

The CNA1 Histone of the Ciliate *Tetrahymena thermophila* Is Essential for Chromosome Segregation in the Germline Micronucleus[□]

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Ciliated protozoans present several features of chromosome segregation that are unique among eukaryotes, including their maintenance of two nuclei: a germline micronucleus, which undergoes conventional mitosis and meiosis, and a somatic macronucleus that divides by an amitotic process. To study ciliate chromosome segregation, we have identified the centromeric histone gene in the *Tetrahymena thermophila* genome (*CNA1*). CNA1p specifically localizes to peripheral centromeres in the micronucleus but is absent in the macronucleus during vegetative growth. During meiotic prophase of the micronucleus, when chromosomes are stretched to twice the length of the cell, CNA1p is found localized in punctate spots throughout the length of the chromosomes. As conjugation proceeds, CNA1p appears initially diffuse, but quickly reverts to discrete dots in those nuclei destined to become micronuclei, whereas it remains diffuse and is gradually lost in developing macronuclei. In progeny of germline *CNA1* knockouts, we see no defects in macronuclear division or viability of the progeny cells immediately following the knockout. However, within a few divisions, progeny show abnormal mitotic segregation of their micronucleus, with most cells eventually losing their micronucleus entirely. This study reveals a strong dependence of the germline micronucleus on centromeric histones for proper chromosome segregation.

INTRODUCTION

All eukaryotic chromosomes rely on the fidelity of chromosome segregation machineries to faithfully transmit genetic information from one generation to the next. Centromeres are the chromosomal sites of microtubule attachment that mediate this segregation. Despite the universal requirement of faithful chromosome segregation, different eukaryotic lineages have remarkable diversity in the individual components and steps that characterize mitosis and meiosis. First, centromere size can range from just ~125 base pairs in budding yeast, to several hundred kilobases in mammals (Henikoff *et al.*, 2001; Malik and Henikoff, 2002). In these “complex” centromeres, defining the centromeric boundaries is technically challenging and has only been accomplished in a few select organisms (Schueler *et al.*, 2001; Sun *et al.*, 2003; Nagaki *et al.*, 2004). In some holokinetic organisms like nematodes, “mini-centromeres” organize throughout the length of interphase chromosomes, coalesce during prophase, and segregate as chromosome-long centromeres during anaphase (Buchwitz *et al.*, 1999). Second, chromo-

some segregation is typically accompanied by a breakdown of the nuclear membrane in most eukaryotes. However, the nuclear envelope does not break down during cell division in yeasts and ciliated protozoans (“closed mitosis”), affecting the chronology of events from kinetochore assembly to microtubule attachment. For instance, yeast kinetochores maintain microtubule attachment for the bulk of the cell cycle, whereas in plants and animals, microtubule attachment only takes place after breakdown of the nuclear envelope. Third, eukaryotes undergo several different versions of meiosis. Animals and plants undergo both a traditional “male meiosis” (a symmetric process in which all meiotic products are retained) and “female meiosis” (an asymmetric process in which only one of four meiotic products is retained). Budding yeasts only have “symmetric meioses,” ciliated protozoans like *Tetrahymena thermophila* only have “asymmetric meioses” (Martindale *et al.*, 1982), whereas asexual organisms like bdelloid rotifers appear to lack meiosis altogether (Welch and Meselson, 2000; Mark Welch *et al.*, 2004).

In addition to these differences, a striking specialization to emerge in the eukaryotic kingdom is the invention and copropagation of two nuclei in ciliated protozoans (Katz, 2001). Germline function is restricted to the diploid, largely transcriptionally inert micronucleus, which undergoes “closed” meiosis and mitosis (Davidson and LaFountain, 1975). In contrast, the somatic macronucleus is highly polyploid and consists of amplified, highly rearranged segments of the micronuclear genome (Woodard *et al.*, 1972; Yao *et al.*, 1984). The macronucleus is transcriptionally active and is responsible for most gene expression in ciliates. Chromosome segregation in the macronucleus is believed to be

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amitotic and not subject to the same quality checks as the micronucleus. For instance, during the amitotic division of the macronucleus, DNA is randomly segregated (Orias and Flacks, 1975) and can lead to phenotypic assortment where an allele can be lost stochastically.

Ciliated protozoans like *T. thermophila* thus present several unique features of chromosome segregation in the eukaryotic lineage. Key to studying chromosome segregation in ciliates is the identification of a marker that unambiguously marks centromeres at all stages of the cell cycle. The chief determinants of centromere identity are the centromeric histones, which were first identified in mammals and yeast (Palmer *et al.*, 1987, 1991; Stoler *et al.*, 1995), and are now known to be present in all eukaryotic genomes (Henikoff and Malik, 2002; Malik and Henikoff, 2003). Centromeric histones (hereafter referred to as CenH3s) are expressed from a single-copy gene and bear strong homology to canonical histone H3 proteins, although CenH3s evolve much more rapidly (Henikoff *et al.*, 2001; Malik and Henikoff, 2001; Talbert *et al.*, 2002). Blocks of CenH3-containing nucleosomes (that lack canonical H3) physically identify the centromeric chromatin (Blower *et al.*, 2002). These blocks recruit and organize kinetochore proteins, which will form microtubule attachment sites at mitosis and meiosis. The identification of centromeric histones in different eukaryotic lineages has greatly facilitated the study of centromere organization and function in diverse eukaryotes. Antibodies to centromeric histones have provided a direct means to look at chromosome segregation processes in various eukaryotes (Earnshaw *et al.*, 1986; Schatten *et al.*, 1988; Buchwitz *et al.*, 1999; Henikoff *et al.*, 2000; Talbert *et al.*, 2002; Nagaki *et al.*, 2004). Use of centromeric histones in chromatin immunoprecipitation experiments have led to the identification of satellite DNA repeats that are truly centromeric, separate from highly similar, adjacent heterochromatic counterparts in "complex" centromeres (Vafa *et al.*, 1999; Schueler *et al.*, 2001; Nagaki *et al.*, 2004). Additionally, disruptions of centromeric histone genes or depletion of their products have delineated previously inscrutable processes of kinetochore assembly (Stoler *et al.*, 1995; Buchwitz *et al.*, 1999; Howman *et al.*, 2000; Blower and Karpen, 2001; Moore and Roth, 2001; Oegema *et al.*, 2001).

Here, we identify the *T. thermophila* centromeric histone from the recently released genome sequence. In keeping with the nomenclature guidelines for *Tetrahymena* researchers (Allen, 2000), we have named this gene CNA1 (for centromeric protein A like). Using specific antibodies raised to a CNA1p peptide, we describe in detail the cytology of mitosis and meiosis in the ciliate germline micronucleus. Most features of chromosome segregation are found to be similar to other eukaryotes, although striking differences in the localization of CNA1p to the meiotic chromosomes and spindle can be seen. In addition, we describe CNA1p localization to centromeres as one of the first steps that distinguish nascent micronuclei from macronuclei. We also show that germline knockouts of CNA1 have an exclusively micronuclear effect, with many cells losing their micronucleus entirely within 8–9 cell divisions. However, macronuclei in these cells remain unaffected despite the cell lacking most micronuclear chromosomes. Thus, the centromeric histones of ciliated protozoans provide unprecedented insight into the functional diversification of their two nuclei.

MATERIALS AND METHODS

Strains

The wild-type strains CU427_{PB}C, CU428_{PB}N1 and B2086 II were obtained from Peter Bruns (Cornell University, Ithaca, NY). For conjugation, cells were

washed in 10 mM Tris-HCl, pH 7.5, resuspended in 10 mM Tris-HCl, pH 7.5, at 2×10^5 /ml, and incubated at 30°C for 20 h and then strains of different mating types were mixed.

Evolutionary Analyses

The CNA1 open reading frame was identified using TBLASTN searches against the *Tetrahymena* (macronucleus) genome database housed at The Institute for Genome Resources (TIGR). Preliminary sequence data for *T. thermophila* was obtained from The Institute for Genomic Research website at <http://www.tigr.org> and for *Paramecium tetraurelia* from <http://www.genoscope.cns.fr>. The *Stylonchia lemnae* sequence (Bernhard, 1999) was obtained from GenBank. We used PCR amplification on genomic DNA to confirm the sequence of the gene from *T. thermophila* genomic DNA and RT-PCR using RNA derived from vegetatively growing cells to confirm the boundaries of the coding sequence. The predicted protein encoded by CNA1 is 187 amino acids in length and has a nonconserved N-terminal tail that cannot be aligned to canonical H3s (Figure 1A). However, the initial five amino acids ("MARKA...") are quite similar to canonical H3s ("MARTK...") as has been previously seen in the *Arabidopsis* CenH3 (Talbert *et al.*, 2002), providing further support that we have correctly identified the coding boundaries of CNA1. The gene sequence has been deposited in GenBank under sequence accession no. DQ126145. The histone-fold domains of selected canonical and centromeric histones were aligned using ClustalX (Thompson *et al.*, 1997) and a neighbor-joining phylogeny was constructed using the PAUP* suite of programs (Swofford, 2000).

Northern Analysis

RNA samples were collected using Qiagen RNeasy Mini Kit (Chatsworth, CA). DNA Probes were prepared from gel purified PCR products of the coding sequence of CNA1. A probe to the gene that codes the ribosomal rpL21 protein (Strausberg *et al.*, 2002) was used as a loading control. PCR products were used for random prime labeling and unincorporated radionucleotides were removed with Amersham MicroSpin 325 columns (Piscataway, NJ). The membrane was hybridized at 65°C in Church's buffer, exposed to film, and developed.

Production of CNA1 Antibody

Previously, specific antibodies to CenH3s in *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, and *Oryza sativa* were successfully generated using polyclonal antibodies raised to a peptide at the amino-terminal end of the protein (Buchwitz *et al.*, 1999; Henikoff *et al.*, 2000; Talbert *et al.*, 2002; Nagaki *et al.*, 2004). We used a similar strategy to create an affinity-purified polyclonal antibody specific to CNA1p, using the amino-terminal peptide sequence ARKAYQPKRRNSNSQNQQC. Although this peptide sequence had lower than ideal sequence complexity, TBLASTN searches had indicated that it was likely to be very specific to CNA1p. Antibodies to CNA1p were made using the Premium Affinity protocol from Quality Controlled Biochemicals (QCB, Hopkinton, MA). The N-terminal peptide was produced and purified using HPLC, conjugated to the KLH carrier and used to immunize two rabbits. Bleeds from the rabbits were screened by ELISA assays and affinity-purified using the peptide.

To test the specificity of the antibody for CNA1p, we expressed the CNA1 coding sequence in an in vitro transcription-translation system. Because ciliates employ a slightly altered genetic code, in which codons TAA/TAG encode for glutamine (Horowitz and Gorovsky, 1985), it was necessary to change 11 TAA/TAG codons to CAA/CAG within the coding sequence before we could use the in vitro expression system. The CNA1 open reading frame was subcloned into the pCR T7/TOPO TA cloning vector. Untagged and unmodified CNA1 protein was produced from this plasmid (or no DNA mock control) using an in vitro-coupled transcription-translation kit (TnT Coupled Reticulocyte Lysate, Promega, Madison, WI).

Western Analysis

Tetrahymena cultures were collected during early log, 24 h in starvation, and at various time points during conjugation. Cell cultures were centrifuged and washed in 10 mM Tris-HCl, pH 7.5. Pellets were either frozen on dry ice or processed immediately by boiling for 5 min in ~5 volumes of 2× SDS loading buffer (4% SDS, 160 mM Tris-HCl, pH 6.8, 20% glycerol, Bromophenol blue, 10% [vol/vol] 2-mercaptoethanol). Immediate processing of vegetatively growing *Tetrahymena* cells was essential to avoid protein degradation. Protein extracts were loaded onto either 12% or 4–12% gradient gels (Invitrogen, Carlsbad, CA) for SDS-PAGE analyses. Equivalent loading was monitored in all cases by Coomassie staining of duplicate gels (unpublished data). Gels were blotted onto nitrocellulose in blotting solution (20% methanol, 25 mM Tris-HCl, 192 mM glycine, pH 8.3). Filters were blocked in 10% milk, 0.3% Tween-20, 1× phosphate-buffered saline (PBS) and incubated with affinity-purified primary antibody (1: 1000 dilution in 1/10 blocking solution, 1× PBS). Blots were washed in 1× PBS, 0.3% Triton, 5 M NaCl. Secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham) was used at 1:3000 dilution followed by washing as before and detection by ECL (Amersham). Blots were typically exposed for 2 s to reveal the

modified form of CNA1 and for 1–2 min to visualize nonspecific bands as well as the unmodified CNA1 expressed *in vitro*, that is also present in *Tetrahymena* extracts.

Gene Disruption

The *neomycin* cassette under the control of the Metallothionein promoter, flanked by CNA1 flanking sequence was cloned into the pMNB vector (gift from M. Gorovsky, University of Rochester, Rochester, NY). The vector was digested to release the neo fragment before biolistic transformation of *Tetrahymena* (Bruns and Cassidy-Hanley, 2000). The *neo* cassette was inserted into the CNA1 locus by homologous recombination replacing the entire CNA1 coding sequence. Transformed cultures were kept in 10 mM Tris overnight at room temperature then spun down and resuspended in SPP. After 6 h in SPP, 100 μ g/ml paramomycin was added to the cultures and plated in 96-well plates. Heterozygous germline transformants were carried through genomic exclusion to create heterokaryons that are homozygous knockout in the germline nucleus and wild type in the macronucleus (Hai and Gorovsky, 1997). Insertion and deletion were verified by PCR and Southern analyses. After mating, nonmated cells were selected against using cycloheximide, as previously described (Bruns and Brussard, 1974; Orias *et al.*, 2000).

For Southern analyses, genomic DNA was collected from wild-type and knockout progeny after >10 and 8 divisions, respectively (Blomberg *et al.*, 1997). DNA was digested with *Xba*I overnight, run on 0.8% agarose gel, and transferred by pressure blotting to a nylon membrane. PCR products corresponding to the *neo* gene, the CNA1 coding sequence, and to the genomic region upstream of CNA1, were gel-purified using Qiagen QIAquick PCR purification, radioactively labeled as above, and hybridized in Denhardt's buffer. The membrane was exposed to film and developed.

Microscopy

Immunofluorescence using antibodies recognizing the CNA1p was adapted from previous studies (Stuart and Cole, 2000; Cole *et al.*, 2002). Cells were grown to early log phase, $\sim 2 \times 10^5$ /ml, 5 ml of cells was washed in 10 mM Tris, resuspended in 0.5 ml 10 mM Tris, and then 2 ml 1% paraformaldehyde in PHEM buffer was added to cells and left at room temperature for 10 min. After fixation cells were washed twice in PBS, resuspended in 2 ml PBS, and dropped onto coverslips. Coverslips were allowed to dry at room temperature and then stored at 4°C. Coverslips were allowed to warm to room temperature then set in PBS, 0.3% Tween-20 (PBS-T) for 45 min, blotted, and inverted onto 100 μ l PBS-T with primary rabbit polyclonal CNA1p antibody (1:600) in a weighboat and incubated at 4°C overnight. After incubation at 4°C, coverslips were set in PBS-T for 45 min, blotted, and incubated with secondary goat anti-rabbit FITC conjugated antibody (Sigma, St. Louis, MO) at 4°C overnight in the dark. After incubation with secondary antibody, coverslips were set in PBS-T for 30 min and PBS-T with DAPI at room temperature in the dark for 10 min, PBS-T at room temperature in the dark for 10 min, and finally inverted onto 7 μ l DABCO and sealed with nail polish. Slides were stored at 4°C.

Immunofluorescence of CNA1p along with α -tubulin was done as above except paclitaxel was added (final 10 μ M) to fixation and PBS washing steps before dropping onto coverslips. The anti- α -tubulin antibody 12G10 developed by J. Frankel and E. M. Nelsen was obtained from the Developmental Studies Hybridoma Bank developed under the auspice of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). We used a 1:25 dilution of the 12G10 antibody and a 1:100 dilution of the secondary anti-mouse Texas Red-conjugated antibody (Molecular Probes, Eugene, OR; Invitrogen). The micronuclear specific linker histone δ and Pdd1p antibodies (gift from Emily Wiley and Dave Allis) and was used at a dilution of 1:100 along with the secondary goat anti-rabbit FITC-conjugated antibody at a dilution of 1:100.

Slides were viewed on an Olympus IX70 Deltavision microscope (Applied Precision, Issaquah, WA) with a 60 \times PlanApo 1.40 numerical aperture oil immersion lens. Images were collected with a Photometrics CH350 digital camera (Tucson, AZ), deconvolved using the Softworx program (Applied Precision), and processed using Photoshop (Adobe Systems, San Jose, CA).

RESULTS

Identification of CNA1

All eukaryotic genomes sequenced to date have a single variant histone H3 gene that encodes the centromeric histone (CenH3) protein (Malik and Henikoff, 2003). Bioinformatic criteria refined by using yeast, animal, and plant CenH3s (Malik and Henikoff, 2003) were used to screen the histone H3 homologues that were identified in the *T. thermophila* genome database. This allowed the unambiguous identification of a single gene, CNA1, which had all the hallmarks of being a CenH3 (Figure 1) in spite of the fact that ciliate histones appear to

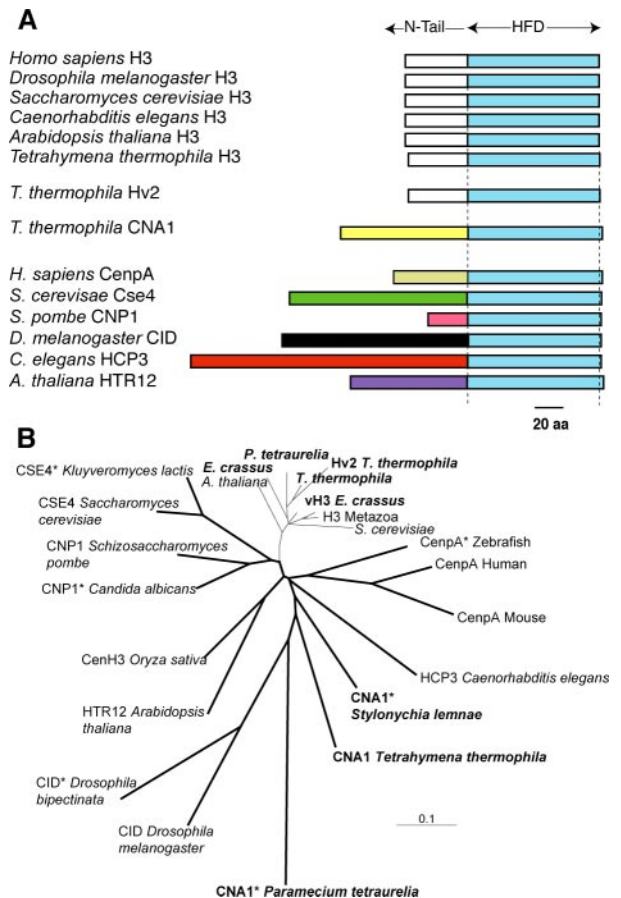


Figure 1. CNA1 is the *T. thermophila* centromeric histone gene. (A) Schematic comparison of selected canonical histone H3s from eukaryotes showing the conservation of the canonical histones in their histone-fold domains (HFD) and N-terminal tails. In contrast, the centromeric histones do not have a well-conserved N-terminal tail, and this evolves rapidly in both size and sequence. The three histone H3-like genes from *T. thermophila* are schematized, including the canonical H3, the H3.3 like replacement histone variant hv2 and the centromeric histone CNA1. (B) A neighbor-joining phylogeny of the histone-fold domains of selected canonical and centromeric histone proteins (bold lines) is shown. Asterisks are used to indicate those proteins that, based on bioinformatic or evolutionary criteria, are thought to be the centromeric histone although this has not been directly tested. This includes putative centromeric histones from other ciliate species, *P. tetraurelia* and *S. lemnae* (ciliate histones are shown in bold lettering). In comparison to yeast, vertebrate and plant CenH3s, those from ciliates appear to be rapidly evolving.

evolve more rapidly (Bernhard, 1999; Katz *et al.*, 2004). The histone-fold domain of CNA1p is only $\sim 50\%$ identical to other histone H3 variants in *Tetrahymena*, and like other CenH3 proteins, is evolving more rapidly than canonical H3 proteins (Figure 1B). Using TBLASTN searches, we have also obtained the sequence of the histone-fold domain from CenH3s of two other ciliates, *P. tetraurelia* and *S. lemnae* (Bernhard, 1999) to look at the evolution of CenH3s in ciliates. From this cursory analysis, the ciliate CNA1 proteins appear to be evolving more rapidly than most CenH3s, on par with what has been seen previously for the rapidly evolving *Drosophila* CID proteins (Malik and Henikoff, 2001; Malik *et al.*, 2002).

CNA1 Expression

Northern analysis was used to determine the expression of CNA1 during vegetative growth and conjugation. Express-

sion of *CNA1* is not detectable in either growing cells, where only a small fraction of cells are dividing at any given moment, or in cells starved for 24 h. However, when starved cells of different mating types were mixed together to induce conjugation synchronously, we found that *CNA1* was strongly expressed (Figure 2A). At 4 h in conjugation, at the time of meiosis, a strong band of ~1 kb is apparent but expression steadily decreases by 6 h in conjugation, and is just detectable by 16 h in conjugation (Figure 2A).

A polyclonal rabbit anti-CNA1p antibody was generated and tested against in vitro-expressed CNA1p. Western blots show a single band at the expected ~22 kDa from in vitro expressed CNA1p, but no bands in the mock in vitro expression (Figure 2B). We tested the anti-CNA1p antibody against protein extracts from *Tetrahymena* during vegetative growth (Figure 2C). In addition to the 22-kDa band that comigrates with the in vitro expressed protein, the antibody also recognizes a very prominent band ~5 kDa larger than the expected size in all extracts with high specificity. The unmodified form of CNA1p is readily visible with an overexposure. However, the bulk of the CNA1p appears to be posttranslationally modified. The nature of this modification is unknown but posttranslational modifications have been previously seen in practically all CenH3s in Western analyses (Zeitlin *et al.*, 2001; Talbert *et al.*, 2002; Collins *et al.*, 2004; Sullivan and Karpen, 2004). We could show that both these bands correspond to the CNA1p protein because their recognition was specifically outcompeted by the original peptide to which the antibody was generated, but nonspecific bands of higher molecular weight were not affected (Figure 2C). Thus, the polyclonal antibody has high specificity for the CNA1p protein. We then used this antibody to screen extracts of *T. thermophila* from vegetative growth, starvation, and conjugation in a Western blot (Figure 2D). Although we had expected to see a large increase in CNA1p during conjugation based on the Northern analysis (Figure 2A), we could detect no appreciable differences in standing levels of CNA1p. This implies that most of the increased transcription seen at the onset of meiosis does not lead to increased steady state protein levels, either due to translational or posttranslational control. Rigorous posttranslational control of CenH3 levels has been previously reported in the case of the budding yeast Cse4 (Collins *et al.*, 2004; Pearson *et al.*, 2004).

CNA1p Localization during Mitosis

During vegetative growth, the germline micronucleus divides by mitosis, whereas the somatic macronucleus is believed to divide by an amitotic process that leads to random segregation of the macronuclear chromosomes. This random segregation of macronuclear chromosomes (Orias and Flacks, 1975) had led to the assumption that macronuclei do not contain centromeres. However, this was an indirect conclusion because of the lack of centromeric markers or centromeric satellite sequences. Localization of CNA1p gave us the first opportunity to verify this assumption. Using the antibody, we could show that CNA1p staining is completely absent from the somatic macronucleus (Figure 3A), confirming the absence of macronuclear centromeres.

CNA1p localizes to peripheral, clustered dots that mark the centromeres around the germline micronucleus during interphase. *T. thermophila* has five pairs of metacentric chromosomes in the micronucleus and we never see more than 10 "dots." When micronuclei undergo closed mitosis (no nuclear envelope breakdown), CNA1p staining identifies pairs of centromeres that remain peripheral, aligning in a "ring" during metaphase (Figure 3B). Centromere pairs separate as anaphase progresses, and centromeres can be seen

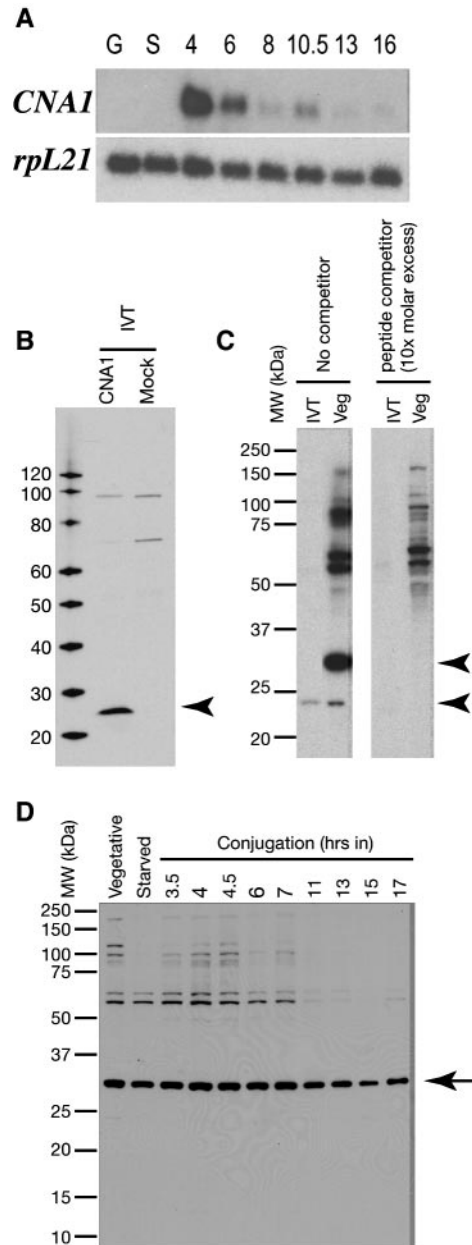


Figure 2. CNA1 expression. (A) On a Northern blot probed with the CNA1 coding sequence there is no detectable transcript during vegetative growth (G) or starvation (S) but high levels of expression are seen during meiosis (4 h) in conjugation. Expression continues to decrease as conjugation progresses. The *rpl21* gene was used as a loading control. (B) Western blot shows that the polyclonal antibody raised to the CNA1 peptide specifically recognizes in vitro-translated (IVT) CNA1p, but no band is detected in the mock expression. (C) The CNA1 antibody recognizes two bands in vegetatively growing *T. thermophila* cells. The lower band corresponds to the unmodified protein as seen in the in vitro expression and can only be visualized by overexposure, whereas the predominant upper band is ~5 kDa higher and corresponds to an unknown posttranslationally modified form of CNA1p. Neither band is detected when the antibody staining is competed with a 10-fold molar excess of peptide competitor. (D) CNA1p levels as detected by the Western blot appear stable in vegetative and starved cells, and during conjugation, in stark contrast to the Northern analysis in A.

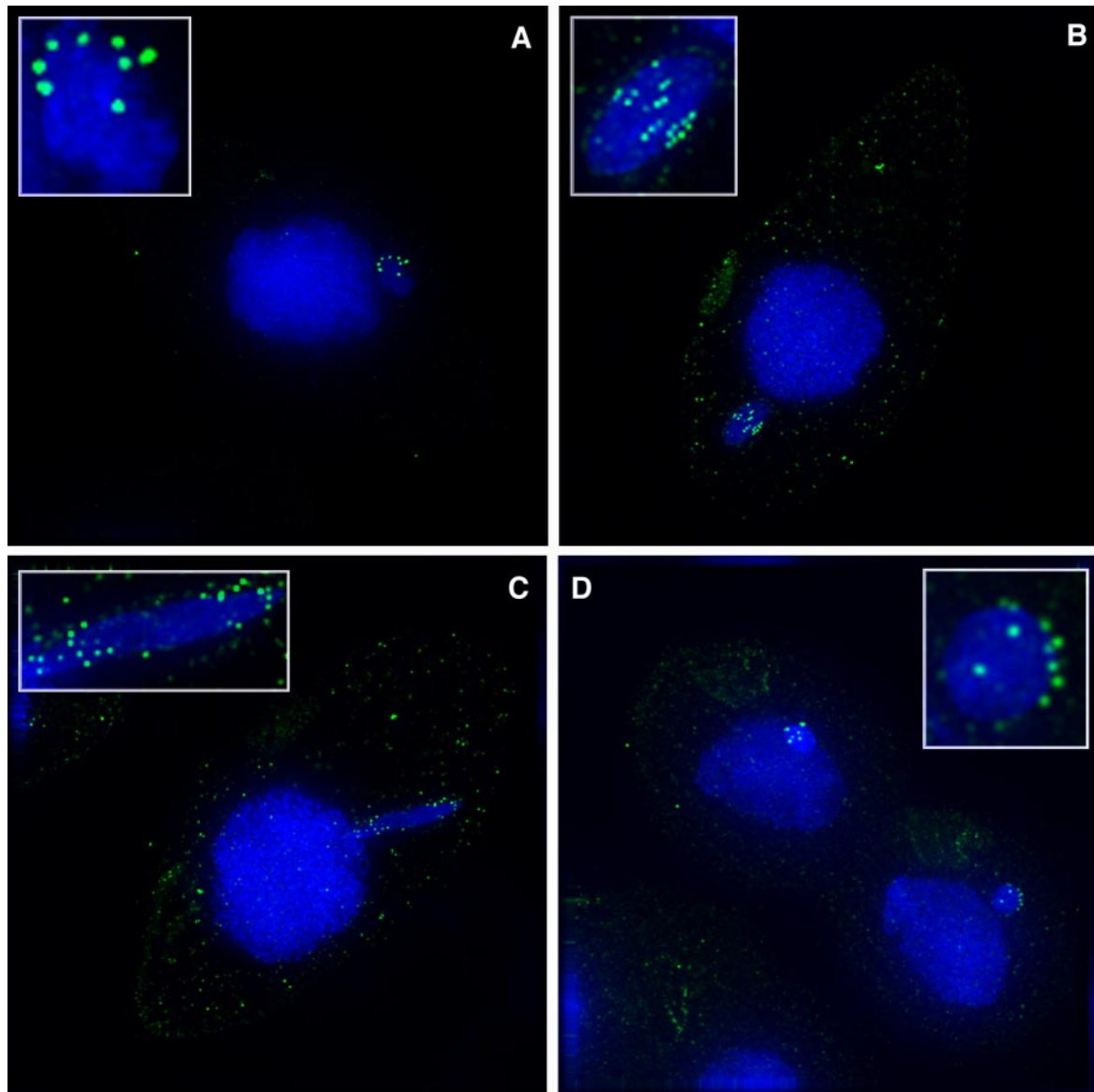


Figure 3. CNA1p localization during mitosis. (A) Immunofluorescence using CNA1p antibody during mitosis shows CNA1p is found only at the centromeres in the micronucleus. During interphase, the centromeres are clustered and peripheral. (B) Centromeres remain peripheral during metaphase when pairs of centromeres align along the middle of the chromatin. (C) The centromeres are on the leading edge of segregating chromosomes in anaphase. (D) By telophase the centromeres are peripheral again. CNA1p staining is shown in green, whereas DNA is stained with DAPI (blue). A weak background staining of basal bodies is seen with the CNA1 antibody, which occasionally outline the cellular boundaries.

on the leading edge of the DNA (Figure 3C). By telophase and later, the centromeres have returned to a random, peripheral position with respect to the nuclear envelope (Figure 3D). Our results with the CNA1p antibody are in agreement with previous results based largely on electron microscopy (LaFountain and Davidson, 1979, 1980). The absence of a suitable centromere marker and lack of obvious chromosome morphology in mitosis had previously precluded the correct assignment of mitotic stages in *Tetrahymena*. The availability of the CNA1p antibody and assignment of centromere positions will now allow another means to reevaluate existing mutants to determine the exact stage of mitotic arrest (Kirk *et al.*, 1997; Petcherskaia *et al.*, 2003).

CNA1p Localization during Meiosis

In *Tetrahymena*, conjugation is an extremely dynamic process involving (sequentially) nuclear elongation, meiosis, nuclear selection, and two rounds of postzygotic mitosis followed by genomic rearrangement (meiosis and fertilization are schematized in Figure 4A; Martindale *et al.*, 1982). Unlike mitosis during vegetative growth, we found that localization of CNA1p during conjugation is highly dynamic. Soon after pairing of cells of opposite mating type, early meiotic prophase is distinguished by the micronucleus taking on a crescent shape that is almost twice the length of the cell. Early in crescent formation the micronucleus moves away from the macronucleus and is elongated to an egg shape, with the centromeres clustered at the apical end of the

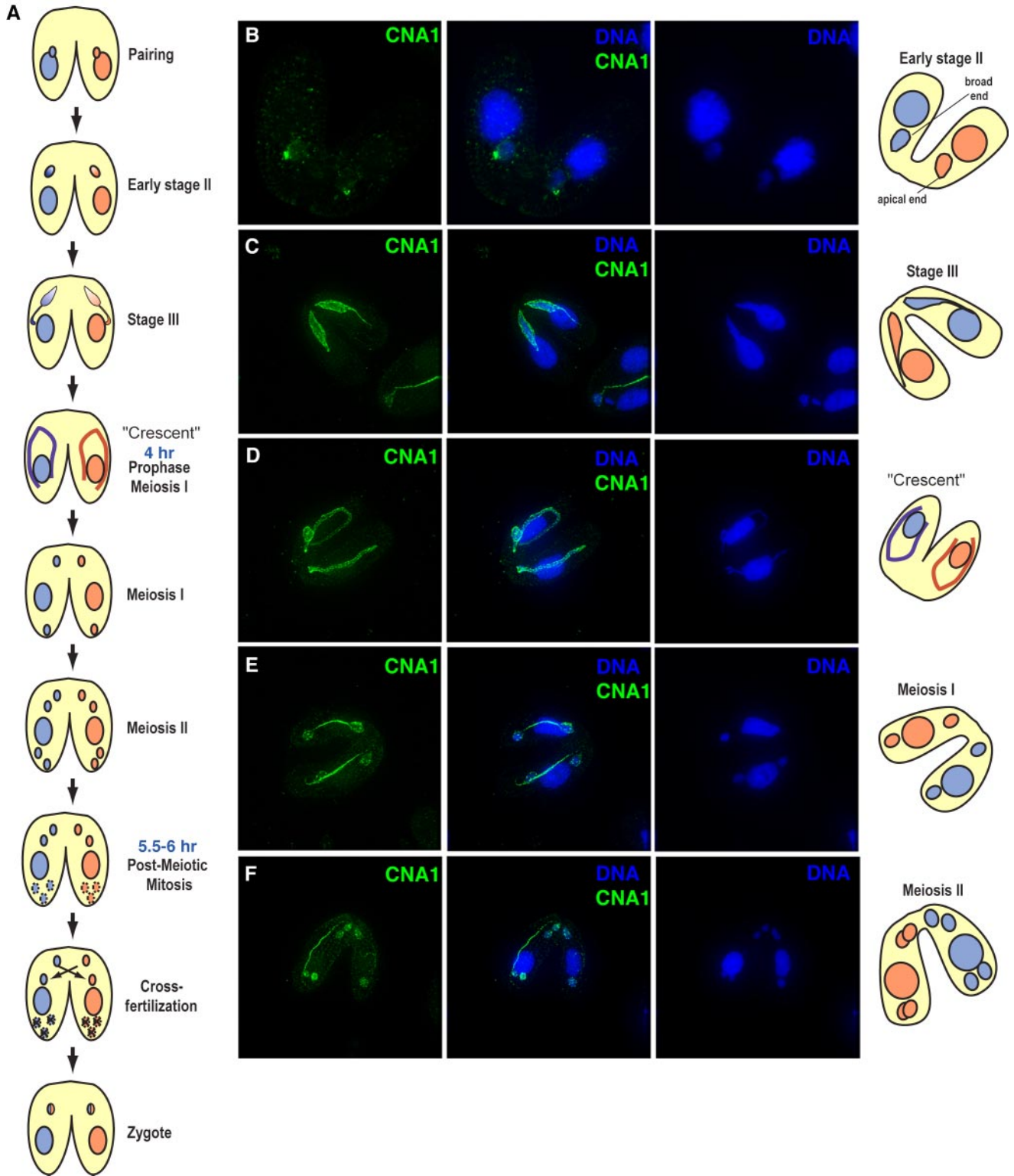


Figure 4. Early events of conjugation. (A) Conjugation in *Tetrahymena* is schematized to highlight the major events starting from pairing of cells of different mating types (shown as blue or red) to the formation of the zygote. The diploid micronucleus undergoes meiosis, and one of the four haploid meiotic products is chosen, whereas the other three move to the bottom of the cell and are degraded. The chosen meiotic product divides mitotically and the two cells exchange one haploid nucleus each and fuse to give rise to the zygote (shown as a chimeric red/blue hereafter). (B) During prophase, the micronucleus goes through an egg shape (early stage 2). At this stage, centromeres are clearly visible at the apical end of the nucleus (telomeres have been shown to localize to the basal end at this stage). Micronuclei are then gradually stretched into stage III (C) and eventually the "crescent" shape (D). As the crescent elongates, CNA1p localization can be seen throughout the length of the crescent. (E and F) As meiosis progresses CNA1p remains punctate throughout the micronuclei and also localizes to the meiotic spindle. Distinct centromeres are visible in late anaphase of meiosis I and II. (CNA1p, green; DNA-DAPI, blue). Tubulin staining at these stages of meiosis are presented in Supplementary Information.

micronucleus (Figure 4B). Centromere location can be seen precisely coincident with microtubules, suggesting microtubule anchoring of centromeres determines their position. Previous work has shown that telomeric repeats cluster at the broad end of the micronucleus (Loidl and Scherthan, 2004), implying that centromeres and telomeres are on opposite ends at this stage of meiosis. During full crescent phase CNA1p is seen in a punctate pattern throughout the length of the crescent (Figure 4, C and D) and this pattern remains so throughout meiosis (Figure 4, C–G). During telophase of meiosis I and II, CNA1p localization is diffuse throughout the DNA as well as punctate along the spindle (Figure 4, E and F).

The localization of CNA1p during the closed meiosis of *Tetrahymena* is remarkable in two respects. The first novel feature of CNA1p deposition during meiosis is how strongly CNA1p appears to be “mislocalized” throughout the DNA. Such mislocalization has only been previously achieved by means of strong overexpression (Ahmad and Henikoff, 2002; Jager *et al.*, 2005) or by eliminating turnover of the CenH3 (Collins *et al.*, 2004). This deposition appears to be less stable than at centromeres, however, because the CNA1p is quickly displaced from noncentromeric chromatin as the “stretched” crescent chromosomes revert to normal in telophase. The second novel feature of CNA1p localization is that during meiosis, the CNA1 protein appears to move to the meiotic spindle during anaphase (Figure 4, E–G). Although other kinetochore proteins have been previously found as “passenger proteins” on the spindle (Wang, 2001; Vagnarelli and Earnshaw, 2004), this represents the first case where a centromeric histone has been found as a passenger protein on the meiotic spindle. This localization is not an artifact introduced by the antibody because we could see a similar pattern using a GFP-tagged CNA1 (gift from B. Cui and M. A. Gorovsky). The localization is also completely coincident with microtubules as stained by α -tubulin (tubulin staining for all stages is provided in Supplementary Material). We cannot formally rule out the possibility that there is a small amount of DNA remaining in the area between the divided nuclei that was not detectable by DAPI staining. However, the pattern persists up to nuclear separation when DNA segregation is complete, making this a highly unlikely possibility.

CNA1p Distinguishes Developing Micronuclei and Macronuclei in Late Conjugation

The completion of meiosis sets up a cascade of events that leads up to the formation and diversification of the germline micronucleus and somatic macronucleus (Figures 4A and 5A). One of four meiotic products is selected as a gamete and undergoes one round of mitosis. The paired cells exchange gametic nuclei and then the two gametic nuclei, one from each parent cell, fuse forming the diploid zygotic nucleus. After formation of the zygote, the new zygotic micronucleus undergoes two rounds of mitosis, producing four new diploid nuclei per cell. At this stage, at 8 h in conjugation there are five nuclei per cell: one parental macronucleus and four zygotic nuclei (Figure 5A). The parental macronucleus moves to the bottom of the cell and eventually goes through apoptotic degradation.

Of the four genetically identical zygotic nuclei, two become somatic macronuclei, whereas the other two remain germline micronuclei (Wenkert and Allis, 1984). Strikingly, at or very soon after this decision point, the CNA1p localization in the two nuclei destined to become micronuclei reverts to the localization pattern seen in interphase micronuclei. In these two nuclei, CNA1p is seen in fewer than 10

dots at the periphery of the nucleus (Figure 5B). However, in the nuclei destined to become macronuclei, CNA1p remains punctate throughout the nucleus, steadily decreasing as conjugation progresses until the time of genomic rearrangement when the staining disappears entirely within the developing macronuclei (Figure 5, C and D). Meanwhile, bright staining of CNA1p can be seen late in the apoptotic degradation of the parental macronucleus, when most of the DNA has been degraded (Figure 5C). It is possible that this intense CNA1p staining corresponds to CNA1p protein that moves from the developing macronucleus to the old, parental macronucleus to be degraded. Previous reports have shown that the Pdd1p protein that is involved in DNA elimination in the developing macronucleus, is similarly localized to the parental macronucleus before being degraded (Madireddi *et al.*, 1996).

Thus, CNA1p joins a select list of early markers that can help distinguish between the two fates of zygotic nuclei in *Tetrahymena*. Previously, events that were known to contribute to this early differentiation included the removal of the micronuclear-specific linker histone, the appearance of a macronuclear histone H2A variant, and acetylation of histone H4 in those nuclei destined to become macronuclei (Chicoine *et al.*, 1985; Lin *et al.*, 1989; Pfeffer *et al.*, 1989). All these events involved changes specific to those nuclei destined to become macronuclei, from the common stage. These results suggested that micronuclear fate was the default status of these zygotic nuclei, which was overwritten by epigenetic events only in macronuclei. Our results show that CNA1p localization is specifically altered only in those nuclei destined to become micronuclei, implying that micronuclei do not necessarily represent the default fate of zygotic nuclei. Thus, altered CNA1p localization is the first known identifying marker for micronuclear fate.

Knockouts of CNA1 Have Micronuclear Chromosome Segregation Defects

We next wanted to investigate the role of CNA1 in mediating chromosome segregation, by making gene knockouts. There are two means of introducing knockouts in the binucleate *Tetrahymena*, in either the somatic macronucleus or the germline micronucleus. Gene knockout in the somatic macronucleus affects the expression of the gene while leaving it unchanged in the germline. Because the somatic macronucleus contains ~50 copies of each macronuclear chromosome, we replaced one copy of the CNA1 gene with a *neomycin* cassette. During division of the macronucleus the macronuclear chromosomes are segregated randomly which can lead to loss of one allele of a particular gene (Orias and Flacks, 1975; Merriam and Bruns, 1988). Growing cells in increasing amounts of the drug paramomycin will select for cells with more copies of the *neomycin* cassette and fewer wild-type copies of the CNA1 gene. For nonessential genes this “phenotypic assortment” can lead to complete loss of the wild-type gene. Despite several attempts, we could not obtain greater than 50% replacement of the CNA1 knockout. Even at this 50% replacement stage, there were defects in chromosome segregation during mitosis and cells would eventually cease division. When these “knockdown” cells were stained with DAPI, they showed lagging DNA during mitosis and many of the cells had smaller micronuclei than wild type (unpublished data). Thus the CNA1 gene, like CenH3s in other eukaryotes (Stoler *et al.*, 1995; Buchwitz *et al.*, 1999; Howman *et al.*, 2000; Blower and Karpen, 2001), is essential for *Tetrahymena* viability. This further suggests that levels of CNA1 expression play a critical role in chromosome segregation, as we are seeing defects manifest in what is the macronuclear equivalent of a heterozygous state.

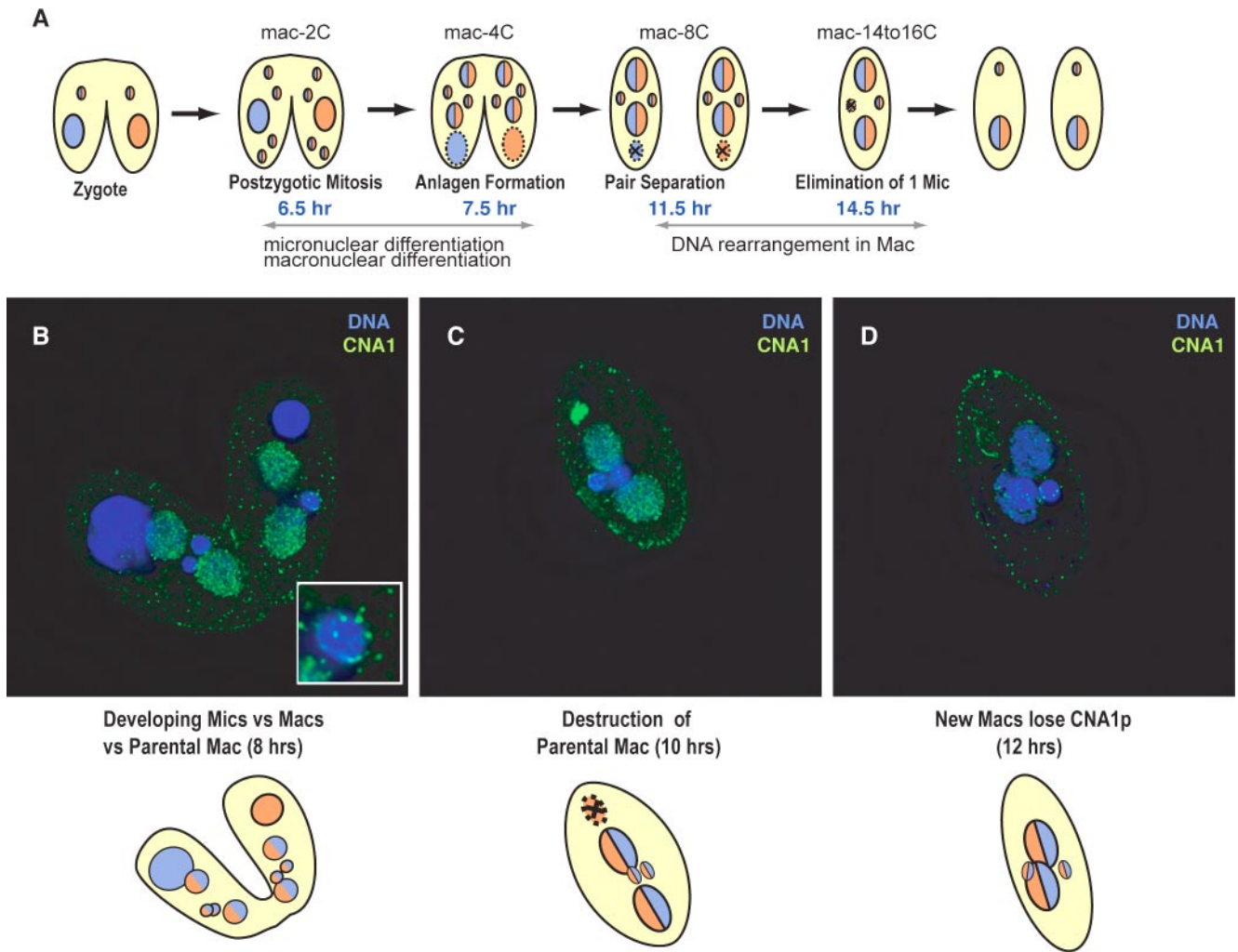


Figure 5. CNA1 localization during late events in conjugation. (A) Conjugation events leading from zygote formation to progeny cells are schematized. The zygotic nucleus undergoes two rounds of mitosis producing four nuclei (6.5 h), two become micronuclei, whereas two become developing macronuclei (anlagen) and undergo genomic rearrangement and endoduplication. The active parental macronucleus remains in the middle of each cell until the new zygotic macronuclei become active, at ~7.5 h, and then the parental macronucleus moves to the bottom of the cell and undergoes apoptotic degradation. (B) Eight hours into conjugation CNA1p localization reverts to the peripheral centromeres in the developing micronuclei (inset) but remains diffuse throughout the developing macronuclei. At this time cells remained paired, the parental or old macronucleus has migrated to the bottom of the cell and is beginning apoptotic degradation. (C) By 10 h, CNA1p is concentrated in the old macronucleus that has little DNA remaining. CNA1 localization is decreased in the developing macronuclei, whereas it remains constant at the centromeres in the micronuclei. (D) At the time of genomic rearrangement CNA1p is largely absent from the cell except at the centromeres in the micronuclei, similar to vegetative cells (Figure 3A) (CNA1p, green; DNA- DAPI, blue)

Because we could not accomplish a complete somatic knockout of the *CNA1* gene, we decided to replace the *CNA1* gene in the germline nucleus. The advantage of this approach is that because the germline nucleus is silent during vegetative growth, crosses can be engineered to create homozygous germline knockouts. Using biolistic transformation, we replaced the entire *CNA1* coding region with a *neomycin* cassette. Two clones that segregated the *neomycin* cassette were taken through a mating of genomic exclusion where each clone is mated with a cell that does not contain a functional germline micronucleus leading to whole genome homozygotes (Kahn *et al.*, 1993; Cassidy-Hanley *et al.*, 1997; Hai and Gorovsky, 1997). These cells are phenotypically wild type, because gene expression is only from the wild-type macronucleus, whereas the homozygous knockout micronucleus is transcriptionally silent. This facilitates the study of the *CNA1* knockout by mating two homozygous

knockouts of different mating types. By mating two homozygous knockouts of different mating types we were able to observe the phenotype of the resulting *CNA1* null progeny (hereafter simply referred to as $\Delta CNA1$).

Conjugation was found to proceed normally in mating of two homozygous knockouts. No delay was detected in early events of meiosis and nuclear differentiation and markers of DNA elimination were identical to wild type and production of exconjugants was similar to a wild-type mating (unpublished data). In addition, the pattern of CNA1p localization throughout conjugation was identical in the knockout mating compared with wild type. Thus, any defects that arise from the *CNA1* knockout only manifest in the cell divisions that follow conjugation, when all zygotic transcription relies solely on the new knockout macronucleus.

We ascertained whether the *CNA1* gene had been completely replaced with the *neo* cassette in the progeny of the

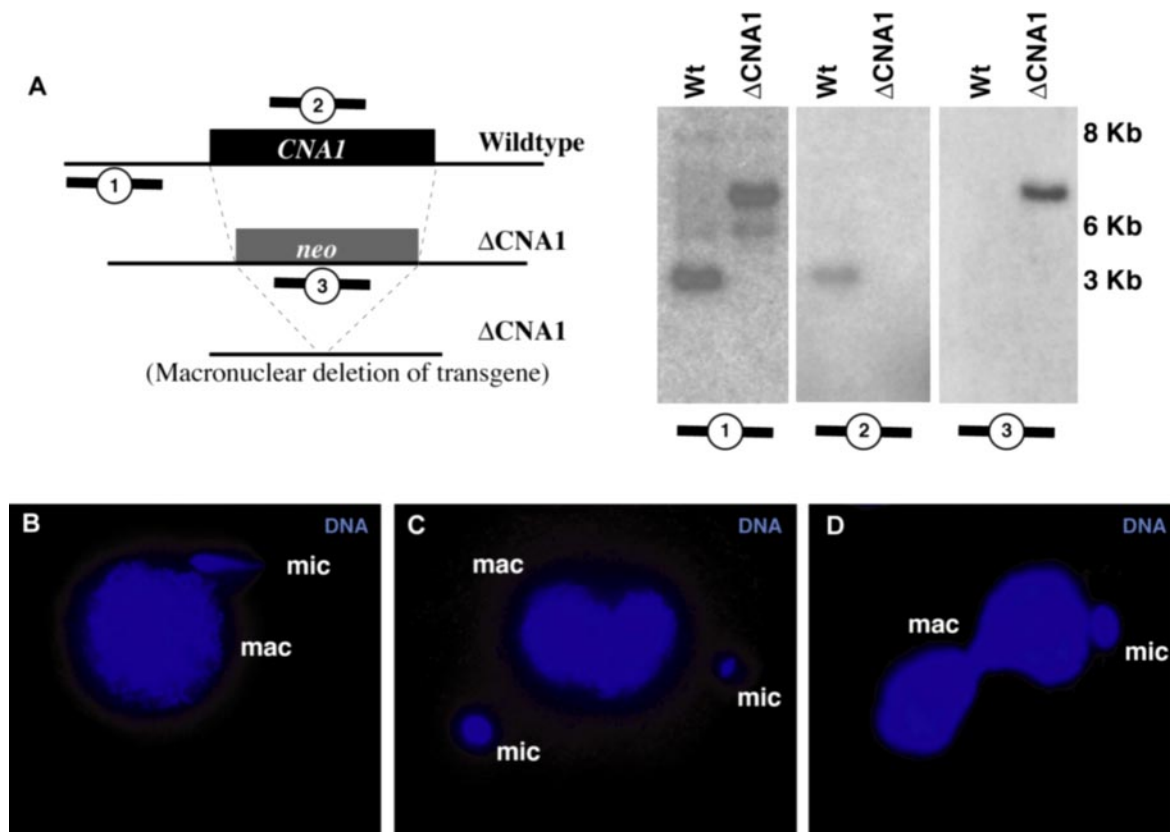


Figure 6. Germline knockouts of *CNA1* show micronuclear chromosome segregation defects. (A) Southern blots confirming the complete replacement of the *CNA1* coding region by the *neo* cassette in $\Delta CNA1$ cells. Genomic DNA from wild-type and $\Delta CNA1$ cells was digested with *XbaI* and probed with the upstream genomic region (1), the *CNA1* coding sequence (2), or the *neo* gene (3). The upstream probe detects the wild-type band of 4.1 kb only in wild type. In the $\Delta CNA1$ cells the wild-type band is absent, the most prominent band is the expected 6.2-kb band for the knockout and a small amount of transgene deletion at 5.4 kb was apparent (Yao *et al.*, 2003). The *CNA1* gene was absent in the $\Delta CNA1$ cells (middle panel), whereas the *neo* gene was only detectable in the $\Delta CNA1$ cells (right panel). (B and D) A variety of micronuclear chromosome segregation defects are seen in $\Delta CNA1$ cells, including asymmetric segregation of micronuclear DNA in early anaphase (B) and telophase (C). This asymmetry can lead to cells where the macronuclei divide even though the micronucleus has not (D), whereas typically macronuclear division occurs much later than micronuclear division in wild-type cells.

knockout matings ($\Delta CNA1$) using Southern blots. After 2 d in growth media, genomic DNA was collected from progeny of both wild-type cells and those from knockout matings. Southern analysis was carried out to ascertain that the *CNA1* gene had been completely replaced (Figure 6A). When we used a probe to the *CNA1* gene, a band of the expected size was seen only in the wild-type sample. Similarly, a probe for the *neo* coding sequence was only seen in the knockout progeny. Finally, a probe to the genomic region upstream of the *CNA1* gene confirmed that the *CNA1* gene had been completely replaced with the *neo* cassette.

$\Delta CNA1$ cells initially grew like wild-type cells, but they ceased to grow after 2 d. To further investigate the nature of defects in the $\Delta CNA1$ cells, single conjugating pairs were cloned into drops and progeny scored for cytological defects using antibodies to CNA1p and a micronuclear-specific linker histone δ (Sweet *et al.*, 1996). We found that the progeny retained parental CNA1p for at least eight divisions. The $\Delta CNA1$ progeny cells completed ~ 3 –4 divisions normally but subsequent divisions showed unequal DNA segregation during mitosis, showing a range of defects that are represented in Figure 6, B–D. $\Delta CNA1$ cells ceased dividing after ~ 10 divisions. This limited number of divisions after a depletion of centromeric histones is in remarkable agreement with results from a number of diverse eukaryotes (Collins *et al.*,

2004; Pearson *et al.*, 2004; Regnier *et al.*, 2005). By the time the $\Delta CNA1$ cells stopped dividing, a few cells had micronuclei that were now the size of a macronucleus (Figure 7, A and B), whereas 70% of the cells did not have a detectable micronucleus (Figure 7C). The large micronuclei were found to stain with a micronuclear specific histone and still retain CNA1p although the localization patterns were clearly aberrant in being more peripheral and having more than ten dots (Figure 7, A and B). Cells without a detectable micronucleus (represented in Figure 7C) arise because micronuclear chromosome segregation is blocked but cytokinesis proceeds normally resulting in one daughter cell that does not inherit any micronuclear chromosomes. From these studies, we conclude that CNA1 is essential for normal micronuclear DNA segregation during mitosis. Such aberrant micronuclear chromosome segregation patterns have not been previously observed in *T. thermophila* (Shen *et al.*, 1995).

Despite these dramatic defects in micronuclear division, we were not able to observe any obvious defects, such as lagging DNA or macronuclei being cut by the cleavage furrow of cytokinesis, in the somatic macronucleus in these cells. Previously, mutations that disturb macronuclear division have been shown to result in large extrusion bodies that contain macronuclear DNA (Wiley *et al.*, 2005). We found no indication of extrusion bodies in the $\Delta CNA1$ cells. Together,

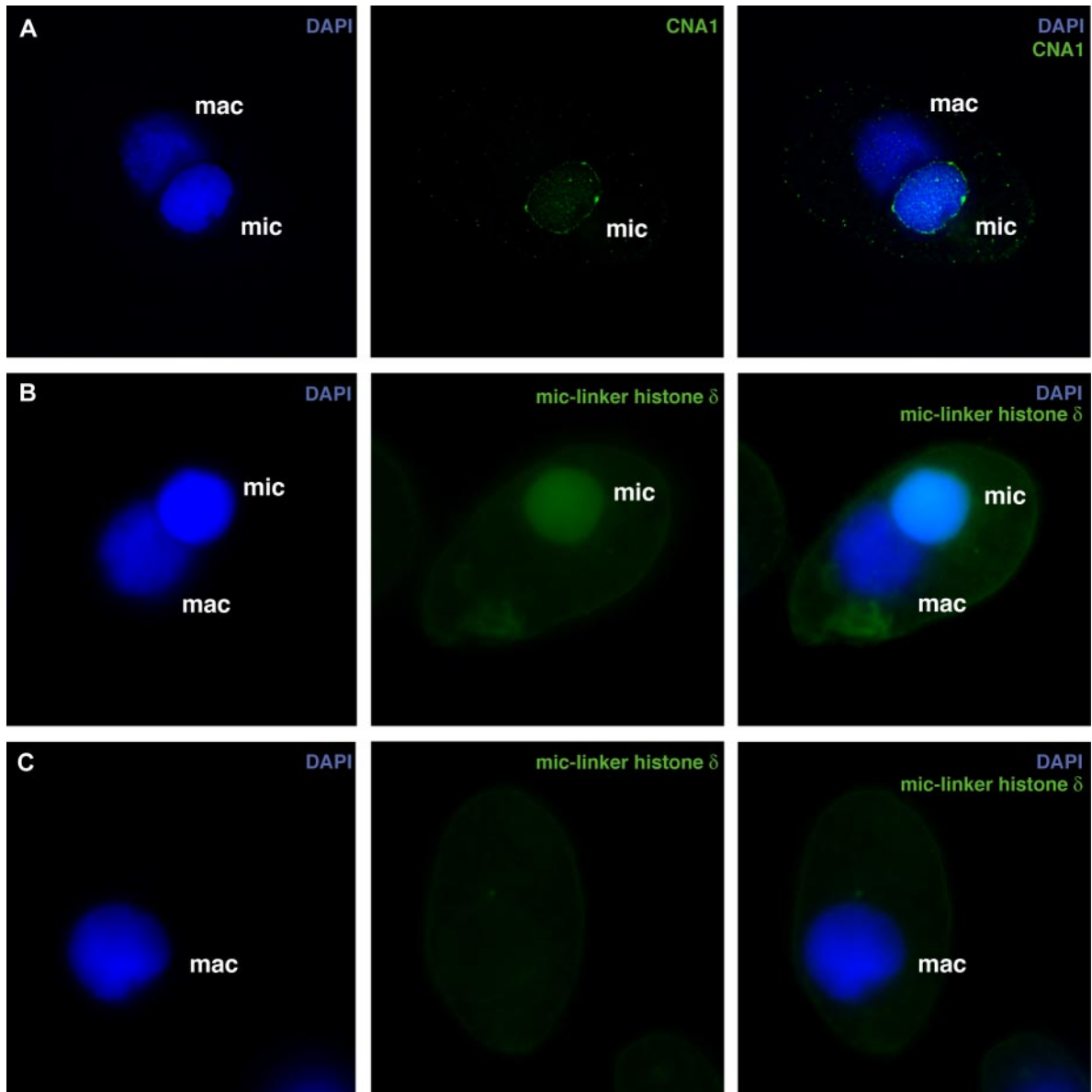


Figure 7. Germline knockouts of *CNA1* lead to cells with either large micronuclei or no micronuclei. Micronuclear chromosome segregation defects in $\Delta CNA1$ cells lead to cells that either have abnormally large micronuclei (A and B) or have completely lost their micronucleus (C). Large micronuclei have aberrant CNA1p localization (A). (B) In cells with two large nuclei, large micronuclei were unambiguously identified using antibodies to the micronuclear-specific linker histone δ (Sweet *et al.*, 1996). (C) In cells lacking a cytologically visible micronucleus, no staining with the linker histone δ or CNA1p (unpublished data) was seen. (CNA1p or Linker histone δ , green; DNA-DAPI, blue)

these results provide the strongest evidence to date, that macronuclear chromosome segregation in ciliates proceeds independent of any centromere function.

DISCUSSION

Centromeres in Closed Mitosis

Our identification of CNA1 as the *T. thermophila* centromeric histone has provided us with a unique tool to study centromeres in one of the earliest branches of eukaryotes. It affords us an unprecedented visualization of chromosome segregation in the ciliate micronucleus, a process that has been traditionally difficult to study because it is not accompanied by a nuclear envelope breakdown. Centromeres in the mi-

cronucleus are found to have a peripheral localization (as opposed to randomly dispersed in the interior of the nucleus) at practically all stages of the cell cycle (Figure 3). This localization pattern is similar to that of other organisms with closed mitoses, like budding and fission yeasts (Funabiki *et al.*, 1993; Goshima and Yanagida, 2000; He *et al.*, 2001; Pearson *et al.*, 2004) but not similar to organisms known to have an “open” mitosis, like *Drosophila* and humans (Henikoff *et al.*, 2000). In yeasts, it is believed that this peripheral localization is on account of the centromere attachment to the nuclear envelope by means of nuclear microtubules (Funabiki *et al.*, 1993; Goshima and Yanagida, 2000; He *et al.*, 2001; Pearson *et al.*, 2004). Similar attachments between the kinetochores and nuclear envelope have been previously

proposed for the *Tetrahymena* kinetochores based on electron microscopy (LaFountain and Davidson, 1979). Microtubule-based anchoring of kinetochores should not be a necessary prerequisite for closed chromosome segregation. Nonetheless, our results suggest that diverse eukaryotic lineages with closed mitosis appear to have adopted the same strategy of anchoring kinetochores to the nuclear membrane. Presumably, this obviates the requirement of microtubule “capture” and makes the process more efficient.

CenH3 Deposition Outside Centromeres

The intriguing localization of CNA1p during meiotic prophase also provides support to hypotheses concerning the determinants of CenH3 deposition. Previous work has highlighted the unique nucleosome and histone modifications present at centromeric regions. But the core issue of what targets CenH3s to centromeres has been a mystery. Even under normal expression levels, CenH3s can target euchromatin in addition to centromeres. In mammalian cells, CenH3 overexpression can lead to localization in euchromatin but this does not lead to the formation of a functional kinetochore at ectopic locations (Shelby *et al.*, 1997; Van Hooser *et al.*, 2001; Ahmad and Henikoff, 2002). Similarly, in instances where the turnover of centromeric histones is impaired in budding yeast, CenH3s can localize throughout the DNA. Under normal conditions, however, this localization appears to be transient and CenH3s are only stably maintained at centromeric regions. One determinant for this stability and increased deposition at centromeres has been proposed to be the tension resulting from microtubule attachments to the centromere (Mellone and Allshire, 2003; Henikoff and Dalal, 2005). The crescent phase of meiosis in *Tetrahymena* provides an unexpected albeit indirect insight in favor of this hypothesis.

The crescent phase of meiosis in *Tetrahymena* is a unique structure that stretches the nucleus up to 50-fold compared with interphase with the nucleus growing $\sim 0.8 \mu\text{m}/\text{min}$ (Wolfe *et al.*, 1976). In contrast to the distinct centromeres seen during mitosis, CNA1p is distributed throughout the length of the micronuclear chromosomes as they are stretched in the crescent formation. Indeed, at full crescent we were not able to distinguish the position of the centromeres because of diffuse staining of CNA1p. Telomeres continue to be clustered at one end of the nucleus throughout prophase (Loidl and Scherthan, 2004). We can only assume that the centromeres remain clustered at the opposite end. It is reasonable to suggest that the high degree of “stretching” exerted on the meiotic chromosomes during the formation of the crescent generate torsional stresses analogous to the “tension” generated by the pulling forces on centromeres at anaphase, triggering the deposition of CNA1p along the length of the micronuclear chromosomes. At the later stages of conjugation, micronuclear fate is marked by the removal of CNA1p except for at the centromeres. Results obtained in budding yeasts (Collins *et al.*, 2004; Pearson *et al.*, 2004) and *Tetrahymena* suggests that although CenH3s may deposit in euchromatin, they are quickly replaced by canonical histones after telophase, except at centromeres where they are maintained by the anchoring of kinetochores to the nuclear envelope.

We do not believe that the “ectopic” CNA1p deposition implies that *Tetrahymena* meiotic micronuclear chromosomes are holocentric. Although we are not able to see individual chromosomes during mitosis or meiosis, the micronuclear chromosomes unambiguously behave as metacentric chromosomes during mitosis and meiosis. CNA1p localization remains diffuse throughout meiosis but distinct centromeres are apparent at anaphase I. Other reports show

chromosomes segregating in a “V” shape during meiosis as expected for metacentric chromosomes with centromeres leading (Cole, 1991). This is in contrast to the movement of holocentric chromosomes, which would segregate as an “|” shape. Furthermore, it is unlikely that the CNA1p along the chromosomes leads to the formation of a functional kinetochore. In mammalian cells when the centromeric protein is overexpressed and localized to euchromatin, only a few kinetochore proteins are recruited to the ectopic loci but this does not produce a functional kinetochore (Van Hooser *et al.*, 2001).

CNA1p: A Meiotic Passenger Protein?

Remarkably, in late anaphase I and II, CNA1p is also localized on the meiotic spindle akin to what has been previously described for passenger proteins. In mammals and yeast, the Aurora B kinase has been found to phosphorylate CenH3 (Buvelot *et al.*, 2003; Parra *et al.*, 2003; Vagnarelli and Earnshaw, 2004). Aurora is a chromosomal passenger protein that moves from positions on the chromosomes to the central spindle during late anaphase I. The functional significance of CNA1p’s localization to the spindle is unclear. We suggest that CNA1p’s association with another passenger protein contributes to its localization, rather than it directly being selected for some downstream role in nuclear division (at the spindle or mid-body), as is the case for “authentic” passenger proteins.

Depletion of CNA1p from Centromeres

Our finding that progeny of CNA1 germline knockouts are able to complete mitosis several times before we observe segregation defects is consistent with results from other organisms. When CenH3 expression is blocked either by mutation or RNAi, it takes several divisions until a defect in mitotic chromosome segregation is obvious (Collins *et al.*, 2004; Regnier *et al.*, 2005). In experiments using RNAi to deplete CenH3, it was found that as CenH3 is replaced by H3 at centromeric regions, centromere integrity is lost (Buchwitz *et al.*, 1999; Oegema *et al.*, 2001; Blower *et al.*, 2002). However, the use of degen-associated Cse4 (the CenH3 is *Saccharomyces cerevisiae*) suggests that the effect of Cse4 depletion can be seen in the course of a single cell cycle in budding yeasts (Collins *et al.*, 2005). The difference between diverse eukaryotes may be partly attributed to the number of CenH3 nucleosomes at centromeres. Budding yeasts have a simple 125-base pair centromere that is believed to package a single CenH3-nucleosome (Keith and Fitzgerald-Hayes, 2000), depletion of which would have instant repercussions for chromosome segregation. However, many higher eukaryotes have complex centromeres with blocks of CenH3 nucleosomes. Depletion of CenH3s in these organisms would lead to a dilution of CenH3 because of random assortment after DNA replication. Our analysis of the ΔCNA1 progeny supports the idea that there is a threshold of CenH3 required at the centromere for proper chromosome segregation, below which faithful chromosome segregation cannot be sustained. On the basis of this and the data on CNA1p localization in mitosis, we conclude that *T. thermophila* centromeres might be as complex as those in higher eukaryotes.

What leads to the arrest in ΔCNA1 cells? It has been previously shown in *Tetrahymena* that although cells with a diminished micronucleus can proceed through additional cell divisions, cells that lack a micronucleus will cease dividing. This paradigm is true for all but one *Tetrahymena* laboratory strain (Karrer *et al.*, 1984) although the reasons behind this are still unclear. Nevertheless, as segregation defects become manifest in ΔCNA1 cells and as the majority

of cells lose all micronuclear chromosomes, we suggest that this results in their arrest.

Absence of Centromeres in the Ciliate Macronucleus

The traditional model for chromosome segregation in the ciliate macronucleus has been that of amitotic division during vegetative growth, wherein chromosomes are subject to random segregation. The absence of requirements for centromeres in random segregation has supported the suggestion that centromeres are deleted from the developing macronucleus during genomic rearrangement. However, one unsatisfying aspect of this model had been the lack of direct evidence from known centromeric determinants. Our localization of CNA1p helps provide the most direct support to the hypothesis that the ciliate macronucleus does not employ centromeres at any stage of its development or propagation. CNA1p is not detected in the macronucleus during vegetative growth when centromeres are clearly visible in the micronucleus. The strongest piece of evidence comes from our somatic and germline CNA1 knockout experiments, where we can show that chromosome segregation in the micronucleus has clearly gone awry. Nonetheless, chromosome segregation in the macronucleus appears to proceed normally. Thus, the ciliate macronucleus, bereft of germline responsibilities, now lacks the means (and dependence on the means) adopted by all other eukaryotes to accurately transmit genetic information.

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