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

Naomi R. Stevens,1 Jeroen Dobbelaere,1 Alan Wainman,2 Fanni Gergely,3 and Jordan W. Raff1,2

1The Gurdon Institute, Cambridge CB2 1QN, England, UK
2Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, England, UK
3Cancer Research UK Cambridge Research Institute, Cambridge CB2 0RE, England, UK

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.
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 ~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 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
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).

(Ana3SH0558; 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, Ana3G18168, 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 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 µm; (C' and D') 20 µm.
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

\[\text{Ana3 is not essential for centriole duplication.}\]

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). We 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
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. 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 µm.

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. 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 µm.

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...
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 structure.
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 Rtn mutant mice indicate that Ana3 homologues are likely to perform a similar function in other species (Mellony et al., 1998; Faisst et al., 2002; Chatterjee et al., 2007). Therefore, we predict that mutations in human Rtn 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 Zea 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 w1118 or w1118 flies as WT controls. We obtained the Ana3G18168 mutation and the Df[2R]Exel6061 chromosome from the Bloomington stock center and Ana3exad3 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 anAna3/G18168 stock) were selected and transferred to a Petri dish at 20°C. These cells were identified using DNA morphology and PH3 staining (see Live analysis in embryos). Neuroblasts were identified semiautomatically using Volocity in fixed samples of WT and mutant third instar larval brains. Stacks of confocal images spanning the entire centrosomal volume were taken at 0.2-µm intervals using the aforementioned confocal system (seeLive analysis in embryos), imported into Photoshop (CS2; Adobe), and adjusted to use the full range of pixel intensities.

Generation of antibodies
Maltose-binding protein (MBP; New England Biolabs, Inc.) fusions of the following regions of the Ana3, Rtn, Ana1, and Asterless (Asl) proteins were purified according to the manufacturer’s instructions: Ana3 (aa 2–300), Rtn N terminal (aa 343–584), Rtn Mid (aa 1,347–1,591), Ana1 (aa 790–1,089 of short B form), and Asl (aa 1–333). Antiseras 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 volatol 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 (Ccrs 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-propyligalactate).

Larval brains were dissected in PBS and fixed in PBS + 4% formaldehyde for 20 min. Brains were transferred to 45% 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 centrosomes 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 contained 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 centrosome 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 GTUB8*. 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 (GTUB8*) centrioles were measured from five testes for each condition.
The following antibodies were used at a 1:1,000 dilution: rabbit anti-D-PLP (Martinez-Campos et al., 2004), mouse monoclonal anti-α-tubulin (DM1α; 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-γ-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.

Electrophoresis and immunoblotting
WT and Ubaq-Ana3-GFP methanolixed 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 µg/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), 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. Rtn proteins from many species were identified in round 2, and Rtn was the top human hit (E value = 2 × 10−3). A reciprocal position-specific iterated BLAST using human Rtn 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 Blosum62 coloring scheme.

Human tissue culture
HeLa cells were cultured in DME supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. HB2 mammary epithelial cells were grown in DME supplemented with 10% FBS, 5.0 µg/ml hydrocortisone, penicillin, and 100 µg/ml streptomycin. HB2 mammary epithelial cells were identified in round 2, and Rttn was the top human hit (E value = 2e−17) from the National Center for Biotechnology Information was obtained from the National Center for Biotechnology Information and visualized in Jalview (Waterhouse et al., 2009) using the Blosum62 coloring scheme.

Transfection
HeLa cells were transfected with SMARTpool (Thermo Fisher Scientific) siRNAs targeting Rtn 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 days after transfection.

Retroviral shRNA
The shRNA targeting Rtn (target sequence 5′-GGAGTAAATCCAGAGATG-3′) was cloned into the modified MSCVmR3puro 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 × 10⁵ cells were plated in a 10-cm dish 24 h before transfection by calcium phosphate precipitation with 15 µg 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-µm filter (Millipore), and supplemented with polybrene (Sigma-Aldrich) to a final concentration of 5 µg/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 µg/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 Rtn centrosome staining
The intensity of Rtn staining at metaphase centrosomes was measured in fixed samples of control (empty vector) and Rtn 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 cellular bodies. Fig. S2 shows that Ana3 mutant centrioles appear structurally abnormal both by immunofluorescence and EM. Fig. S3 shows that Rtn antibodies recognize Rtn 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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