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Progeny of germ line knockouts of *ASI2*, a gene encoding a putative signal transduction receptor in *Tetrahymena thermophila*, fail to make the transition from sexual reproduction to vegetative growth

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Abstract: *The ciliated protozoan Tetrahymena has two nuclei: a germ line micronucleus and a somatic macronucleus. The transcriptionally active macronucleus has about 50 copies of each chromosome. At sexual reproduction (conjugation), the parental macronucleus is degraded and new macronucleus develops from a mitotic product of the zygotic micronucleus. Development of the macronucleus involves massive genome remodeling, including deletion of about 6000 specific internal eliminated sequences (IES) and multiple rounds of DNA replication. A gene encoding a putative signal transduction receptor, ASI2, (anlagen stage induced 2) is up-regulated during development of the new macronuclei (anlagen). Macronuclear ASI2 is nonessential for vegetative growth. Homozygous ASI2 germ line knockout cells with wild type parental macronuclei proceed through mating but arrest at late macronuclear anlagen development and die before the first post-conjugation fission. IES elimination occurs in these cells. Two rounds of postzygotic DNA replication occur normally in progeny of ASI2 germ line knockouts, but endoreduplication of the macronuclear genome is arrested. The germ line ASI2 null phenotype is rescued in a mating of a knockout strain with wild type cells.*

Introduction

Tetrahymena thermophila is a unicellular eukaryote, with two nuclei (reviewed in Karrer, 2000). The germ line micronucleus is diploid and transcriptionally silent in vegetatively dividing cells. The somatic macronucleus contains about 50 copies of each macronuclear chromosome. It is transcriptionally active, and responsible for the phenotype of vegetatively growing cells.

In rich medium, *Tetrahymena* cells reproduce by asexual fission, during which the micronucleus divides mitotically and the macronucleus divides amitotically. That is, there are no functional centromeres in the macronucleus and macronuclear alleles are distributed to asexual progeny at random (reviewed in Frankel, 2000).

Under conditions of starvation, *Tetrahymena* cells of different mating types pair and initiate sexual reproduction, which is called conjugation (Fig. 1). The micronucleus undergoes meiosis. Three of the meiotic products degenerate and the fourth undergoes prezygotic mitosis. Mating

cells exchange pronuclei and fertilization occurs when the migratory nucleus from the mating partner fuses with the stationary pronucleus. The zygotic nuclei undergo two postzygotic mitoses to produce four nuclei in each cell, two of which develop into new macronuclei (macronuclear anlagen) and two into new micronuclei. The parental macronucleus is degraded apoptotically. Finally, one of the new micronuclei degenerates and the other divides mitotically, initiating the first vegetative cell division (Reviewed in Karrer, 2000)

One of the key events in the development of the macronuclear anlagen is the deletion of approximately 6000 specific sequences from the macronuclear genome. In most cases this deletion is accompanied by the ligation of flanking sequences, and thus the elements are referred to as internal eliminated sequences (IES). The protein Pdd1p (for programmed DNA degradation) is required for IES elimination (Coyne et al., 1999), which occurs via a RNAi-like mechanism (Mochizuki et al., 2002; Yao et al., 2003). Pdd1p appears early in conjugation and first accumulates in the parental macronucleus, where it is thought to be associated with small RNAs (scRNA) generated by a dicer-like protein (Mochizuki and Gorovsky, 2005; Malone et al., 2005). Subsequently, Pdd1p is concentrated in the “conjusome”, a non-membrane bound, electron-dense particle in the anterior cytoplasm of postzygotic pairs, (Janetopoulos et al., 1999). Eventually, Pdd1p is transferred to the macronuclear anlagen. Staining with anti-Pdd1p reveals a punctate pattern showing colocalization of Pdd1p with the IES (Madireddi et al., 1996). This is thought to establish a specialized chromatin structure, further characterized by methylation of histone H3 at the K9 residue (Liu et al., 2004), that is required for DNA elimination.

Much of sexual reproduction is driven by transcription in the parental macronucleus. Several genes have been shown to be up-regulated during meiosis, before the new macronucleus develops (Martindale and Bruns, 1983). Four genes involved in excision of the IES, PDD1, PDD2, TWI1, and DCL1, are required in the parental macronucleus. Cells that are somatic knockouts of those genes do not complete DNA rearrangement and fail to make viable progeny (Coyne et al., 1999; Mochizuki and Gorovsky, 2005; Nikiforov et al., 1999). PDD1 is also transcribed in the macronuclear anlagen. The function of the zygotic Pdd1p, if any, is unknown. The present study describes a gene, *AS/2* (anlagen stage induced gene 2) encoding a putative signal transduction receptor. As its name implies, the abundance of *AS/2* mRNA peaks at 9 h of mating, early in macronuclear anlagen development. *AS/2* is required in the macronuclear anlagen for sexual reproduction. Cytological analysis of matings between germ line *AS/2* knockouts shows the progeny develop new macronuclei, the parental macronuclei degenerate, and the cells separate to produce exconjugants. The molecular events of macronuclear anlagen

development leading up to and including IES excision occur normally. However, endoreduplication of DNA in the macronuclear anlagen arrests in the early stages and progeny die prior to the first vegetative fission.

Materials and methods

Cell culture

Tetrahymena thermophila cultures were maintained in 1% or 2% PPYS (proteose peptone, yeast extract, and sequestrene) at 30 °C (Orias et al., 1999).

Construction of *Tetrahymena ASI2* knockout lines

Tetrahymena knockout strains were obtained by biolistic transformation with the BioRad Particle Delivery System (Cassidy-Hanley et al., 1997). An *ASI2* knockout construct was made, containing the *neo2* cassette (Nikiforov et al., 1999) flanked by approximately 1 kb of genomic DNA 5' and 3' to *ASI2*. Cells from a mating between strains CU428 (VII) and B2086 (II) were bombarded at the crescent micronucleus stage. Transformants, in which the *ASI2* gene was replaced with the *neo2* cassette, were selected on the basis of resistance to paromomycin. Two types of transformants were obtained. Somatic (macronuclear) transformants resulted from bombardment of unpaired cells in the mating. A single germ line (micronuclear) transformant was identified as a progeny of the mating on the basis of 6-methylpurine resistance and subsequently confirmed as a germ line *ASI2* knockout. (The genotypes and phenotypes of all strains used in this study are provided in Table 1).

Northern blots

4–5 µg poly(A) mRNA isolated from *Tetrahymena* at different stages of conjugation, and from log phase and starving cells. RNA samples were treated and run on formaldehyde gels according to established protocols (Sambrook et al., 1989). The RNA was transferred to GeneScreen Plus nylon membrane (NEN) with 20× SSC for about 4 h. A lane containing 5 µg of RNA marker (Promega) was cut from the filter and stained with 0.04% methylene blue in 0.5 M sodium acetate pH 5.0 for 15 min, then de-stained for about 10 min in DEPC-treated dH₂O.

Southern blots

Tetrahymena macronuclear DNA (2–4 µg) was digested with restriction enzymes and the fragments were separated by electrophoreses on 0.7% agarose gels. DNA fragments were transferred to GeneScreen Plus nylon membrane (NEN) with 10× SSC overnight. The DNA was crosslinked to the membrane in a UV Stratalinker (Stratagene). The membranes were pre-hybridized and hybridized as described previously (Wuitschick et al., 2002).

Inverse PCR

About 3–5 µg of macronuclear DNA from strain CU428 were digested with the appropriate restriction enzymes and circularized by ligation at a concentration of 10 ng/µl. Inverse PCR products were gel purified using QIAquick Gel Extraction Kit (QIAGEN) and cloned into the pGEM-T Easy vector (Promega). All primers used in this study are listed in Table 2.

Real-time reverse transcriptase (RT)-PCR

Real-time reverse transcriptase reactions were performed in triplicate. For the RT reaction, 1 µg of DNase (RNase-free, Roche) treated total RNA was mixed with 10 pM first strand primers p5 for *AS/2* and p6 for the 17S rRNA gene (Table 2). 1 µl 25 mM dNTPs and DEPC/dH₂O were added to bring the volume to 31 µl. The samples were incubated at 65° for 5 min, and then chilled on ice. 8 µl 5× RT buffer (5× RT buffer: 250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂, 50 mM DTT) was added and the samples were incubated at 42° for 2 min. 1 µl M-MLV reverse transcriptase (Promega) was added and the samples were incubated at 42° for 1.5 h. RT enzyme was heat inactivated at 80° for 15 min. A minus RT reaction mix was used as the negative control. The PCR reaction was carried out in an iCycler (BioRad) real-time PCR machine with primers p9 and p10 for *AS/22* mRNA and primers p7 and p8 for 17S rRNA. The relative amount of *AS/2* message versus 17S rRNA was determined according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Single cell PCR

Single cell PCR reactions were done as described previously (Coyne et al., 1999). Single pairs of cells were isolated into drops of 1% PPYS and the cells separated after about 24 h. One exconjugant from each single pair was placed in 1 µl of lysis buffer (0.5% NP-40, 0.5% Tween-20, 20 mM Tris–HCl (pH 8.0), 0.5 mg/ml proteinase K, 50 mM KCl, 1 mM EDTA (pH 8.0)). The mating partner was maintained in 1% PPYS to determine whether the pair had completed mating. In the case of matings between *AS/2* germ line knockouts, the partner cell died. Exconjugants of matings between *AS/2* wild type cells that were heterokaryons for mutations conferring drug resistance survived and were identified as sexual progeny by inheritance of the resistant phenotype. A first round PCR reaction of 40 cycles was performed in a total volume of 25 µl. 5 µl of the first PCR reaction mixture was used as template in a second round PCR of 40 cycles with nested primers.

Cytology

A 100 µl aliquot of cells at 2.5×10^5 cell/ml was dried on a slide and fixed with 95% ethanol at room temperature for 30 s. The fixed cells were incubated with 100 ng/ml DNA-specific dye

4',6-diamidino-2-phenylindole (DAPI, Roche) in 70% ethanol/300 mM NaCl for 1 min. Excess dye was removed by dipping the slide into 70% ethanol for 15 s, and 35% ethanol for 15 s (Stuart and Cole, 2000). The slides were air dried and staining was observed with a Nikon E600 upright fluorescence microscope (EX 330-380, Barrier filter 435–485).

Flow cytometry

Flow cytometry was done as described previously (Nikiforov et al., 1999). In particular, *Tetrahymena* cells were lysed in 0.25 M sucrose, 10 mM MgCl₂, and 0.5% NP-40 at a concentration of 1.5×10^6 cells/ml. The DNA-specific dye propidium iodide was then added to 50 µg/ml and nuclei were stained for about 1 h before flow cytometry analysis using a Becton Dickinson FACSCalibur flow cytometer at the Medical College of Wisconsin.

Feulgen staining and cytophotometry

Cells were fixed and Feulgen stained as described previously (Marsh et al., 2001). The Schiff's reagent (MP biomedical, LLC) was prepared according to the supplier's instructions. Cells from matings between knockout lines and wild type lines were dropped on the same slide to equalize hydrolysis and staining. Staining was quantitated with a Zeiss Axioskop microscope and a Zeiss MSP-21 photometer as described previously (Marsh et al., 2001). Ten nuclei were analyzed for each stage at each time point.

Immunocytochemistry

Mating cells were fixed for immunocytochemistry as described previously (Liu et al., 2004). Primary antibody for Pdd1p staining was a generous gift from D. Allis (Rockefeller University). The secondary antibody was Cy3conjugated goat-anti-rabbit IgG obtained from Jackson ImmunoResearch Laboratories.

Results

ASI2* is up-regulated during sexual reproduction in *Tetrahymena thermophila

The *Tetrahymena* gene *ASI1* (anlagen stage induced 1), was isolated from a cDNA library of genes that are up-regulated during development of the macronuclear anlagen (Udani and Karrer, 2002). Downstream of *ASI1* was a region that was relatively high in GC content, a characteristic of exons in *Tetrahymena* (Wuitschick and Karrer, 1999). To determine whether this region was transcribed, poly(A)⁺ mRNA was isolated from starved cells, vegetatively growing cells, and from a population of mating cells at 6 h, 9 h, and 12 h post-mixing. Northern blot analysis revealed a message of about 2.7 kb that was up-regulated in mating cells, beginning at meiosis and with maximal abundance of message early in macronuclear anlagen development (Fig. 2B).

The gene encoding this RNA was designated *ASI2*, for anlagen stage induced 2.

Because the cloned DNA contained only about 350 bp of the *ASI2* open reading frame, two rounds of inverse PCR were done to obtain the full *ASI2* gene (Materials and Methods). Inverse PCR products were cloned and sequenced (GenBank accession no. AF435076). Open reading frame analysis showed a single ORF of about 2.3 kb (Fig. 2A), which was in good agreement with the size of the mRNA estimated from the northern blot.

In order to determine the structure of the mature *ASI2* mRNA, RT-PCRs were done and the cDNAs of *ASI2* were cloned and sequenced. Comparison of the cDNA sequence with the genomic DNA sequence revealed one intron of 147 bp. The intron has a low GC content (12%), characteristic of non-coding DNA in *Tetrahymena* (Wuitschick and Karrer, 1999) and the canonical 5'-GU and AG-3' splice sites. In order to estimate the length of the 5' and 3' UTRs, PCR reactions were done using a cDNA library of mRNAs up-regulated during macronuclear anlagen development as the template. For the 3' end, PCR analysis using oligo-dT and a primer in the second exon showed a 3'UTR of 441–444 bp with a putative polyA addition signal 30–33 bp upstream of the polyA tail. For the 5' end, one primer was complementary to sequences in the cloning vector and the second primer was from the first exon of *ASI2*. The sequence of the PCR cloned product suggested the minimal length of the 5' UTR for *ASI2* is 129 bp.

Although *ASI2* mRNA was not seen in RNA from starved cells in the blot shown in Fig. 2B, it was clearly detected in later experiments (data not shown). Due to the low abundance of *ASI2* mRNA, it was necessary to isolate poly(A)⁺ RNA for the northern blots. However, *Tetrahymena* rRNA is very AT-rich and a significant (and variable) amount of the rRNA is retained in the poly(A)⁺ mRNA isolations. In order to avoid this problem, real-time RT-PCR was performed using total RNA as template. The relative abundance of *ASI2* mRNA at different stages of development was determined, setting the signal in vegetatively growing cells at 1 (Fig. 2C). The result confirmed the presence of *ASI2* message in the starved cells, at a level about 3.3-fold higher than that in vegetatively growing cells. The abundance of *ASI2* message decreased early in the mating, but increased again during meiosis. In agreement with the Northern analysis, the major peak in mRNA abundance was at 9 h post-mixing, when there was about 14 times as much *ASI2* mRNA as in vegetatively growing cells. Thus, *ASI2* mRNA abundance peaks during macronuclear anlagen development, suggesting that Asi2p may play a role in that process.

Sequence analysis of the deduced Asi2p suggests that *ASI2* may encode a signal transduction receptor

The deduced Asi2p is a 719 amino acid protein with a molecular weight of 84 kDa.

BLASTp analysis (Altschul et al., 1997) of the NCBI database indicated that Asi2p is similar to a group of hypothetical Cache domain containing proteins of *Tetrahymena* which includes Asi1p (Udani and Karrer, 2002). Asi1p and Asi2p have 22% identity and 40% similarity over a region of 684 amino acids (Fig. 3A). A BLASTp search of the NCBI data base detects a Cache domain in Asi1p and suggests that Asi1p has some similarity to bacterial methyl-accepting chemotaxis proteins (MCPs), at an expect value of $6e^{-6}$. There were no highly significant similarities between *ASI2* and other known proteins.

Sequence analysis of the deduced Asi2p suggests it may be a signal transduction receptor. A domain search of the predicted gene product using the SMART program (Letunic et al., 2004) suggested there are two transmembrane domains and two coiled coil domains in Asi2p (Fig. 3A). Pfam analysis (Bateman et al., 2004) detected a partial Cache domain between the transmembrane domains. Cache domains with a consensus sequence of ~80 amino acids are found in bacterial MCPs, a class of prokaryotic chemotaxis receptors. Although it is not clear what the function(s) of these domains are, it was proposed that they bind small molecules in a pocket formed by the predicted β sheet and function as the sensory domains in the chemotaxis receptors (Anantharaman and Aravind, 2000). Alignment of Asi1p and Asi2p with the first 41 amino acids of the consensus Cache domain and with four MCPs is shown in Fig. 3B. There was an additional block of 18 conserved amino acids that Asi1p and Asi2p shared with the MCPs that was not included as part of the Cache domain in the bioinformatics analysis. This region with similarity to Cache domains is proposed to be the sensory domain of Asi2p.

The deduced Asi2p does not contain a MCP signaling domain, nor were any putative protein kinase domains identified in Asi2p. However, Asi2p does have a motif DQEDDDYDENGSNQ (amino acids 600–614) that is among the 0.074% best matches to Nck SH2 (Src homology group 2) binding sites in Scansite (Obenauer et al., 2003); and Y607 of that motif is a predicted target for phosphorylation, with a score of 0.994 (Blom et al., 1999). This suggests that phosphorylated Asi2p may interact with the SH2 domain of a noncatalytic SH2/SH3 adaptor protein of the Nck1 class. These adaptor proteins typically link cell surface receptors, via their SH2 domains, with downstream effectors, bound to the SH3 domains (Wei et al., 2001). Taken together, these structural features suggest that Asi2p may function as a signal transducer.

***ASI2* is not essential for vegetative growth**

The function of Asi2p was analyzed in gene knockout strains. An *ASI2* knockout construct was made, containing the *neo2* cassette (Nikiforov et al., 1999) flanked by approximately 1 kb of genomic DNA 5' and 3' to *ASI2* (Fig. 4A). The construct was introduced into starved cells by

biolistic bombardment. Several independent somatic knockout cell lines were obtained, three of which were used for further study. Line MU111 was obtained by transformation of the heterokaryon line CU428, transformant lines MU112 and MU113 were obtained by transformation of the wild type strain B2086. The genotypes of cell lines used in this study are listed in Table 1. Insertion of the *neo2* cassette at the *AS/2* locus was confirmed by PCR analysis of transformant lines using one primer in the 5' flanking region of *AS/2* and one primer within the *neo2* cassette (p11 and p12 in 4A). The expected 1.6 kb PCR products were obtained from somatic knockout cell lines (data not shown).

The macronucleus of *Tetrahymena* contains about 50 copies of the genome and divides amitotically, without disassembly of the nuclear membrane. There are no functional centromeres in the macronucleus and macronuclear alleles are distributed to asexual progeny at random (Reviewed in Frankel, 2000). Due to the inability to partition alleles equally, individual cells assort to purity for one allele or the other over 100–200 vegetative fissions, a phenomenon known as phenotypic assortment (Orias and Flacks, 1975). Somatic transformation replaces one or a few wild type alleles with the *neo2* cassette via homologous recombination. The *neo2* cassette confers resistance to the drug paromomycin. Thus, in the presence of the paromomycin, cells that have a higher proportion of an allele that confers drug resistance are favored in growth. Nonessential genes can be identified as those that assort to purity of the knockout allele.

Somatic knockout *AS/2* transformants were grown in increasing concentrations of paromomycin until all the wild type *AS/2* alleles were replaced by the knockout allele. Complete replacement of endogenous *AS/2* genes was confirmed by Southern blot analysis of macronuclear DNA isolated from the wild type CU428 and *AS/2* knockout strains MU111, MU112 and MU113 (Fig. 4B). Macronuclear DNA from somatic knockout strains contained only the *AS/2::neo2* allele (1.3 kb) and undetectable amount of the wild type allele (3.1 kb), suggesting that the wild type *AS/2* allele in macronuclear DNA was completely replaced by the *neo2* cassette in the somatic knockout strains.

To ensure that all wild type alleles had been lost from the somatic knockout strains, cultures which had been maintained in the absence of drug for 10 months were examined to determine whether any cells had assorted back to paromomycin sensitivity. Forty single cell clones were established from each strain (MU111, MU112 and MU113). The clones were plated into 500 µg/ml paromomycin. This concentration of drug killed two control paromomycin sensitive strains within 1 day, whereas all of the somatic knockout cell clones continued to divide rapidly.

In order to determine whether *AS/2* somatic knockout cells were defective in vegetative

growth, the fission rate of *AS/2* somatic knockout cells was measured and compared to that of the *AS/2* wild type cell line CU428. Cultures were inoculated with log phase cells at a density of 10^4 cells per milliliter and incubated at 30°C for 9 h. The cell density was measured every 2.5 h (data not shown). The growth curves indicated that the somatic *AS/2* knockouts and the wild type cells had similar fission times of 3.5 and 3.6 h respectively. Thus *AS/2* is not essential for vegetative growth, which is consistent with the low abundance of the *AS/2* message in vegetatively growing cells (Figs. 2B and C).

***AS/2* germ line knockout cells do not produce viable progeny**

The abundance of the *AS/2* transcript in wild type cells is maximal in mating cells at about 9 h after mixing of the two mating types (Fig. 2). At this time the cells are in the early stages of macronuclear anlagen development, and the new macronucleus is becoming transcriptionally active (Bruns and Brussard, 1974; Mayo and Orias, 1986; Stargell et al., 1990). Germ line (micronuclear) *AS/2* knockout strains were constructed to determine whether transcription of *AS/2* in the developing macronucleus is required to complete conjugation.

A single heterozygous *AS/2* germ line knockout line MU114 was obtained by replacement of the endogenous *AS/2* gene with a neo construct via homologous recombination. Homozygous germ line *Tetrahymena* strains are constructed by a mating known as Round I genomic exclusion (Allen, 1967; Doerder and Shabatura, 1980). This is a specialized mating between a cell line that is heterozygous for the allele of interest and a “star” strain. (Fig. 5). Star strains have a defective micronucleus which degenerates in the first round of genomic exclusion mating. The heterozygous partner undergoes meiosis and three of the haploid nuclei degenerate (Fig. 1). Since the selection of the surviving micronucleus is random, it can have either of the two alleles; in this case, the wild type *AS/2* allele or the knockout allele. It divides mitotically to produce two gametic pronuclei, one of which is transferred to the star cell. Since the star cell has no micronucleus to transfer in return, both cells are haploid. At this point, the cells separate, retaining the parental macronuclei and their micronuclei endoreduplicate. Thus the progeny of Round I genomic exclusion pair are identical whole genome homozygotes in their micronuclei, and they are sexually mature since they have retained their parental macronuclei. Cell lines designated syncrones can be established from isolated pairs of cells. After expanding the syncrone, the cells can be starved a second time to induce the Round II genomic exclusion. In wild type cells, this mating goes to completion and produces progeny that are whole genome homozygotes.

In order to produce strains homozygous for the germ line *AS/2* knockout allele, the heterozygous germ line knockout with a somatic knockout macronucleus MU114 was crossed to

strain B*7 and synclones were established from Round I pairs (Fig. 5). Clonal cell lines derived from four pairs were designated Clones D–G.

As a preliminary test for the ability of *AS/2* germ line knockouts to produce progeny, the synclones were tested in a Round II cross for the ability to produce sexual progeny. Single pairs were isolated from each of the Round II crosses. For two synclones, D and E, the viability of isolated pairs was high (82% and 95% respectively). In addition, all of the clones established from those pairs were paromomycin sensitive (Table 3), indicating that the progeny of the Round II cross had degraded the parental macronuclei and produced new macronuclei, as expected for a Round II genomic exclusion mating (Fig. 5). Since all of the progeny were paromomycin sensitive, the micronuclei in the Round I clones were deduced to be homozygous for the wild type *AS/2* allele.

In the case of Round I synclones F and G, the viability of pairs isolated from the Round II mating was very low (3% and 0% respectively) (Table 3). This was consistent with the hypothesis that Round I clones F and G were homozygous for the *AS/2* knockout allele, and that *AS/2* germ line knockouts are zygotic lethal. The single surviving Round II clone, which was paromomycin resistant, could be explained in two ways. One possibility was that the *AS/2* zygotic lethal phenotype was not completely penetrant. More likely, the pair that produced the viable clone was paired only a short time before the pairs were isolated. If refed within an hour of pairing, early pairs of *Tetrahymena* abort mating, retain the parental macronucleus, and resume vegetative growth.

The hypothesis that the null allele of *AS/2* is zygotic lethal predicted that Round I clones D and E were homozygous for the wild type *AS/2* allele in the mic and that clones F and G were homozygous for the *asi2::neo2* knockout allele. The genotype of the Round I clones deduced from the experiment shown in Table 3 was confirmed genetically. Individual cells were isolated from the Round I clones and descendants of the non-B* cell were identified on the basis of paromomycin resistance. Strain MU120 was derived from clone D, which produced viable Round II progeny in the genomic exclusion mating (Fig. 5). The micronuclear genotype of MU120 was determined by crossing it to strain CU427 (Fig. 6A). Mating pairs were isolated into individual drops of medium. True progeny of the test cross all inherit the dominant *chx1-1* allele from CU427 and were identified on the basis of cycloheximide resistance. All of the cycloheximide resistant progeny from the mating of MU120 with CU427 were paromomycin sensitive. Since the neo allele in the knockout cassette is dominant, this confirmed that MU120 was homozygous for the wild type *AS/2* allele in the micronucleus (Table 4). Strains MU119.1 and MU119.2 were established as single cell clones of Round I synclone G (Fig. 5), which did not produce viable progeny in

Round II genomic exclusion. All of the cycloheximide resistant progeny from the mating between these strains and CU427 were also paromomycin resistant. Thus the MU119 lines were homozygous for the *ASI2* knockout allele in the micronucleus (Fig. 6B, Table 4).

In the test crosses between the MU119 clones and strain CU427, the micronucleus of one cell (CU427) is homozygous for the wild type *ASI2* allele and the mating partner (MU119) is homozygous for the *asi2::neo2* knockout allele (Fig. 6B). Since the viability of the clones from these crosses was very similar to the viability of pairs from the cross between cells with wild type alleles in both micronuclei (Table 4), the germ line wild type allele shows cytoplasmic dominance to the null allele of *ASI2*.

In the Round II genomic exclusion cross with strain MU119 (Fig. 5), both of the mating partners were germ line *ASI2* knockouts but one of them (MU119) was a somatic knockout as well. In order to determine whether lack of *ASI2* in the germ line was sufficient to account for the lethality without the contribution of the somatic knockout, strain MU121.3 was constructed that was a homozygous germ line *ASI2* knockout with the wild type *ASI2* allele in the macronucleus (Fig. 7). Strain MU119 was crossed to CU427. This resulted in a strain that was heterozygous for the wild type and knockout *ASI2* alleles in the micronucleus with mixed alleles in the macronucleus. The cells were allowed to mature and a progeny clone was identified with a mating type different from that of the parents. This clone was crossed to B*7 in a Round I genomic exclusion mating to bring the micronucleus to homozygosity. Pairs of mating cells were isolated from the Round I cross. The clones were expanded and progeny that were homozygous for the *asi2::neo* allele were tentatively identified as those that were unable to produce Round II progeny. A single cell clone, MU121, from the non-B* side of the Round I cross was identified on the basis of the dominant paromomycin resistance phenotype.

The progeny of the Round I genomic exclusion was assorted to purity and clones were identified with either the wild type or knockout allele of *ASI2* in the macronucleus as determined by sensitivity to paromomycin. These strains, designated MU121.3 and MU121.1, respectively, were shown to be homozygous in the micronucleus for the *asi2::neo* allele in test crosses to CU427. That is, all of the progeny of the test crosses, identified on the basis of cycloheximide resistance, were also resistant to paromomycin.

A genomic exclusion cross was done to determine whether MU121.3 could produce Round II progeny. The clonal lines MU119, MU120 (Fig. 5) and strain CU427, which is wild type for *ASI2*, were crossed to strain B*7 as controls. MU119 produced no viable Round II progeny, as observed previously. Similarly, no viable Round II progeny were produced in a cross between

MU121.3 and B*7 (Table 5). Control Round II genomic exclusion matings resulted in high survival of the isolated pairs. The percentage of true progeny from these matings was determined on the basis of cycloheximide resistance for CU427 × B*7 and paromomycin sensitivity for MU120 × B*7. These experiments suggested that transcription of *AS/2* in the developing macronuclear anlagen is required to complete sexual reproduction. A full complement of wild type *AS/2* alleles in the parental macronuclei is not sufficient to rescue the zygotic lethal knockout phenotype.

In order to ensure that the B* macronucleus did not contribute to the zygotic lethal phenotype, strain MU121.3 was crossed to MU119. In this cross, somatic *AS/2* was supplied by MU121.3, but both cells in the pair were null for micronuclear *AS/2*. Of the 89 pairs isolated from this cross, none produced viable clones, as compared to 53% of the pairs from the control cross between CU427 and CU428 (Table 5). Thus the zygotic lethal phenotype observed in the genomic exclusion matings could not be attributed to the B* macronucleus.

Matings between *AS/2* germ line knockouts produce exconjugants with macronuclear anlagen

Cytological analysis was done to determine at what stage of conjugation zygotic *AS/2* was required. Under conditions of continuous starvation, wild type cells proceed through all of the cytological stages of mating and arrest as exconjugants with two macronuclear anlagen and a single micronucleus. This was confirmed in a control mating between strains CU428 and B2086 (Fig. 8B). The experimental cross was a genomic exclusion mating between MU119 and B*7. A Round I pair was isolated. The clone was expanded and the cells were starved to induce Round II mating. The kinetics of the experimental cross were somewhat delayed relative to the wild type control because the wild type cells were starved before mixing and the cells derived from the Round I clone required a period of starvation to initiate Round II mating. However, the Round II mating produced a distribution of cells at very similar stages to that observed in wild type cells by 24 h after starving, including exconjugants with two anlagen and a single micronucleus (Fig. 8A). Thus, the cytological events of the Round II mating were normal, including development of macronuclear anlagen, resorption of the parental macronucleus and the degradation of one of the micronuclei.

Single pairs of *AS/2* germ line knockout Round II mating pairs were isolated into drops of 1% PPYS medium. At about 16 h after refeeding, the cells in the pairs had separated, but no more than two cells were present in the drops, whereas cells from Round II matings with wild type *AS/2* micronuclei had completed the first cell division. Exconjugants from Round II matings of *AS/2* germ line knockouts did not undergo cytokinesis; they rounded up and died within 48 h.

Macronuclear anlagen development in matings between *AS/2* germ line knockouts

Cytological analysis showed development of macronuclear anlagen in matings between germ line *AS/2* knockouts, but did not reveal which, if any, of the molecular events of macronuclear anlagen development were completed. One well-studied hallmark of macronuclear anlagen development is the elimination of IES and the events leading up to it, including the synthesis of Pdd1p. A Round II genomic exclusion mating between MU121.3 and B*7 was done and aliquots of cells were removed every 3 h. The cells were stained with DAPI to visualize the nuclei and with antibody to Pdd1p. The synthesis and accumulation of Pdd1p in the *AS/2* germ line knockout mating was very similar to that previously described for wild type cells (Coyne et al., 1999)(Fig. 9). The macro- and micronuclei of unpaired cells and premeiotic pairs showed no staining for Pdd1p. (An unpaired cell is in the same field as the pair shown at early anlagen stage in Fig. 9. The macronucleus is stained with DAPI, but not anti-Pdd1p.) There was faint, punctate staining with anti-Pdd1p in the parental macronucleus of cells in meiosis I. The staining of the parental macronucleus increased in intensity as the cells progressed through prezygotic mitosis. During the postzygotic mitosis the conjusome stained intensely. At the stage of early anlagen development, the parental macronucleus no longer stained with α Pdd1p, but there was bright staining of the macronuclear anlagen. The Pdd1p staining in the anlagen took on a punctate appearance by the time the parental macronucleus had degenerated. In some cells, the staining was particularly intense around the periphery of the macronuclear anlagen.

As a second test for macronuclear anlagen development, elimination of an IES, the M element, was assayed directly. Single mating pairs of Round II genomic exclusion mating between MU119 and the B*7 were isolated into 1% PPYS medium. The exconjugants were allowed to separate. One of the cells was placed into lysis medium for single cell PCR and the mating partner was maintained in growth medium to determine whether mating was complete. Exconjugants from a mating between wild type cells were isolated as a control.

Elimination of the M element produces two different products in wild type cells. The deletions have a common right boundary, but there are two alternative left boundaries, resulting in elimination of 600 bp or 900 bp of DNA respectively (Fig. 10A) (Austerberry et al., 1984). Single cell PCRs were done on exconjugants from a mating between strains CU427 and CU428 as a control. Both parental strains had the 560 bp band characteristic of the 600 bp deletion in the macronucleus. The appearance of the 250 bp band in DNA from the exconjugants showed that the 900 bp deletion in the developing macronuclei was detectable by this procedure (Fig. 10B). In a Round II genomic exclusion mating between *AS/2* germ line knockout strain MU119 and B*7,

the PCR reaction produced only the 250 bp product, showing that the parental cells contained only the allele with the 900 bp deletion. However, the 560-bp band characteristic of the 600-bp deletion was detected in DNA from the exconjugants (Fig. 10B). This suggested that the M element was deleted from the developing macronuclei of *AS/2* germ line knockout exconjugants.

Replication of DNA in the macronuclear anlagen of *AS/2* germ line knockout progeny

Endoreplication of the DNA late in macronuclear anlagen development increases the DNA content of the macronucleus to 128C when exconjugants are fed (Marsh et al., 2001). The macronuclear DNA content diminishes over the next dozen divisions, producing the 45–50C characteristic of postdivision, vegetatively growing cells (Woodard et al., 1972). The intensity of the signals in single cell PCR experiments on exconjugants from Round II genomic exclusion matings of *AS/2* knockouts was consistently weaker than the signals from exconjugants of control matings between wild type cells (Fig. 10). This suggested that there was less DNA in the anlagen of the mutant cells, possibly due to a failure of the *AS/2* knockout cells to replicate the DNA in the macronuclear anlagen.

To determine whether DNA replication is affected in the anlagen of *AS/2* germ line knockout exconjugants, the relative DNA content of nuclei in mutant and wild-type cells were compared using flow cytometry. Cells were collected at 24 h post-mixing, by which time most of the mating cells had separated to produce exconjugants. The cells were lysed in buffer containing the DNA-specific dye propidium iodide, and subjected to FACS analyses. Micronuclei were isolated from strain CU428 as standard for DNA micronuclear ploidy (data not shown). Because replication of micronuclear DNA coincides with micronuclear anaphase preceding cytokinesis, micronuclei from vegetatively growing cells are typically at G2 of the cell cycle with 4C DNA content (Allis and Dennison, 1982; Charret, 1969, Doerder, 1980 #434). Since *Tetrahymena* are starved to induce mating, nuclei from starved, nonmating, cells of strain CU428 were examined as a second control (Fig. 11A). The two peaks in the analysis of starved cells correspond to the 4C micronucleus and the 50C postdivision macronucleus.

In two matings between *AS/2* wild-type cells, two peaks with intermediate DNA contents were observed. One of the experiments is shown in Fig. 11B. These data suggested that under conditions of continuous starvation, the macronuclear anlagen from wild type cells undergo at least two rounds of DNA replication. In these experiments the pairing was very efficient (~ 95%). By this late stage of conjugation the parental macronuclei in wild type cells were resorbed. Thus, the peak corresponding to the DNA content expected for mature macronuclei was very small.

In four independent experiments, the peaks characteristic of early rounds of DNA

replication were not detected in matings between *AS/2* germ line knockout cells at 24 h after mixing. Two of the experiments are shown in Fig. 11C. In each case, there was a peak corresponding to the 4C micronuclei and the ~ 50C macronuclei. The micronuclear peaks were somewhat broader than those in the control experiments, suggesting that this peak may include macronuclear anlagen that have initiated endoreduplication. Large peaks with DNA content expected for mature macronuclei were also observed. The efficiency of pairing was only ~ 80% in these experiments. This was probably due to somewhat unequal growth rates of the progeny from the two cells from the Round I pair during expansion of the Round I clones. Thus, a relatively large peak representing the macronuclei from unpaired cells was expected. The notable absence of peaks representing the early rounds of DNA replication in the FACS analysis was consistent with the hypothesis that the progeny of *AS/2* germ line knockouts failed to replicate the DNA in the macronuclear anlagen.

In order to avoid the ambiguities created by the background of nuclei from unpaired cells in the FACS analysis, the DNA content of individual nuclei was determined. A Round II genomic exclusion mating was done between strains MU121.3 and B*7. The exconjugants were fixed and stained quantitatively with the Feulgen reaction and the relative DNA contents of micronuclei and macronuclear anlagen were analyzed cytophotometrically. The staining intensity of micronuclei from exconjugants of matings between *AS/2* wild type cells was measured as a control and their DNA content was set at 4C.

The DNA content of developing macronuclear anlagen under conditions of continuous starvation was assessed at four cytological stages (Fig. 12). During stage 1, the two nuclei located in the anterior of the cell after the second postzygotic division begin to swell and differentiate into macronuclear anlagen. At stage 2, the macronuclear anlagen and the new micronuclei take up a more central location in the cell. The parental macronucleus condenses and moves toward the posterior of the cell. Anlagen in pairs of cells in which the parental macronucleus was degraded were designated as stage 3, and macronuclear anlagen in exconjugants were stage 4.

In wild type cells under continuous starvation, stage 1 macronuclear anlagen were not significantly different in DNA content from the postzygotic nuclei, suggesting that the increase in size of the nuclei precedes detectable DNA replication (Fig. 12). By stage 2, when the anlagen have moved toward the center of the cell, endoreduplication of the macronuclear genome has begun such that the average DNA content of stage 2 nuclei from cells at 10–14 h of mating was about 6.5C. A similar DNA content of 6.6C was measured in stage 3 nuclei at 14 h of mating, but by 18 h the stage 3 nuclei were at 9.4C. This was in good agreement with a previous FACS

analysis of purified macronuclear anlagen, showing that replication from 4C to 8C occurs between 12 h and 15 h after mixing of mating cells (Madireddi et al., 1994).

The mean DNA content of stage 4 nuclei in wild type cells was 10.6C at 18 h, and it increased to a maximum of 14.3C by 24 h. Since ~ 15% of the germ line DNA sequences are eliminated during the first round of endoreduplication, the expected C value after 2 rounds of DNA replication is 13.6C (16C minus 15%). This is in good agreement with our FACS analysis, and supports the conclusion that wild type cells under conditions of continuous starvation complete about 2 rounds of DNA replication in the anlagen. By 30 h of mating, the mean DNA content decreased to 9.3C and by 36 h to 7.2C (data not shown). This suggested that by 40 h of continuous starvation (starving and mating), the cells begin to cannibalize the macronuclear anlagen.

The postzygotic nuclei in matings between *ASI2* knockouts had a DNA content very similar to the micronuclei of wild type cells. The DNA content of micronuclei in the *ASI2* knockout exconjugants was 3.99C after the first postzygotic division and 4.75C after the second postzygotic division, indicating that DNA replication was normal in the two postzygotic mitoses.

The DNA content of stage 1 and stage 2 macronuclear anlagen in progeny of matings between *ASI2* germ line knockouts was also similar to that of wild type cells (Fig. 12). However, no further increase in the C value was detected in stage 3 or stage 4 nuclei even as late as 24 h of mating. This suggested that replication of the genome may be initiated in the macronuclear anlagen, which is consistent with the broadening of the micronuclear peak in the FACS analysis. However, endoreduplication ceased in *ASI2* germ line knockout progeny during the first round.

Discussion

***ASI1* and *ASI2* may have evolved from a gene duplication**

ASI1 and *ASI2* have similarities in amino acid sequence and are located within 1.5 kb of each other on the right arm of chromosome 3 (E. Hamilton and E. Orias, personal communication). One possibility is that they evolved from a gene duplication. Although these two genes have similar developmental expression profiles, they are not redundant in function, because the *ASI2* phenotype is observed in a wild-type *ASI1* background.

A small family of potential signal transduction receptors in the *Tetrahymena* genome

The linear arrangement of predicted transmembrane, Cache, and coiled coil domains in Asi2p (Fig. 3A) is similar to the order of those domains in methyl-accepting chemotaxis receptors in bacteria (Stock et al., 1992), suggesting the Asi2p is a signal transduction receptor. Although

we have no experimental data regarding the subcellular localization of Asi2p, we propose that it is a plasma membrane protein. In analogy to the MCPs, the Cache domain may be the extracellular sensory domain. The coiled coils and the SH2 domain of Asi2p are proposed to be cytoplasmic. The presence of the coiled coil domains suggests Asi2p is involved in protein–protein interactions. In the bacterial MCPs, these domains are thought to facilitate the formation of homodimers (Stock et al., 1992).

A tBLASTn search of the annotated *Tetrahymena* genome data base found seven genes (including *ASI1* and *ASI2*) that were identified as encoding Cache domain containing proteins. The deduced proteins encoded by these genes were all moderately large, ranging in size from 666 to 984 amino acids, and they all contain putative transmembrane domains. In five cases, the putative Cache domain lies between two putative transmembrane domains. We propose that at least some of these genes may encode a class of signal transduction receptors in *Tetrahymena*. A search of the *Paramecium* genome data base (<http://paramecium.cgm.cnrs-gif.fr/ptblast>) suggested there may be at least one protein with a Cache domain in *Paramecia*.

ASI2* and development in *Tetrahymena thermophila

Homozygous *ASI2* germ line knockout cells do not produce viable progeny. Exconjugants were observed in cytological analysis of matings between germ line *ASI2* knockouts (Fig. 8), but the progeny cells rounded up and died without completing the first cell division. This showed that expression of zygotic *ASI2*, most likely in the developing macronuclear anlagen, is required to make the transition from the program of sexual reproduction to vegetative growth.

ASI2 is the second gene shown to be required in the macronuclear anlagen in order for *Tetrahymena* to produce sexual progeny. *RAD51* germ line knockouts have a phenotype similar to the *ASI2* knockouts in that they cannot make the transition from conjugal development to vegetative cell division (Marsh et al., 2001). However, there are important differences between the two mutants. First, exconjugants of the *ASI2* germ line knockout cells usually die within 48 h, whereas the progeny of *RAD51* nulls survive as exconjugants for up to 2 weeks. More importantly, DNA replication in *RAD51* exconjugants brings the cells to normal ploidy levels whereas endoreduplication does not occur in the anlagen of the progeny of *ASI2* germ line knockouts. This implies that the requirement for *ASI2* may be upstream of the cell cycle block in *RAD51* exconjugants.

Another important difference between *RAD51* and *ASI2* is that somatic *RAD51* is required for maintenance of the micronucleus. In a *RAD51* somatic knockout strain, the micronuclear chromosomes undergo rapid deterioration (Marsh et al., 2000). Round I clones D and E, derived

from a genomic exclusion mating between MU114 and B*7 (Fig. 5) showed high viability and fertility in the Round II crosses (Table 3). Since the micronucleus of the B*7 strain makes no contribution to the progeny of these crosses, the development of the macronuclei of the progeny is entirely dependent on the micronucleus that was maintained in the presence of the somatic *ASI2* knockout macronucleus. Thus, macronuclear *ASI2* is not required for maintenance of the micronucleus.

The nature of the putative signal transmitted by *Asi2p* and its role in macronuclear anlagen development is unknown. It is important to note that *Asi2p* is not required for DNA replication in vegetatively growing cells, because the somatic knockouts are viable and divide at a rate comparable to that of wild type cells. Thus, *ASI2* is not required for DNA replication per se. It may be that *Asi2p* is required to make the transition from the program of sexual reproduction to vegetative growth.

It has long been known that DNA rearrangements occur at the early stages of endoreduplication, when the developing macronuclear anlagen are between 4C and 8C (Brunk and Conover, 1985; Yokoyama and Yao, 1982). Deletion of IES occurs when DNA replication is inhibited with aphidicolin (Nikiforov et al., 1999), suggesting that IES excision is independent of DNA replication. The converse question is whether IES excision is required for *Tetrahymena* to complete endoreduplication of the macronuclear DNA. This was suggested by the observation that in matings between somatic knockouts of *PDD1* or *PDD2*, two genes required for efficient IES elimination, the DNA fails to replicate in the macronuclear anlagen. The experiment described here shows that IES excision is not sufficient for triggering endoreduplication of anlagen DNA and suggests that the cells monitor additional components of the developmental program before making the transition to vegetative growth.

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Appendix

Table 1

Genotypes and phenotypes of *T. thermophila* strains

Strain	Miconuclear genotype	Macronuclear genotype	Phenotype
CU427	<i>chx1-1/chx1-1</i>	<i>CHX1</i>	cy-s, VI
CU428	<i>mpr1-1/mpr1-1</i>	<i>MPR1</i>	mp-s, VII
B2086	Wild type	Wild type	cy-s, mp-s, II
B*7	Star	Wild type	cy-s, mp-s, VII
MU114	<i>ASI2/asi::neo, CHX1/CHX1, mpr1-1/MPR</i>	<i>asi2::neo, mpr 1-1</i>	pm-r, mpr-r, II
MU 119	<i>asi2::neo/asi2::neo, CHX1/CHX1, MPR1, MPR1</i>	<i>asi2::neo, mpr 1-1</i>	pm-r, mp-r, II
MU120	<i>ASI2/ASI2, CHX1/CHX1</i>	<i>asi2::neo, mpr 1-1</i>	pm-r, mp-r, II
MU121.1	<i>asi2::neo/asi2::neo, chx1-1/chx1-1, MPR/MPR</i>	<i>asi2::neo</i>	pm-s, cy-s, other II, VI, VII
MU121.3	<i>asi2::neo/asi2::neo, chx1-1/chx1-1, MPR/MPR</i>	<i>ASI2</i>	pm-s, cy-s, other II, VI, VII

Macronuclear phenotype designations: -r: resistant, -s: sensitive. Phenotypes of mutant genes are as follows: *mpr1-1*: 6-methylpurine (mp) resistant; *chx1-1*: cycloheximide (cy) resistant; : *Asi2::neo*, paromomycin (pm) resistant. Mating types are designated by Roman numerals.

Table 2
Primers

p1	5' AATAAACATTCCATTCACAATAATAC 3'
p2	5' ATTAGGGTGTTTTGTGTTCTACTT 3'
p3	5' TCATTCCAATTTTCAAGTGTA 3'
p4	5' AAAGTATAACAAGCACGATGA 3'
p5	5' ACCAAATCCAAGAACCAATAA 3'
p6	5' AGAAATATGAAAAGAACCACAA 3'
p7	5' TCATCTAAGTTTCTGCCCTAT 3'
p8	5' GTTTCTCAGGCTCCTTCTCC 3'
p9	5' TACAATTACCAGTCCCTATCC 3'
p10	5' ATTCACCATCTTTTTCCTGC 3'
p11	5' TATTGAGTTGTTTATTCTGAA 3'
p12	5' TATTTATCTTCTTTTCTGCTA 3'

Table 3

Determination of micronuclear genotype of Round I progeny from *ASI2* germ line heterozygotes

Round I syncclone	Viability of round II syncclones	pm-s round II syncclones
D	90/110 (82%)	90/90 (100%)
E	37/39 (95%)	37/37 (100%)
F	1/33 (3%)	0/1 (0%)
G	0/116 (0%)	N.A.

N.A., not applicable.

Table 4

Test crosses to determine the genotype of Round I clones

Strains	Viability	cy-r	pm-r, cy-r
MU120 X CU427	37/54 (69%)	35/37 (95%)	0/35 (0%)
MU119.1 x CU427	76/114 (67%)	66/76 (87%)	66/66 (100%)
MU119.2 x CU427	41/65 (63%)	35/41 (85%)	35/35 (100%)

Table 5

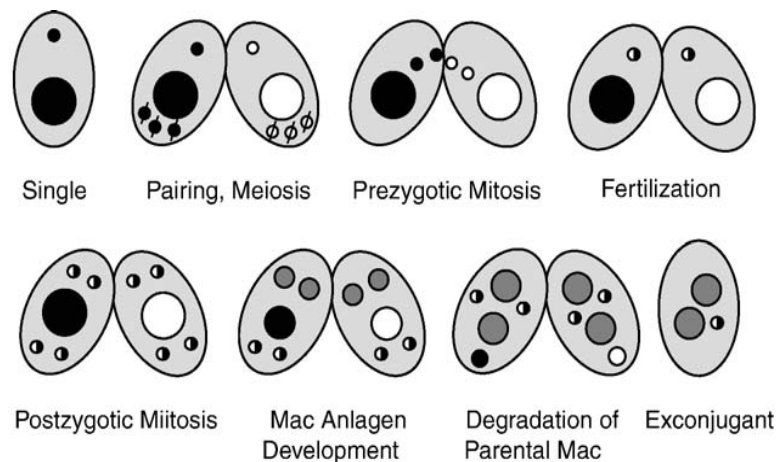
***ASI2* germ line knockout cells do not produce viable progeny**

Cells lines	Pair survival	Progeny
MU119 x B*7	0/116 (0%)	N.A.
MU121.3 x B*7	0/71 (0%)	N.A.
MU120 x B*7	90/110 (82%)	90/90 (100%)
CU427 x B*7	63/76 (83%)	59/63 (94%)
MU119 x MU121.3	0/89 (0%)	N.A.
CU427 x CU428	48/91 (53%)	36/48 (75%)

N.A., not applicable.

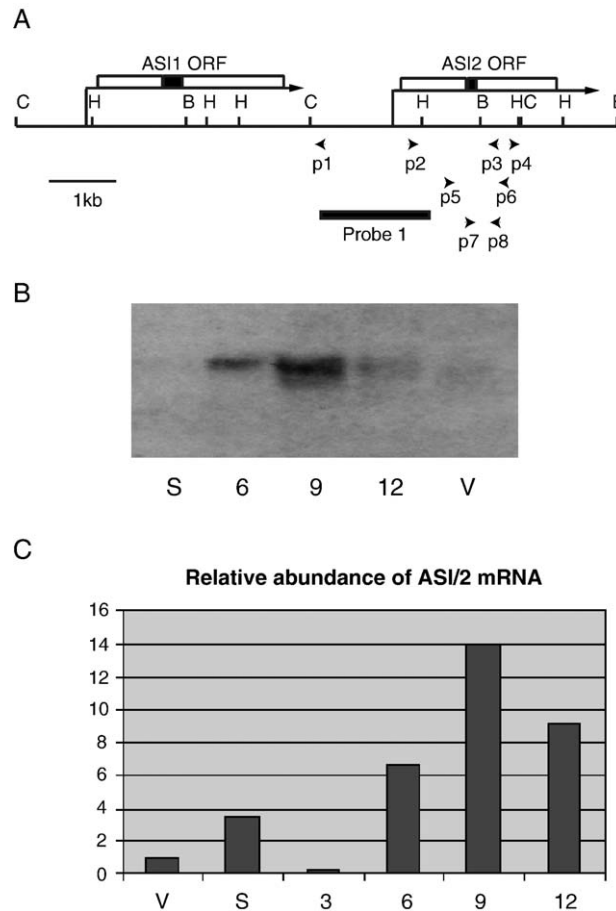
Figure 1

Sexual reproduction in *Tetrahymena*



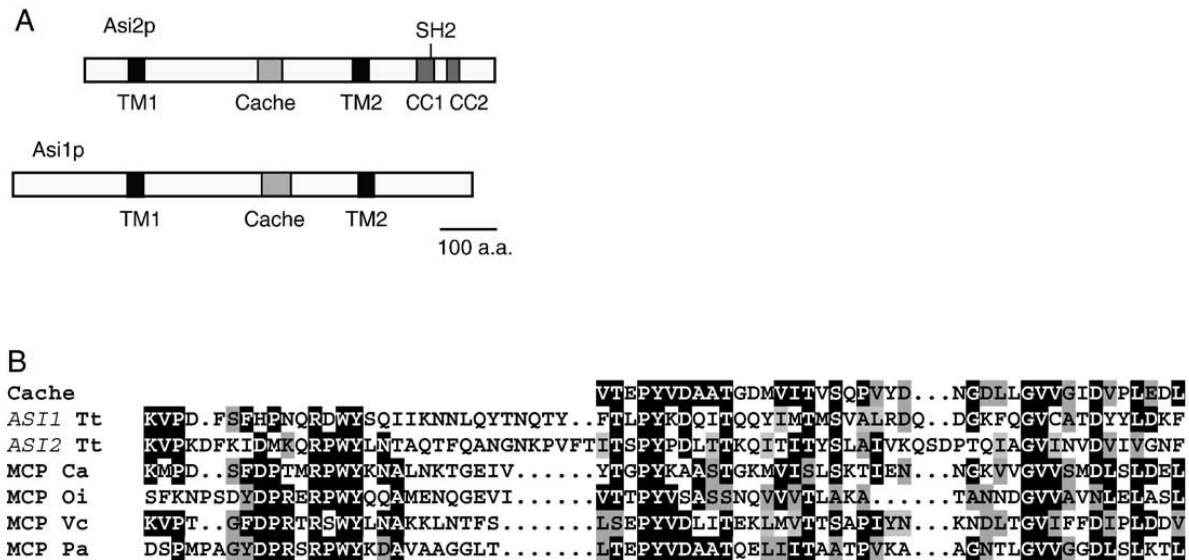
Black and white indicate two different alleles of a gene. Gray represents mixed alleles in the macronuclear anlagen.

Figure 2
***ASI2* mRNA abundance during the development**



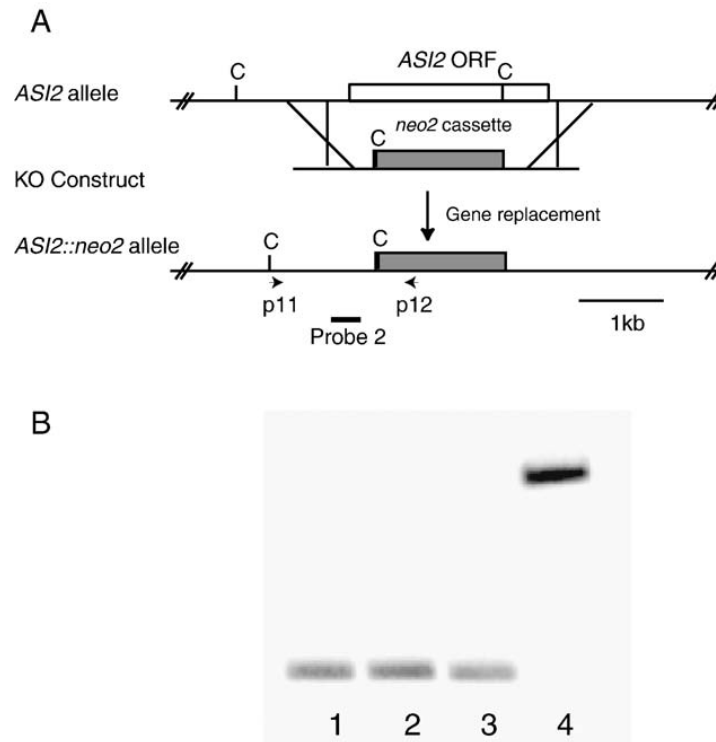
(A). Genomic map of *ASI1* and *ASI2*. The transcripts are represented as angled arrows: open bars, exons; filled bars, introns. The sequence of the *ASI2* gene was obtained by sequencing PCR products from two rounds of inverse PCR, using primers p1 and p2 on DNA digested with *ClaI* in the first round and primers p3 and p4 on DNA digested with *BglII* in the second. The RT-PCR product generated with primers p5 and p6 revealed the intron. The primers for real-time PCR were p7 and p8 for *ASI2* and p9 and p10 for 17S rRNA (Table 5). The Northern blot in panel B was probed with Probe 1. B, *BglII*; C, *ClaI*; H, *HindIII*. (B). Northern blot. Poly(A)⁺ RNA was isolated from *Tetrahymena* cells that were S, starved; conjugating cells at 6 h, 9 h, and 12 h post-mixing of the two complementary mating types; and V, in vegetative growth. (C). Real-time RT-PCR. Total RNA was isolated from vegetatively growing cells, starved cells and conjugating cells at 3 h, 6 h, 9 h, and 12 h post-mixing. The data are the average of two experiments, each done in triplicate.

Figure 3
Structure of the deduced Asi2p



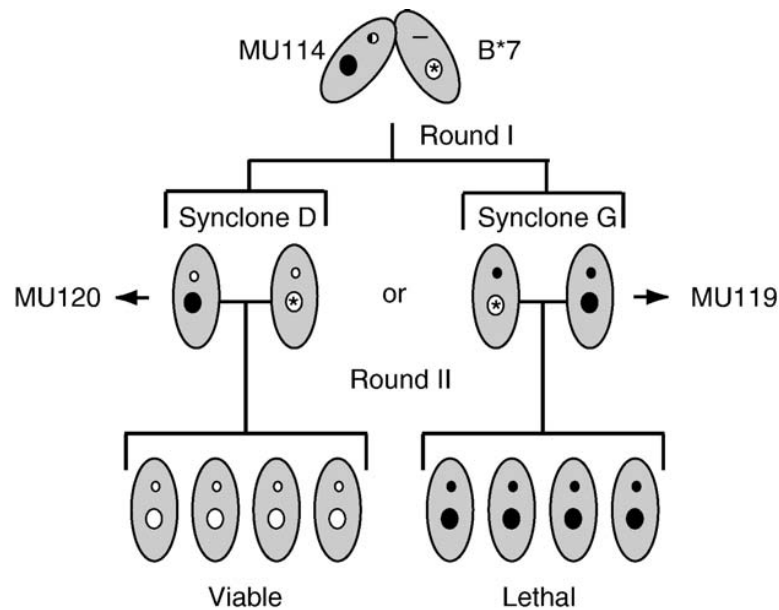
(A) Asi1p and Asi2p domain comparison. TM, transmembrane domains; Cache, putative Cache sensory domain; CC, coiled coil domains; SH2, SH2 receptor binding site. (B) Alignment of the Cache domains of *ASI1* and *ASI2* with the consensus Cache domain and the domains of four methyl-accepting chemotaxis proteins. Cache, Cache consensus from the Pfam program. The genes, organisms and accession numbers are: *ASI1*, AAL37738 and *ASI2*, AAR83913; Tt, *Tetrahymena thermophila*; MCP, Methyl-accepting chemotaxis protein; Ca, *Clostridium acetobutylicum*, B96999; Oi, *Oceanobacillus iheyensis*, BAC14058; Vc, *Vibrio cholerae*, L25660.1; Pa, *Pseudomonas aeruginosa*, BAA29579. Shaded letters are conserved in the majority of the sequences. Black, identical amino acids; gray, similar amino acids. *ASI1* and *ASI2* were aligned by placing gaps in two locations where they also occur in the alignment of the signature proteins. The signature proteins are the first five in the CDD alignment of *ASI1*. The Cache domain of *ASI2* was identified in Pfam with an expect value of 6.4×10^{-5} for the first 40 amino acids (300–339 in *ASI2*). The expect value for *ASI1* is 4×10^{-4} over 80 amino acids.

Figure 4
Somatic *AS/2* is not essential for vegetative growth



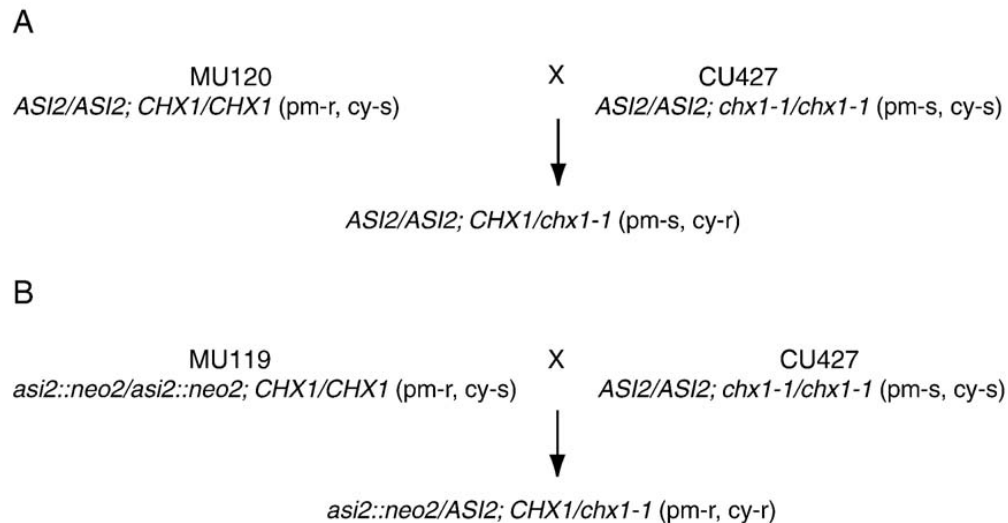
(A). A knockout construct containing *neo2* cassette with about 1 kb of flanking DNA from the *AS/2* locus on each side was introduced into *T. thermophila* by biolistic bombardment. The *neo2* cassette replaced the endogenous *AS/2* gene by homologous recombination, producing the knockout chromosome. p11 and p12, primers used in PCR to confirm the location of the *neo2* cassette; C, *Clal*. The Southern blot in panel B was probed with probe 2. (B). Macronuclear DNA was isolated from somatic knockout cell lines MU111, MU112 and MU113 (lanes 1–3) and wild-type cell line CU428 (lane 4) and a Southern blot of the DNA digested with *Clal* was done. Probe 2 detected the fragment of 3.1 kb expected for the endogenous gene in wild type cells, and a 1.3 kb fragment expected for the knockout allele in DNA from the transformants.

Figure 5
Genomic exclusion matings between *ASl2/asi2::neo2* heterozygote with strain B*7



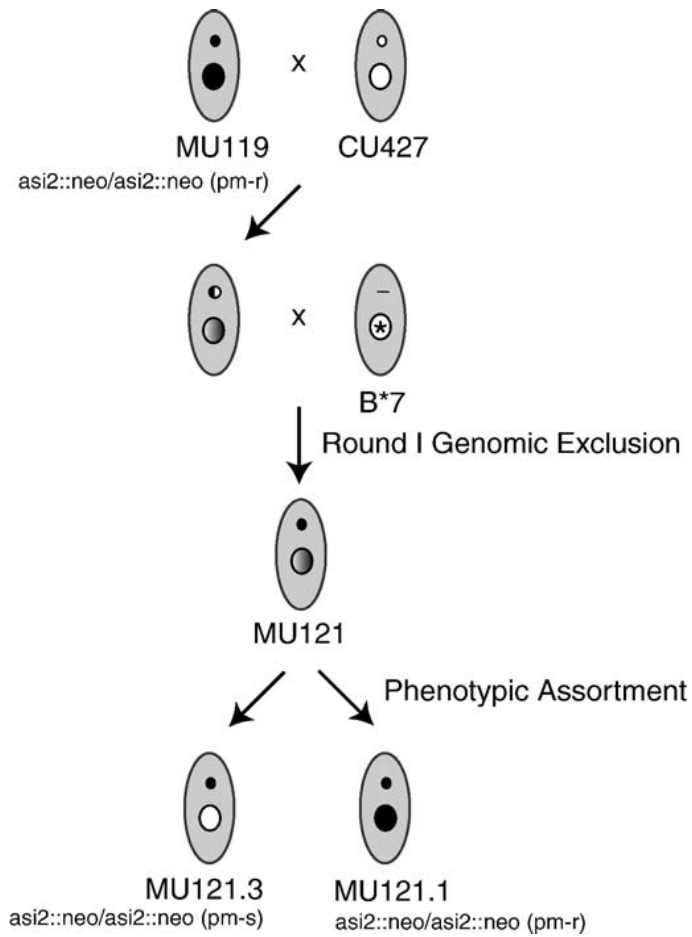
Black, *asi2::neo2* knockout allele; white, *ASl2* wild type allele; * star strain macronucleus.

Figure 6
Test crosses for genotype of Round I clones of the mating between MU114 and B*7



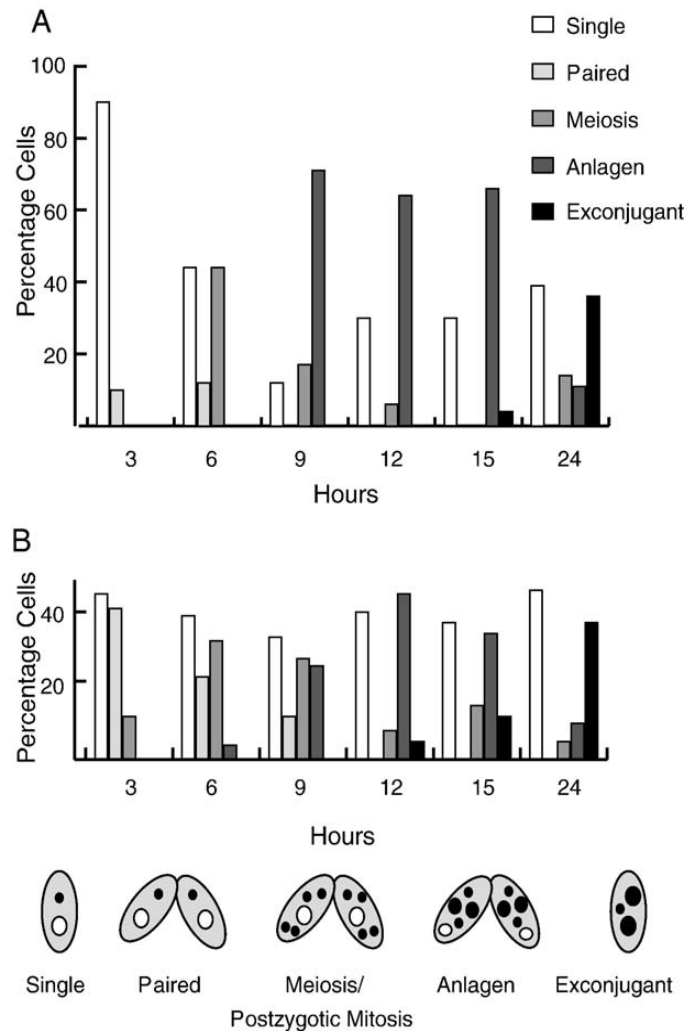
Micronuclear genotypes are shown followed by the phenotype expressed by the macronuclei in parentheses.

Figure 7
Construction of ASI2 germline knockout strain with a wild type macronucleus



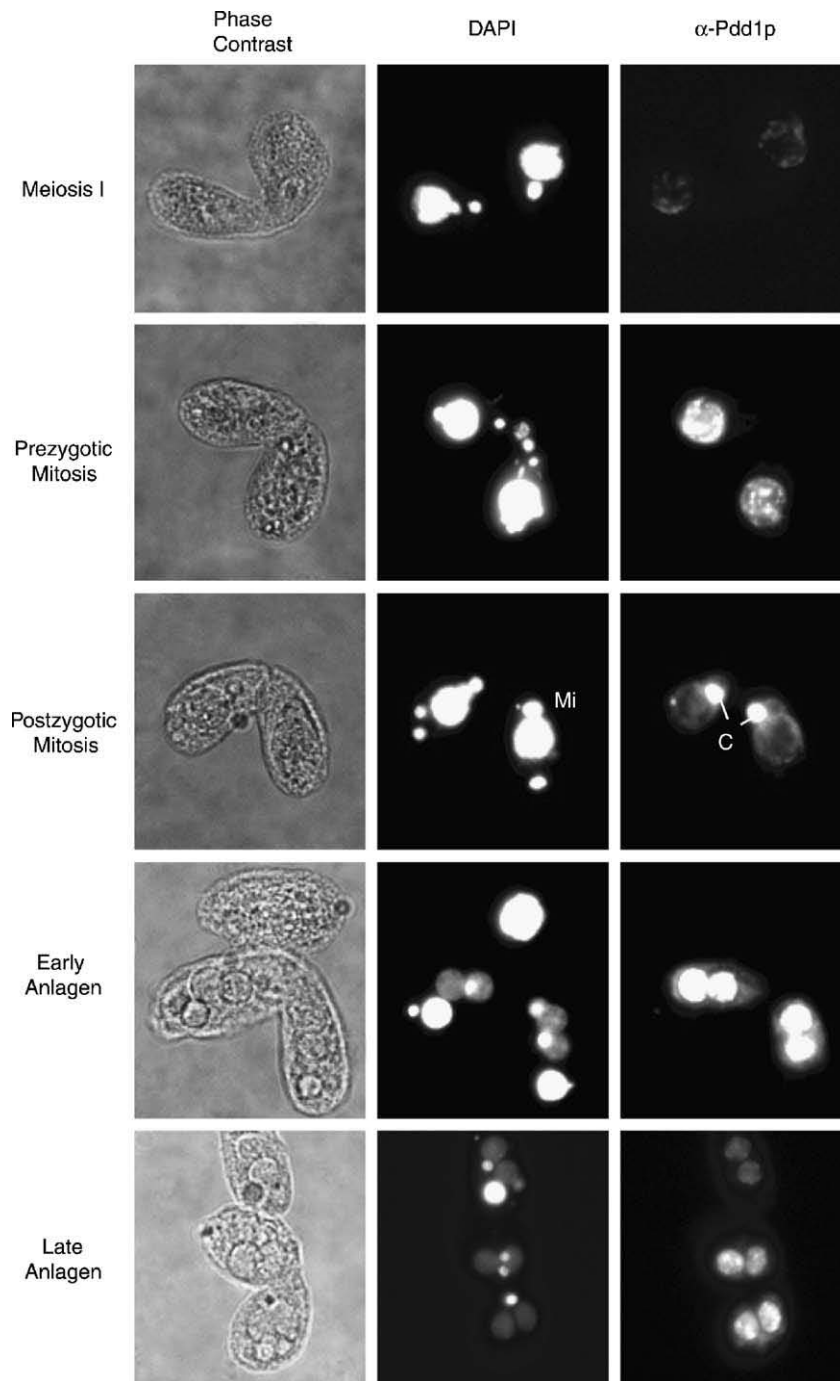
Black, *asi2::neo2*; white, *ASI2* wild type allele; shaded, macronuclei with mixed alleles.

Figure 8
Cytology of (A) a Round II genomic exclusion mating of *ASI2* germ line knockouts and (B) a control mating between *ASI2* wild type cells



The drawing below the histogram depicts the various cytological stages. Black, germ line micronuclei of the parental cells and zygotic nuclei of the exconjugants; white, parental macronuclei.

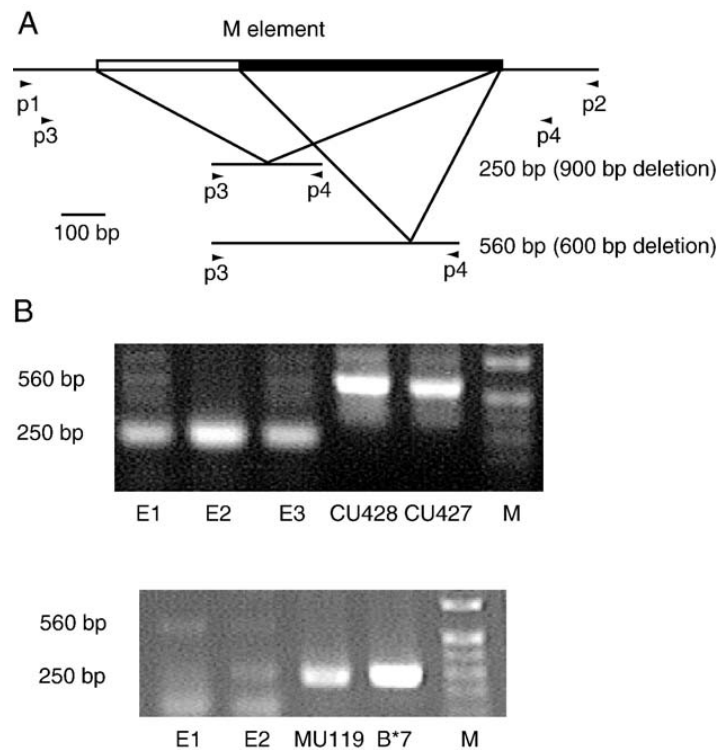
Figure 9
Immunostaining of matings between *ASI2* germ line knockout strains for Pdd1p



To facilitate identification of the nuclei, phase contrast photographs were taken with filters to show both DAPI staining and immunostaining with α Pdd1p. Mi, micronucleus; C, conjugosome.

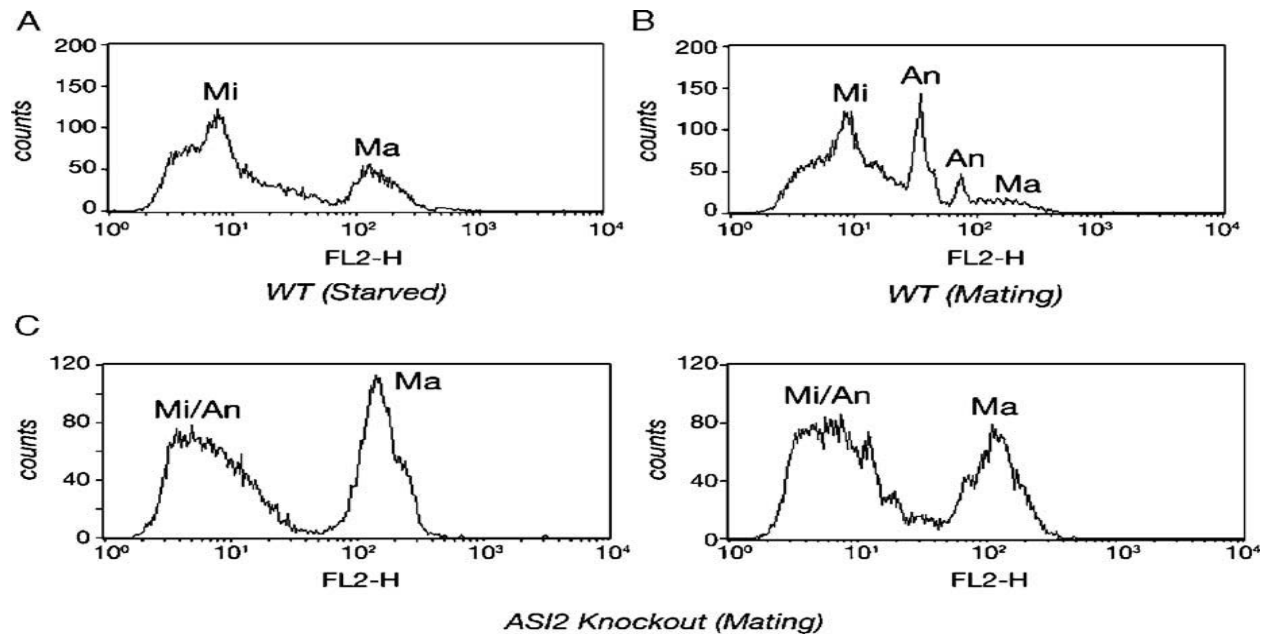
Figure 10

Deletion of the M element from the macronuclei of *AS/2* germ line knockout exconjugants



(A) Two alternative deletions of the M element from the micronuclear chromosome eliminate the region depicted by the black box or the region including both the black box and the open box. Single cell PCR was done with primers p1 and p2 in the first reaction and the nested primers p3 and p4 in the second reaction, producing PCR fragments of 250 bp or 560 bp from the macronuclear chromosome with the corresponding deletion. (B) Single-cell PCR products. CU427 and CU428, parental strains for the control cross; MU119 and B*7, parental strains for the *AS/2* knockout Round II genomic exclusion mating; E1–E3, exconjugants; M, Hi-Lo DNA marker (Minnesota Molecular).

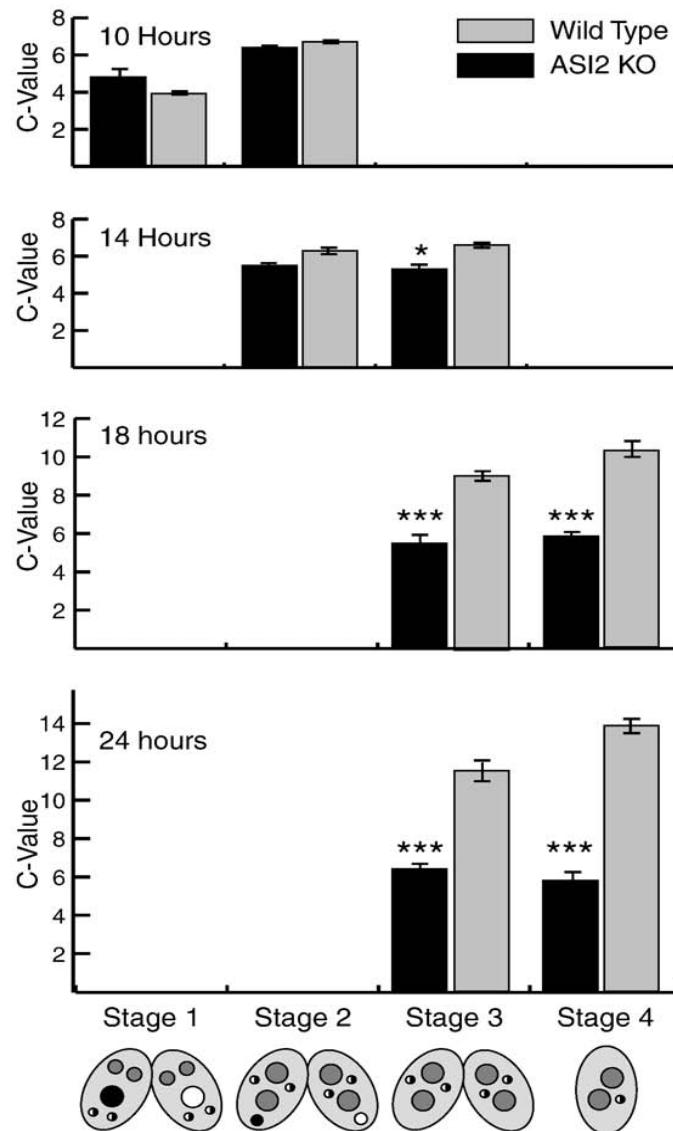
Figure 11
FACS analysis of matings between germ line *ASI2* knockout cells



(A) Nuclei from wild type, starved cells. (B) Nuclei isolated from a mating between wild type cells. (C) Nuclei isolated from a Round II genomic exclusion mating of *ASI2* germ line knockouts with B*7.

Figure 12

DNA content of macronuclear anlagen in a Round II genomic exclusion matings of an *ASI2* germ line knockout strain and in wild type controls



Error bars indicate the standard error *, statistically significant difference from wild type progeny at a confidence level of $P < 0.02$; ***, statistically significant difference at a confidence level of $P < 0.001$.