubated in 1 mg/ml RNAseA for 1 hour, then incubated in primary antibody overnight at 4°C, washed,
then incubated in Cy2 and/or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch) for 3 hours at RT. Embryos were stained with propidium iodide and mounted in clearing solution (Fenger et al., 2000) or in Prolong-Gold with DAPI (Life Technologies). Embryos were visualized with a Nikon Eclipse 80i microscope equipped with a CoolSNAP ES camera (Photometrics). Statistical analysis of imaging quantifications was performed using the Fisher Exact test. Primary antibodies used were anti-α-Tubulin YL1/2 (AbD Serotec) or anti-Tubulin DM1α (Sigma-Aldrich), anti-Centrosomin (gift from W. Theurkauf, University of Massachusetts Medical School), anti-PH3 (06-570, Millipore), anti-Rod (Scaerou et al., 1999), anti-Zw10, and anti-Zwilch (gifts from M. Goldberg, Cornell University).

**Embryo Immunoblotting**

0-1 hour embryos were homogenized in non-denaturing lysis buffer (NDLB) (50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100). For immunoprecipitations, 500 μg of embryo lysate was incubated with 5 μl anti-Rod serum (Scaerou et al., 1999) or normal rabbit serum overnight at 4°C. Lysates were then incubated with 25 μl of Protein G Sepharose beads (GE Healthcare) for 3 hours at 4°C. Beads were pelleted, washed three times in NDLB, and boiled in 30 μl 6X Sample Buffer. The resulting supernatant and 20 μg of input lysate were analyzed by SDS-PAGE and by immunoblotting using standard techniques. HRP-conjugated secondary antibodies and chemiluminescence were used to detect primary antibodies.

For sucrose gradient analysis, 2 mg of embryo lysate was layered on top of a sucrose gradient column of 5%, 10%, 20%, and 30% sucrose, then centrifuged in a L8-70M ultracentrifuge (Beckman) with a SW55Ti rotor for 4 hours at 46,000 rpm and 4°C. 19 fractions were collected and 8% of each fraction was analyzed by SDS-PAGE and immunoblotting.

**Neuroblast Cytology**

Immunostaining used the method described previously (Williams et al., 2007). *Drosophila* third instar larval brains expressing Spc25-RFP were dissected in 0.7% NaCl and treated 15 minutes in 10^{-4} M colchicine. After formaldehyde fixation and methanol dehydration, brains were immunostained with a mouse monoclonal anti-RFP (Abcam, ab658556) 1:500 and rabbit anti-Rod 1:1000. Secondary antibodies were Alexa-594 anti-
mouse and Alexa-488 anti-rabbit 1:500 (Life Technologies). Images were acquired with a
Zeiss Axio Imager.Z1 equipped with a Plan-Apo 100X NA 1.4 oil objective and an apotome
module. Acquisition times were 400 ms for Alexa 594 and 300 ms for Alexa 488 and images
were stacks of 5 planes at 1 μm intervals. Fluorescence was quantified using ImageJ.
For determination of aneuploidy rates and assay the SAC response, third instar larval
brains were fixed and stained in aceto-orcein following incubation in colchicine (10^-4 M) for
5, 30 or 60 min, and the mitotic density (the average number of mitotic figures per
microscope field with at least one mitosis) was determined as described previously (Buffin, et
al 2007).

**In vivo observation of larval neuroblasts**

Larval brains were prepared as described (Emre et al., 2011). Fluorescent time-lapse
videos were acquired with an Olympus IX-70 inverted microscope, a focused xenon lamp
and an OrcaER camera (Hammamatsu), piloted by the Cell-R hardware and software system
(Olympus). Acquisition times per frame were 100 ms for GFP-Zw10 and 150 ms for RFP-
Rod, and images were obtained as stacks of 5 planes at 1 μm intervals taken every 15 seconds
with a 60 X NA 1.4 oil objective.

**FRAP (Fluorescence Recovery After Photo-bleaching) analysis**

Neuroblasts from third instar larval brains were treated 15 minutes with colchicine at
10^-4 M. FRAP experiments were performed using a confocal microscopy system (Zeiss
Axiovert with LSM780) equipped with a Plan-Apo 63X NA 1.4 oil objective (during
experiments with Mad1-GFP, Spc25-GFP and GFP-Rod) or a Plan-Apo 40X NA 1.3 oil
objective (during experiments with GFP-Zw10). Imaging was controlled by Zeiss confocal
software (Zen 2012). A circular area of 0.9 μm (GFP-Zw10) or 4 μm (Mad1-GFP, Spc25-
GFP) diameter was bleached with the 488 nm and 514 nm lines of an Argon laser at 25 mW.
Images were acquired with a temporal resolution of 1 second (for Mad1-GFP) or 5 seconds
(for Spc25-GFP and GFP-Zw10) with a GaAsP detector (490-650nm). Fluorescence intensity
in a region of interest was quantified using ImageJ. The exponential kinetics of FRAP was
analyzed by nonlinear regression fitting using Origin software.

**Assay for perdurance of maternally supplied Rod**
To test the hypothesis that perdurance of a small amount of WT Rod (protein or mRNA), initially loaded into the cytoplasm of the egg laid by a rod^{Z3}/+ heterozygous mother, might be responsible for the relatively mild phenotype of homozygous rod^{Z3} larvae, we assayed for GFP-Rod+ of strictly maternal origin in third instar larval brains. From the cross y w; GFP-Rod/y^+Cy; +/+ X y w/Y, phenotypically y^+ larvae should not inherit the GFP-Rod transgene. Third instar larval brains from these y^+ larvae were examined both by immunoblotting, probing with anti-GFP antibody, and by fluorescence microscopy following colchicine treatment, for evidence of GFP signal on kinetochores. Neither approach detected a positive signal of GFP-Rod.
RESULTS

rod^{Z3} is a novel maternal-effect lethal allele of rod with abnormal syncytial mitoses and defective PB M-phase maintenance.

In a screen for new genes in *Drosophila* affecting PB maintenance, we identified a novel allele of rod in a large maternal-effect lethal collection (Koundakjian et al., 2004). Flies homozygous for this allele, called rod^{Z3}, are viable and appear normal. However, they are completely sterile, the females laying eggs that fail to hatch (Table I). Examination of 3-5 hour eggs revealed a number of early developmental defects. None of the embryos had reached gastrulation and most presented highly abnormal distribution of blastoderm nuclei and unusually large internal nuclei, suggesting problems with the syncytial mitoses (Table I, Fig. 1A, and S1 in supplementary material). In contrast, nearly all the wild type (WT) embryos at this stage had either begun gastrulating (73%) or were cellularizing (22%) (Table I).

In younger (0-2 hour) syncytial stage rod^{Z3}-derived embryos, the nuclei were frequently unevenly spaced and asynchronously cycling (Fig. 1A,B. Table I). The majority of the mitotic figures in these embryos were abnormal, including multipolar spindles with multiple centrosomes and poorly focused spindles lacking one or both centrosomes (Fig. 1B). In fact, the overall phenotype of these embryos is similar to that reported for germline clones of zw10 null mutants (Williams and Goldberg, 1994).

In nearly all the eggs derived from rod^{Z3} females, the PB formed a large indistinct mass of interphase-like decondensed chromatin instead of the starburst configuration of condensed chromosomes found in WT embryos (Table II, Fig. 1A,C). The increased size of these PBs and the intensity of their DNA stain compared to WT suggest an increase in DNA content, presumably due to additional rounds of DNA replication. Expression of the rod^{+C3L9} transgene, which contains the WT rod genomic region, in the rod^{Z3} background rescued this PB defect, the embryonic lethality (Table I), and the mitotic phenotypes described below, confirming that the mutation in rod was responsible for the phenotypes.

Immunostaining rod^{Z3}-derived embryos for phosphorylated Histone H3 (PH3), a marker of M-phase chromosome condensation, distinguished two classes of decondensed PBs. Roughly 40% of the PBs were PH3-negative with rounded, interphase-like chromatin (Fig. 1C, Table II); the rest were PH3-positive with irregularly-shaped and partially decondensed chromatin. WT PBs were PH3-positive as has been reported by others (Fischer et al., 2004; Perez-Mongiovi et al., 2005). The irregularly-shaped, semi-decondensed, PH3-positive PBs of rod^{Z3}-derived embryos also labeled with Spc25-RFP (a marker of the outer
kinetochores, present only during M-phase), indicating that kinetochores were intact.

Interestingly, these PBs contained more than the expected 12 kinetochore signals (Fig. 3A), suggesting that they had at least partially replicated their DNA and returned to an M-phase-like state. The more rounded interphase-like PBs lacked Spc25-RFP (Fig. 3A), consistent with the interpretation that they were in interphase. These results suggest that the PBs of rodZ3-derived embryos may be cycling in and out of M-phase, as has been proposed for other mutants (Fischer et al., 2004; Perez-Mongiovi et al., 2005).

**The SAC is required for PB maintenance.**

Decondensed PBs have been reported previously in mutants of mps1 and bubR1 (Fischer et al., 2004; Perez-Mongiovi et al., 2005) two other genes encoding SAC components. rod is the third SAC gene to be associated with this phenotype. To determine whether rodZ3-derived syncytial embryos could undergo SAC-dependent mitotic arrest in response to spindle damage, syncytial-stage embryos were incubated with or without colchicine for 30 minutes, and the fraction of mitotic embryos was determined (see Methods for details). Whereas nearly all WT embryos had become mitotic (Fig 1D), neither rodZ3 nor mad2-null (mad2P) -derived embryos (a negative control) displayed any increase in the fraction of mitotic embryos in response to colchicine treatment. These results indicate that the SAC is defective in rodZ3-derived embryos and suggests that the failure of PBs to remain in M-phase is due to the defective SAC.

However, Mps1, BubR1 and the RZZ complex all participate in other mitotic functions that do not involve the SAC (Starr et al., 1998; Ditchfield et al., 2003; Jones et al., 2005; Lampson and Kapoor, 2005; Maure et al., 2007; Jelluma et al., 2008; Hewitt et al., 2010; Santaguida et al., 2010; Slieedrecht et al., 2010; Althoff et al., 2012; Wainman et al., 2012). It is therefore conceivable that the PB decondensation is not specifically connected to a defect in SAC activity. To confirm the role of the SAC in the maintenance of PB condensation, we examined embryos laid by mad2P females. *Drosophila* mad2-null mutants have a defective SAC, but do not exhibit the mitotic defects associated with mutations in other SAC genes (Buffin et al., 2007). We found that most of the PBs in mad2P-derived embryos were large with semi- or fully decondensed chromatin (Table II), similar to those of rodZ3. This result supports the hypothesis that SAC activity is required to maintain PB condensation, presumably by stabilizing Cyclin B (Fischer et al., 2004; Perez-Mongiovi et al., 2005). To more rigorously test this latter point, we generated rodZ3 mutants carrying one
copy of a lethal allele of the *shattered* (*shtd*) gene encoding APC/C subunit APC1 in
*Drosophila*. We also directly increased Cyclin B levels in *rod*^{23} flies by introducing two
additional copies of the WT *Cyclin B* gene. Both modifications partially suppressed the PB
phenotype of *rod*^{23}-derived embryos (Table II). The frequency of abnormal polar bodies fell
to 69% in *shtd*/+; *rod*^{23}-derived embryos, and to 53% in 2x*Cyclin B*/+; *rod*^{23}-derived
embryos. These results argue that WT *rod* is required to maintain PB M-phase through its
role in the SAC, which in turn inhibits APC/C-regulated degradation of Cyclin B.

**rod*^{23} does not affect RZZ assembly.**

Sequencing the *rod*^{23} genomic DNA revealed a point mutation in *rod* that substitutes
a glutamic acid for glycine 1973, predicted to disrupt a short α–helix in the relatively
conserved C-terminal region of the 2089 residue Rough Deal (Rod) protein. *Drosophila* Rod
has an overall predicted structure similar to that described for human Rod (Civril et al., 2010)
with N-terminal β–folds and a long α-solenoid region extending to the C-terminus. Although
not conserved in vertebrate Rod (where it is a tyrosine or a serine), the glycine at position
1973 has been conserved since the divergence of mosquitos and *Drosophila*, over 250 million
years (Wiegmann et al., 2011) (Fig. 2A).

The RZZ complex, with an apparent molecular weight of 600-700 kDa, is believed to
contain two copies of each of the three subunits, Rod, Zw10 and Zwilch, with Rod serving as
the scaffold (Scaerou et al., 2001; Williams et al., 2003; Civril et al., 2010). Immunoblotting
revealed that levels of Rod, Zw10, and Zwilch in total extracts of *rod*^{23}-derived embryos
were similar to those in WT embryos (Fig. 2B). More importantly, Zw10 and Zwilch co-
immunoprecipitated with Rod equally well in extracts of WT or *rod*^{23}-derived embryos (Fig.
2B). Fractionating lysates from WT and *rod*^{23}-derived syncytial embryos by sucrose density
gradient centrifugation revealed that the *rod*^{23} mutation does not affect Rod’s capacity to
form high molecular weight complexes (Fig. 2C). These data argue that the *rod*^{23} mutation
does not affect RZZ complex formation or steady-state levels.

**RZZ is not recruited to kinetochores in *rod*^{23}-derived embryos but is recruited at
reduced levels in *rod*^{23} neuroblasts.**

With the knowledge that the RZZ complex was intact, we next examined its behavior
in *rod*^{23}-derived syncytial embryos by following GFP-tagged Zw10. WT embryos expressing
GFP-Zw10 and Spc25-RFP contained RZZ foci at the kinetochores of the PB and mitotic
chromosomes (Fig. 3A,B) as well as along metaphase spindle fibers (Fig. 3B), reflecting the streaming of RZZ from attached kinetochores (Wojcik et al., 2001; Basto et al., 2004). In contrast, no GFP signal was detected on any mitotic structure in rod^{23}-derived embryos (Fig. 3A,B). These data suggest that the rod^{23} mutation interferes with kinetochore recruitment of the RZZ complex.

Although the absence of RZZ recruitment provided an explanation for the severity of the phenotype in embryos laid by homozygous rod^{23} mothers, it was also intriguing, since the homozygous rod^{23} offspring of heterozygous parents were apparently normal. A mutation blocking RZZ kinetochore recruitment would normally be a zygotic lethal. Indeed, null mutations in any component of RZZ are homozygous lethal, dying as a consequence of profound defects in chromosome segregation during postembryonic development (Karess and Glover, 1989; Williams et al., 1992; Williams and Goldberg, 1994; Williams et al., 2003; Karess, 2005). We therefore examined RZZ behavior in dividing tissues of homozygous rod^{23} third instar larvae (Fig. 3C). Unlike the rod^{23}-derived embryos, rod^{23} larval neuroblasts displayed significant but limited recruitment of RZZ to kinetochores during unperturbed mitosis. Average RZZ kinetochore levels (normalized to the Spc25-RFP signal) were approximately one-third that of WT (Fig. 3D). In addition to the reduction of RZZ recruitment to kinetochores, there was little or no RZZ on the spindle fibers and poles of rod^{23} neuroblasts, suggesting a problem with streaming (see below and Fig. 5).

In WT cells, a brief colchicine or nocodazole treatment to depolymerize kinetochore microtubules greatly increases the levels of several outer kinetochore proteins, including RZZ, Mad1 and Mad2 (Hoffman et al., 2001; Williams and Goldberg, 1994; Basto et al., 2004; Buffin et al., 2005). Strikingly, colchicine treatment did not significantly increase the kinetochore signal of GFP-Zw10 in rod^{23} neuroblasts; whereas in WT cells, the signal more than doubled (Fig. 3D). This reduced recruitment of RZZ in rod^{23} neuroblasts was confirmed by quantitation of Rod levels by immunostaining with anti-Rod antibody (data not shown).

To ask if this limited RZZ recruitment is compatible with a functional SAC, we assayed the ability of colchicine-treated rod^{23} larval brains to accumulate mitotic cells (Fig. 3E). Both rod^{23} and WT larval brains more than doubled their density of mitotic cells after 1 hour incubation in colchicine, whereas, rod null brains did not. These results indicate that the SAC is properly functioning in rod^{23} larval neuroblasts.

Examination of karyotypes of mitotic cells in third instar rod^{23} larval brains revealed relatively little aneuploidy (Table III). Live imaging of dividing rod^{23} neuroblasts (Fig. 3F),
however, detected an elevated frequency of anaphases in which some kinetochores migrated poleward more slowly than the others. Indeed, the fraction of anaphases with at least one lagging kinetochore was nearly as high in rodZ3 as in rod or mad1 nulls (Emre et al., 2011), although only rarely was more than one kinetochore involved (Table III). As is the case for mad1 null mutants (Emre et al., 2011), these lagging chromatids were in most cases correctly segregated, despite their delay in migration (since overall aneuploidy levels are low). In several cases, the lagging chromatids appeared to be attached to spindle fibers emanating from both poles (merotelic attachment), which is also a feature associated with mitosis in mad1 null mutants (Emre et al., 2011).

In summary, RZZ retains a limited capacity to associate with the mitotic apparatus in rodZ3 larval neuroblasts, but apparently adequate for mounting a SAC-mediated mitotic arrest and providing sufficient functionality for maintaining reasonably accurate mitosis. In contrast, RZZ appears to be incapable of kinetochore recruitment in early embryos derived from rodZ3 mothers. These results largely explain the different mitotic phenotypes of these two tissues.

### Mad1 kinetochore recruitment rate and accumulation is reduced in rodZ3 neuroblasts.

We next asked if rodZ3 affected the recruitment of Mad1 and Mad2, since kinetochore levels of the Mad1-Mad2 complex depend in part on the presence of functional RZZ (Buffin et al., 2005; Kops et al., 2005). In untreated rodZ3 neuroblasts, levels of Mad1 (Fig. 4A) and Mad2 (not shown) were often low, and sometimes undetectable on prometaphase mitotic figures. On average, Mad1-GFP kinetochore signal in rodZ3 was not significantly different from that in rod null cells (Fig. 4B). The low levels of Mad1 may therefore help explain why rodZ3 displays the elevated rate of lagging chromatids also seen in mad1 mutants. However, when treated with colchicine, rodZ3 neuroblasts did accumulate Mad1 and Mad2 at kinetochores (Fig. 4C). Both the rate of this accumulation and the final level attained were reduced; reaching approximately one-third of the levels found in WT after 8-10 minutes. This degree of Mad1 and Mad2 recruitment must be sufficient to elicit the robust SAC response of colchicine-treated rodZ3 larval brains (Fig. 3E).

The relatively low levels of Mad1 recruitment in colchicine might be a quantitative consequence of the reduced RZZ at rodZ3 kinetochores or a qualitative consequence of the structural change caused by the rodZ3 mutation (or both). To gain insight into this question, we simultaneously measured the fluorescent signals from GFP-Zw10 and Cherry-Mad1 on...
the kinetochores of individual colchicine-treated neuroblasts and displayed the values in a scatter plot (Fig. 4D). The plot shows that the highest RZZ and Mad1 signals are (not surprisingly) only found in the WT cells. However, considering only the range of RZZ values overlapping between WT and rod\textsuperscript{23} (included within the box of Fig. 4D), the distribution of Mad1 values was not significantly different and the linear regression curves which fit the two data sets had similar slopes. This result indicates that the recruitment levels of Mad1 are “normal” in rod\textsuperscript{23} cells, given the reduced amount of RZZ on unattached kinetochores.

To determine whether the rod\textsuperscript{23} mutation is affecting steady-state turnover of Mad1 at unattached kinetochores, we subjected Mad1-GFP to FRAP analysis (Fig. 4E). The Mad1-GFP that had accumulated on rod\textsuperscript{23} kinetochores after 15 minutes in colchicine displayed the same turnover dynamics as in WT. A fast turnover pool, about 40\% of the total, recovered with a half-life of 10-12 seconds, while the remaining 60\% was essentially stable, showing little or no recovery. Thus, the steady-state turnover properties of Mad1 are not disrupted in rod\textsuperscript{23} neuroblasts.

In summary, the primary cause of the reduced kinetochore Mad1 signal in colchicine-treated rod\textsuperscript{23} neuroblasts is the low levels of RZZ recruitment itself, and not a specific effect of the rod\textsuperscript{23} mutation on the ability of RZZ to recruit Mad1. Nevertheless, the systematic reduction in kinetochore Mad1 signal seen in unperturbed mitosis (Fig. 4A) to levels found in rod null cells, and the fact that Mad1 is slower to accumulate on unattached kinetochores (Fig. 4C), suggests the rod\textsuperscript{23} mutation may specifically alter the rate of Mad1 accumulation.

RZZ streaming is disrupted in rod\textsuperscript{23} neuroblasts.

Several transient outer kinetochore components, including RZZ, and Mad1-Mad2 display a characteristic movement from kinetochores to poles along kinetochore fibers called shedding, stripping or streaming (Howell et al., 2000; Howell et al., 2001; Wojcik et al., 2001; Karess, 2005; Famulski et al., 2011). This dynein-dependent process is believed to contribute to the extinction of the SAC signal following proper K-MT attachment. In live images of GFP-Zw10 expressed in rod\textsuperscript{23} neuroblasts (Fig. 3C), this streaming appeared to be significantly reduced. Because streaming is highly variable, even in WT, we quantitated this apparent difference by scoring mitotic cells into three classes according to their degree of streaming: none (all GFP-Zw10 signal restricted to kinetochores), low (GFP-Zw10 signal mostly on kinetochores) and high (signal on kinetochores, spindle and poles). By these criteria, rod\textsuperscript{23} neuroblasts consistently failed to show any streaming (Fig. 5A).
Since streaming is a dynein-dependent process and RZZ is required for the recruitment of dynein-dynactin complex to kinetochores (Starr et al., 1998; Bader and Vaughan, 2010; Raaijmakers et al., 2013), we asked if rodZ3 specifically reduced kinetochore dynein levels. We measured accumulation of GFP-DLIC2, a dynein subunit, relative to Spc25-RFP, on mutant or WT cells incubated 15 minutes in colchicine (Fig. 5B). In 90% of rodZ3 cells, GFP-DLIC2 was detectable at kinetochores, averaging about half the signal found in WT. As was the case for kinetochore Mad1-GFP, the range of GFP-DLIC2 levels was very broad and probably reflects the variation in RZZ recruitment levels.

Since dynein recruitment is only partially reduced, whereas dynein-dependent streaming is entirely absent, we considered the possibility that the RodZ3 protein was directly interfering with the ability of RZZ to stream. Consistent with this idea, rodZ3/+ heterozygous neuroblasts also displayed reduced streaming relative to WT (Fig. 5A), suggesting that rodZ3 has a dominant effect in disrupting RZZ function. To test this idea, we co-expressed two Rod proteins with different fluorescent tags in a rod null background: Cherry-Rod+ and either GFP-Rod+ or GFP-RodZ3, expressed from a transgene containing the identical mutation as the rodZ3 allele (Fig. 5B). When two WT transgenes were expressed, both green and red particles of RZZ streamed on K-MTs, whereas when GFP- RodZ3 was co-expressed with Cherry-Rod+ the streaming of both the red (WT) and green (mutant) RZZ was greatly reduced (Fig. 5C). These results are consistent with a partially functional GFP- RodZ3 subunit assembling into the multi-subunit RZZ complex and inhibiting its ability to be transported from kinetochores.
DISCUSSION

The SAC is required for PB M-phase maintenance in Drosophila.

In a search for maternal effect mutants affecting the regulation of early embryonic divisions, we found a unique allele of rod, called rod\textsuperscript{Z3}, which profoundly affects the ability of RZZ to be recruited to kinetochores in the early embryo. In the discussion below, we will refer to the mutant form of the complex as R\textsuperscript{Z3}ZZ.

Although previous studies have shown that Cdk1 and Cyclin B activity are required for PB condensation, we have established that PBs maintain Cdk1-Cyclin B activity through activation of the SAC. We show here that the RZZ complex, like Mps1 and BubR1, localize to kinetochores in PBs and is required to maintain PB condensation in Drosophila. rod\textsuperscript{Z3}-derived embryos, like those of mad2, mps1 and bubR1 mutants, contain large interphase-like polar bodies.

Perez-Mongiovi et al. (2005) found evidence that in bubR1\textsuperscript{Rev1}-derived embryos, PBs cycle between periods of condensation and decondensation with accompanying DNA replication. They suggested that these PB cycles are under the control of the embryonic mitotic oscillator. Because PBs in rod\textsuperscript{Z3} embryos are not always fully-decondensed, it is possible that they undergo similar cycles in DNA replication and condensation. The fact that the semi-decondensed polar bodies have more than the expected 12 kinetochores (labeled with the outer kinetochore protein Spc25; (Fig. 3A), is consistent with this idea.

Directly or indirectly increasing Cyclin B levels in rod\textsuperscript{Z3}-derived embryos partially suppresses PB condensation defects. This result argues that Rod and the other SAC components maintain PB condensation by inhibiting the APC/C-mediated degradation of Cyclin B. The Png kinase complex is also required to maintain Cyclin B and thus PB condensation (Lee et al., 2001). However, rod and png regulate Cyclin B levels through different mechanisms. During embryogenesis, PNG kinase promotes the translation of Cyclin B by regulating the polyadenylation of its mRNA and antagonizing the translational repressor Pumilio (Vardy and Orr-Weaver, 2007). The SAC components, on the other hand, recognize the PB kinetochores as improperly attached to the spindle, and generate the inhibitor of APC/C mediated proteolytic degradation of Cyclin B.

rod\textsuperscript{Z3} sterility is due to defects in RZZ function beyond its role in the SAC.

rod\textsuperscript{Z3}-derived embryos undergo aberrant syncytial mitosis and a developmental arrest prior to gastrulation. These embryos accumulate asynchronously dividing nuclei with...
centrosome attachment defects throughout the syncytial divisions. Defects in mitotic spindle function during syncytial embryogenesis can uncouple the nuclear and centrosomes cycles (Archambault and Pinson, 2010). The resulting replication of detached centrosomes and fusion of free centrosomes to neighbouring mitoses leads to additional aberrant mitoses. Furthermore, centrosome detachment during syncytial embryogenesis inhibits the nuclear positioning and migration required for morphogenesis, all of which may contribute to the developmental arrest of rod\textsuperscript{zz}-derived embryos.

In Drosophila, inactivation of the SAC has only minor consequences on mitosis and on embryonic viability (Buffin et al., 2007). Comparing the embryonic phenotype of rod\textsuperscript{zz} with that of mad2 nulls is instructive. Both lack a functional SAC in embryos and consequently the PB fails to arrest in M-phase. However, although 60-70% of mad2 embryos hatch and reach adulthood (Buffin et al., 2007), no rod\textsuperscript{zz}-derived embryos reach gastrulation stage. The different phenotypes of mad2 and rod\textsuperscript{zz}-derived embryos argue strongly that it is not the absence of the SAC per se that is responsible for the lethality of rod\textsuperscript{zz}-derived embryos, but rather the combination of defects caused by simultaneously removing the SAC and non-SAC functions of RZZ, the latter regulating dynein and K-MT attachments (Starr et al., 1998; Yang et al., 2007; Gassmann et al., 2008; Gassmann et al., 2010).

rod\textsuperscript{zz} affects the recruitment and behavior of RZZ and Mad1.

The primary defect we have identified in R\textsuperscript{zz}-ZZ is its failure to associate with syncytial and PB kinetochores in the early embryo. Most of the other phenotypes can be explained as secondary consequences of this recruitment failure. The striking difference between the maternal and zygotic phenotypes of rod\textsuperscript{zz} can be ascribed to the fact that R\textsuperscript{zz}-ZZ partially retains its ability to be recruited to kinetochores in dividing larval tissues.

Why is R\textsuperscript{zz}-ZZ capable of kinetochore recruitment in larval neuroblasts and not in maternally-derived early embryos? One possibility is that the unusual rapidity of syncytial mitosis might render it more sensitive to the structural change in R\textsuperscript{zz}-ZZ. However, the fact that R\textsuperscript{zz}-ZZ is also absent from PBs argues that this is unlikely to be a complete explanation, since the PB does not rapidly cycle but remains in M-phase. It would, in this scenario, still have enough time to recruit R\textsuperscript{zz}-ZZ.

We suggest that there are factors involved in recruiting RZZ to kinetochores that subtly differ in syncytial stage and postembryonic mitoses. For example, there may be two, partially redundant, recruitment pathways for RZZ. One would be employed in
postembryonic mitoses, such as in larval neuroblasts, but not in the early embryo. The other
recruitment pathway would normally be used in both tissues, but is damaged in rod^{Z3}. These
two “pathways” could be as simple as two different kinetochore-binding domains of RZZ and
two corresponding docking sites on kinetochores. This idea may also provide an explanation
for the second striking feature of R^{Z3}ZZ behavior: in larval neuroblasts, it shows no further
kinetochore accumulation beyond the basal level when microtubules are depolymerized with
colchicine. This supplemental RZZ binding may also depend on the second recruitment
pathway, explaining why it is defective in rod^{Z3}. The mutation in rod^{Z3} may thus affect a
residue important to RZZ kinetochore binding, about which very little is known, particularly
in Drosophila. Kinetochore proteins implicated in RZZ recruitment in vertebrates such as
Zwint1 (Starr et al., 2000; Famulski et al., 2008) and Cenp-I (Matson and Stukenberg, 2014),
have not been found in the fly genome (Przewloka et al., 2007).

During unperturbed mitosis, kinetochore Mad1 levels are as low in rod^{Z3} as they are
in rod null larval neuroblasts (Fig 4A,B). We have previously reported that mad1 null
neuroblasts display a relatively mild mitotic phenotype. Despite having a defective SAC, they
display little aneuploidy, but have a high incidence of lagging chromatids and merotely
(Emre et al., 2011). It is therefore not surprising that we find a similar phenotype in rod^{Z3}
cells, which have greatly reduced kinetochore Mad1 levels.

The mutation in rod^{Z3} does not seem to alter the ratio of Mad1 recruited per unit
RZZ. On unattached kinetochores, given enough time, Mad1 accumulates to levels
approximately proportional to the amount of RZZ present (Fig 4C,D). The recruited Mad1
also displays the same steady-state turnover dynamics as in WT cells (Fig 4E). However, the
time required to reach that maximal recruitment is longer than in WT (Fig 4C). We speculate
that the slower recruitment rate of Mad1 may explain its low levels on kinetochores during
unperturbed mitosis. There is little known about the molecular mechanism by which RZZ
helps to recruit Mad1. It is not even known if they interact directly, although a fraction of
RZZ and Mad1 can be co-immunoprecipitated from mitotic cells (Défachelles et al, in
preparation).

Finally, rod^{Z3} has a perceptible dominant effect on the dynein-dependent streaming of
RZZ. This can best be seen in Fig 5C, where RFP-Rod^{Z3} co-expressed with GFP-Rod+
interferes with the streaming of both tagged proteins from kinetochores. The RZZ complex is
believed to contain two copies of each of its three subunits, with Zw10 and Zwilch binding to
the amino-terminal half of the Rod protein (Starr et al., 1998; Scaerou et al., 2001; Williams
et al., 2003; Civril et al., 2010; Cheerambathur et al., 2013). This stoichiometry means that a given RZZ particle could contain one WT and one mutant copy of RZZ, if both versions are present. Since the effect of \(rod^{23}\) on streaming is more severe than its effect on dynein recruitment, we suggest that the mutation in \(rod^{23}\) may also perturb an interaction with the dynein motor complex.

In summary, the mutation in \(rod^{23}\) affects three activities of RZZ: its own kinetochore recruitment, its capacity to rapidly recruit Mad1, and its capacity to be removed from attached kinetochores by dynein-dependent streaming. The G to E mutation of \(rod^{23}\) at residue 1973 resides in the most highly conserved portion of the Rod protein, known as Rod_C domain (pfam10493), predicted to fold into several short a-helices. This domain is only found in known or suspected homologues of Rod, although no specific function or interacting proteins have been associated with it. Thus, characterization of \(rod^{23}\) provides a unique opportunity to examine how the Rod C-terminus contributes to RZZ function.
ACKNOWLEDGEMENTS

The following grants from the National Institutes of Health supported this work:
GM074044 (to L.A.L.), and GM008554 (to S.G.H.). L.D. was supported by fellowships from
the French “Ministère Français de l’Enseignement Supérieur et de la Recherche”, and the
Association pour la Recherche sur le Cancer. R.K. was supported by Ligue Nationale Contre
le Cancer équipe labellisée LIGUE. Thanks to the ImagoSeine facility, a member of France
BioImaging infrastructure, supported by the French National Research Agency (ANR-10-

FOOTNOTES

Competing interests

The authors have no competing interest to declare.

Author contributions

All authors contributed to the conception and design of experiments, analysis of data,
and manuscript preparation. Experiments were performed principally by L.D. and S.G.H.
A.M. contributed to the initial analysis of the rod<sup>iz3</sup> zygotic phenotype.
**FIGURE LEGENDS**

**Fig. 1** Embryos laid by rod$^{23}$ mothers are defective in syncytial mitosis, PB maintenance, and the SAC.

(A) Representative images of DNA-stained early (left) and late (right) syncytial embryos from wild-type (WT) and rod$^{23}$ females. rod$^{23}$-derived embryos contain large decondensed polar bodies (left). Also note the asynchrony of mitotic stages and the irregular distribution of nuclei in the rod$^{23}$-derived embryos (right). Scale bar, 50 µm. Insets, larger images of PBs (marked by arrows). (B) Representative images of prophase and metaphase figures from WT and rod$^{23}$-derived syncytial embryos stained for tubulin (green), centrosomin (red), and DNA (blue). rod$^{23}$ mitotic figures have abnormal centrosome attachments. Broad acentrosomal and multipolar spindles are common. Scale bar, 10 µm. (C) Details of PBs from WT and rod$^{23}$-derived syncytial embryos stained for PH3 and DNA. rod$^{23}$ polar bodies are either PH3-positive (partially decondensed) or PH3-negative (fully decondensed). Scale bar, 10 µm. (D) The SAC is not functional in rod$^{23}$-derived embryos. Relative change in the fraction of mitotic syncytial embryos after 30 min incubation in colchicine, (untreated= 1), for the indicated genotypes. rod$^{23}$-derived embryos, like those derived from mad2 null mothers (mad2$,^P$, negative control), show no significant increase after treatment. **p<0.0001, *p<0.005. n=80 embryos, except rescue-derived embryos (n=140).

**Fig. 2** The rod$^{23}$ mutation changes a conserved glycine in the Rod_C domain, but does not affect RZZ assembly. (A) The mutation in rod$^{23}$ results in a G to E change at residue 1973 in a conserved C-terminal region of the protein. A Clustal alignment around the mutated region of Rod from mammals and dipterans shows that G1973 (red box) has been conserved since the divergence of mosquitoes and flies. The corresponding position in mammalian Rod is always a polar residue. (B) Levels of Rod, Zw10, and Zwilch are similar in extracts of WT and rod$^{23}$-derived syncytial embryos and immunoprecipitation of Rod brings down Zw10 and Zwilch equally well in both WT and rod$^{23}$ extracts. Immunoblots for Rod, Zw10, and Zwilch in input lysates of WT or rod$^{23}$ derived embryos (left) (Tubulin is a loading control). Immunoprecipitates using anti-Rod, (middle), or control non-immune rabbit serum (right). The lower molecular weight band in the Zwilch blots is an unrelated contaminant. (C) RZZ from WT and rod$^{23}$-derived syncytial embryos both form high molecular weight complexes. Fractions of a sucrose density gradient centrifugation of embryo extracts immunoblotted for Rod. Arrows indicate migration of size markers.
Fig. 3 RZZ kinetochore recruitment is absent in rod^{23}-derived embryos but present, though reduced, in homozygous rod^{23} neuroblasts. (A) RZZ does not localize to PBs in rod^{23}-derived embryos. PBs from fixed WT and rod^{23}-derived syncytial embryos expressing Spc25-RFP (a kinetochore marker, red), GFP-Zw10 (an RZZ component, green) and stained for DNA (blue). Scale bar, 10 µm. p<0.0001. n=12 PBs. (B) RZZ does not localize to metaphase kinetochores or spindle fibers in rod^{23}-derived embryos. Scale bar, 10 µm. p<0.0001. n=30 metaphases, from 12 embryos. (C) RZZ is recruited to kinetochores in rod^{23} larval neuroblasts, but at reduced levels. In WT or rod^{23} heterozygous neuroblasts GFP-Zw10 localizes to kinetochores (marked by Spc25-RFP) and spindle fibers. In homozygous rod^{23} cells (bottom), GFP-Zw10 is detectable at kinetochores, but at lower intensity. Particles of RZZ are visible between the kinetochores (white arrow) and the poles (yellow arrows) in WT and heterozygous rod^{23}, but not in homozygous rod^{23}, suggesting a problem with dynein-dependent streaming (See also Fig 5). Scale bars, 2 µm. (D) Quantification of RZZ levels on kinetochores. GFP-Zw10 signal, normalized to that of Spc25-RFP, was determined for WT (blue), rod^{23} homozygous (red) or rod^{23}/+ heterozygous (green) cells. Closed circles and open circles indicate cells treated or untreated, respectively, with colchicine for 15 minutes. Horizontal bars represent the mean. * p<0.05, ** p<0.01 (E) The SAC is functional in rod^{23} larval brains. Relative mitotic density in larval brains as a function of time in colchicine, WT (black), rod^{23} (light gray), rod null (dark gray). Bars indicate S.E.M. * p<0.05. In rod^{23}, the mitotic density increases as a function of time in colchicine similar to WT, whereas in SAC-defective rod null brains, mitotic cells do not accumulate, even after 1 hour in colchicine. (F) Lagging chromatids are common in rod^{23} neuroblast mitosis. Representative images of single live neuroblasts of the indicated genotypes in metaphase and anaphase. Kinetochores are visualized with Spc25-RFP (red) and the spindle is marked with GFP-Jupiter (green). Indicated times (minutes: seconds) are relative to anaphase onset. Scale bars, 2 µm. (See also Table III).

Fig. 4 Mad1 kinetochore recruitment is slow, but its steady state dynamics are normal in rod^{23} neuroblasts. (A) Images of live WT (top) or rod^{23} (bottom) prometaphase neuroblasts expressing Mad1-GFP and Spc25-RFP. Scale bars, 2 µm. (B) Quantification of Mad1-GFP kinetochore recruitment, normalized to Spc25-RFP in untreated WT (blue), rod^{23} (red), or rod null (grey) cells. Horizontal bars represent the mean. In most rod^{23} cells, little...
or no Mad1 is detectable at kinetochores, similar to *rod* null. ** p<0.01. ns= not significant.

(C) Mad1-GFP (top) and GFP-Mad2 (bottom) accumulate slowly on kinetochores, but reach significant levels in colchicine-treated *rod*²³ neuroblasts. GFP signals of kinetochore Mad1 or Mad2 were monitored in individual, colchicine-treated WT (blue) or *rod*²³ (red) neuroblasts, normalized to Spc25-RFP, and plotted as a function of time after nuclear envelope breakdown (NEB). The maximum accumulation is about 35% that of WT. p<0.05. (D) The relationship between Mad1 and RZZ levels is unchanged in *rod*²³ cells. Larval brains expressing Cherry (Ch)-Mad1 and GFP-Zw10 were imaged after a 30 minute colchicine treatment. The intensities of kinetochore Ch and GFP were quantified in individual WT (blue) or *rod*²³ (red) cells. Levels of GFP-Zw10, representing RZZ, were plotted on the X-axis and those of Ch-Mad1 were plotted on the Y-axis. The two data sets show a similar correlation between Mad1 and RZZ. The regression curves shown for WT and *rod*²³ fit the data points within the black box, which correspond to kinetochores displaying the range of GFP-Zw10 signals common to the two genotypes. The difference in slope is not significant (ANOVA test). (E) *rod*²³ does not alter turnover dynamics of Mad1-GFP on unattached kinetochores. (*Left*) FRAP analysis of WT (blue) or *rod*²³ (red) neuroblasts expressing Mad1-GFP (top) or GFP-Spc25 (bottom) treated with colchicine for 15 minutes. Fluorescence recovery was monitored over 200 seconds and normalized to the pre-bleach signal. Neither the size of the fast-turnover pool of Mad1 (about 40%) nor its half-life (about 10 seconds) are altered in *rod*²³ cells. The stable outer kinetochore component Spc25 was used as a control. (*Right*) Representative images of Mad1-GFP in (a) WT, (b) *rod*²³ cells, and (c) GFP-Spc25 in WT cells, before and after photobleaching kinetochores. Bleached area is circled in yellow. Scale bars, 5 µm.

**Fig. 5** The mutation in *rod*²³ affects RZZ streaming in neuroblasts.

(A) Quantification of RZZ streaming in WT, *rod*²³, and *rod*²³/+ neuroblasts. Cells were classed by intensity of GFP-Zw10 streaming: high, low, and no streaming. RZZ streaming is not observed in homozygous *rod*²³ cells and is significantly reduced in *rod*²³/+ cells. (B) Dynein recruitment is reduced but not eliminated in *rod*²³ neuroblasts. Images of GFP-DLIC2 at kinetochores in live WT, *rod*²³, and *rod* null cells treated with colchicine. Dynein is recruited to unattached kinetochores in WT and *rod*²³ cells, but not in *rod* null cells. Scale bars, 2 µm. (right). (C) Quantification of GFP-DLIC2 at kinetochores, normalized to Spc25-RFP, in colchicine treated WT (blue), *rod*²³ (red), or *rod* null (grey) cells. In 90% of *rod*²³...
cells, GFP-DLIC2 is detectable at kinetochores but its signal is reduced compared to that in WT cells. Horizontal bars represent the mean. (D) RodZ3 dominantly disrupts RZZ streaming. (Top) Metaphase spindles of neuroblasts expressing two different colors of WT Rod (Ch-Rod+ and GFP-Rod+, first row) or one WT copy and one mutant copy (Ch-Rod+ and GFP-RodZ3, second row) in a rod null background. In cells expressing only WT Rod, streaming is normal, and particles containing both colors are found on the spindle fibers. When GFP-RodZ3 is co-expressed with Ch-Rod+, the streaming of both is reduced. Scale bars, 2 µm. (Bottom) Quantification of Rod streaming, using criteria described in (A). The fraction of cells with robust streaming is significantly reduced when GFP-RodZ3 is present.
REFERENCES


transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. The Journal of Cell Biology 155, 1159-1172.


TABLE I: Maternal-Effect Embryo Phenotype of \( \text{rod}^{23} \)

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Percent of Embryos</th>
<th>Hatched (n)</th>
<th>Normal Gastrulation at 3-5 hr (n)</th>
<th>Normal Cellular Blastoderm at 3-5 hr (n)</th>
<th>Abnormal Syncytial Divisions (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>94 (2355)</td>
<td>73 (290)</td>
<td>22 (290)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>( \text{rod}^{23} )</td>
<td></td>
<td>0 (945)</td>
<td>0 (235)</td>
<td>0 (235)</td>
<td>60 (99)</td>
</tr>
<tr>
<td>( \text{rod}^{23} ); ( \text{rod}^{23} )</td>
<td></td>
<td>73 (1737)</td>
<td>-</td>
<td>-</td>
<td>8 (101)</td>
</tr>
</tbody>
</table>
TABLE II: Polar Body Phenotype of *rod*<sup>z3</sup>

<table>
<thead>
<tr>
<th>Maternal Genotype (n)</th>
<th>PB Phenotype</th>
<th>Percent Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent Normal Condensed PBs</td>
<td>Decondensed PBs</td>
</tr>
<tr>
<td>WT</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td><em>rod</em>&lt;sup&gt;z3&lt;/sup&gt;</td>
<td>6</td>
<td>94&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>rod</em>&lt;sup&gt;z3&lt;/sup&gt;; <em>rod</em>&lt;sup&gt;z3&lt;/sup&gt;</td>
<td>77</td>
<td>23</td>
</tr>
<tr>
<td><em>shtd</em>&lt;sup&gt;3&lt;/sup&gt;; <em>rod</em>&lt;sup&gt;z3&lt;/sup&gt;</td>
<td>31</td>
<td>69&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2XCyclinB; <em>rod</em>&lt;sup&gt;z3&lt;/sup&gt;</td>
<td>47</td>
<td>53&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>mad2</em>&lt;sup&gt;p&lt;/sup&gt;</td>
<td>18</td>
<td>82</td>
</tr>
</tbody>
</table>

(a, b) These differences are significant (*p*<0.0001)
Table III: Mitotic phenotype of rod<sup>Z3</sup> larval neuroblasts.

<table>
<thead>
<tr>
<th>Zygotic Genotype</th>
<th>Percent aneuploid (s.d.)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent abnormal anaphase (one or more lagging kinetochore)&lt;sup&gt;b&lt;/sup&gt; (n)</th>
<th>Average number of laggards per abnormal anaphase</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.2 (0.22)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.5 (16)</td>
<td>1</td>
</tr>
<tr>
<td>rod&lt;sup&gt;Z3&lt;/sup&gt;</td>
<td>2.9 (1.2)</td>
<td>64 (22)</td>
<td>1.8</td>
</tr>
<tr>
<td>rod&lt;sub&gt;null&lt;/sub&gt;</td>
<td>37 (4.5)</td>
<td>70 (23)</td>
<td>2.3</td>
</tr>
<tr>
<td>mad1</td>
<td>Less than 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>63 (28)</td>
<td>2.3</td>
</tr>
</tbody>
</table>

a) % Aneuploidy (>2n only) determined on fixed tissue, aceto-orcein stained.

b) Anaphases determined on live cells.

c) From Buffin et al. (2007).

Figure 1
Fig 3