

# Basal Body Duplication and Maintenance Require One Member of the *Tetrahymena thermophila* Centrin Gene Family<sup>□</sup>

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**Centrins, small calcium binding EF-hand proteins, function in the duplication of a variety of microtubule organizing centers. These include centrioles in humans, basal bodies in green algae, and spindle pole bodies in yeast. The ciliate *Tetrahymena thermophila* contains at least four centrin genes as determined by sequence homology, and these have distinct localization and expression patterns. *CEN1*'s role at the basal body was examined more closely. The Cen1 protein localizes primarily to two locations: one is the site at the base of the basal body where duplication is initiated. The other is the transition zone between the basal body and axoneme. *CEN1* is an essential gene, the deletion of which results in the loss of basal bodies, which is likely due to defects in both basal body duplication and basal body maintenance. Analysis of the three other centrins indicates that two of them function at microtubule-rich structures unique to ciliates, whereas the fourth is not expressed under conditions examined in this study, although when artificially expressed it localizes to basal bodies. This study provides evidence that in addition to its previously known function in the duplication of basal bodies, centrin is also important for the integrity of these organelles.**

## INTRODUCTION

Centrins, also called caltractins, are widely conserved members of the EF-hand superfamily of calcium-binding proteins (Salisbury, 1995). Although sharing ~50% identity with the ubiquitous calcium sensor calmodulin, centrins perform distinct functions within the eukaryotic cell. In particular, centrins have a variety of roles at microtubule organizing centers (MTOCs) and microtubule-based structures (Salisbury, 1995; Vaughn and Harper, 1998; Chapman *et al.*, 2000). Additionally, centrins are constituents of calcium-responsive contractile fibers found in many protists (Salisbury *et al.*, 1984; Wright *et al.*, 1985).

One of the widely recognized roles for centrin is in the duplication of microtubule organizing centers, including centrosomes, basal bodies, and the yeast spindle pole body (Baum *et al.*, 1986; Salisbury *et al.*, 2002; Koblenz *et al.*, 2003). Centrosomes consist of two orthogonally oriented centrioles surrounded by amorphous pericentriolar material from which spindle and astral microtubules are organized. Centrioles are distinguished by 9-fold symmetry of microtubules in a “flower” arrangement, with the majority of metazoans displaying triplet microtubules at these positions. Basal bodies are structurally similar to centrioles and differ primarily in the manner in which they organize microtubules. Cilia and flagella, which consist of nine doublet microtubules surrounding a central microtubule pair, are nucleated from the distal end of the basal body (reviewed in Beisson and Wright, 2003). In some situations, it is the identical organelle that acts as a centriole and then is converted to a basal body, or vice versa, at particular points in the life cycle of the cell. This is the case in many vertebrate cells where the oldest centriole is capable of forming a single “9 + 0” cilium (known as the primary cilium) that is assembled during the G<sub>1</sub> phase of the cell cycle and disassembled during mitosis (Jensen *et al.*, 1987). The budding yeast spindle pole body is structurally distinct from the centriole and basal body. This trilaminar structure remains embedded within the nuclear envelope throughout the cell cycle. Spindle and astral microtubules emanate from the inner and outer plaques, respectively. Although dissimilar in appearance to the centrosome, the spindle pole body performs the same task of organizing mitotic and meiotic spindles, and some components of these organelles, including centrin, are conserved between these divergent organelles (Adams and Kilmartin, 2000).

Temperature-sensitive mutations in the yeast centrin gene *CDC31* result in failure in an early step of spindle pole body duplication and a cell cycle arrest in G<sub>2</sub>/M phase, such that cells arrest with a single, enlarged spindle pole body (SPB) (Byers, 1981; Baum *et al.*, 1986). Like SPB duplication, basal body and centriole duplication is dependent on centrin function, as demonstrated by RNA interference (RNAi) experi-

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ments in both human cells and *Chlamydomonas*. Thus far, three centrin genes have been cloned from human cells (Lee and Huang, 1993; Errabolu *et al.*, 1994; Middendorp *et al.*, 1997). When human centrin2 levels are reduced by RNAi, cells exhibit defects in centriole duplication (Salisbury *et al.*, 2002). The same is true when levels of the *Chlamydomonas* centrin (Vfl2) are decreased to 5% of the normal level. In these cells, the number of basal bodies is reduced to ~0.3 per cell from the normal condition of two per cell, indicating a failure in basal body duplication (Koblenz *et al.*, 2003). Defects also were observed in the structure of remaining basal bodies, indicating a possible role for centrin in basal body maturation or stability.

In addition to its essential role in MTOC duplication, centrin is involved in other cellular processes. In yeast, Cdc31 is required for Kic1 kinase activity that is important for proper cell wall development (Sullivan *et al.*, 1998). Cdc31 also is involved in mRNA export at the nuclear pore complex (Fischer *et al.*, 2004). The contractile properties of centrin are important in a number of structures, including the nucleus-basal body connector and stellate fibers associated with *Chlamydomonas* basal bodies (Sanders and Salisbury, 1989; Taillon *et al.*, 1992; Sanders and Salisbury, 1994), and the infraciliary lattice, a contractile cortical network in *Paramecium* (Madeddu *et al.*, 1996; Klotz *et al.*, 1997). Centrin also has been associated with the inner dynein arm important for axonemal beating (LeDizet and Piperno, 1995; Guerra *et al.*, 2003).

In some organisms, such as yeast and *Chlamydomonas*, the product of a single centrin gene carries out multiple functions. However, other species have multiple centrin genes that may encode products that are specialized to carry out different tasks or to carry out similar tasks at different stages of the life cycle. In humans, centrin1 expression is confined to the testes and retinal cells, whereas centrin2 and 3 are more ubiquitously expressed (Hart *et al.*, 1999; Laoukili *et al.*, 2000). Strikingly, when a *Leishmania* centrin gene was deleted, one of the two lifestyle forms of this parasite was unable to duplicate its basal bodies and was unable to progress through mitosis, whereas the other was unaffected, suggesting that a different centrin was active during this stage of its life cycle (Selvapandiyar *et al.*, 2004).

Because the ciliate *Tetrahymena thermophila* has ~750 basal bodies per cell (Frankel, 2000; Frankel and Nelsen, 2001) and sophisticated genetic and molecular biological techniques are available for its study (Bruns and Cassidy-Hanley, 2000a,b; Gaertig and Kapler, 2000; Hai *et al.*, 2000), we turned to *Tetrahymena* to gain insights into basal body/centriole duplication. Centrin has been localized to basal bodies and cilia in *Tetrahymena*, and a centrin gene has been cloned (Guerra *et al.*, 2003). We independently cloned this centrin gene, called *CEN1*, and have begun characterizing it and three other centrin genes we identified through sequence similarity in the recently sequenced *Tetrahymena* genome. By using fusions with the green fluorescent protein (GFP) (Chalfie *et al.*, 1994), we localized Cen1 and the second of the centrin genes (*CEN2*) to basal bodies. We created a null mutation in *CEN1* and have shown it is required for basal body duplication and stability. Furthermore, we found that the third centrin (*CEN3*) localizes to specialized structures in the oral apparatus, a complex network of cilia, and accessory structures that sweeps food into the cell. The fourth centrin (*CEN4*) also localizes to these structures in the oral apparatus as well as to contractile vacuole pores (the openings for the contractile vacuoles that maintain the proper osmolarity of the cells) and to basal bodies (Frankel, 2000).

## MATERIALS AND METHODS

### Strains, Culture Conditions, and Matings

*T. thermophila* strains B2086, CU427, and CU428 (all generous gifts from Dr. Peter Bruns, Cornell University, Ithaca, NY) were the starting point for new strain development. Strains with constructs encoding GFP-Centrin fusion proteins were created as described below. UCB8 and UCB9 are *CEN1* knockout heterokaryons (described below). B\*VI (gift from Dr. Aaron Turkewitz, University of Chicago, Chicago, IL) was used for star crosses in strain construction (Hai *et al.*, 2000). All were grown in SSP (2% proteose peptone, 0.1% yeast extract, 0.2% glucose, 0.003% FeEDTA) at 30°C. Starvation media consisted of 10 mM Tris, pH 7.5 (Orias *et al.*, 2000). For matings, an equal number of cells of different mating types, previously starved for 18 h, were mixed (Gaertig and Kapler, 2000).

### Gene Identification and Sequence Analysis

To search for centrin genes in *Tetrahymena*, PCR using degenerate primers EIK-F and VAK-R, designed to represent regions of high conservation between centrin genes, was conducted (Supplemental Table 1 lists all primers used in this study). Standard molecular biology techniques were used to isolate a centrin gene, which was subsequently reported by Guerra *et al.* (2003). To search for additional centrin genes, preliminary sequence data were obtained from The Institute for Genomic Research Web site at <http://www.tigr.org>. *Tetrahymena CEN1* translated sequence was used to query the *Tetrahymena* database using the tblastn program. *CEN2* was found on sequence #8254664 between positions 498633 and 499370, with possible introns spanning 498790–498909, 499005–499063, and 499256–499320. *CEN3* was found on sequence #8253915 between positions 239917 and 240426. *CEN4* was found on sequence #8254437 between positions 298144 and 298659. Sequences were compared and analyzed at the Pasteur Bioweb site ([bioweb.pasteur.fr](http://bioweb.pasteur.fr)). ClustalW (Thompson *et al.*, 1994) was used to align the sequences, and BOXSHADE (written by Hofmann and Baron) was used to create the image of the alignment. Using PHYLIP (Phylogeny Inference Package), protein distances were calculated with PROTDIST (Jones–Taylor–Thornton matrix), and a tree was constructed using NEIGHBORS followed by DRAWGRAM for image generation. For statistical analysis, 1000 replicates were performed and bootstrap values were calculated. (Felsenstein, J. 2004. PHYLIP version 3.6. Distributed by the author, Department of Genome Sciences, University of Washington, Seattle, WA)

### Plasmid Construction

As a starting point for the creation of a *CEN1* knockout plasmid, pHM74 was constructed. A 2.1-kb fragment containing *CEN1* was amplified from genomic *Tetrahymena* DNA by PCR (Saiki *et al.*, 1988) using primers RM13 and RM14, which were engineered to include unique *Bam*HI and *Sac*II sites at their respective 5' ends. This fragment, which begins 1 kb upstream of the *CEN1* start codon, was cloned into the *Bam*HI and *Sac*II sites of p4T2-1 (Gaertig *et al.*, 1994a) downstream of the NEO2 cassette. Primers RM11 and RM12, which were engineered to contain unique *Kpn*I and *Xho*I sites at their respective ends, were then used to amplify a 1.6-kb genomic fragment upstream of *CEN1*. This fragment was cloned into *Kpn*I/*Xho*I-digested p4T2-1, upstream of the NEO2 cassette, to create pHM74. pHM74 was digested with *Kpn*I and *Bam*HI, eliminating the NEO2 cassette and the 1.6-kb *CEN1* upstream region. A *Kpn*I-*Bam*HI adapter from EZCloneSystems (New Orleans, LA) was used to recircularize the plasmid. This construct was digested with *Eco*RI to remove most of the *CEN1* coding sequence, including the start codon, and blunted. A *Sma*I, *Eco*RV fragment from pHM74 containing the NEO2 cassette was inserted into this plasmid, creating pCentrin:NEO 1. To obtain more sequence downstream of the *CEN1* gene, a library of 3- to 4-kb *Pst*I, *Eco*RI-digested genomic DNA was created in pBluescript KSII<sup>-</sup> (Stratagene, La Jolla, CA) and transformed into *Escherichia coli* for colony lifts. A PCR fragment generated by amplification of pHM74 DNA with primers RM10 and T7 was used as a probe. Lifts and labeling of the probe were conducted as per instructions for Hybond-N<sup>+</sup> nylon membrane and the AlkPhos direct labeling system (Amersham Biosciences, Piscataway, NJ). A clone was recovered with ~3 kb of sequence downstream of *CEN1*, and a *Bgl*III, *Sac*I fragment was cloned from this into pCentrin:NEO 1 to create pCentrin:NEO1+48. To use all of the upstream sequence available, a PCR fragment from genomic DNA spanning the site where the NEO2 cassette had been inserted in the sequence upstream of *CEN1* was amplified with primers NEORemove1 and NEORemove2, digested with *Bcl*I and *Spe*I, and inserted into similarly digested pHM74, creating pHM74-NEORemove. Finally, a *Bcl*I, *Kpn*I fragment from this plasmid was ligated into similarly digested pCentrin:NEO1+48 to create pMicCentrinKO1.

For the plasmids encoding the GFP-centrin fusions, genomic DNA was amplified with *Xho*I and *Apa*I sites engineered into the primers to allow cloning into similar sites in pVGF-1 (Wiley *et al.*, 2000). Primers were used as follows: for *CEN1*, primers *Xho*I-5'Centrin and *Apa*I-3'Centrin; for *CEN2*, primers 5'*Xho*I TtCentrin2 and 3'*Apa*I TtCentrin2; for *CEN3*, primers 5'*Xho*I TtCentrin3 and 3'*Apa*I TtCentrin3; and for *CEN4*, primers 5'*Xho*I TtCentrin4 and 3'*Apa*I TtCentrin4. Clones were sequenced at Macrogen (Seoul, Korea) to confirm that errors had not been introduced by PCR.

To create a version of *CEN1* that could be expressed in *E. coli*, a PCR fragment containing the coding region of *CEN1* was amplified from a *Tetrahymena* cDNA library (generous gift from Dr. Aaron Turkewitz) using primers 5'*Bam*HI-Centrin and 3'*Pst*I-Centrin, which were engineered to contain the indicated restriction sites. This fragment was cloned into similarly digested pUC18. Because *Tetrahymena* uses an alternative genetic code, in which UAA and UAG represent glutamine codons, it was necessary to modify the *CEN1* sequence to allow expression in *E. coli*. Four glutamine codons were changed to the canonical CAA or CAG codons using the U.S.E. site-directed mutagenesis system (Amersham Biosciences, Piscataway, NJ) with the following primers: CentSw1, CentSw2, CentSw3, and TMSelect. The resulting plasmid (pUC18-TiCentrinMFE) was digested with *Bam*HI and *Pst*I, and the *CEN1* fragment was ligated into the pQE10 6-His fusion expression vector (QIAGEN, Valencia, CA), creating pQE10-CentrinMFE.

To create the *CEN1* rescuing construct, a 1.0-kb *Bam*HI, *Mfe*I *CEN1* promoter region was excised from pHM74 and cloned into similarly digested pUC18-TiCentrinMFE. *CEN1*, with its promoter, was then excised from this construct with *Sma*I and *Sph*I, blunted, and ligated into the blunted *Bsm*BI site of grl3/4:BLAST (a construct that replaces the *GRL3* and *GRL4* promoter region with the *Bsr* gene, which provides resistance to Blastocidin S, a generous gift from Aaron Turkewitz). Finally, the *CEN1* 3' untranslated region (UTR) was amplified by PCR of genomic DNA using primers Centrin\_BstXI and *Bsm*BI-dsCentrin and ligated into *Bst*XI (partial), *Bsm*BI-digested grl3/4:BLAST-PrCEN1 to create grl3/4:BLAST-PrCEN1-3'UTR. The resulting plasmid was sequenced to ensure no errors had been introduced.

### Protein Expression and Antibody Generation

pQE10-CentrinMFE was transformed into the *E. coli* strain M15, and a culture was grown in 500 ml of Luria Broth + 0.2% glucose with 50  $\mu$ g/ml kanamycin and 100  $\mu$ g/ml ampicillin. At an OD<sub>600</sub> of 0.4, cells were cooled to room temperature for 1 h. Isopropyl  $\beta$ -D-thiogalactoside was added to 0.3 mM and induction was allowed to proceed for 2.5 h. Cells were pelleted, washed once with phosphate-buffered saline (PBS), and stored frozen. Pellets were thawed, and resuspended in 10 ml of PBS containing 1 mM phenylmethylsulfonyl fluoride, 0.5  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml pepstatin A. Lysozyme was added to 200  $\mu$ g/ml before a 10-min incubation on ice. Cells were sonicated five times for 15 s each with 1-min intervals of rest on ice and centrifuged 15 min at 10,000  $\times$  g at 4°C. The supernatant was loaded onto a column containing Talon resin (BD Biosciences Clontech, Palo Alto, CA) equilibrated in PBS, and fractions were eluted in PBS + 200 mM imidazole. Fractions with Cen1 were pooled, and 1 mg was sent to Animal Pharm (Healdsburg, CA), for antibody generation in rabbits.

### Northern Blot Analysis

RNA was isolated using the TRI Reagent (Molecular Research Center, Cincinnati, OH) as per the manufacturer's instructions. Samples (each containing 10  $\mu$ g of RNA) were run on formaldehyde gels and transferred to Hybond N<sup>+</sup> (Amersham Biosciences) in 20 $\times$  SSC (Ausubel *et al.*, 1997). Northern blots were probed with randomly primed [ $\alpha$ -<sup>32</sup>P]dATP-labeled fragments (Feinberg and Vogelstein, 1983), which were obtained by amplification by PCR of genomic DNA with the primers used to create the GFP fusions described above. Probes were specific for the target sequences as determined by Southern blot analysis of total DNA (our unpublished data).

### Western Blot Analysis

Cells were lysed in buffer as described previously (Williams, 2000), and whole cell extracts were run on 12.5% or 11.5% standard Laemmli acrylamide gels (Ausubel *et al.*, 1997) along with BenchMark prestained molecular weight standards (Invitrogen, Carlsbad, CA). Proteins were electrophoretically transferred to Immobilon-P membrane and blotted according to the manufacturer's instructions (Millipore, Billerica, MA). Membranes were incubated with antibodies diluted in Tris-buffered saline containing 0.05% Tween and 1% bovine serum albumin for 1 h at room temperature. Primary antibodies (either serum from bleed 4 of a Cen1-injected rabbit, an anti-GFP monoclonal antibody [mAb] [B34 from Abcam, Cambridge, MA]), or an anti- $\alpha$ -tubulin mAb (12G10; a kind gift of Joseph Frankel, University of Iowa, Iowa City, IA) were diluted 1:2000, 1:5000, and 1:1000, respectively. Secondary antibodies (anti-rabbit IR800 [Li-Cor Biosciences, Lincoln, NE], anti-mouse Alexa680 [Molecular Probes, Eugene, OR], anti Mouse horseradish peroxidase [HRP], and anti-rabbit HRP [Pierce Chemical, Rockford, IL]) were diluted 1:10,000. Blots were visualized either on a Li-Cor Odyssey scanner or by SuperSignal West Pico chemiluminescent substrate (Pierce Chemical) and exposure to film.

### Macronuclear Transformations

Plasmids encoding GFP-centrin fusions were transformed into the developing macronucleus during late stages of conjugation of strains B2086 and CU428 by electroporation (Gaertig and Kapler, 2000). Paromomycin-resistant cells were selected and viewed on the fluorescence microscope. The *cen1* $\Delta$  rescuing construct was transformed into the developing macronucleus of the strains UCB8 and UCB9 during conjugation by electroporation, using *Kpn*I, *Xho*I-

digested grl3/4:BLAST-prCEN1-3'UTR, followed by selection with Blastocidin S (InvivoGen, San Diego, CA).

### Micronuclear Knockout and Heterokaryon Generation

pMicCentrinKO1 was digested with *Sac*I and introduced into the micronucleus of B2086- and CU428-conjugating cells by biolistic bombardment as described previously (Bruns and Cassidy-Hanley, 2000a). To check for proper integration of the *CEN1* knockout construct, total *Tetrahymena* DNA was isolated (Gaertig *et al.*, 1994b) and digested with *Nsi*I and *Pst*I. Standard Southern blot analysis was performed using a probe representing sequence upstream of the transforming fragment. The probe was generated by PCR amplification with primers CentrinUS1 and CentrinUS2 and labeled by the AlkPhos Direct (Amersham Biosciences) labeling system. Crosses to confirm that the transformation occurred in the micronucleus were performed, and the knockout heterokaryon was constructed, as described previously (Hai *et al.*, 2000) with the modification that an outcross was performed on the original transformant strains to rid them of an apparent micronuclear aneuploidy before mating with B\*VI.

### Analysis of *cen1* $\Delta$ Cells

Cultures containing equal numbers of UCB8 and UCB9 cells were starved and mixed. Ten hours after mixing, an equal volume of 2 $\times$  SPP (1% proteose peptone, 0.1% yeast extract, 0.2% glucose, 0.003% FeEDTA final) was added. Seventeen hours after mixing, paromomycin was added to 120  $\mu$ g/ml to select against cells that had not gone through conjugation. Samples were removed for Western blotting at 6-, 25-, 50-, 75-, and 96-h time points. Samples were removed from separate mating cultures and prepared for immunofluorescence microscopy at 17-, 24-, 48-, and 72-h time points. Wild-type (WT) cells were treated with paromomycin as a control. To determine whether cells could continue to grow without Cen1, individual conjugating pairs were isolated into 30- $\mu$ l drops of SPPA media, and cells were counted at 50 and 75 h.

### Fluorescence Microscopy

Cells containing the GFP-Centrin fusions were placed on slides and allowed to dry just until cells no longer swam. GFP-Cen1 cells were from logarithmic cultures, GFP-Cen2 cells were from dense cultures, and GFP-Cen3 and GFP-Cen4 were from starved cultures, as dense and starved cultures had less nonspecific background signal. Immunofluorescence microscopy was performed as described previously (Stuart and Cole, 2000). Cells were fixed with both formaldehyde and EtOH as described previously. Fixed cells were visualized on Antibody slides (Bellco Glass, Vineland, NJ). Antibodies were diluted in PBS + 1% bovine serum albumin. *Tetrahymena* Cen1 antibody was diluted 1:1000, the 20H5 monoclonal centrin antibody raised against *Chlamydomonas* centrin (a generous gift from Dr. J. Salisbury, Mayo Clinic, Rochester, MN) was diluted 1:2500, the antibody against polyglutamylation (a generous gift from Dr. M. Gorovsky) (Shang *et al.*, 2002) was diluted 1:500, and the 12G10 mAb against  $\alpha$ -tubulin (a generous gift of Dr. J. Frankel, University of Rochester, Rochester, NY) was diluted 1:50. Secondary antibodies were used at a 1:1000 dilution and included goat anti-rabbit Alexa594, goat anti-rabbit Alexa488, and donkey anti-mouse Alexa488 (Molecular Probes). Antibody incubations were carried out at room temperature for 1 h, or alternatively, overnight at 4°C. To visualize DNA, 4,6-diamidino-2-phenylindole (DAPI) was applied to the cells at 1  $\mu$ g/ml for 3 min. After each antibody incubation, cells were washed six times with PBS + 0.1% bovine serum albumin. Standard fluorescence microscopy was carried out using a Leica DMRXA/RF4/V automated microscope with a Cooke Sensi-Cam digital camera. Images were collected and subjected to no neighbors or nearest neighbors deconvolution algorithms using the Slidebook software package (Intelligent Imaging Innovations, Denver, CO).

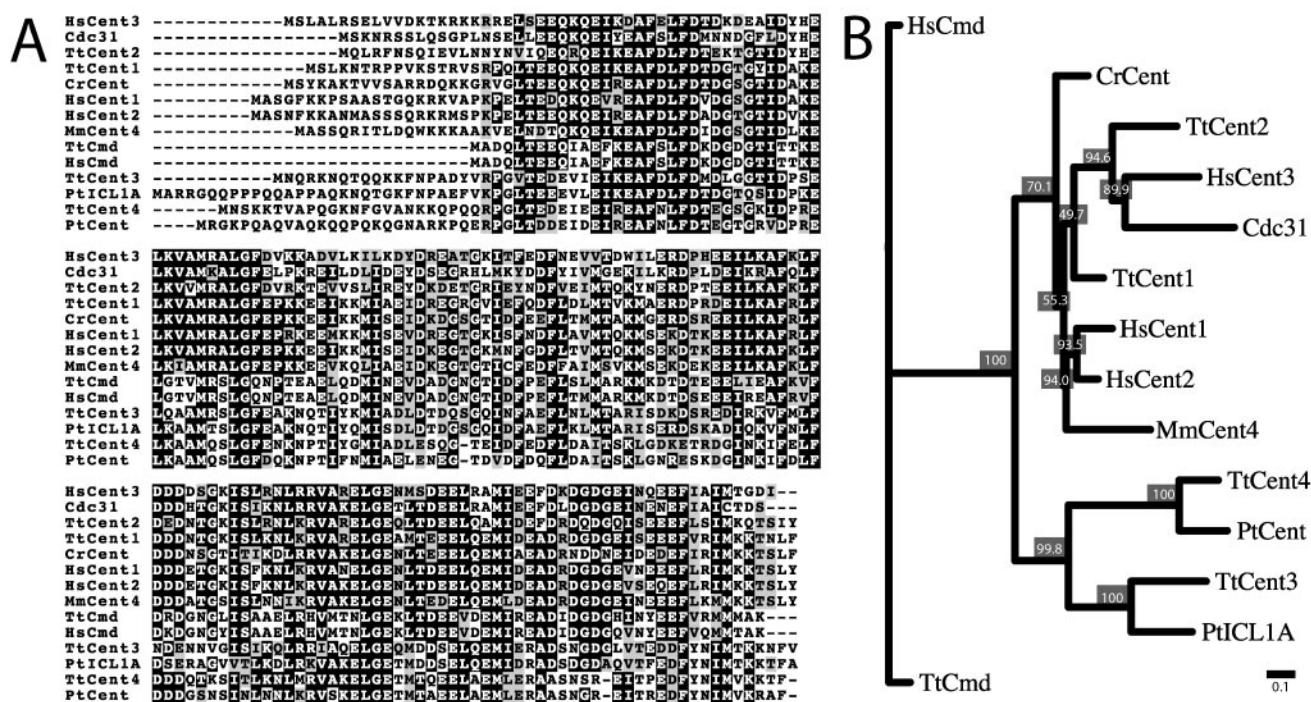
### Immunoelectron Microscopy

*Tetrahymena* cells were pelleted, frozen under high pressure in a BAL-TEC HPM-010 high-pressure freezer, freeze substituted in 0.25% glutaraldehyde/0.1% uranyl acetate, and embedded in Lowicryl HM20. Serial sections (55 nm) were taken and labeled with 1:200 dilutions of primary antibody in 1% nonfat milk phosphate-buffered saline/Tween 20, which included an affinity-purified rabbit anti-GFP primary antibody (a gift from Jason Kahana, Ludwig Institute for Cancer Research, New York, NY, and Pamela Silver, Dana-Farber Cancer Institute, Boston, MA) and the Cen1 antibody described above. A 1:20 dilution of 15-nm gold-conjugated goat anti-rabbit secondary antibody (Ted Pella, Redding, CA) was used. Grids were stained with uranyl acetate and lead citrate and were visualized in a Philips CM10 electron microscope.

## RESULTS

### Identification of a *Tetrahymena* Centrin Gene Family

We wished to isolate the *T. thermophila* centrin gene for analysis with respect to its potential role at basal bodies. A



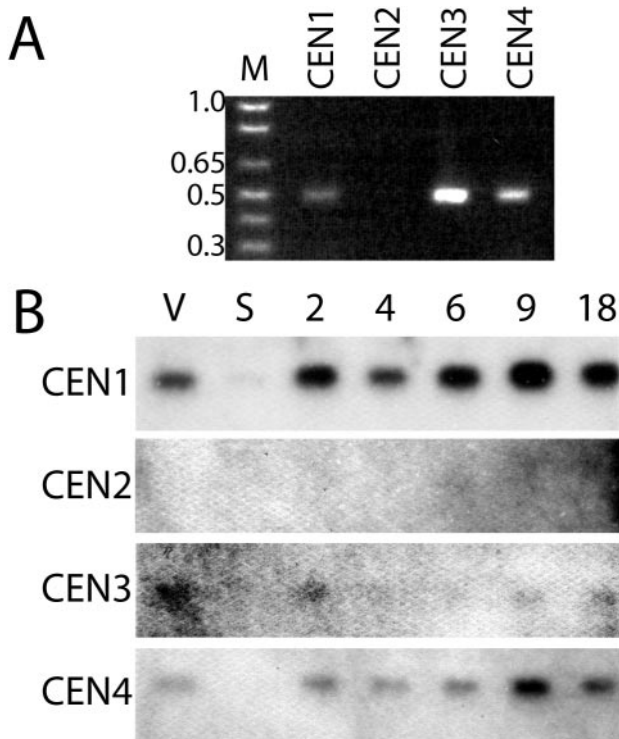
**Figure 1.** Comparison of centrins from different organisms. Tt, *T. thermophila*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Cr, *Chlamydomonas reinhardtii*; Pt, *P. tetraurelia*; Cdc31 from *S. cerevisiae*; Cmd, calmodulin. (A) Twelve representative centrins were aligned along with two calmodulin proteins. Residues where 50% or more are identical between the proteins are shaded black, and residues where 50% or more are similar are shaded gray. (B) The centrins and calmodulin sequences were subjected to phylogenetic analysis using the Neighbor-Joining method; 1000 replicates were performed, and a representative unrooted tree is displayed as a dendrogram. Bootstrap values are displayed as percentages at each node and indicate how often the separation delineated by the branching occurred. Bar = 0.1 substitutions per one amino acid (see *Materials and Methods*).

fragment was amplified by PCR from *Tetrahymena* genomic DNA using degenerate primers designed based on the alignment of several published centrins (see *Materials and Methods*). Flanking sequences were cloned by inverse PCR, and DNA sequencing revealed the fragment to be a centrins gene. During the course of this work, this centrins gene was independently reported by Guerra *et al.* (2003). We refer to this gene as *CEN1*.

The corresponding protein sequence of *CEN1* was used as a query to search the recently sequenced *Tetrahymena* genome at The Institute for Genomic Research (TIGR, www.tigr.org), and three additional potential centrins genes were identified. Two of these genes, called *CEN3* and *CEN4* herein, contain 510 and 515 base pairs, respectively, of uninterrupted protein coding sequence. The remaining gene, *CEN2*, contains 492 base pairs of coding sequence with three introns predicted by the protein alignment and standard splicing rules (Alberts *et al.*, 2002). All three centrins had predicted molecular masses of 19.5 kDa, which is similar to *Cen1*'s molecular mass of 19.4 kDa. The translated sequences were aligned with a variety of characterized centrins as well as the *Tetrahymena* and human calmodulins and placed in a phylogenetic tree (see *Materials and Methods*) (Figure 1). The phylogenetic analysis revealed three distinct branches: one for the calmodulins, one for two centrins identified in the ciliate *Paramecium tetraurelia* and *CEN3* and *CEN4* from *Tetrahymena*, and the third for all the remaining centrins included in the analysis. Of the *Tetrahymena* centrins, *Cen1* is most similar to the *Chlamydomonas* centrins and human *Centrin2*, which are involved in duplication of basal bodies and centrioles, respectively (Salisbury *et al.*, 2002;

Koblenz *et al.*, 2003). *Cen2* is most closely related to budding yeast *Cdc31*, which is essential for spindle pole body duplication, and to human *Centrin3*. *Cen3* and *Cen4* are similar to centrins found in the infraciliary lattice of the ciliate *P. tetraurelia* (Klotz *et al.*, 1997), and this more distantly related branch of the centrins family may be unique to the ciliates.

We checked for expression of these sequences to confirm that they encoded genes. Initially, PCR was carried out on a cDNA library generated from vegetatively growing cells, and an uncloned cDNA preparation also from vegetatively growing cells. In both cases, amplification of sequence resulting from primers specific to *CEN1*, *CEN3*, and *CEN4* was observed, but not for primers specific to *CEN2*, which are capable of amplification of *CEN2* from the genome (Figure 2A; our unpublished data). Five clones from each of these PCR products were sequenced, confirming that the primers used were specific for the targeted sequences. To determine whether *CEN2* was expressed during other stages of the *Tetrahymena* life cycle and to examine expression patterns for the three other centrins, RNA was isolated from vegetatively growing cells, cells in starvation media, and cells at various time points through the mating cycle (conjugation) for Northern analysis with probes derived from each of the four centrins genes (Figure 2B). *CEN1*, *CEN3*, and *CEN4* have similar expression profiles: bands of the expected size are observed during vegetative growth, and the intensity of these bands decreases in cells in starvation media, which were no longer dividing. During conjugation, which occurred in a starvation medium, expression increases to levels approximately twice those seen during logarithmic growth, peaking 9 h after initiation of mating. During conjugation,

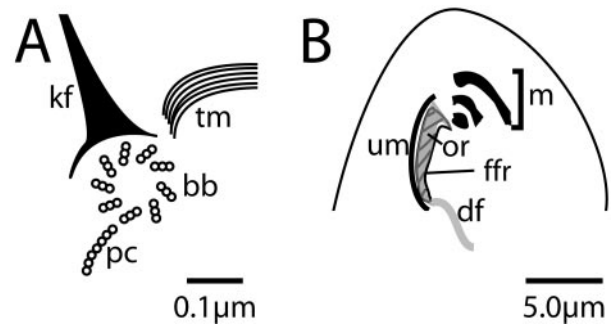


**Figure 2.** Expression of genes in the *Tetrahymena* centrin family. (A) PCR products using primers specific for *CEN1*, *CEN2*, *CEN3*, and *CEN4* on cDNA from cells during vegetative growth displayed on an agarose gel. Sizes of select marker (M) bands are displayed in kilobase pairs. (B) Northern blot analysis of RNA from vegetatively growing cells (V), starved cells (S), and conjugating cells at various time points after initiation of mating (2, 4, 6, 9, 18 h) (see *Materials and Methods*). Similar amounts of total RNA were loaded as assessed by absorbance spectroscopy and ethidium bromide staining our unpublished data).

cells lose the oral apparatus, and its reformation is an early event after conjugants separate (Kiersnowska *et al.*, 1993); the increase in the centrin's expression during later stages of conjugation could play a role in this process. Among the centrin genes, *CEN1* seems to have the highest expression levels, followed by *CEN4* and then *CEN3*, although it is possible that differences in band intensity may be the result of the ability of each probe to recognize the appropriate message. Because mRNA was detected for *CEN1*, *CEN3*, and *CEN4*, these sequences represent three bona fide members of a Centrin family in *Tetrahymena*. *CEN2* expression was not detected for this limited set of conditions, although this result does not preclude expression during other untested growth conditions.

#### GFP Tagging and Localization of *Tetrahymena* Centrin Family Members

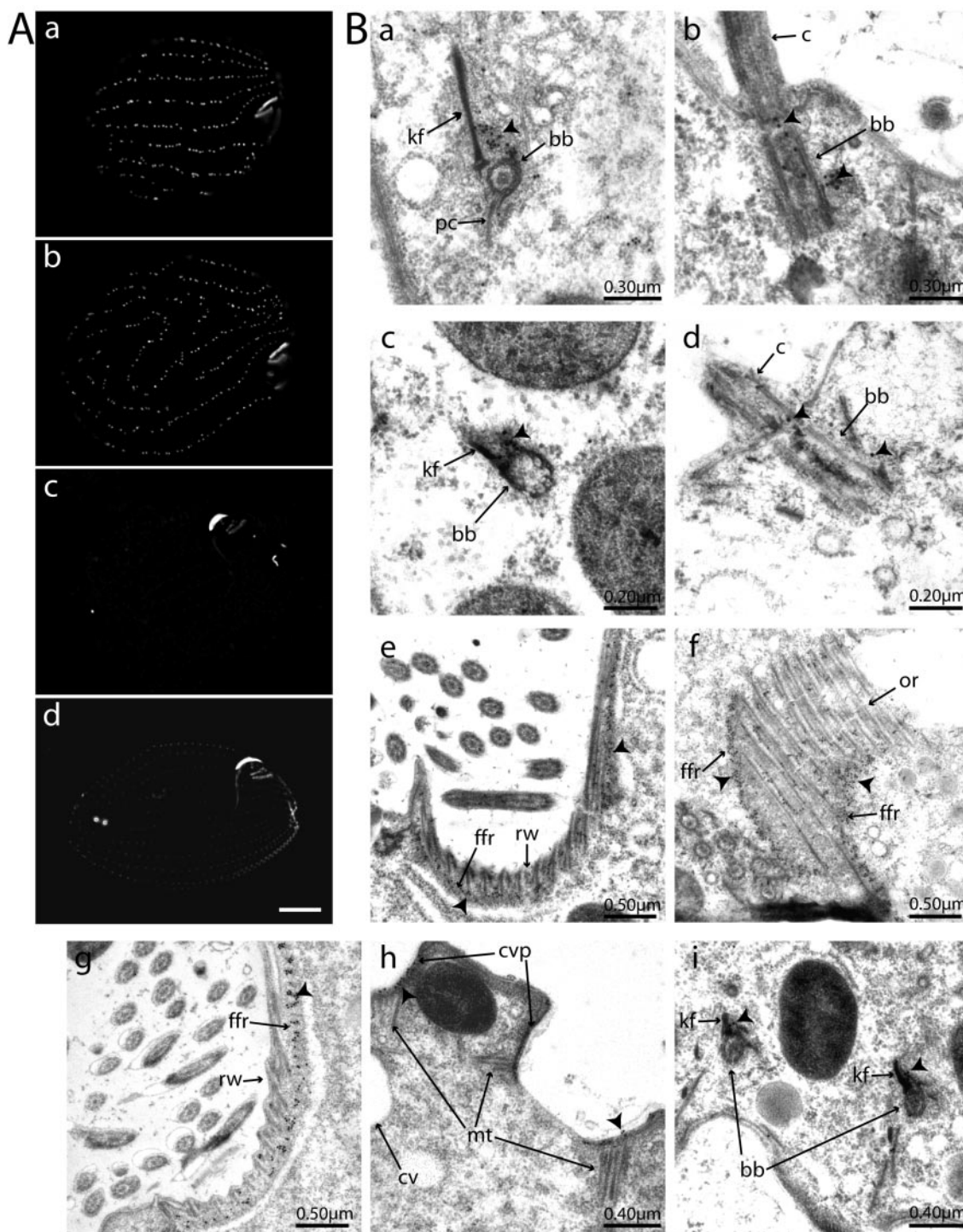
To understand more about the functions of the centrin genes, we attempted to localize their products within the cell. Each gene was fused at its N terminus to the GFP (Chalfie *et al.*, 1994) in the vector pVGF1 (Wiley *et al.*, 2000). This vector is based on a ribosomal DNA processing vector in which rDNA sequences on the plasmid, into which the GFP fusion is inserted, are processed in the developing macronucleus, resulting in amplification and strong expression (Karrer, 2000). An N-terminal GFP-centrin fusion protein has been reported previously to localize correctly to



**Figure 3.** Schematic drawings of centrin-containing structures. (A) A basal body from a cortical row is represented along with some of the structures associated with it. bb, basal body; kf, kinetodesmal fiber; tm, transverse microtubules; pc, postciliary microtubules. (B) Some of the structures within the oral apparatus are represented. Um, undulating membrane; m, membranelles, of which there are three; or, oral-rib (gray in diagram); df, deep-fiber; ffr, fine-filamentous reticulum (striped in diagram). The undulating membrane and membranelles contain basal bodies and cilia. The oral-rib is a sheet of microtubules along the inside of the oral apparatus cavity beneath the undulating membrane, edged with ribbed walls (not labeled). The deep-fiber consists of a subset of microtubules that continue inward from the oral-rib into the cell. The fine-filamentous reticulum lies beneath the oral ribs.

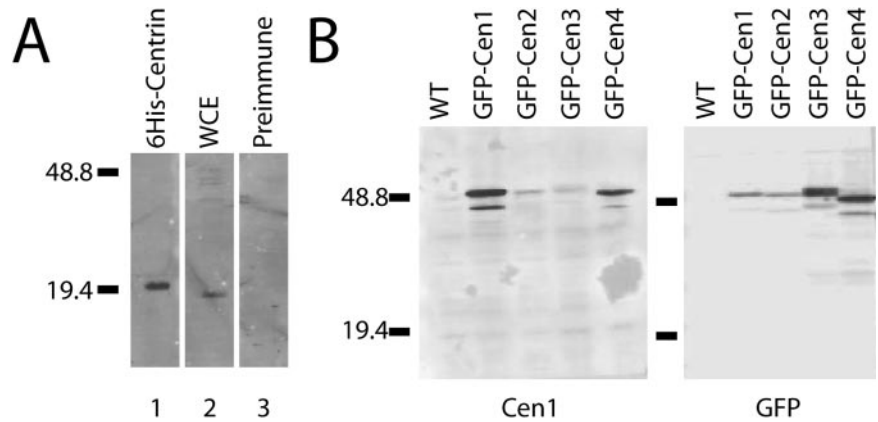
centrioles in cell culture, and a C-terminal centrin-GFP fusion protein localized to *Chlamydomonas* basal bodies (White *et al.*, 2000; Ruiz-Binder *et al.*, 2002), although these fusions are probably not fully functional (Ruiz-Binder *et al.*, 2002; Kilmartin, 2003). Although *Cen2* mRNA is not normally detectable during vegetative growth, we tagged this protein along with the others to gain some understanding of where it might function if expressed. Transformants were recovered for each construct, and live cells were imaged by fluorescence microscopy. GFP-*Cen1* and GFP-*Cen2* both localized to the oral apparatus, a complex cytoskeletal structure rich in basal bodies, microtubule structures, and other non-microtubule-based filaments (Sattler and Staehelin, 1976; Frankel, 2000) and to cortical rows in a pattern similar to that observed for basal bodies. Interestingly, transformation of both constructs resulted in an apparent disorganization of the cortical rows, such that the rows sometimes ended prematurely, or curled around in unusual patterns, suggestive of a dominant-negative phenotype (Figure 4A, a and b). Consistent with these constructs being deleterious alleles, the transformants grew slowly, and neither construct was stably maintained over the course of extended vegetative growth. Nonetheless, *Cen1* and *Cen2* seem to localize to basal bodies.

In contrast, GFP-*Cen3* and GFP-*Cen4* transformants did not have any obvious growth defects. The fluorescence signal for *Cen3* and *Cen4* fusion proteins also was strikingly different from that of the other centrin fusions. GFP-*Cen3* localized primarily to the oral apparatus. However, this fluorescence signal was distinct from the GFP-*Cen1* and GFP-*Cen2* signal at the oral apparatus, which was punctate and in all likelihood originated from GFP signal at the oral apparatus basal bodies. Rather, the GFP-*Cen3* signal was found in a crescent-shaped region to the cell's left of the undulating membrane and extended along the deep rootlet. Signal also was present at the three membranelles of the oral apparatus, and in a crescent-shaped band at the apical tip of the cell (Figures 3B and 4A, c). GFP-*Cen4* also localized to the structures where GFP-*Cen3* was observed as well as to



**Figure 4.** Localization of GFP-centrin fusion proteins. (A) Fluorescence signal of live cells: a, GFP-Cen1; b, GFP-Cen2; c, GFP-Cen3; and d, GFP-Cen4. The oral apparatus is on the right side in each image. In a and to a greater extent b, cortical rows are affected by the overexpression of the GFP fusion protein. The strong signal in c is at the oral apparatus primarily in the oral crescent, internal to the undulating membrane, and in the apical band. These same regions are visible in d. The contractile vacuole pores are the two small circles near the posterior of the cell. Bar, 10  $\mu\text{m}$ . (B) Immunoelectron microscopy with a GFP antibody and a 15-nm gold-conjugated secondary antibody. Arrowheads indicate representative concentrations of gold particles. a and b, GFP-Cen1 localization; c and d, GFP-Cen2 localization. kf, kinetodesmal fiber; pc, postciliary microtubules; bb, basal body; c, cilia. Representative cross-sectional (a and c) and longitudinal views (b and d) of basal bodies are shown. e and f, localization to the fine-filamentous reticulum of GFP-Cen3, with f also showing some localization along the oral ribs. ffr, fine-filamentous reticulum; rw, ribbed wall; or, oral-ribs. GFP-Cen4 localization is shown in g, h, and i. g, localization in the fine filamentous reticulum of the oral apparatus. h, sections of both contractile vacuole pores as well as some associated microtubules. cvp, contractile vacuole pore; mt, microtubules; cv, contractile vacuole. h, two basal bodies in cross section with signal apparent along the kinetodesmal fibers.

**Figure 5.** Reactivity of the Cen1 antibody. (A) Western immunoblot analysis using the Cen1 antibody as a probe on recombinant 6-histidine-tagged Centrin (lane 1) and *Tetrahymena* whole cell extract (wce) from cells in logarithmic growth (lane 2). The reactivity of the preimmune serum on wce also is shown (lane 3). (B) Reactivity of the Cen1 antibody with the overexpressed GFP-centrin fusion proteins. Whole cell extracts from strains expressing different fusion proteins were probed with either the Cen1 antibody (left) or a GFP antibody (right). Because the fusion proteins are overexpressed, the signal overwhelms the endogenous Centrin signal, which is visible with longer exposures. Select marker bands are noted and sizes are in kilodaltons.



the contractile vacuole pores (CVPs) through which the contractile vacuole expels fluid at the posterior of the cell (Frankel, 2000). Additionally, faint signal was observed along the cortical rows, reminiscent of basal body localization (Figure 4A, d).

We examined cells by immunoelectron microscopy to better resolve the localization of the four centrin fusions. Cells were prepared for electromicroscopy by high-pressure freezing and freeze substitution, and sections were stained using an antibody that recognizes GFP followed by a secondary antibody conjugated to 15-nm gold particles (see *Materials and Methods*). GFP-Cen1 is abundant at the base of the basal body adjacent to the kinetodesmal fiber, a nonmicrotubule-based striated rootlet that extends from the basal body toward the anterior of the cell, at the location where new basal body formation occurs (Figures 3A and 4B, a and b) (Allen, 1969). Additionally, signal was observed along the length of the basal body, with a second concentration at the transition zone between the basal body and the ciliary axoneme. Transition zone localization for centrin has been reported in *Chlamydomonas* (Sanders and Salisbury, 1994). The overall signal from GFP-Cen2 was significantly weaker than that for GFP-Cen1 in this experiment, but when multiple images were analyzed signal was detected in a pattern similar to that of GFP-Cen1 (Figure 4B, c and d). GFP-Cen3 and GFP-Cen4 both localized primarily to the region directly underneath the ribbed wall and the oral-ribs within the oral apparatus that corresponds to the fine filamentous reticulum (Williams and Luft, 1968; Williams and Bakowska, 1982) (Figures 3B and 4B, e–g), and GFP-Cen4 signal was clearly detected at the contractile vacuole pores (Figure 4B, h). Weak GFP-Cen4 signal also was observed at the basal bodies, primarily on the kinetodesmal fiber (Figure 4B, i). When a strain not expressing GFP was submitted to identical antibody incubations, there was almost no background signal apparent, supporting the authenticity of the localization data (our unpublished data).

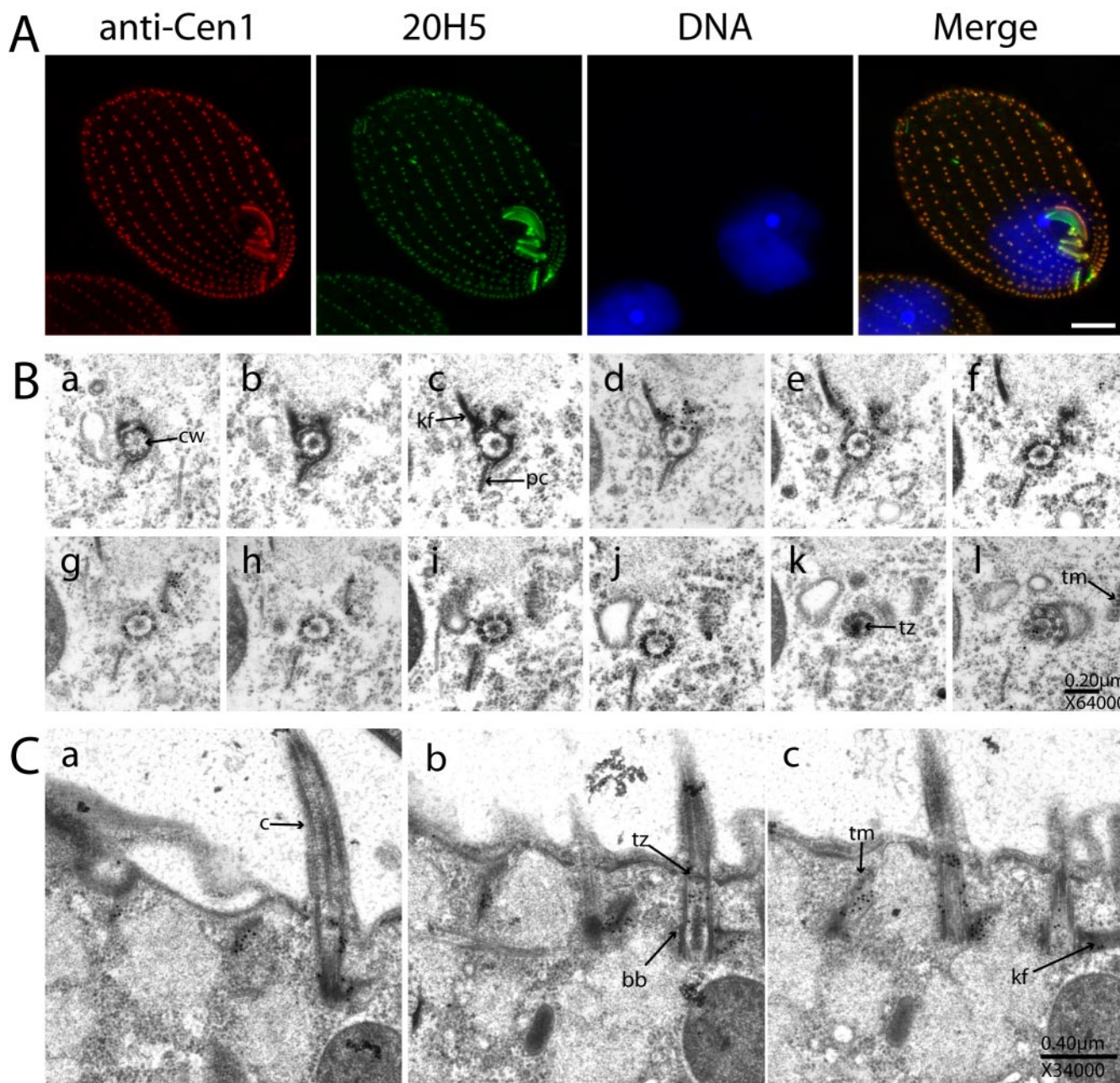
Together, the localization of the GFP fusion proteins and the expression patterns of the different centrins suggest that each member of the *Tetrahymena* centrin family has a unique role within the cell. The possibility remains that there is some redundancy among the centrin genes, particularly between *CEN3* and *CEN4*, because their protein products have similar distributions within the oral apparatus. Because our primary interest is the role of centrin in basal body duplication during vegetative growth, we chose to focus on Cen1. Of the four *Tetrahymena* centrins, Cen1 is most similar to human centrin2, which is required for centriole duplication. Furthermore, we have shown that Cen1 is expressed

strongly during logarithmic growth, and a GFP fusion protein localizes to basal bodies at the precise location where initiation of basal body duplication is thought to occur (Allen, 1969).

#### Basal Body Localization of Endogenous Cen1

A polyclonal antibody was raised against full-length 6-histidine-tagged Cen1 (see *Materials and Methods*) and initially characterized by Western blot analysis. The antibody detected recombinant 6-histidine-tagged Cen1 (Figure 5A, lane 1), and a band the size expected for centrin (19.5 kDa) in a whole cell *Tetrahymena* extract (lane 2). We then took advantage of the GFP-centrin fusion strains, to examine the antibody's reactivity with each individual *Tetrahymena* centrin family member. GFP adds approximately 27 kDa to the molecular mass of the protein, thus allowing for easy separation of the tagged allele from the endogenous proteins, which all have a molecular mass of 19.5 kDa. To control for the individual expression level of each protein, antibody recognizing the GFP tag was used as a control. As expected, the Cen1 antibody strongly recognized GFP-Cen1 (Figure 5B, left). The antibody had minimal cross-reactivity with GFP-Cen2 and slightly more cross-reactivity with GFP-Cen3 and GFP-Cen4 compared with the GFP probed immunoblot. Endogenous centrin was clearly visible with the Cen1 antibody on longer exposures (our unpublished data). We therefore concluded that our antibody is largely specific to Cen1, but it may interact with the other centrins at a low-to-moderate level.

To examine localization of endogenous Cen1, we used the Cen1 antibody for immunofluorescence and immunoelectron microscopy. Immunofluorescence was performed on cells from a logarithmic culture (Figure 6A) using our rabbit polyclonal antibody (anti-Cen1), and a mAb (20H5) that recognizes centrins from a variety of species and has previously been used to localize centrin in *Tetrahymena* (Jerka-Dziadosz *et al.*, 1995; Guerra *et al.*, 2003). Staining patterns from each antibody were very similar but not identical. Both recognized the basal bodies along the cortical rows and in the oral apparatus, but the 20H5 antibody recognized additional structures that the Cen1 antibody did not. The signal from 20H5 was observed at the oral crescent (corresponding to the fine filamentous reticulum) and also at the apical filamentous band (Jerka-Dziadosz, 1981; Jerka-Dziadosz *et al.*, 1995). Both these structures were revealed by GFP-Cen3 and GFP-Cen4. However, the 20H5 antibody did not recognize the contractile vacuole pores, raising the possibility that this antibody can recognize Cen1 and Cen3, but not Cen4, or that Cen4 is not accessible to the antibody at the CVPs.



**Figure 6.** Cen1 localization at basal bodies. (A) Immunofluorescence microscopy using the Cen1 antibody and the monoclonal centrin antibody 20H5 raised against *Chlamydomonas* centrin. DNA is visualized with DAPI. Bar, 10  $\mu\text{m}$ . (B) Serial section immunoelectron microscopy of a basal body in cross section with the Cen1 antibody. Panels proceed from the axoneme distal part of the basal body (a) and track upwards through the structure. Figure 3A serves as a reference for some of the basal body associated structures that are present. Certain basal body associated structures are identified in sections where they are most prominent, although this does not preclude these same structures from being visible in other sections. cw, cartwheel; kf, kinetodesmal fiber; pc, postciliary microtubules; tz, transition zone; tm, transverse microtubules; c, cilia; bb, basal body. Centrin concentrates on the transverse microtubules that are seen obliquely, on the kinetodesmal fiber, and at the transition zone (k). (C) Serial section immunoelectron microscopy of three basal bodies in longitudinal section.

Consistent with this interpretation, the 20H5 antibody strongly recognized Cen1 and Cen3 by Western blot analysis, whereas showing moderate affinity to Cen4 and virtually no affinity for Cen2 (our unpublished data). Like the GFP-Cen1 data, data from the Cen1 antibody indicates that Cen1 localizes specifically to basal bodies.

To map Cen1 localization within basal bodies, immunoelectron microscopy was performed on serial sections of

cells from a logarithmic culture using the Cen1 polyclonal antibody. This analysis on endogenous levels of Cen1 revealed a similar localization pattern to that observed with the GFP-Cen1 strain. Serial sections through six basal bodies were examined. At the basal body, there are two major centers of Cen1 concentration. The first is at the base of the basal body, along and adjacent to the kinetodesmal fiber (Figures 3A, 6B, a–e, and 6C, a–c). At the very base of the

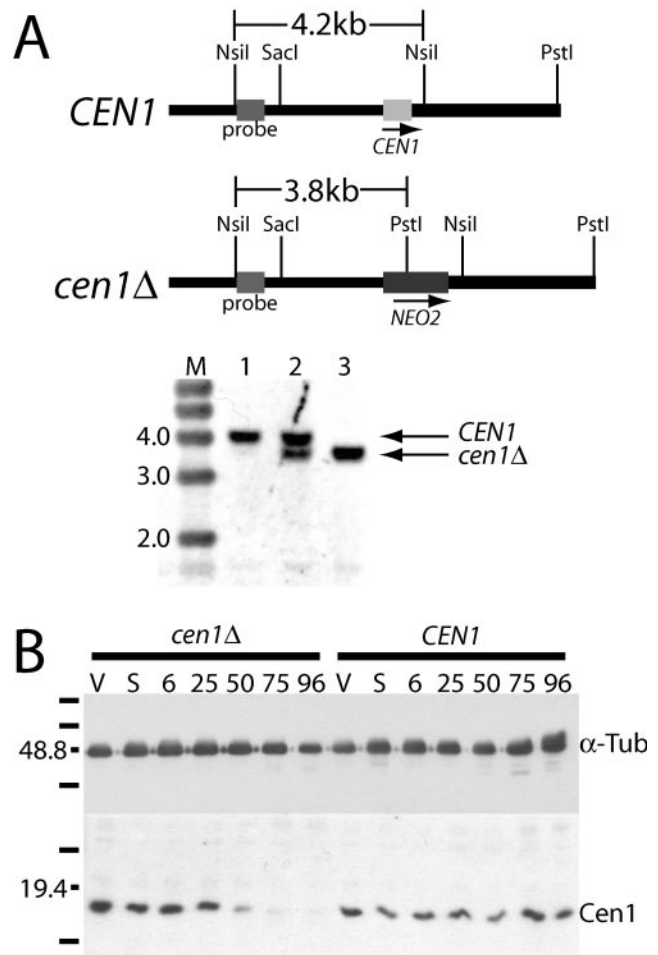


basal body (most distal to the axoneme), this signal is immediately adjacent to the basal body (Figure 6B, a), the site of new basal body formation (Allen, 1969). As the cross-sectional serial sections are tracked through the basal body, this signal moves away from the basal body, following a path along the transverse microtubules that extend to the adjacent cortical row (for reference, see Figures 3A and 6B, c–j). There is also a moderate amount of signal from the Cen1 antibody within the basal body cylinder that extends the length of the basal body immediately adjacent to basal body microtubules (Figure 6B, g–j). The second concentration of Cen1 occurs at the transition zone between the basal body and the axoneme (Figure 6B, k). These localization patterns also were observed in longitudinal sections (Figure 5C, a–c).

### *Cen1 Is Required for Basal Body Duplication and Maintenance in Tetrahymena*

Both Cen1's localization to the base of the basal body where duplication is initiated, and the disruption of the cortical rows caused by overexpression of the GFP-Cen1 fusion protein, are suggestive that Cen1 has an important role at the basal body. To assess this more directly, a null strain was created. We replaced *CEN1* with the NEO2 drug resistance cassette, which provides resistance to paromomycin (Gaertig *et al.*, 1994a), in the diploid micronucleus by homologous recombination (Bruns and Cassidy-Hanley, 2000a), as confirmed by Southern analysis (Figure 7A). This strain was then used to create a knockout heterokaryon (Hai *et al.*, 2000), a strain homozygous for the knockout in the micronucleus, but containing only wild-type *CEN1* in the expressed macronucleus. When two such strains with different mating types are mated to each other, a new macronucleus is formed based on the DNA of the germline micronuclei of the parent strains. The resulting progeny lack the *CEN1* gene, having the NEO2 gene cassette in its place (Figure 7A). *CEN1* knockout heterokaryon strains UCB8 and UCB9 were mated to each other, and 120  $\mu\text{g}/\text{ml}$  paromomycin was added to select for cells that had proceeded successfully through mating and development of progeny. The cell density of the culture increased only slightly by 72 h after initiation of mating (starting at  $2.5 \times 10^5$  cells/ml, and increasing to  $2.8 \times 10^5$  cells/ml), and eventually all cells died, either due to sensitivity to paromomycin (nonmating, parental cells) or due to the lack of Cen1 (progeny). No "survivors" were observed in these cultures, indicating that none of the other centrins can substitute for Cen1 in its essential function. Western blot analysis confirmed that Cen1 protein levels were reduced significantly by 50 h after mating and that the reduction in Cen1 protein levels continued over time (Figure 7B). These Western blot results further indicate that either the antibody is very specific for Cen1 and/or Cen1 is the predominantly expressed centrin in cells, because the decrease in Cen1 was not masked by the other centrins.

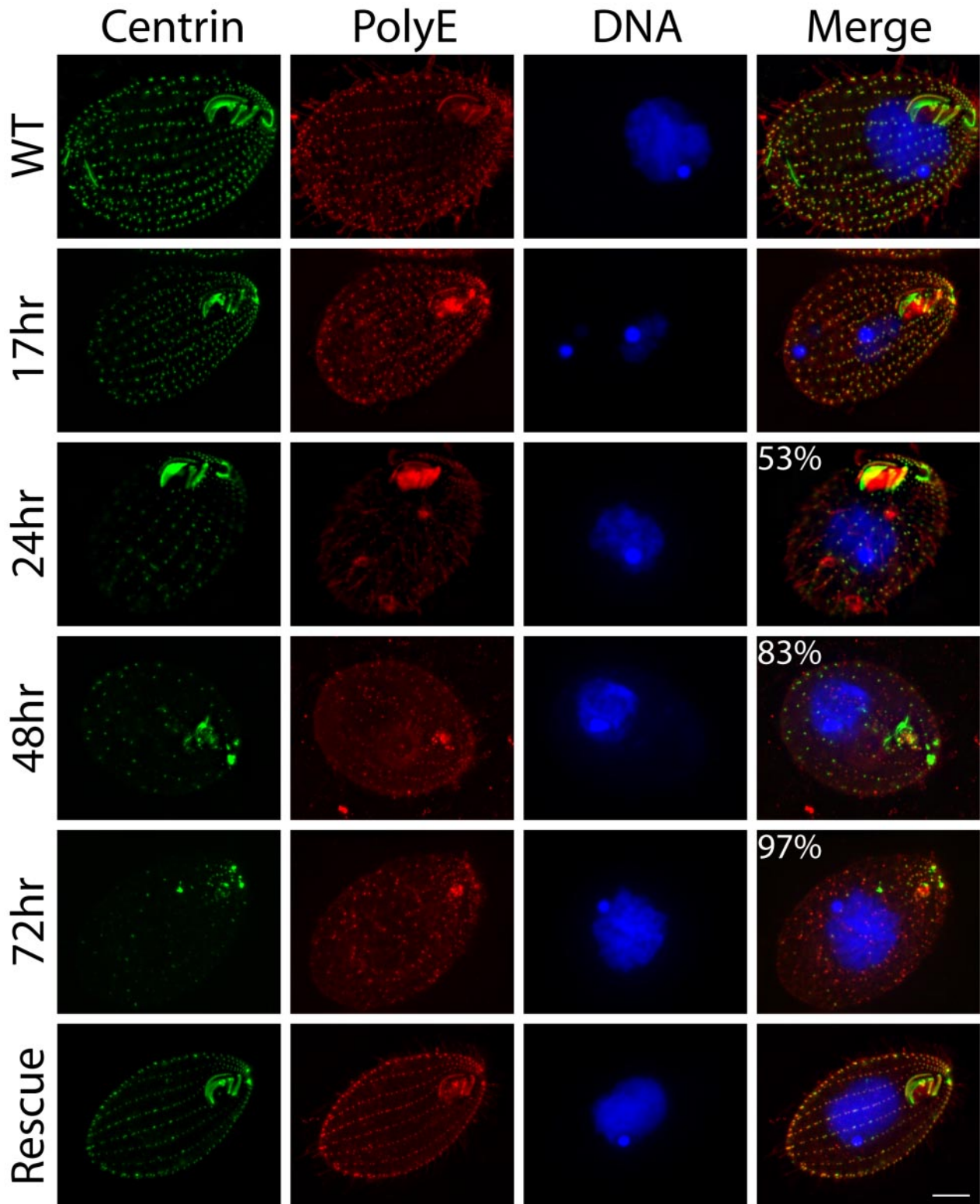
The ability of *cen1* $\Delta$  cells to divide was tested by mating UCB8 and UCB9 *cen1* $\Delta$  knockout heterokaryon strains and isolating individual conjugating pairs into small drops of media. As a control, B2086 and CU428 cells (both wild-type for *CEN1*) were subjected to the same procedure. Cells were counted after 50 h of growth and checked again at 75 h, and an estimate as to how many divisions each cell had completed was made. Both wild-type and *cen1* $\Delta$  cells had a significant population of cells that were completely unable to divide after conjugation (29 and 55%, respectively). Most wild-type cells went on to form stable vegetative cultures (62%). In contrast, none of the *cen1* $\Delta$  cells progressed to form



**Figure 7.** Cen1 protein levels decrease when *CEN1* is deleted. (A) Southern blot analysis of the *CEN1* locus. The fragment used for transformation extends from the *SacI* site to the *PstI* site with the NEO2 cassette replacing *CEN1*. Total *Tetrahymena* DNA from wild-type cells (lane 1), the micronuclear transformant (lane 2), and progeny from the knockout heterokaryon strains rescued by *CEN1* transformed at a second genomic location (lane 3) was digested with *NsiI* and *PstI*. The sizes of select marker bands (M) are noted in kilobase pairs. (B) Western blot analysis of progeny from the *cen1* $\Delta$  knockout heterokaryon strains UCB8 and UCB9 (*cen1* $\Delta$ ) and of the progeny from wild-type strains B2086 and CU428 (*CEN1*). Cen1 is visualized with the Cen1 polyclonal antibody, and a mAb to  $\alpha$ -tubulin is used as a loading control. V, vegetative growth; S, starvation; numbers correspond to hours after parental cells were mixed. Marker bands are noted, and sizes are in kilodaltons.

a stable vegetative culture. However, of the 45% that did divide after conjugation, more than half divided approximately three times. Therefore, at least some of the *cen1* $\Delta$  strains were able to undergo several cell divisions before arresting and eventually dying.

The effect of the lack of Cen1 on basal body integrity was observed by fixing and staining cells at various time points after mating with both the 20H5 monoclonal centrin antibody and an antibody raised against a polyglutamic acid peptide that recognizes a tubulin modification, polyglutamylation, that is abundant at basal bodies (Shang *et al.*, 2002) (Figure 8). The number of cells with an obviously reduced number of basal bodies compared with wild-type was counted at each time point. Cells proceeded through



**Figure 8.** Deletion of *CEN1* results in a failure to duplicate basal bodies. *cen1* $\Delta$  knockout heterokaryon strains UCB8 and UCB9 were mated, and their progeny were analyzed at 17-, 24-, 48-, and 72-h time points by immunofluorescence microscopy. The monoclonal centrin antibody 20H5 was used along with an antibody that recognizes polyglutamic acid, which is prominent on modified tubulin at basal bodies. DNA is visualized with DAPI. WT is strain CU428 in logarithmic growth. Percentages refer to the proportion of cells with reduced numbers of basal bodies, with the images shown being representative ( $n = 200$ ). Rescue refers to UCB8  $\times$  UCB9 progeny that were rescued by *CEN1* transformed into a second site in the genome. In the WT and 17-h samples, 100% of cells had an apparently normal number of basal bodies. The oral apparatus is oriented on the right side of each panel. Bar, 10  $\mu$ m.

conjugation without apparent problems, and 17 h after initiation of mating the cells had separated and looked normal. However, 24 h after mating, some cells displayed reduced centrin signal and fewer basal bodies along the cortical rows. By 48 h, cells had fewer basal bodies, and by 72 h most of the cells showed a severe phenotype, including loss of most basal bodies, disorganization of the remainder along the cortical rows, and loss of the oral apparatus. Cell division intermediates were observed in the samples, and although these cells looked capable of completing the division cycle, because they did not accumulate at later time points, they were unable to form a new oral apparatus and had fewer basal bodies, especially in the posterior half of the cell (Supplemental Figure 1A). Additionally, there was signal from the polyglutamic acid antibody at locations where there was no centrin signal, indicating that there was no longer enough Cen1 to localize to all of the remaining basal bodies.

To ensure that the loss of basal bodies was due to the lack of Cen1, and not the result of the introduction of paromomycin to the cultures, three controls were performed. First, UCB8 and UCB9 *cen1Δ* knockout heterokaryon strains were mated to each other, and the resulting culture was allowed to progress in the absence of drug, allowing parental, unmated cells to largely overtake the population. Nevertheless, the loss of centrin signal and basal bodies was still observed in some cells, whereas in a wild-type control, cross-centrin signal remained high throughout the population (Supplemental Figure 1B; our unpublished data). In the second control, a wild-type strain was subjected to 120 μg/ml paromomycin and observed at time points after addition of drug similar to those used in the experiment with the knockout strains. Although dying cells often had disorganized basal bodies, these cells were distinct from *cen1* knockout cells in that centrin signal remained strong, and these cells were small and rounded (Supplemental Figure 1C). In the third control, a construct that reintroduces *CEN1* at the *GRL3/4* locus (Verbsky and Turkewitz, 1998) rescued *cen1Δ* cells. UCB8 and UCB9 cells were mated, and this construct was transformed into the developing macronucleus by electroporation. Transformants were recovered that were resistant both to blasticidin S, which selects for the *bsr* gene used in this construct, and to paromomycin, which selects for the *NEO2* cassette used to disrupt *CEN1* (Figure 7A; our unpublished data). Rescued cultures grew normally and were subjected to immunofluorescence microscopy (Figure 8, bottom). The cortical rows and oral apparatus in these cells were indistinguishable from wild type, indicating that when Cen1 is restored to these cells, they no longer have basal body defects.

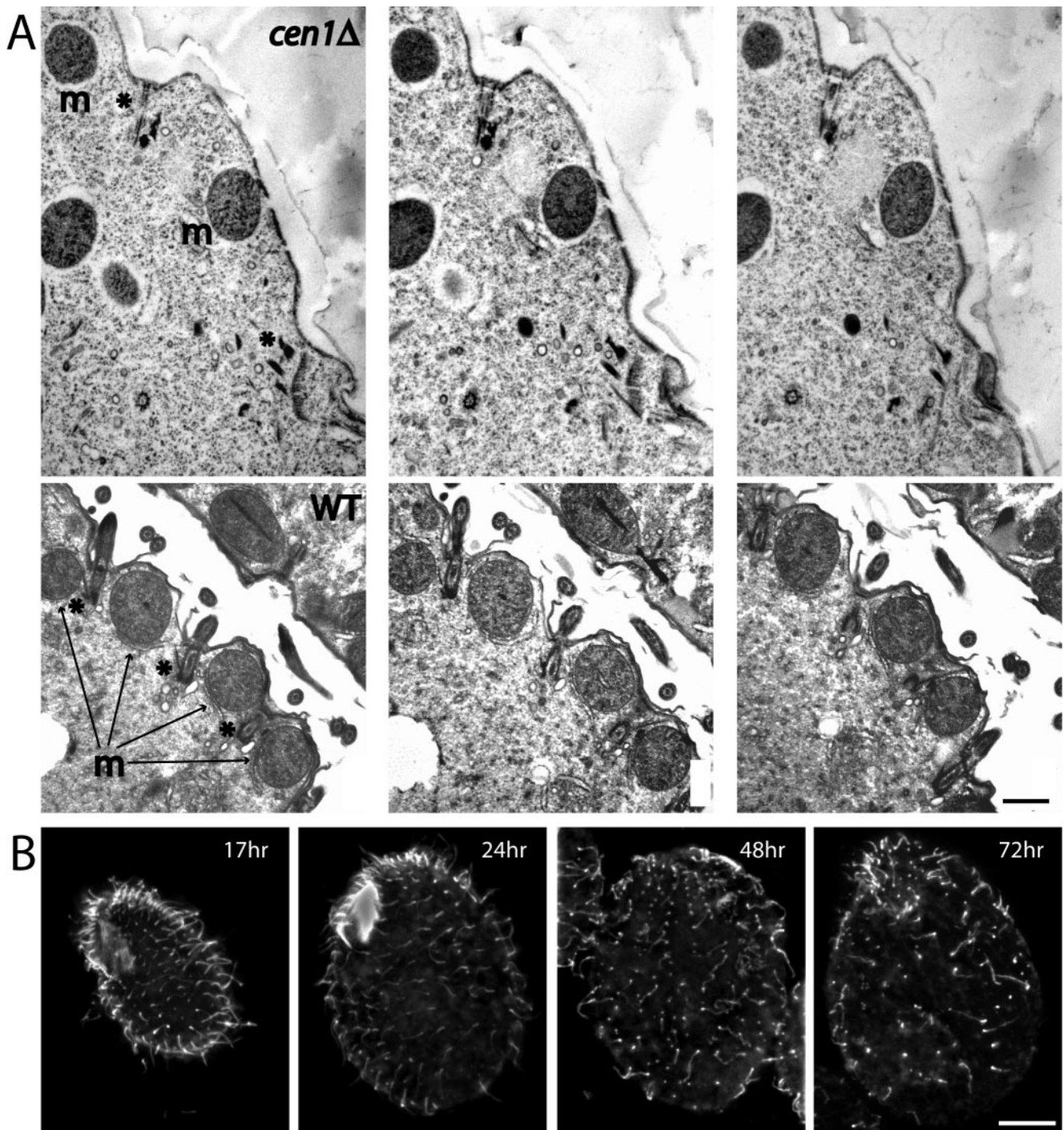
The immunofluorescence data on the *cen1Δ* strain strongly suggests that basal bodies are absent from these cells at later time points. However, fluorescence signal at late time points was still observed, more so with the antibody to polyglutamic acid, and the question remained whether this signal was at basal bodies, or some identifiable remnant of basal bodies. To address this issue, electron microscopy was performed on cells processed 68 h after the initial mixing of the two knockout heterokaryon strains. Fewer basal bodies were observed in sections from the *cen1* knockout strains compared with a wild-type control cross. When basal bodies were detected, they often seemed incomplete without cilia attached (Figure 9A). Occasionally the electron dense structures associated with the base of the basal body were present without all of the microtubules that normally comprise a basal body (our unpublished data). Immunoelectron microscopy with the Cen1 antibody revealed that basal bodies with more Cen1 were better preserved than basal bodies with less

antibody signal (our unpublished data). The possibility that Cen1 is important for maintenance of basal bodies was addressed further by immunofluorescence microscopy using an antibody to  $\alpha$ -tubulin, which detects cilia, a marker for a functioning basal body, as well as basal bodies. By 48 h after the initiation of mating of *cen1Δ* cells, many punctate spots reminiscent of basal bodies remained but a large percentage of these did not have attached cilia, indicating the absence of functional basal bodies at these sites (Figure 9B). Together, the results from the micronuclear knockout strains indicate that centrin is important for both the duplication and stability of basal bodies.

## DISCUSSION

We report the initial characterization of a family of four centrin genes in the ciliate *T. thermophila*. Two of these genes are closely related to the majority of the centrin genes found in other organisms, whereas the other two are related to the centrin genes identified in another ciliate, *P. tetraurelia*. Ciliates contain many distinct and highly specialized cytoskeletal structures. In *Tetrahymena*, these include basal bodies, cilia, micronuclear spindles, macronuclear microtubule arrays, a cortical network organized around the basal bodies of the ciliary rows, the contractile vacuole pores, and the complex microtubule and filamentous structures that comprise the oral apparatus (Gaertig *et al.*, 1993; Frankel, 2000). Because centrins play multiple roles at structures associated with microtubules (Taillon *et al.*, 1992), it would not be surprising if specialized centrins evolved along with some of these structures unique to ciliates. For example, GFP-Cen3 localizes predominantly to the fine filamentous reticulum of the oral apparatus and a band at the apex of the cell, and GFP-Cen4 localizes primarily to these structures and to the contractile vacuole pores. These structures are involved in dynamic processes: the undulating membrane of the oral apparatus sweeps media (food) into the oral cavity, whereas when the contractile vacuole expands, contracts, and ruptures, fluid is expelled through the CVPs maintaining the cell's proper osmolarity. The function of the apical band is not known; it is distinct from the contractile division furrow ring, although it, too, may be contractile and involved in regulating cell shape (Jerka-Dzidosz, 1981). In *Paramecium multimicronucleatum*, the number of contractile vacuoles increases in response to an increased amount of calcium in the environment (Iwamoto *et al.*, 2003). The assumption is that this is a response with which the cell rids itself of excess calcium, but it is possible that calcium, through centrin, more directly stimulates the assembly of this structure. Although the roles for Cen3 and Cen4 at the fine filamentous reticulum and the contractile vacuole pore are not known, it is possible the contractile properties of centrin affect the dynamic properties of these structures (Salisbury *et al.*, 1984). Additionally, *Tetrahymena* Cen1 along with inner arm dynein is involved in ciliary beat changes in response to calcium (Hennessey *et al.*, 2002; Guerra *et al.*, 2003), and perhaps centrins at the fine filamentous reticulum perform a similar task for this structure.

*Tetrahymena* *CEN1* and *CEN2* are on branches of the centrin tree that are most widely conserved throughout the eukaryotic world, and here we examined the function of Cen1 in MTOC duplication. Centrin is required for this process in species as diverse as yeast, green algae, and humans (Baum *et al.*, 1986; Salisbury *et al.*, 2002; Koblenz *et al.*, 2003). Although GFP-Cen1 and GFP-Cen2 both localize to basal bodies, *CEN2* is not normally expressed during vegetative growth, or conjugation. This situation may be



**Figure 9.** Cen1 is required for basal body maintenance. (A) Serial sections of a *cen1Δ* cell (top) and a cell from a wild-type control cross (bottom). The asterisks (\*) (top left) indicate the location of one incomplete basal body and a second site of interest. The basal body (top left) does not nucleate a cilium and seems disorganized at its base. The material at the bottom right is unrecognizable as a basal body, although it is present where a basal body would be expected given the location of the mitochondrion on the right (m) and the indentation in the cell surface. In the wild-type panels, three basal bodies (\*), two with cilia clearly attached, are visible. Basal bodies are between mitochondria (m), and the cilia emerge from indentations in the cell surface. Bar, 0.5  $\mu\text{m}$ . (B)  $\alpha$ -Tubulin localization in *cen1Δ* cells. Times are after mating initiation of the *cen1Δ* knockout heterokaryon strains, and cells were stained with a mAb raised against  $\alpha$ -tubulin (12G10). By 48 h, many apparent basal bodies do not have cilia attached. Bar, 10  $\mu\text{m}$ .

similar to that in humans, in which centrin1 expression is limited to the testes and retinal cells (Hart *et al.*, 1999). We have not determined whether *CEN2* is expressed under different conditions, or not at all, although the similarity to

other centrioles, in particular yeast Cdc31 and human centrin3, leads us to suspect that it is a functional gene. *CEN1*, however, is highly expressed in vegetative and mating cells. Localization of the endogenous protein places the majority

of Cen1 at two positions on the basal body. One is at the transition zone between the basal body and the axoneme of the cilium, as has been reported in *Chlamydomonas* (Sanders and Salisbury, 1994). The second is at the base of the basal body adjacent to the kinetodesmal fiber where probasal body formation is initiated (Allen, 1969). Disruption of *CEN1* led to the progressive loss of basal bodies. Of the basal bodies that remained, many were defective and unable to act as MTOCs for the cilia. Cen1 is a stable molecule, and small amounts of Cen1 remained even 72 h after mating initiation of the knockout heterokaryon strains. Therefore, our experiments examine the results of a slow depletion of Cen1. As Cen1 decreased, cells were unable to fill their cortical rows with the proper number of basal bodies, or to form a new oral apparatus, suggesting the requirement for Cen1 in the duplication of basal bodies.

The deterioration of the remaining basal bodies indicates that Cen1 also is required for proper basal body maintenance. The depletion of  $\gamma$ -tubulin yielded a related phenotype, demonstrating the necessity for this protein in basal body maintenance as well as basal body duplication. When  $\gamma$ -tubulin is depleted, oral apparatus basal bodies are lost before cortical basal bodies, suggesting that turnover of  $\gamma$ -tubulin is more rapid in the oral apparatus than at the basal bodies of the cortical rows, and required for these organelles' stability (Shang *et al.*, 2002). The *CEN1* knockout strain had no such hierarchy of loss, indicating that turnover of Cen1 is not significantly different at basal bodies along the cortical rows and basal bodies at the oral apparatus. Instead, Cen1 seems to be very stably associated with basal bodies and plays some role in preserving these organelles. Hence, a *cen1Δ* cell preparing for division can have one existing oral apparatus that seems normal and a highly aberrant developing oral apparatus (Supplemental Figure 1A). We do not know whether Cen1's roles in basal body duplication and maintenance can be separated, but it is tempting to speculate that Cen1 localization to the base of the basal body is important for duplication, whereas localization to the transition zone is important for basal body maintenance and function. Future work on Cen1 binding partners will shed light on this issue.

Our experiments did not reveal any association between the *Tetrahymena* centrin proteins and the micronucleus. This is somewhat surprising because centrins are found at mitotic spindle poles in a wide range of organisms. This supports the previous finding that centrin was not detected with  $\gamma$ -tubulin at the micronucleus (Shang *et al.*, 2002). However, centrin antibodies tested to date have not revealed centrin localization at the contractile vacuole pores, and our analysis of Cen4 indicates that it is likely present at this structure. Thus, the possibility remains that the experiments have not been sensitive enough to reveal centrin at spindle poles. Also, the GFP tag could interfere with centrin localization to the spindle pole in *Tetrahymena*. Another possibility is that there are yet more *Tetrahymena* centrin genes encoding isoforms that are at the spindle pole. Finally, it is possible that *Tetrahymena* does not require centrin for its mitotic apparatus. Few components of the *Tetrahymena* mitotic apparatus have been identified, and this issue may take some time to resolve.

We have shown there exists a family of *Tetrahymena* centrin genes with at least three bona fide members based on expression. Two of the centrins likely function at specialized fibrillar structures in the oral apparatus and the apical tip, and one of these also may act at the contractile vacuole pore. The predominant centrin, encoded by *CEN1*, localizes to basal bodies, and is required for their duplication and main-

tenance. The genetic malleability of these cells will likely lead to further insights into the mechanisms by which Cen1 functions at the basal body, and Cen3 and Cen4 function at their respective locations in the cell.

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