

# Ana3 is a conserved protein required for the structural integrity of centrioles and basal bodies

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Recent studies have identified a conserved “core” of proteins that are required for centriole duplication. A small number of additional proteins have recently been identified as potential duplication factors, but it is unclear whether any of these proteins are components of the core duplication machinery. In this study, we investigate the function of one of these proteins, *Drosophila melanogaster* Ana3. We show that Ana3 is present in centrioles and basal bodies, but its behavior is distinct from that of

the core duplication proteins. Most importantly, we find that Ana3 is required for the structural integrity of both centrioles and basal bodies and for centriole cohesion, but it is not essential for centriole duplication. We show that Ana3 has a mammalian homologue, Rotatin, that also localizes to centrioles and basal bodies and appears to be essential for cilia function. Thus, Ana3 defines a conserved family of centriolar proteins and plays an important part in ensuring the structural integrity of centrioles and basal bodies.

## Introduction

Centrioles are microtubule (MT)-based structures that are required for the formation of two important cellular organelles, centrosomes and cilia. Within the centrosome, centrioles are arranged as an orthogonal pair and normally organize an amorphous meshwork of proteins called the pericentriolar material (PCM). This surrounds the centrioles and contains factors involved in nucleating and regulating MTs; in this way, centrosomes function as major MT-organizing centers in multiple cell types (Doxsey et al., 2005). In many noncycling cells, the centrioles migrate to the cell cortex where the older, mother centriole forms a basal body that organizes a cilium. Like centrosomes, cilia have diverse roles in development, and defects in both centrosome and cilia function are associated with a wide variety of human diseases (Badano et al., 2005; Sharma et al., 2008).

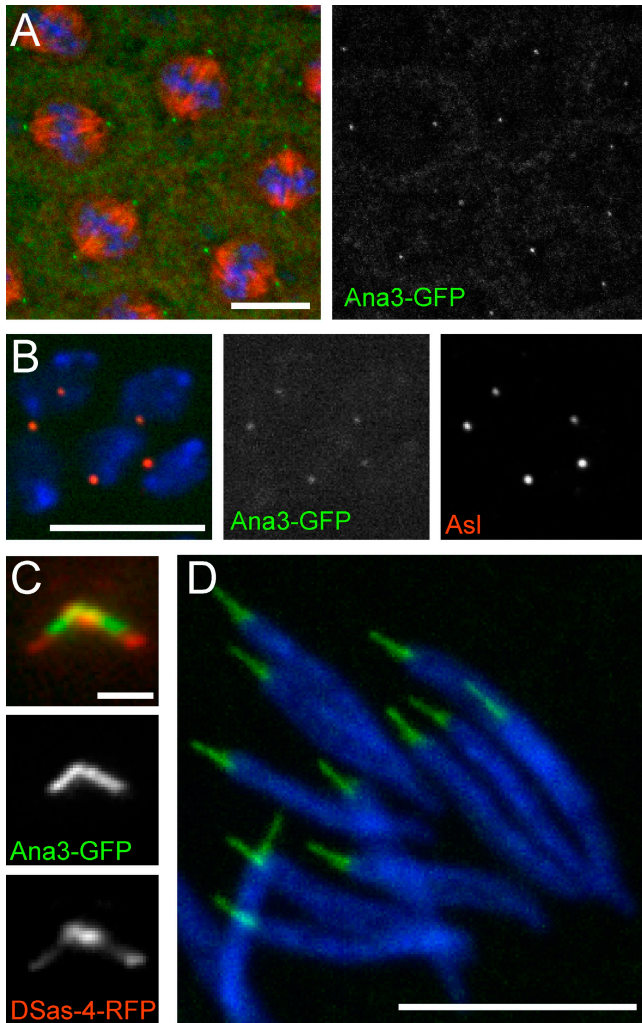
Therefore, it is essential that centriole numbers are tightly regulated, with each centriole duplicating once and only once per cell cycle. Studies in worms have identified just five proteins that are essential for centriole duplication: SPD-2, ZYG-1, SAS-5, SAS-6, and SAS-4 (O’Connell et al., 2001; Kirkham et al., 2003;

Leidel and Gönczy, 2003; Dammermann et al., 2004; Delattre et al., 2004; Kemp et al., 2004; Pelletier et al., 2004; Leidel et al., 2005). Proteins related to ZYG-1, SAS-6, and SAS-4 have a conserved role in centriole duplication in other systems, leading to the idea that these proteins form a conserved “core” machinery for centriole duplication (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Strnad et al., 2007). However, recent studies in fly and human cells have identified a small number of additional proteins that are potentially required for centriole duplication (Goshima et al., 2007; Kleylein-Sohn et al., 2007; Dobbelaere et al., 2008). For example, in a genome-wide RNAi screen designed to identify proteins involved in mitotic spindle function in cultured *Drosophila melanogaster* cells, Ana3 was identified as a potential centriole duplication factor because its depletion led to an increased level of anastral spindles, which is suggestive of a defect in centrosome assembly (Goshima et al., 2007). Ana3 was also picked up in a screen specifically designed to find factors required for centriole duplication in which its depletion led to a reduced number of centrioles in cultured *Drosophila* cells (Dobbelaere et al., 2008). In this study, we set out to investigate the function of Ana3 in vivo.

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Abbreviations used in this paper: Asl, Asterless; Cnn, Centrosomin; D-PLP, *Drosophila* pericentrin-like protein; MBP, maltose-binding protein; MT, microtubule; PCM, pericentriolar material; Rtn, Rotatin; shRNA, short hairpin RNA; Ubq, ubiquitin; WT, wild type.

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**Figure 1. Ana3 is a component of centrioles and basal bodies, but its localization is distinct from the core duplication proteins.** (A) A syncytial embryo expressing Ana3-GFP (green) stained for tubulin (red) and DNA (blue). Ana3-GFP localizes to centrosomes throughout the cell cycle, which is shown at metaphase. (B) Interphase brain cells expressing Ana3-GFP (green) stained for the centriole marker Asl (red) and DNA (blue). Ana3-GFP localizes to centrosomes throughout the cell cycle, indicating that Ana3 is a centriolar component. (C) A large, v-shaped centriole pair from a primary spermatocyte expressing Ana3-GFP (green) and DSas-4-RFP (red). Ana3-GFP is distributed evenly along the centriole barrels. In contrast, DSas-4-RFP, like the other conserved core duplication proteins, is enriched at the proximal and distal ends of the centrioles. (D) Spermatids expressing Ana3-GFP (green) stained for DNA (blue). Ana3-GFP localizes to the basal bodies attached to the elongating nuclei. Bars: (A, B, and D) 10  $\mu$ m; (C) 2  $\mu$ m.

## Results and discussion

### Ana3 is a component of centrioles and basal bodies, but its localization is distinct from the core centriole duplication proteins

The localization of Ana3 has not previously been reported (Goshima et al., 2007; Dobbelaere et al., 2008), so we generated transgenic fly lines carrying a full-length Ana3-GFP fusion protein under the control of the ubiquitously active ubiquitin (Ubq) promoter. The fusion protein localized to centrosomes throughout the cell cycle in embryos and larval brain cells (Fig. 1, A and B; and Video 1). As the centrioles in larval brain

cells organize little or no PCM during interphase (Martinez-Campos et al., 2004), this observation indicates that Ana3 is a centriolar component.

To further define the localization of Ana3, we investigated its distribution in the giant centrioles of primary spermatocytes. Three conserved core centriole duplication proteins, DSas-4, DSas-6, and Sak (the functional homologue of ZYG-1), have been identified in *Drosophila*, and all three exhibit a characteristic localization to the proximal and distal ends of these centrioles (Peel et al., 2007; Rodrigues-Martins et al., 2007a). In contrast, *Drosophila* pericentrin-like protein (D-PLP) and DSpd-2, which are not required for centriole duplication, localize all along the centriole barrel (Martinez-Campos et al., 2004; Dix and Raff, 2007). We found that Ana3-GFP, like D-PLP and DSpd-2, was distributed evenly along the length of the centriole (Fig. 1 C). After meiosis, each spermatid inherits a single centriole, which acts as a basal body to nucleate the flagellar axoneme. Ana3-GFP continued to localize along the length of the basal body (Fig. 1 D), which again contrasted with the core duplication proteins (Blachon et al., 2009). We confirmed this localization using affinity-purified antibodies raised against Ana3 (Fig. S1). Collectively, these data demonstrate that Ana3 localizes to centrioles in a manner that is distinct from the known core duplication proteins.

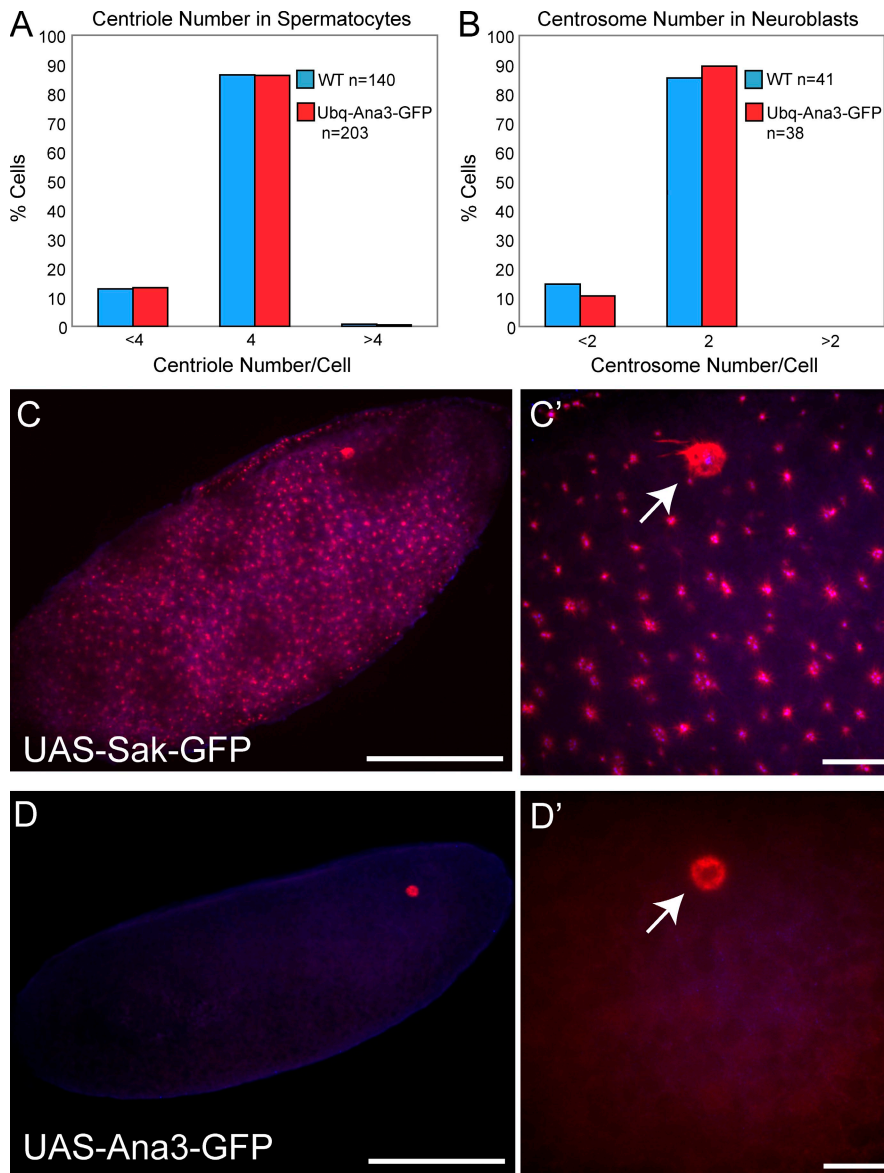
### Ana3 overexpression does not drive centrosome amplification or de novo centriole formation

The core centriole duplication factors DSas-6 and Sak can drive centriole overduplication in various tissues when overexpressed with the Ubq promoter (Peel et al., 2007). To test whether this was the case for Ana3, we counted centriole numbers in wild-type (WT) and Ubq-Ana3-GFP-expressing spermatocytes and neuroblasts (Fig. 2, A and B). In both cases, the expression of Ana3-GFP did not lead to the presence of extra centrioles or centrosomes. Moreover, we followed  $\sim$ 350 centriole duplication events in eight living embryos overexpressing Ana3-GFP and did not observe a single instance of centriole overduplication (Video 1).

We next investigated the ability of Ana3 to drive de novo centriole formation in unfertilized eggs. Centrioles are normally destroyed during oogenesis so that mature eggs contain no centrioles (Peel et al., 2007). Strikingly, however, all of the known core centriole duplication factors in *Drosophila* can induce the assembly of centriole-like structures in unfertilized eggs when highly overexpressed using the UAS-GAL4 system; these structures recruit PCM and nucleate MT asters (Fig. 2 C; Peel et al., 2007; Rodrigues-Martins et al., 2007b). In contrast, the high level overexpression of Ana3-GFP did not drive the de novo formation of centriole-like structures in any unfertilized eggs ( $n = 206$ ; Fig. 2 D). Thus, unlike Sak-GFP, GFP-DSas-6, or DSas-4-GFP, Ana3-GFP is unable to drive the de novo formation of centriole-like structures in eggs or centriole overduplication in any tissues we tested.

### Ana3 is required for basal body formation/maintenance in sensory neurons

To further investigate the function of Ana3, we obtained a stock with a P-element inserted 3 bps upstream of the initiating ATG



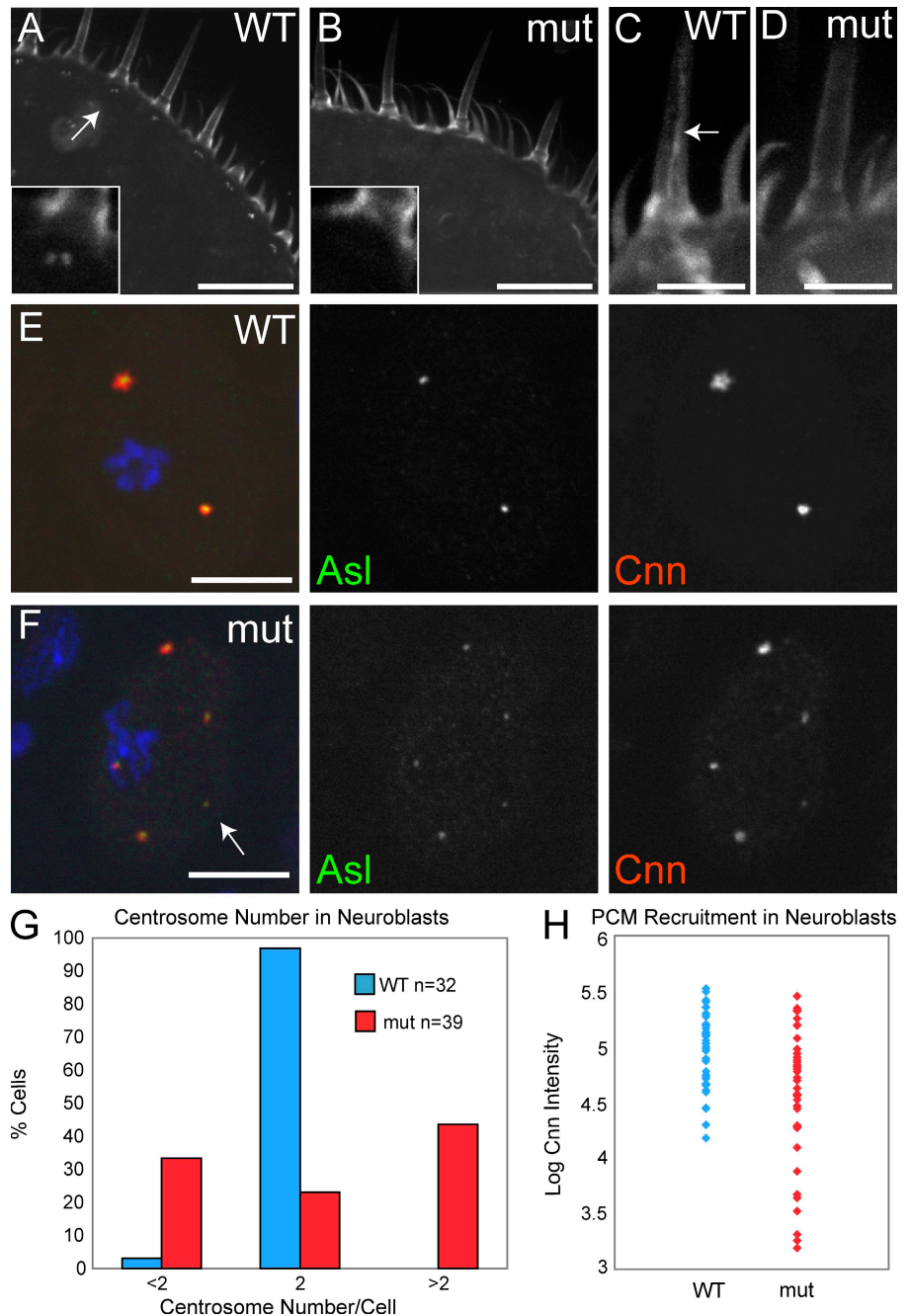
**Figure 2. Ana3 overexpression does not drive centrosome amplification or de novo formation.** (A) Centriole numbers (marked by GFP-PACT) in WT and Ana3-overexpressing G2 primary spermatocytes. Centrioles were counted in a total of 140 WT and 203 Ubq-Ana3-GFP cells from five testes per condition. (B) Centrosome numbers (identified by colocalization of D-PLP and Cnn) in WT and Ana3-overexpressing prophase neuroblasts. Centrosomes were counted in a total of 41 WT and 38 Ubq-Ana3-GFP cells from four brains per condition. (C and D) Unfertilized eggs laid by UAS-Sak-GFP (C) and UAS-Ana3-GFP (D) mothers stained for tubulin (red) and D-PLP (blue). The high level overexpression of Sak, like the other core duplication proteins, induces de novo assembly of centriole-like structures, which nucleate MT asters (magnified in C'). In contrast, high level overexpression of Ana3 does not induce de novo centriole formation, and the only MTs visible are those around the polar bodies (C' and D', arrows). Bars: (C and D) 100  $\mu$ m; (C' and D') 20  $\mu$ m.

(*Ana3*<sup>SH0558</sup>; hereafter referred to as the *Ana3* mutation). The phenotypes described in this study were indistinguishable between flies homozygous for this mutation and flies trans-heterozygous for the mutation and a deficiency uncovering the *Ana3* region. We also failed to detect any Ana3 protein on Western blots of *Ana3* mutant brains or by immunofluorescence at basal bodies in *Ana3* mutant testes (Fig. S1). These observations show that the *Ana3* mutation is at least a strong hypomorph. The phenotypes we describe in this study were completely rescued by coexpression of the Ubq-Ana3-GFP transgene (see Fig. 4 F) and reverted by precise excision of the P-element, demonstrating that it is the mutation in *Ana3* that causes these defects. We also recently obtained a second stock, *Ana3*<sup>G18168</sup>, with a P-element in the *Ana3* coding region (3 bps after the initiating ATG), and this exhibited very similar phenotypes to those described in this study (unpublished data).

*Ana3* mutant flies were viable but severely uncoordinated and died shortly after eclosion, as they became stuck in their food. This phenotype is often associated with defective cilia in type I sensory neurons (Dubruille et al., 2002) and is shown by mutants

for other centriolar components, including the core centriole duplication factors *Sak*, *DSas-4*, and *DSas-6* (Bettencourt-Dias et al., 2005; Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007a) as well as those required for the maintenance of basal body structure such as *D-PLP* (Martinez-Campos et al., 2004). To investigate the origin of this phenotype in the *Ana3* mutant, we examined the pupal third antennal segment, where the basal bodies of the sensory cilia can be observed using the markers GFP-PACT (Fig. 3 A) or DSas-4-GFP. In the *Ana3* mutant, basal bodies were undetectable with either of these markers (Fig. 3 B). We observed at least five WT and mutant antennae per experiment, and this was repeated four times with GFP-PACT and three times with DSas-4-GFP. We conclude that Ana3 is essential for proper basal body formation and/or maintenance in these sensory neurons. This defect would be expected to lead to a lack of cilia in *Ana3* mutant sensory neurons, which would explain the uncoordinated phenotype of the mutant flies. We confirmed the absence of cilia using the membrane marker mCD8-GFP (Fig. 3, C and D).

**Figure 3. *Ana3* is essential for basal body formation/maintenance in sensory neurons but not for centriole duplication.** (A and B) WT (A) and *Ana3* mutant (B) third antennal segments expressing GFP-PACT. In WT, this marker reveals the basal bodies at the base of each sensory bristle (arrow; magnified in insets). Basal bodies are undetectable in the mutant (mut). (C and D) WT (C) and *Ana3* mutant (D) third antennal segments expressing the membrane marker mCD8-GFP. In WT, this marker reveals a cilium, a thin line extending into the bristle (C, arrow). Cilia are undetectable in the mutant. (E and F) WT (E) and *Ana3* mutant (F) mitotic neuroblasts stained for DNA (blue), the centriole marker *Asl* (green), and the PCM protein *Cnn* (red). WT cells contain two centrosomes, whereas mutant cells frequently contain too few or too many centrosomes. The depicted mutant cell has five centrosomes with variable amounts of *Cnn*. The dimmest centriole (arrow) has the least *Cnn*. (G) Centrosome numbers (identified by colocalization of D-PLP and *Cnn*) in WT and *Ana3* mutant prophase neuroblasts. Centrosomes were counted in a total of 32 WT and 39 mutant cells from four brains per condition. (H) Quantification of *Cnn* levels at metaphase centrosomes in WT and *Ana3* mutant brain cells. Total fluorescence intensity was measured for a total of 40 centrosomes from four brains per condition. Bars: (A and B) 20  $\mu$ m; (C–F) 10  $\mu$ m.

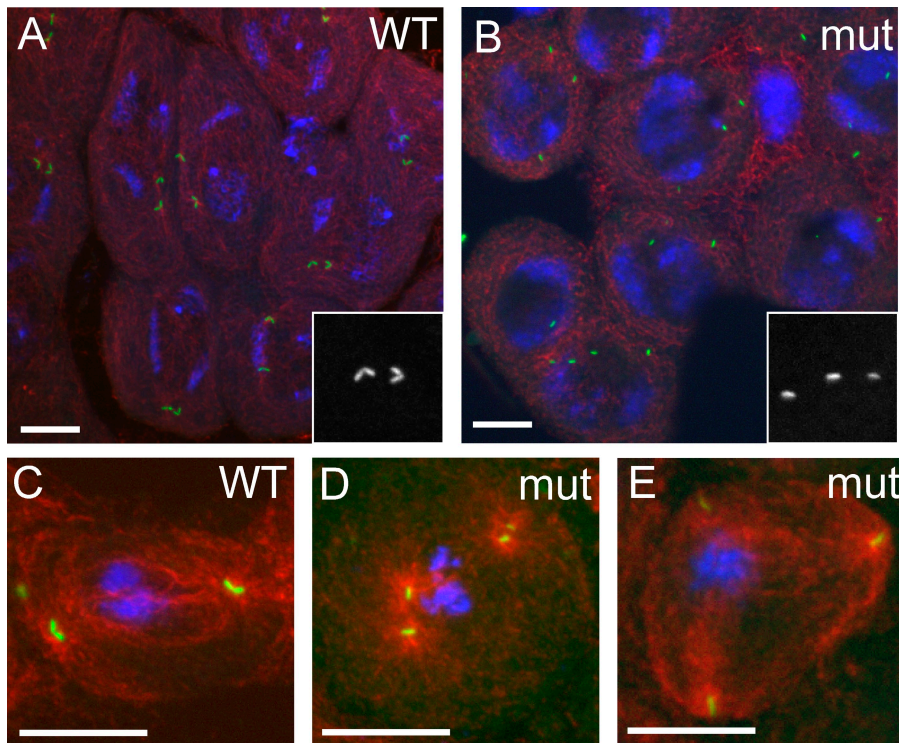


### **Ana3 is not essential for centriole duplication**

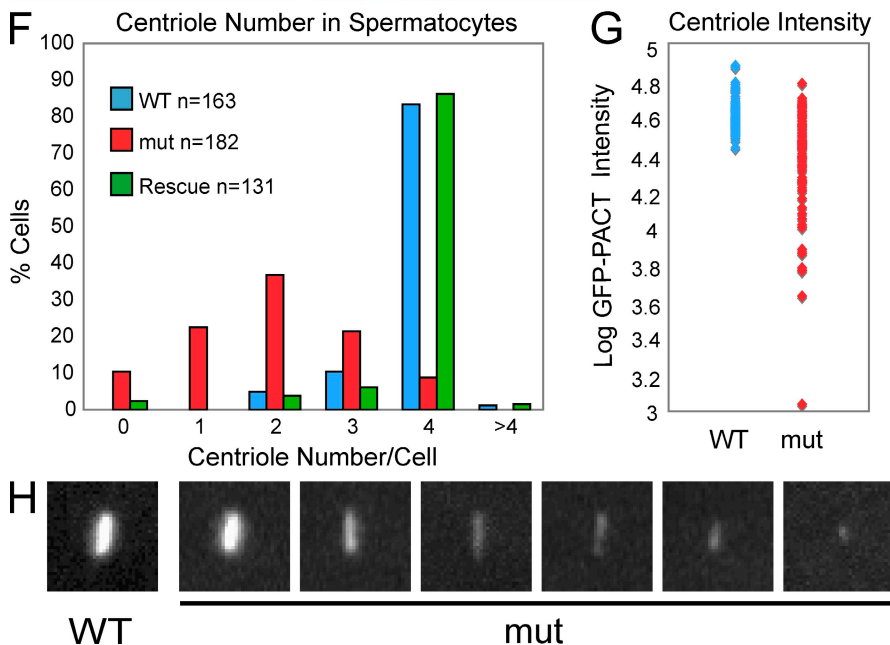
The lack of basal bodies in the sensory neurons of *Ana3* mutants suggested that *Ana3* could be essential for centriole duplication, as these cells may lack basal bodies because the flies lack centrioles, as is the case in *Sak*, *DSas-4*, and *DSas-6* mutants (Bettencourt-Dias et al., 2005; Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007a). To test this possibility, we stained mutant larval brain cells with various centriolar and centrosomal markers. To our surprise, we found a large variation in centriole and centrosome number, with a third of cells having too few centrioles and centrosomes and 44% of cells having too many (Fig. 3, E–G). In fact, the mean number of centrosomes per cell actually increased slightly in *Ana3* mutant brain cells (from 2 to 2.4). This is in stark contrast to previous observations in *Sak*, *DSas-4*, and *DSas-6*

mutant brains in which centriole and centrosome numbers are dramatically reduced (Bettencourt-Dias et al., 2005; Basto et al., 2006; Peel et al., 2007; Rodrigues-Martins et al., 2007a). We conclude that the primary defect in *Ana3* mutant brain cells is not a failure in centriole duplication.

It has previously been shown that a failure to recruit enough PCM to the centrioles during mitosis can lead to centrosome missegregation, which results in some cells having too many centrosomes and some having too few (Lucas and Raff, 2007). To test whether PCM recruitment occurred normally in *Ana3* mutants, we compared the levels of the PCM protein Centrosomin (*Cnn*) at metaphase centrosomes in WT and mutant brain cells. We found that most centrioles in mutant cells recruited normal amounts of PCM, but there was a class of



**Figure 4. Ana3 is required for centriole structure and cohesion.** (A–E) WT (A and C) and *Ana3* mutant (mut; B, D, and E) primary spermatocytes expressing GFP-PACT (green) stained for tubulin (red) and DNA (blue). (A and C) WT primary spermatocytes contain two v-shaped centriole pairs (A; magnified in inset), and the centrioles in each pair remain tightly associated until the end of meiosis I (C). (B) In the *Ana3* mutant, centriole numbers are reduced, and the centrioles (magnified in the inset) are almost never paired. (D and E) Prematurely separated centrioles nucleate asters during meiosis (D), leading to multipolar spindles (E). (F) Centriole numbers in WT, *Ana3* mutant, and *Ana3* mutant expressing *Ana3*-GFP (rescue) G2 primary spermatocytes. Centrioles were counted in a total of 163 WT, 182 mutant, and 131 rescue cells from at least seven testes per condition. (G) Quantification of centriole intensity (marked by GFP-PACT) in WT and *Ana3* mutant meiosis II spermatocytes. A total of 71 centrioles were measured from five testes per condition. (H) A WT centriole and six mutant centrioles (marked with GFP-PACT) from spermatocytes in prophase of meiosis II. Bars, 10  $\mu$ m.



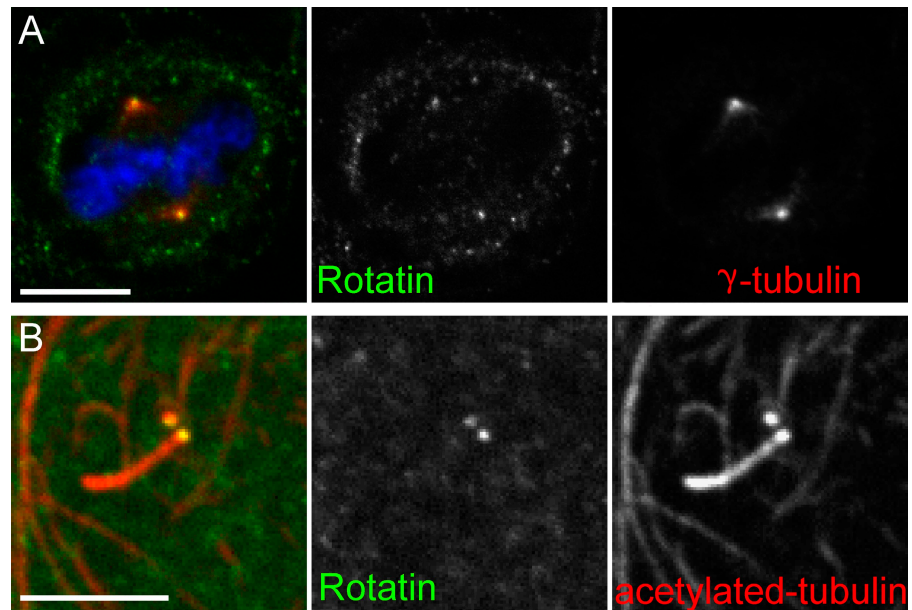
centrioles that recruited only very small amounts (Fig. 3 H). When we stained these cells with antibodies against centriolar proteins, the centrioles that organized the least PCM often appeared to be smaller and dimmer than normal (Fig. 3 F). Thus, it appears that at least some of the centrioles in *Ana3* mutant brain cells are abnormal and, consequently, recruit less PCM. This may lead to centrosome segregation defects, thus explaining, at least in part, the uneven distribution of centrioles and centrosomes.

#### **Ana3 is required to establish and/or maintain centriole structure and cohesion**

However, centrosome missegregation alone cannot explain the mean increase in centriole and centrosome numbers observed

in *Ana3* mutant brain cells. We reasoned that this might arise if the centriole pairs tended to separate prematurely. In brain cells, however, it is not possible to distinguish whether the dots we observe with centriole markers are single centrioles (as normally found in G1 cells) or closely apposed duplicated centrioles (diplosomes, as normally found in S and G2 cells). Therefore, to assess centriole cohesion, we examined the centrioles in WT and *Ana3* mutant spermatocytes. In mature primary spermatocytes, individual centrioles are easily resolved, and WT centrioles remain tightly paired in a v-shape arrangement until the end of meiosis I (Fig. 4, A and C). In contrast, virtually none of the centrioles in *Ana3* mutant spermatocytes

Figure 5. **Rttn localizes to the centrosome and basal bodies.** (A) A metaphase HeLa cell stained for Rttn (green),  $\gamma$ -tubulin (red), and DNA (blue). Rttn antibodies costain with  $\gamma$ -tubulin at the centrosome but label a smaller dot, suggesting that Rttn localizes to centrioles. (B) An interphase HB2 cell stained for Rttn (green) and acetylated tubulin (red). Rttn antibodies stain the basal bodies at the base of the cilium marked by acetylated tubulin. Bars, 10  $\mu$ m.



were paired (Fig. 4 B). Centriole numbers were reduced in the mutant, but  $\sim$ 30% of spermatocytes still had three or four centrioles (Fig. 4 F), allowing cohesion to be assessed. In 55 such G2 cells containing a total of 181 centrioles, we observed only 1 centriole pair from an expected 71 pairs. The prematurely separated centrioles remained competent to nucleate astral MTs during meiosis (Fig. 4 D), leading to multipolar spindles (Fig. 4 E). Thus, Ana3 is normally required to establish and/or maintain the connection between centrioles in spermatocytes. We suspect that this is also true in brain cells, which could explain the apparent increase in centrosome number in these cells in *Ana3* mutants.

The large size of the spermatocyte centrioles also allowed us to investigate the effect of loss of Ana3 function on centriole structure. We quantified the centriolar fluorescence intensity of the centriole markers GFP-PACT, Ana1, and GTU88\* and found that a large proportion of centrioles in mutant spermatocytes fell below the normal range (Fig. 4, G and H; and Fig. S2, A and B). We also analyzed the distribution of DSas-6, which we found at the proximal and distal tips of 84% ( $n = 88$ ) of WT primary spermatocyte centrioles (Fig. S2 C); in 16% of WT centrioles, DSas-6 was only detected at one tip. In contrast, in *Ana3* mutant primary spermatocytes, DSas-6 was only detected at both centriole tips in 36% ( $n = 88$ ) of centrioles; in the majority of centrioles, DSas-6 was undetectable at one (43%) or both (21%) tips (Fig. S2, D–F). Thus, centrioles in *Ana3* mutant spermatocytes recruited less of every centriole marker we tested, strongly suggesting that these centrioles are structurally abnormal.

We also observed structurally abnormal centrioles and axonemes in *Ana3* mutant testes by EM (Fig. S2, G–N). Indeed,  $\sim$ 50% of mutant axonemes were missing between one and seven MT doublets. As Ana3-GFP does not localize to the axoneme and Ana3 antibodies do not stain the axoneme (Fig. 1 D and Fig. S1 B), it seems unlikely that Ana3 plays a direct role in axoneme structure. Thus, the axoneme defects are likely to be a

consequence of the abnormalities we have observed in centriole/basal body structure.

#### Ana3 defines a conserved family of centriolar proteins that appear to be required for cilia function

We wanted to see whether Ana3 has a conserved role in centriole and/or basal body function. Using an iterative BLAST search based on the Ana3 homologues we identified in other insect species, we found significant homology to the Rotatin (Rttn) family of proteins first identified in mice. Moreover, the reciprocal iterative BLAST search, starting with human Rttn, identified Ana3 as the most similar *Drosophila* protein. Overall, Ana3 is 19% identical and 34% similar to human Rttn.

Interestingly, homozygous *Rttn* mutant mice show a variety of defects characteristic of defective cilia function, such as randomized left–right asymmetry and neural tube abnormalities (although the potential link between these phenotypes and cilia malfunction was not appreciated at the time of these studies; Melloy et al., 1998; Faisst et al., 2002; Chatterjee et al., 2007). To test whether Rttn localizes to centrosomes, we raised and affinity purified rabbit polyclonal antibodies against two regions of the human protein. We found that both antibodies stained centrosomes in HeLa cells and basal bodies in HB2 cells that had formed cilia (Fig. 5, A and B). To demonstrate the specificity of our antibodies, we depleted Rttn using siRNAs and by generating clonal HeLa and HB2 cell lines expressing short hairpin RNAs (shRNAs) targeting *Rttn* (Fig. S3 A). We quantified Rttn centrosome staining in one of our HeLa shRNA lines, and the mean fluorescence intensity was reduced to 56% of levels in control (empty vector) cells (Fig. S3, B–D). We were unable to completely deplete Rttn and did not observe an obvious phenotype. Nevertheless, the centrosomal localization of Rttn, combined with the previous mouse mutant studies, indicates that Rttn could perform a similar function to Ana3 in mammalian cells. Thus, we propose that Ana3 defines a new family of proteins required for centriole and basal body function.

## Conclusions

Our results from both gain and loss of function experiments strongly suggest that Ana3 is not a core centriole duplication protein. Although Ana3 appears to be dispensable for centriole duplication, it is required for the structural integrity of centrioles and basal bodies and for centriole cohesion. We cannot be certain whether the role played by Ana3 in centriole structure is in assembly or maintenance. We favor the latter possibility, as we have observed centrioles in the *Ana3* mutant that appear normal, indicating that centrioles may assemble correctly without Ana3. However, we propose that these centrioles are structurally unstable, leading to their gradual disintegration. The structural defects may then cause a failure in centriole cohesion, although it is also possible that Ana3 plays a direct role in cohesion. Previous studies on *Rttm* mutant mice indicate that Ana3 homologues are likely to perform a similar function in other species (Melloy et al., 1998; Faisst et al., 2002; Chatterjee et al., 2007). Therefore, we predict that mutations in human *Rttm* are likely to be associated with the many varied phenotypes of human ciliopathies.

## Materials and methods

### Generation of GFP fusions and transgenic lines

The two Ana3 exons were amplified separately from genomic DNA with *att* sites at either end for Gateway cloning (Invitrogen). The reverse primer for exon 1 and the forward primer for exon 2 also included KpnI sites. These fragments were inserted separately into Gateway pDONR Zeo vectors. Both vectors were digested with KpnI and SmaI, and exon 2 was ligated into the exon 1 vector to produce a vector with the complete coding sequence. This was recombined with Ubq and UASp plasmids (Peel et al., 2007) with the coding sequence placed in frame with GFP at the C terminus. Transgenic lines were generated by standard P-element-mediated transformation by BestGene.

### Fly stocks and methods

We used *w<sup>67</sup>* or *w<sup>f</sup>* flies as WT controls. We obtained the *Ana3<sup>SH0558</sup>* mutation and the Df(2R)Exel6061 chromosome from the Bloomington stock center and *Ana3<sup>G<sup>18168</sup></sup>* from the GenExel *Drosophila* EP collection (Biomedical Research Center, Korea Advanced Institute for Science and Technology). DSas-4-monomeric RFP (Lucas and Raff, 2007), DSas-4-GFP (Peel et al., 2007), and GFP-PACT (Martinez-Campos et al., 2004) transgenic lines all contain GFP or RFP fusions driven by the Ubq promoter, which drives moderate expression in all tissues (Lee et al., 1988). The UASp-Sak-GFP (Peel et al., 2007) and UASp-Ana3-GFP lines were crossed to V32a, which expresses a Gal4/VP16 fusion protein from a maternal tubulin promoter; this drives very high level overexpression in the female germline (Peel et al., 2007).

To assess uncoordination, mutant pupae (nontubby pupae from an *Ana3<sup>SH0558</sup>/Sm6<sup>Δ</sup>Tm6* stock) were selected and transferred to a Petri dish as described previously in Stevens et al. (2007). The eclosed flies were observed to assess their ability to walk, fly, and feed. P-element excision was performed using standard genetic methods and precise excisions confirmed by sequencing.

### Generation of antibodies

Maltose-binding protein (MBP; New England Biolabs, Inc.) fusions of the following regions of the Ana3, Rttm, Ana1, and Asterless (Asl) proteins were purified according to the manufacturer's instructions: Ana3 (aa 2–300), Rttm N terminal (aa 343–584), Rttm Mid (aa 1,347–1,591), Ana1 (aa 790–1,089 of short B form), and Asl (aa 1–333). Antisera were raised against each protein in two rabbits by Eurogentec. To affinity purify antibodies, antisera were first depleted of anti-MBP antibodies by passing over an MBP column (AminoLink; Thermo Fisher Scientific). Specific antibodies were purified by passing each antiserum over the appropriate column of MBP fusion protein. The column was washed with PBS + 0.5 M KCl and antibodies eluted in 0.1 M glycine, pH 2.1. The antibodies were neutralized with 1 M Tris, pH 8.5, and glycerol added to 50%, and they were then stored at –20°C.

### Live analysis in embryos

Embryos expressing Ubq-Ana3-GFP were dechorionated by hand and mounted on a coverslip on a stripe of glue that had been dissolved in heptane. Embryos were covered in voltalef oil and observed at room temperature on a spinning-disc confocal system (UltraView ERS; PerkinElmer) mounted on an inverted microscope (Axiovert 200M; Carl Zeiss, Inc.) with a charge-coupled device camera (Orca ER; Hamamatsu Photonics) using a 63×/1.25 NA objective (Carl Zeiss, Inc.) with Immersol oil (Carl Zeiss, Inc.). Images were acquired using UltraView ERS software and made into videos using Volocity (PerkinElmer).

### Fixed analysis of eggs, embryos, larval brains, pupal testes, and antennal segments

0–4-h collections of eggs and embryos were dechorionated in 60% bleach for 2 min, washed in water + 0.05% Triton X-100, and washed into a small glass bottle with 1 ml heptane. 1 ml methanol + 5% 0.25 M EGTA was added, and the bottle was shaken gently until most eggs/embryos fell into the lower methanol/EGTA layer. Eggs/embryos were stored in methanol at 4°C. For immunostaining, embryos were rehydrated by washing in PBT (PBS + 0.1% Triton X-100), blocked in PBS + 5% BSA, and incubated with primary antibodies at 1–2 µg/ml in PBS/BSA overnight at 4°C. Eggs/embryos were washed in PBT before incubation with secondary antibodies diluted (1:1,000) in PBT for 4 h at room temperature. After final washes in PBT, eggs/embryos were mounted in mounting medium (85% glycerol and 2.5% *n*-propylgallate).

Larval brains were dissected in PBS and fixed in PBS + 4% formaldehyde for 20 min. Brains were transferred to 45% acetic acid for 15 s and then to 60% acetic acid on a coverslip for 3 min. Brains were squashed between slide and coverslip by tapping with a pencil on the coverslip, and slides were flash frozen in liquid nitrogen. Coverslips were removed, and slides were incubated in cold methanol for 8 min at –20°C. Samples were rehydrated by washing in PBT before incubation with the primary antibody under a mounted coverslip in a moist chamber overnight at 4°C. The remaining steps were performed as described for eggs/embryos in the previous paragraph.

Pupal testes were dissected in PBS, placed on a coverslip, and cut open. A slide was placed over the coverslip, and the slide was flash frozen in liquid nitrogen. Coverslips were removed, and slides were incubated for 5 min in methanol at –20°C and in acetone for 1–2 min at –20°C. This was followed by incubation in PBT for 10 min, washes in PBS, and blocking in 1% PBS/BSA. Slides were incubated in primary antibody (diluted in PBS/BSA) as described for brains. After washes in PBS, slides were incubated in secondary antibodies (diluted 1:300 in PBS) for 1 h at 25°C. After final washes, slides were mounted in mounting medium.

Pupal antennal segments were dissected in PBS and fixed in PBT + 4% formaldehyde for 2 h. Antennae were washed in PBS before mounting in mounting medium.

Images were acquired using the aforementioned microscope system (see Live analysis in embryos), imported into Photoshop (CS2; Adobe), and adjusted to use the full range of pixel intensities.

### Quantification of centrosome numbers in neuroblasts

Brains were stained with antibodies against phosphohistone H3, Cnn, and D-PLP. Prophase neuroblasts were selected to ensure that centrosomes were duplicated but extra centrosomes would not be clustered at the spindle poles. These cells were identified using DNA morphology and PH3 staining, and dots were scored as centrosomes only if they costained for Cnn and D-PLP.

### Quantification of PCM recruitment in neuroblasts

The intensity of Cnn staining at metaphase centrosomes was measured in fixed samples of WT and *Ana3* mutant third instar larval brains. Stacks of images spanning the entire centrosomal volume were taken at 0.2-µm intervals using the aforementioned confocal system (see Live analysis in embryos). Centrosomes were identified semiautomatically using Volocity in projections of these stacks. Total fluorescence intensity was measured for a total of 40 centrosomes taken from four brains for each condition.

### Quantification of centriole intensity in spermatocytes

Images were acquired as described in the previous paragraph from fixed samples of WT and *Ana3* mutant pupal testes expressing GFP-PACT or stained for Ana1 or GTU88\*. Individual centrioles from meiosis II cells (in which the centrioles are well separated) were measured as described in the previous paragraph. A total of 71 (GFP-PACT), 86 (Ana1), or 46 (GTU88\*) centrioles were measured from five testes for each condition.

## Antibodies

The following antibodies were used at a 1:1,000 dilution: rabbit anti-D-PLP (Martinez-Campos et al., 2004), mouse monoclonal anti- $\alpha$ -tubulin (DM1 $\alpha$ ; Sigma-Aldrich), mouse anti-phosphohistone H3 (Abcam), guinea pig anti-Cnn (Dix and Raff, 2007), mouse monoclonal anti-acetylated tubulin (6-11B-1; Sigma-Aldrich), mouse monoclonal anti- $\gamma$ -tubulin (GTU88; Sigma-Aldrich), rabbit anti-Dsas-6 (Peel et al., 2007), and GTU88\*, a batch of the GTU88 antibody that cross reacts with centrioles in flies (Martinez-Campos et al., 2004). Alexa Fluor 488, Cy3, and Cy5 secondary antibodies were obtained from Invitrogen or Jackson ImmunoResearch Laboratories, Inc.

## EM

Testes were dissected in phosphate buffer and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, overnight at 4°C. This was followed by three washes in phosphate buffer and postfixation with 1% OsO<sub>4</sub> for 1 h at 4°C. Samples were stained for 1 h in uranyl acetate, dehydrated in a series of ethanol, embedded in Agar 100, and polymerized for 2 d at 60°C. Ultrathin sections were stained with uranyl acetate and lead citrate and observed at room temperature using an electron microscope (Technai 12; Philips) at 80 kV with a charge-coupled device camera (2K US10001; Gatan). Images were acquired using Digital Micrograph software (Gatan).

## Electrophoresis and immunoblotting

WT and Ubq-Ana3-GFP methanol-fixed embryos were rehydrated, and 20 precellularized embryos were selected. 10 WT and 10 Ana3 mutant brains were dissected in PBS. Embryos and brains were homogenized in SDS sample buffer. The proteins were separated in a 3–8% gradient precast NuPAGE (Invitrogen) acrylamide gel and transferred to a Hybond-P membrane (GE Healthcare). After transfer, the membrane was blocked in milk solution (TBS, 10% glycerol, and 3% milk powder) before incubation for 2 h with primary antibodies (diluted to 2  $\mu$ g/ml in milk solution). The membrane was washed in TBST (TBS + 0.1% Tween-20) and incubated with HRP-conjugated secondary antibodies (GE Healthcare) diluted in TBST (1:10,000) for 1 h. Finally, the membrane was washed in TBST, incubated with chemiluminescent substrate (Thermo Fisher Scientific) according to manufacturer's instructions, and exposed to x-ray film.

## Identification of Ana3 homologues and sequence alignments

The position-specific iterated BLAST algorithm (Altschul et al., 1997) obtained from the National Center for Biotechnology Information was used to search for homologues of Ana3. Rttm proteins from many species were identified in round 2, and Rttm was the top human hit (E value =  $2e^{-25}$ ). A reciprocal position-specific iterated BLAST using human Rttm as the input sequence identified Ana3 as the top hit from *Drosophila*. Multiple sequence alignments were performed using ClustalW2 (Larkin et al., 2007) and visualized in Jalview (Waterhouse et al., 2009) using the Blossum62 coloring scheme.

## Human tissue culture

HeLa cells were cultured in DME supplemented with 10% FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. HB2 mammary epithelial cells were grown in DME supplemented with 10% FBS, 5.0  $\mu$ g/ml hydrocortisone, 10  $\mu$ g/ml insulin, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin.

## Transfection

HeLa cells were transfected with SMARTpool (Thermo Fisher Scientific) siRNAs targeting *Rttm* or Silencer negative control siRNAs (Applied Biosystems) using Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were fixed for immunostaining, and protein was extracted for Western blot analysis 3 d after transfection.

## Retroviral shRNA

The shRNA targeting *Rttm* (target sequence 5'-GGAGTAATCAGAAGAG-TAAC-3') was cloned into the modified MSCV-miR30puro vector (provided by S. Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Empty vector was used as a control. Retroviruses were packaged using amphoteric phoenix cells;  $5 \times 10^6$  cells were plated in a 10-cm dish 24 h before transfection by calcium phosphate precipitation with 15  $\mu$ g retroviral vector (for 10 h at 37°C). After 2 d, the virus-containing medium was removed from the phoenix cells, filtered with a 0.45- $\mu$ m filter (Millipore), and supplemented with polybrene (Sigma-Aldrich) to a final concentration of 5  $\mu$ g/ml before being added to the target cells. After two more infections

(at least 4 h apart), the target cells were incubated for 12 h. Cells with stable integration were selected with 3  $\mu$ g/ml puromycin (Sigma-Aldrich) for 3 d. To generate single-cell clones, cells were trypsinized and transferred to 150-mm plates after viral transfection and selected in puromycin for 10 d. Single colonies were transferred to 24-well plates using cloning cylinders (Sigma-Aldrich) and maintained in puromycin.

## Quantification of Rttm centrosome staining

The intensity of Rttm staining at metaphase centrosomes was measured in fixed samples of control (empty vector) and *Rttm* knockdown (shRNA) HeLa cells. Total fluorescence intensity was measured for 35 centrosomes for each condition using the method for Cnn staining described in Quantification of PCM recruitment in neuroblasts.

## Online supplemental material

Fig. S1 shows that Ana3 protein is undetectable in the Ana3 mutant both by Western blotting and by immunofluorescence in spermatid basal bodies. Fig. S2 shows that Ana3 mutant centrioles appear structurally abnormal both by immunofluorescence and EM. Fig. S3 shows that Rttm antibodies recognize Rttm on Western blots and by immunofluorescence. Video 1 shows that Ana3-GFP localizes to centrosomes throughout the cell cycle in syncytial embryos and does not induce centriole overduplication. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200905031/DC1>.

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