ROLES OF THE *Tetrahymena thermophila* TYPE I ELEMENT BINDING FACTOR, TIF1, IN DNA REPLICATION AND GENOME STABILITY

A Dissertation

by

TARA LAINE MORRISON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Medical Sciences

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Approved by:

Chair of Committtee,	Geoffrey M. Kapler
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ABSTRACT

Roles of the *Tetrahymena thermophila* Type I Element Binding Factor, TIF1, in DNA Replication and Genome Stability. (August 2005)

Tara Laine Morrison, B.A., Baylor University

Chair of Advisory Committee: Dr. Geoffrey M. Kapler

The *Tetrahymena thermophila* rDNA minichromosome has been used as a model system for studying DNA replication. Previous studies have identified cis-acting replication determinants within the rDNA origin and promoter region including the type I element that is essential for replication initiation, fork progression and promoter activation. TIF1 is a non-ORC single strand-binding protein that binds the type I element *in vivo*. TIF1 binds opposing strands at the origin and promoter regions indicating that it may play a role in selectively marking these regions. In this dissertation, I use gene disruption to elucidate the role of TIF1 in replication. This work reveals that TIF1 represses rDNA origin firing, and is required for proper macronuclear S phase progression and division. Replication at the rDNA origin initiates precociously despite the observation that TIF1 mutants exhibit an elongated macronuclear S phase and a diminished rate of DNA replication. The amitotic macronucleus also displays delayed and abnormal division even though cells exit S phase with a wild-type macronuclear DNA content. Nuclear defects are also evident in the diploid micronucleus as TIF1 mutants contain fewer micronuclear chromosomes and are unable to pass genetic information to progeny. This defect is progressive as clonal mutant lines exhibit micronuclear instability during subsequent vegetative cell cycling. This work reveals that these macro- and micronuclear phenotypes

may be the result of DNA damage as TIF1 mutants are hypersensitive to DNA damaging agents. This suggests that TIF1 mutants may have defects in the DNA damage response pathway. TIF1-deficient cells also incur DNA damage with no exogenous damaging agents. I propose that micro- and macronuclear defects witnessed in TIF1 mutant cells result from cells exiting S phase with compromised chromosomes due to the accumulation of DNA damage. Furthermore, TIF1 appears to play a role in the prevention, recognition or repair of DNA damage in addition to regulating rDNA replication and cell cycle progression and division. Additionally, TIF1 plays an essential role in the faithful propagation of both the macro- and micronuclear genomes.

DEDICATION

I would like to dedicate this dissertation work to my husband and best friend Dave. I never could have done this without your love, patience, support and understanding. It's been a long hard road but we made it baby. I love you Beloved.

To my parents and sisters, you have no idea how much your encouragement has meant to me. This work is more than just the work of one person. You have all helped me in more ways than you will ever know.

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TABLE OF CONTENTS

CHAPTER Page

CHAPTER Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

BACKGROUND

Overview of DNA replication in eukaryotes

All eukaryotic cells from yeast to humans must undergo DNA replication before cell division in order to maintain their genomes and pass genetic information to progeny cells. This process must be regulated to ensure that the entire genome is replicated once during each cell cycle. Eukaryotic cells initiate DNA replication at specific sites in chromosomes termed origins of replication. These origins contain cis-acting recognition sequences to which trans-acting factors responsible for DNA replication bind. Origins also contain a DNA unwinding-element (DUE) and one or more binding sites for transcription factors (DePamphilis, 1993). These elements work in concert with cell cycle checkpoint proteins to restrict replication of a given DNA segment to once per cell division (Dutta and Bell, 1997). Eukaryotes also have DNA repair pathways that are activated by checkpoint proteins to block progression of the cell cycle when problems arise during S phase.

Eukaryotic replication: replicon organization

Our understanding of DNA eukaryotic replication has primarily been based on studies in genetically tractable organisms such as the yeast *Saccharomyces cerevisiae* and *Saccharomyces pombe*, and other select eukaryotes including *Tetrahymena thermophila*,

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This dissertation follows the style of Cell.

Drosophila melanogaster and *Xenopus laevis* that are amenable to genetic or biochemical analysis. In *S. cerevisiae* origins of replication have been identified as sequence elements that confer the property of autonomous replication onto extrachromosomal plasmids (Newlon and Theis, 1993). The first genetically defined autonomously replicating sequence, *S. cerevisiae* ARS1, spans ~150 base pairs and contains a modular array of short cis-acting functional determinants that co-localize with the initiation site (Marahrens and Stillman, 1992). These elements include the essential A element which is necessary but not sufficient for initiator function as well as three additional elements B1, B2, and B3, which collectively are required for replication origin firing (Figure 1.1A).

The principal regulatory determinant, the A element, contains an 11-base-pair AT-rich region that is conserved across all *S. cerevisiae* ARS elements and is termed the ARS consensus sequence (ACS) (Newlon, 1988). Extensive mutational studies have revealed that all functional replicons in *S. cerevisiae* contain at least one match to this consensus sequence (Rao et al., 1994). The A element along with B1, located 3' of the T-rich strand of A, comprise the core binding site for the six subunit origin of recognition complex (ORC), a central trans-acting player in replication initiation (Bell and Stillman, 1992; Rao and Stillman, 1995). *S. cerevisiae* ORC binds the ACS region in an ATP-dependent manner. The B2 element is proposed to function as a DNA unwinding element (DUE) (Lin and Kowalski, 1997) and the B3 element is a binding site for the transcription factor and replication-enhancer Abf1.

Replicons in the fission yeast, *S. pombe,* are relatively short spanning 500-1000 base-pairs. They are extremely AT-rich and often contain asymmetrically distributed AT clusters, but do not contain highly specific consensus sequences, such as the *S. cerevisiae*

ACS (Kim and Huberman, 1998). Genetic studies have revealed that *S. pombe* replicons contain multiple redundant elements that can be replaced with other AT-rich sequences without substantially affecting origin activity (Okuno et al., 1999). Most identified *S. pombe* origins do not fire in every cell cycle suggesting that there may be more origins than are actually used in a given S phase (Okuno et al., 1997). Replication origins are localized to intergenes which may contain many potential ORC binding sites. Considering that *S. pombe* contains over 5000 intergenes the number of potential ORC binding sites may exceed the number of ORC molecules in the cell. This observation supports a stochastic model of origin firing where ORC may bind to different origins during subsequent cell cycles (Dai et al., 2005).

The *Drosophila* chorion gene locus, which undergoes developmentally programmed gene amplification, is another well-studied higher eukaryotic replicon that spans ~10,000 base-pairs. Transformational studies using deleted and mutated genomic constructs of the third chromosome gene cluster revealed essential and stimulatory cis-acting determinants necessary for gene amplification (Delidakis and Kafatos, 1989; Orr-Weaver et al., 1989). These experiments identified at least five different cis-acting determinants including an essential 320 base-pair region ACE3 and four other unique stimulatory elements, AER-A, AER-B, AER-C and AER-D. (Fig. 1.1B). This replicon contains one primary origin of replication, ori-β, as well as two lesser used origins, ori-α and ori-γ (Heck and Spradling, 1990). The complexity of this replicon is evident in that replication of this region employs multiple origins of replication distributed within the chorion genes over a region of many kilobases (Heck and Spradling, 1990; Delidakis and Kafatos, 1989). Additionally the ACE3 and ori-β elements that are required for chorion

gene amplification (Lu et al., 2001) directly bind *Drosophila* ORC revealing that as in yeast replication initiation is controlled by the interplay of cis-acting determinants and trans-acting factors (Austin et al., 1999).

Contribution of chromatin structure

In addition to cis-acting determinants within the genome, chromatin structure has been shown to affect replication origin firing. This is evident by the observation that euchromatic regions of the genome replicate early during S phase, while heterochromatic regions replicate late (Fangman and Brewer, 1992). In *S. cerevisiae* studies, origins that have been shown to operate well in the context of a plasmid, are poorly utilized in their normal chromosomal context, suggesting that chromatin context can play a repressive role in origin activation (Newlon and Theis, 1993). In support of this, when origins that normally fire early during S phase are placed in the chromosomal context of a late firing origin they adopt the late firing pattern (Friedman et al., 1996). These results indicated that the packaging of DNA into nucleosomes affects origin firing.

The importance of nucleosome positioning was uncovered by biochemical and genetic analyses of *S. cerevisiae* ARS1. The micrococcal nuclease digestion pattern of ARS1 indicated that the A and B3 elements mark the borders for the nucleosome free region (Thoma et al., 1984). When mutations are made that allow nucleosomes to invade the nucleosome-free origin region, origin activity is disrupted (Venditti et al., 1994; Hu et al., 1999). These results suggest that nucleosomes play a role in regulating replication. The B3 element binds to a non-ORC protein, Abf1 (Diffley and Stillman, 1988), and A interacts with scORC (Rao and Stillman, 1995). *In vitro* chromatin assembly experiments revealed that both Abf1 and ORC are necessary for the specific ARS1 chromatin structure

(Lipford and Bell, 2001). This suggests that trans-acting factors play a role in nucleosome positioning and subsequent origin activation.

Chromatin effects on replication have also been observed at the *Drosophila* chorion gene locus. Transformational studies using P-element-mediated gene transfer revealed that the amplification level of chorion genes is extremely sensitive to genomic position suggesting that the chromatin surrounding the replicon can modulate its function (Lu et al., 2001; de Cicco and Spradling, 1984). This is further illustrated by the observation that histone acetylation of nucleosomes in the chorion replicon region increased origin activity (Aggarwal and Calvi, 2004). Additionally, recent studies revealed that DmORC has an ~30 fold higher affinity for negatively supercoiled DNA verses relaxed or linear DNA (Remus et al. 2004). This observation raises the possibility that the topology of DNA at the origin region may also play a role in origin recognition. The collective results indicate that origin recognition in *Drosophila* relies on more than DNA sequence specificity.

The importance of chromatin on origin firing is also observed in *Xenopus laevis.* *Xenopus* undergoes rapid cell cycles early in development during which DNA replication initiates without any detectable sequence specificity (Hyrien and Mechali, 1993). However, origin spacing during early stages of replication is not random as they are spaced between 5-15 kb apart. Additionally, different origin clusters fire at different times, both early and late in S phase (Blow et al., 2001). During this early stage of development cells are not undergoing transcription. However, upon the onset of zygotic transcription and global chromatin restructuring, replication origin usage becomes restricted to intergenic promoter regions (Hyrien et al., 1995) similar to *S. pombe* (Gomez and Antequera, 1999). Recent transformation studies of *Xenopus* eggs by injecting a transcription-inducible

template revealed that, in the presence of corresponding transcription factors, site-specific origin firing is induced. This induction is not dependent on the activation of transcription (Danis et al., 2004). These results suggest that trans-acting proteins, such as transcription factors that have been found to relieve repression due to chromatin structure (Felsenfeld, 1992) may interact with chromatin to promote origin specificity.

Potential contribution of transcription

Transcription and potential transcription factors have also been shown to play a role in replication of the DHFR locus in Chinese hamster ovary (CHO) cells. The DHFR locus was the first identified mammalian replication replicon (Heintz and Hamlin, 1982; Heintz et al., 1983). Its organization and regulation is highly complex, consisting of >20 potential replication initiation sites distributed throughout the 55 kb spacer region between the DHFR and 2BE2121 genes. However, the three identified origins ori-β, ori-β′, and ori-γ are preferred (Kobayashi et al., 1998; Dijkwel et al., 2002). Additionally, initiation does not occur within the DHFR gene itself (Vaughn et al., 1990; and Hamlin and Dijkwel, 1995). Recent studies have revealed that the loss of the DHFR promoter leads to replication initiation within the DHFR coding region, as well as, a decrease in the efficiency of initiation at the DHFR locus. However, the replacement of the DHFR promoter with a *Drosophila* promoter rescued these defects (Saha, et al., 2004). These results suggests that transcription and/or transcription factors may influence the regulation of replication origins in higher eukaryotes. Consequently, replication can be controlled by both local sequences and sequences that are distant from the origin, such as the DHFR promoter.

Similar results have been observed at the human c-myc replicon. This replication locus is located in the 5' flanking region of the human c-myc gene and contains a core 2.4 kb origin segment that displays ARS activity in transfected cells (McWhinney et al., 1995). This core region and flanking DNA contains multiple sites for replication initiation similar to the DHFR locus (Tao et al., 2000). Additionally this region contains multiple cis-acting replicator elements, such as promoters, transcription factor binding sites, and a DNA unwinding element (Michelotti et al., 1996). Deletion of the 3' portion of the c-myc replicator which contains transcription factor binding sites resulted in the loss of origin activity (Liu et al., 2003), suggesting that transcription factors may be important for origin activity. Additionally, replacement of this region with a heterologous transcription factor-binding region restored origin function (Ghosh et al., 2004). This further illustrates the relative complexity of DNA replicons which depend not only on DNA replication origins, but on additional cis-acting determinants that may exist a great distance from origins.

Eukaryotic cell cycle regulation: trans-acting factors

The combination of cis- and trans-acting factors allow cells to switch between being competent for DNA replication initiation and noncompetent. This is essential to ensure that cells only replicate their chromosomes once per cell cycle. Cells become competent for DNA replication during G1 phase with the assembly of specific multiprotein pre-replication complexes (pre-RCs) at DNA replication origins (DePamphilis, 1999). This is followed by the activation of pre-RCs by trans-acting factors that convert them to bidirectional replication forks. After activation of the origin and the initiation of DNA synthesis the replication complex at the origin switches to a post-replication complex

(post-RC) that inhibits further initiation events. This switch is tightly coupled with the initiation of DNA replication and the disassociation of specific pre-RC proteins from the origin such as the MCM complex (Tadokoro etal., 2002). Once this occurs during S phase cells are no longer competent for DNA replication initiation and remain this way until G1.

As with the identification of replication origins, initial studies on replication trans-acting factors were focused on yeast. Homologues for most of the yeast initiation proteins have been identified in *Xenopus, Drosophila*, and mammals. One extremely important trans-acting factor that is essential for initiator function is the six-subunit origin of recognition complex (ORC) (Bell and Stillman, 1992). *S. cerevisiae* ORC binds to replication origins *in vivo* and *in vitro* in an ATP-dependent manner (Bell and Stillman, 1992; Diffley and Cocker, 1992). Mutations in genes encoding the ORC subunits inhibit DNA replication (Foss et al., 1993; Bell et al., 1993). ORC has been found to be a conserved feature of replication in all eukaryotes studied and plays an essential role in recruiting other trans-acting factors necessary for replication initiation and elongation (Dutta and Bell, 1997).

DNA binding proteins: ORC

S. cerevisiae ORC binds in a sequence-specific manner to both the A and B1 elements in the ARS1 replicon (Fig. 1.1A) and remains bound throughout the cell cycle, acting as a marker for replication origins (Diffley and Cocker, 1992). The five largest subunits (Orc1p-Orc5p) are required for DNA binding (Lee and Bell, 1997). Orc6p is not required for DNA binding, however, it is essential for DNA replication and cell viability (Li and Herskowitz, 1993). ScORC binds double-strand DNA showing a preference for the A-rich strand. ssDNA binding allows for the activation of ORC ATPase activity that is

inhibited when bound to dsDNA. A conformational change in ORC is associated with this activation, suggesting that ssDNA binding alters ORC function during the initiation of replication (Lee et al., 2000).

S. pombe differs from *S. cerevisiae* in that their replicons are larger and do not contain specific consensus sequences (Kim and Huberman, 1998). Instead *S. pombe* origin activity depends largely on total AT content (Okuno et al., 1999). Biochemical studies revealed that the amino-terminal domain of SpOrc4p is responsible for targeting SpORC to origins. This domain contains nine AT-hook motifs that are responsible for ORC binding to multiple sites within the origin region (Chuang and Kelly, 1999; Chuang et al., 2001). Although the AT-hook motif has not been found in Orc4p of other systems, it illustrates a novel mechanism for origin-specific targeting.

In higher eukaryotes, such as *Drosophila*, ORC binds to the origin region, ori-β, and essential replication element, ACE3 (Austin et al., 1999). These interactions, unlike that of *S. cerevisiae*, are not sequence specific. DmORC sequence preference is limited to the binding of AT-rich DNA (Zhang and Tower, 2004). This is a reflection of the fact that higher eukaryotes do not contain a consensus ORC binding site as is found in *S. cerevisiae.* Additionally, recent studies have shown that DmORC binds negatively supercoiled DNA ~30 fold over linear or relaxed DNA (Remus et al., 2004). Considering that this preference for supercoiled DNA was only for negatively supercoiled DNA which promotes unwinding, DmORC appears to show a preference for ssDNA character. This observation illustrates that the mechanism of origin recognition by ORC may vary from species to species, despite the fact that the underlying principle that ORC is necessary for DNA replication initiation remains constant.

Non-ORC DNA binding proteins

Non-ORC binding factors, such as transcription factors, may also affect origin firing and ORC binding. The B3 element of the *S. cerevisiae* replicon binds the transcription factor Abf1 (Diffley and Stillman, 1988). Plasmid stability assays revealed that inhibition of Abf1 binding reduced origin efficiency. However, plasmid stability was restored when the Abf1 binding site was replaces with a heterologous transcription factor binding site and subsequent transcription factor binding (Marahrens and Stillman, 1992). This observation suggests that transcription factor binding plays a role in initiation of replication origins in *S. cerevisiae.*

The *Drosophila* transcription factor Myb has also been shown to affect origin activation. Myb, which forms a multiprotein complex *in vivo,* binds to both the ACE3 and Ori-β elements of the chorion gene locus. Deletions in regions essential for Myb binding resulted in a loss of amplification at the *Drosophila* chorion gene locus (Beall et al., 2002). However, both ORC and Cdt1 were localized to the replication locus in the absence of Myb binding. This result suggests that Myb plays an essential role in DNA replication after prereplication complex (pre-RC) formation.

After binding DNA, ORC acts as a recruitment factor for other pre-RC proteins. The first of these proteins to be recruited to the DNA origin is the ATPase Cdc6. In *S. cerevisiae* Cdc6 has been found to modulate the binding affinity of ORC by inhibiting non-specific binding (Mizushima et al.., 2000). Additionally, Cdc6 is necessary for pre-RC formation by recruiting MCM2-7p (Coleman et al.., 1996). This activity is cell cycle regulated in *S. cerevisiae,* as Cdc6 is targeted for degradation by phosphorylation as cells progress into S phase (Drury et al.., 1997). In mammalian cells, Cdc6 is selectively

phosphorylated as cells enter S phase resulting in its export from the nucleus (Saha et al.., 1998). This regulation may play a role in preventing reinitiation events at DNA origins since Cdc6 is an essential part of the pre-RC.

Regulation proteins that do not bind DNA

Another key factor in pre-RC assembly is Cdt1. Cdt1 was first detected in *S. pombe* where mutations resulted in a block of DNA replication and defects in S phase checkpoints (Hofmann and Beach, 1994) SpCdt1 has been shown to associate with SpCdc6 to recruit MCM proteins to the pre-RC (Nishitani et al.., 2000). Cdt1 is conserved in other eukaryotes, such as *Xenopus*, where it associates with XlORC and is required for MCM2-7p recruitment (Maiorano et al.., 2000). Cdt1 is regulated in higher eukaryotes, such as *Xenopus* and humans, by a cell cycle-regulated protein called geminin (Tada et al.., 2001; Wohlschlegel et al.., 2000). Geminin, which is expressed after cells enter S phase and is degraded as cells enter M. Geminin binds to Cdt1 and inhibits its activity suggesting that it plays a role in the prevention of re-replication (McGarry and Kirschner, 1998).

The MCM proteins, of which there are six in all eukaryotes studied, form a multiprotein complex that is recruited to the pre-RC by ORC, Cdc6, and Cdt1. Once the MCM2-7 protein complex has been loaded to the DNA ORC and Cdc6 are no longer necessary for the initiation of DNA replication, suggesting that the primary role of the pre-RC may be MCM2-7p binding (Hua and Newport, 1998; Rowles et al.., 1999). Additionally, subunits of this complex exhibit helicase activity suggesting that it may act as the DNA helicase at the replication fork (Lee et al.., 2000). MCM2-7p is tightly regulated in *S. cerevisiae* as during G2 and M when they are phosphorylated and exported

from the nucleus (Nguyen et al.., 2000; Labib et al.., 1999). In metazoans, however, MCM2-7p proteins remain in the nucleus but their association with chromatin is substantially weakened as cells proceed though S phase, possibly due to their phosphorylation (Lei and Tye, 2001).

Another method for regulating the activation of the pre-RC, which is completed by MCM2-7p binding, involves the utilization of cyclin-dependent protein kinases (CDKs). Genetic studies in *S. cerevisiae* and *S. pombe* revealed that inactivation of CDK in G2/M cells resulted in full re-replication of the genome, suggesting that CDK plays a role in preventing re-replication (Dahmann et al.., 1995; Nishitani and Nurse, 1995). After replication initiation, CDK is responsible for the phosphorylation of Cdc6 which results in degradation (yeast) or export from the nucleus (humans) (Drury et al.., 1997; Saha et al.., 1998). CDK has also been found to affect replication initiation by the phosphorylation of Cdc45p, which leads to its recruitment to chromatin (Zou and Stillman, 1998).

Cdc45p is an essential factor for the transition from pre-RC to replication initiation. In *S. cerevisiae* mutants in Cdc45p result in polymerases α and ε not localizing to replication origins (Aparicio et al.., 1999). Additionally, studies in *Xenopus* have revealed that Cdc45p interacts with DNA pol α and is required for loading it onto chromatin (Mimura and Yakisawa, 1998). Cdc45p is incorporated into the replication fork and has been shown in yeast to co-localize with polymerases (Zou and Stillman, 2000). In addition, Cdc45p has been found to associate with MCM2-7p suggesting that it may play a role in coordinating MCM2-7p and polymerase function (Zou and Stillman, 2000; Kukimoto et al.., 1999).

Another complex important for origin activation, as well as, the recruitment of Cdc45p is the Dbf4-dependent kinase (DDK). This complex is composed of the Cdc7 protein kinase which is constitutively expressed and Dbf4p which is only present during S phase. DDK has been found to associate with chromatin at the beginning of S phase in both *S. cerevisiae* and *Xenopus* (Jares and Blow, 2000; Weinreich and Stillman, 1999). *In vitro* experiments have also revealed that DDK is able to phosphorylate the MCM complex, the catalytic subunit of DNA pol α/primase and Cdc45p (Weinreich and Stillman, 1999; Nougarede et al.., 2000). Additionally, DDK may be responsible for recruiting Cdc45p at the time of initiation. Studies in both *S. cerevisiae* and *Xenopus* have also shown a strong dependence on DDK for the association of Cdc45p with chromatin (Jares and Blow, 200; Zou and Stillman, 2000).

MCM10 is another protein required for the initiation of DNA replication (Maine et al., 1984). Mutants in MCM10 have revealed defects in replication fork progression, including pausing of replication forks (Kawasaki et al., 2000). This suggests that MCM10 may play a role in DNA elongation. Additionally, studies in *Xenopus* have revealed that MCM10p chromatin binding requires bound MCM2-7p and that depletion of MCM10p blocks the loading of Cdc45p (Wohlschlegel et al., 2002). This observation suggests that MCM10p may also play a role at the transition state from pre-RC to DNA elongation.

One protein that functions only during elongation and not initiation is MCM8p. MCM8p has been found in both *Xenopus* and humans and has been shown to bind chromatin later than MCM2-7p (Gozuacik et al., 2003; Maiorano et al., 2005). MCM8p has shown both DNA helicase and DNA-dependent ATPase activity *in vitro*. Additionally, loss of MCM8p *in vivo* results in a decrease in the rate of DNA synthesis suggesting that

MCM8 may play a role in elongation, potentially as a replication fork helicase. This assumption is supported by the observation that MCM8p only associates with chromatin after replication initiation and the recruitment levels of RPA and DNA polymerase α is reduced (Maiorano et al., 2005).

In summary, DNA replication origin recognition and initiation of DNA synthesis is a complex and highly regulated process. This involves specific recruitment of proteins to form the pre-RC complex, as well as the action of multiple cell-cycle regulated proteins to ensure that replication is only initiated once per cell cycle at each origin. The combination of both cis-acting replicator elements and trans-acting regulatory factors work together to promote and regulate DNA replication.

Cell cycle checkpoints and DNA damage

As described in the above section cell cycle control is utilized to guarantee that each segment of the genome is replicated once per cell cycle. However, cell cycle checkpoints are not solely used to regulate origin firing. S phase checkpoints are also activated in response to DNA damage. This is necessary to ensure that cells that have not completed S phase, or that have DNA damaged severely, do not proceed through mitosis or meiosis and subsequent cell division resulting in the propagation of compromised genomes. Eukaryotic cells from yeast to humans have evolved DNA damaged checkpoints at specific points during the cell cycle to monitor genome composition and maintain the integrity of DNA passed to daughter cells.

Cells can incur DNA damage from either cellular metabolites, DNA replication errors or by exogenous DNA-damaging agents such as ionizing or ultraviolet radiation.

Once damage is incurred there are four responses cells may undertake: repair of the damage, arrest of cell cycle, transcribe checkpoint and repair genes or induce apoptosis. The primary repair strategies cells employ involve the direct reversal of DNA lesions, excision of damaged DNA, or rejoining of double strand breaks (Ward and Chen, 2004).

Checkpoint signaling pathways may be activated in order to allow cell the time to repair various types of DNA damage. This occurs by identification of the damage and amplification of the signal so as to transiently arrest or slow cell cycle progression. These pathways may also induce transcriptional programs to enhance DNA repair. Initial recognition of DNA damage is coincident with the formation of large multiprotein complexes that surround the lesion (Nelms et al., 1998). Proteins involved in this initial recognition include the MRN complex, 53BP1, and histone H2AX (Mirzoeva and Petrini, 2001; Rappold et al., 2001; Rogakou et al., 1999).

Two essential players central to all DNA damaged-induced checkpoint responses are ATM and ATR. ATM and ATR are large protein kinases that are conserved from yeast to mammals. ATM is primarily activated in response to DNA double strand breaks (DSB), while ATR is activated by a wider array of damage including stalled replication forks (Suzuki et al., 1999; Unsal-Kacmaz et al., 2002). Upon activation these proteins have been found to associate with DNA at these damaged sites, ATM localizing to DSBs and ATR to stalled replication forks (Tibbetts et al., 2000; Andegeko et al., 2001). Additionally, these activated kinases serve to amplify the DNA damage signal by phosphorylating various substrates.

ATM and ATR are considered to be at the top of the DNA-damage checkpoint signaling pathways acting as sensors for DNA damage. These kinases induce cell cycle

checkpoints by activating the downstream kinases Chk2 and Chk1, respectively (Matsuoka et al., 2000; Brown and Baltimore, 2003; Yarden et al., 2002). Chk2 and Chk1 target cell cycle machinery such as Cdk2/cyclin E to induce cell cycle arrest at the G1/S or intra-S checkpoints (Costanzo et al., 2000; Mailand et al., 2000; Painter and Young, 1980). After phosphorylation by either ATM or ATR, Chk2 and Chk1 phosphorylate Cdc25A resulting in its unbiquitination and degradation (Bartek et al., 2001a). This prevents Cdc25A from activating Cdk2/cyclin E which is responsible for the recruitment of Cdc45 to replication origins. This prevents new origins from firing (Costanzo et al., 2000; Painter and Young, 1980).

ATM and ATR are also found to induce a cell cycle block at the G2/M boundary (Xu et al., 2002; Brown and Baltimore, 2003). This prevents cells from progressing through mitosis with damaged DNA. As in the G1/S and S phase arrests, ATM and ATR activate the downstream kinases Chk2 and Chk1 which in turn phosphorylate the mitosis-promoting Cdc25C (Brown and Baltimore, 2003; Yarden et al., 2002). Phosphorylation of this protein results in its export to the cytoplasm preventing Cdc25C from activating the Cdc2/B1 complex. The loss of Cdc25C activation results in cell cycle arrest in G2 (Peng et al., 1997; Sanchez et al., 1997).

Another important protein complex in DNA damage response is MRN. This complex includes Mre11, Rad50, and Nbs1, all of which are highly conserved. MRN is important for ATM and ATR regulation as the loss of Mre11 blocks the activation of both ATM and ATR (Carson et al., 2003). Additionally, NBS1 is a substrate for ATM and its subsequent phosphorylation is required for G1 and G2 checkpoint arrests (Buscemi et al.,

2001; Yamazaki et al., 1998). MRN is also essential for recognizing and repairing DSB as it forms foci at regions of DNA damage (Mirzoeva and Petrini, 2001).

53BP1 is another ATM substrate that acts early in the DNA damage response. It binds to sites of DSBs by interacting with phosphorylated H2AX (γ-H2AX) (Xia et al., 2001; Ward et al., 2003). H2AX is a histone variant that is randomly incorporated into ~20-30% of nucleosomes and is phosphorylated in response to DNA damage (Rogakou et al., 1998). This phosphorylation is confined to megabase areas surrounding DSBs (Rogakou et al., 1999). 53BP1 is also phosphorylated by ATR in response to replication fork arrest. Additionally, 53BP1 has been found to play a role in the phosphorylation of ATM/ATR substrates perhaps by recruiting them to the sites of double strand breaks (DiTullio et al., 2002).

One more protein that is important for the DNA damage response and repair is Rad51. Rad51 plays and important role in homologous recombination repair as it is required for strand invasion in eukaryotic cells (Baumann et al., 1996). Rad51 catalyzes homologous DNA base pairing and strand exchange at DSBs by first forming a nucleoprotein filament on the resected 3' ssDNA overhang (Baumann and West, 1998). BRCA2 has been found to interact with Rad51 and may play a role in the loading of Rad51 onto ssDNA (Yang et al., 2002).

These are just some of the proteins that have been revealed to play roles in the identification and response to DNA damage. As seen in the case of ATM and ATR, the DNA damage response is not a direct pathway stemming from one overall damage sensor. It is becoming evident that DNA damage response proteins have multiple functions for

both sensing and responding to DNA damage. Additionally, there are many layers of regulation which make the deciphering of these pathways a challenge.

Tetrahymena as a model for DNA replication

Tetrahymena has been used as a model system for studying eukaryotic DNA replication. Studies have centered on the 21 kilobase palindromic ribosomal RNA (rDNA) minichromosome that undergoes programed amplification to 10,000 copies in a single S phase during macronuclear development. Subsequent to this the rDNA minichromosome is maintained vegetatively by being replicated only once per cell cycle. Previous studies have revealed that this chromosome contains two distinct sites for DNA replication initiation (Zhang et al., 1997). These replication origins are localized to two 430 base-pair tandem imperfectly duplicated repeats in the 1.9 kilobase 5' non-transcribed spacer (5' NTS) region and are designated Domains 1 and 2 (D1 and D2; Fig. 1.2). These domains reside in two of three nucleosome-free regions in the 5' NTS that are bracketed by positioned nucleosomes (Palen and Cech, 1984). Together D1 and D2 compose a single replicon that contain several dispersed reiterated cis-acting regulatory elements that are essential for DNA replication and localize to the nucleosome-free regions including the distal rRNA promoter (Reischmann et al., 1999). Several possibly trans-acting proteins have been discovered that bind these essential elements (Mohammad et al., 2000; Mohammad et al., 2003). This section describes in detail what is known about these cis and trans-acting factors.

Cis-acting determinants for DNA replication: the rDNA minichromosome

The origin region of the rDNA minichromosome contains several cis-acting elements that are necessary for DNA replication. Two of the elements that have been studied in detail include the type I and pause site elements (PSE). The type I element is phylogenetically conserved in the rDNA genes of tetrahymenid species (Challoner et al., 1985), and four such elements have been identified in the *T. thermophila* rDNA replicon, one in each replication origin (type IA and 1B) and two that map to the promoter region of the rRNA gene (type IC and ID; Fig. 1.2). Mutations either within or immediately downstream of these elements have been shown to cause rDNA minichromosome maintenance (rmm) defects. These rmm mutant alleles are able to proceed through development normally, but exhibit defects in vegetative propagation of the rDNA chromosome (Kapler et al., 1994; Gallagher and Blackburn, 1998). This phenotype is only evident when rmm mutants are placed in competition with wild-type rDNA alleles. Some of these mutations (rrm1, rmm4, and rmm7) co-localize with the type IB element found in the D2 origin region (Larson, et al., 1986; Yaeger et al., 1989; Zhang et al., 1997) while others (rmm3 and rmm8) are found in the type IC and ID elements in the promoter region (Gallagher and Blackburn, 1998).

Type I elements are essential for rDNA replication (Reischmann et al., 1999) and biochemical studies have revealed that the promoter-proximal elements may act to regulate replication through long-distance DNA-protein/protein-protein interactions (Gallagher and Blackburn, 1998). Additionally mutations in these promoter-proximal type I elements reveal that the type IC and ID elements are part of the basal rRNA gene promoter and consequently regulate rRNA transcription as well as rDNA replication (Pan et al., 1995).

However, mutations in this region affect either replication or transcription suggesting that these processes may be controlled by different DNA-binding factors. Type I elements have also been shown to regulate DNA replication fork movement by causing forks to arrest transiently at specific, conserved nearby PSEs (MacAlpine et al., 1997). This arrest in replication fork movement occurs in an orientation-dependent manner. Together these results indicate that type I elements regulate replication initiation, elongation of replication forks, and rRNA transcription.

In addition to the type I elements, three PSE elements (PSE1-3) are found in the rDNA replicon and coincide with sites of transient replication fork arrest (MacAlpine et al., 1997). These 52 base-pair elements are phylogenetically conserved and contain three blocks of sequence homology separated by two spacers of fixed length. PSE1 and PSE2 reside in D1 and D2 respectively and map to the 5'-border of the nucleosome-free regions of the two DNA replication origins (Fig. 1.2), while PSE3 is promoter-proximal but is not part of the minimal rRNA promoter (Miyahara et al., 1993). Additionally PSE3 has been shown to be essential for DNA replication as transformation studies where this element was replaced with random sequences yielded constructs that were not able to replicate and be maintained in *Tetrahymena* (Saha et al., 2001).

Possible trans-acting determinants for DNA replication: TIF1-4

Four distinct type I element binding factors (TIF1-4) have been identified in *Tetrahymena* extracts using electrophoretic mobility shift assays with type I element oligonucleotides as substrates. These binding activities appear to be distinct due to the fact that they exhibit differentially-regulated expression profiles as well as contrasting

molecular weights for the respective DNA binding subunits. Additionally, the differentially-regulated expression of these factors suggests that these activities could compete for type I binding *in vivo*. All four TIFs bind exclusively to single stranded DNA (Mohammad et al., 2000; Mohammad et al., 2003; Saha and Kapler, 2000).

TIF4 is an ~550 kDa multi-subunit complex found in both micronuclear and macronuclear extracts. TIF4 is an ORC-like factor that binds in a sequence specific manner to the T-rich stand of type I elements. Similar to ScORC, TIF4 demonstrates origin-specific DNA binding, chromatin association, and is a multiprotein complex that contains the Orc2-related subunit, Tt-p69. This subunit cross-reacts with antisera specific for *Xenopus* Orc2 that also recognizes *S. cerevisiae* and *Drosophila* Orc2 proteins. Antisera specific for human Orc2 also recognized this subunit. Tt-p69 associates with DNA in a cell cycle-regulated manner. TIF4 DNA binding activity peaks in S phase when *Tetrahymena thermophila* proteins re-localize from the cytoplasm to the macronucleus (Mohammad et al., 2003). This is similar to recent studies in mammals where Orc1 and Orc2 subunits cycle between chromatin and non-chromatin containing nuclear compartments corresponding with origin regulation (Kreitz, et al., 2001; Laman et al., 2001). Additionally, Tt-p69 is found to be restricted to replicating nuclear compartments during development. The loss of ORC2-Ab staining in the amplification-deficient rmm11 mutant implicates TIF4 in rDNA gene amplification (Mohammad et al., 2003).

TIF2 is an 85 kDa protein that binds to both the A-rich and T-rich strands of the type I element. Binding activity of TIF2 are increased in cells undergoing vegetative replication or rDNA gene amplification (Mohammad et al., 2000).

TIF3 is a 32 kDa protein that also binds to both the A-rich and T-rich strands of the type I element. Binding activities of TIF3 are dramatically increased in non-replicating starved cells. Additionally, binding activity of TIF3 is rapidly lost with the onset of vegetative rDNA replication suggesting that it may play a role in the negative regulation of rDNA replication (Mohammad et al., 2000; Saha et al., 2001).

TIF1 is a 21 kDa protein that forms a homotetramer *in vivo*. TIF1 binds specifically to both the A-rich or T-rich single-strand DNA of the type I element suggesting that it may stabilize unwound DNA in these regions. TIF1 also binds the essential PSE elements as well. Footprinting studies also revealed that TIF1 regulates the occupancy of origin and promoter-proximal type I and PSE elements *in vivo* (Saha et al., 2001) . Additionally, TIF1 binds specifically the A-rich strand at the origin and T-rich strand at the promoter suggesting that TIF1 might play a role in facilitating TIF4 binding to the T-rich origin strand, or function at a later step to regulate origin firing.

Biology of *Tetrahymena thermophila*

In this dissertation I use *Tetrahymena thermophila* as model system to study DNA replication. I sought out to determine the role(s) for a protein, TIF1, that binds to a cis-acting determinant, the type I element, that is essential for the initiation of replication at the well-studied rDNA origin of replication. *Tetrahymena* is a ciliated protozoan, and as is typical of ciliates, contains two structurally and functionally different nuclei, the micro- and macronucleus (Fig 1.3A). The micronucleus functions as the germline nucleus and contains all genetic information necessary for genetic transmission. It contains five diploid chromosomes that are transcriptionally silent (Gorovsky and Woodard, 1969; Yao and

Gorovsky, 1974; Mayo and Orias, 1981). During cell division and conjugation this nucleus undergoes traditional mitosis and meiosis.

The larger macronucleus is the somatic nucleus and is transcriptionally active, expressing all genes necessary for vegetative growth and propagation of the cell (Bruns and Brussard, 1974; Mayo and Orias, 1981). This nucleus does not pass along any genetic information during conjugation. The macronucleus contains ~280 autonomously replicating chromosomes that are derived from site-specific fragmentation of the five micronuclear chromosomes during sexual development. With one exception, chromosomes are present at ~45 copies per macronucleus (Woodard et al., 1972). This nucleus divides amitotically as there is no visible mitotic spindle as seen in traditional mitosis. Instead the macronucleus elongates and constricts, such that approximately half of the macronuclear DNA is distributed to each daughter nucleus during vegetative cell division (Orias, 1991). The absence of a mitotic segregation mechanism allows for phenotypic assortment of differing alleles of a particular locus as minichromosomes segregate randomly during cell division (Sonneborn, 1974).

Tetrahymena divides by binary fission during vegetative growth. This is a completely asexual process and occurs when the micronucleus is in the diploid state (Orias, 1991). *Tetrahymena* also undergoes a sexual phase called conjugation (Martindale et al., 1982; Orias, 1986). In order to perform conjugation cells must first be starved for at least one nutrient, be of different mating types and have reached sexual maturity, which occurs ~70 cell divisions after the prior conjugation (Allewell et al., 1976). Cells conjugate by forming temporary junctions that allow for the reciprocal exchange of gametic pro-nuclei (Wolfe, 1982). Exchange is followed by fertilization and nuclear

differentiation during which the old parental macronucleus is destroyed and replaced by a new macronucleus that is derived from the new micronucleus of progeny.

Nuclear events during Tetrahymena *conjugation*

In this work I utilized matings between TIF1 mutants and wild-type cells to examine the effects of TIF1 depletion on *Tetrahymena* development. By taking advantage of immunofluorescence techniques I was able to observe early events in development including micronuclear meiosis, pronuclear exchange, and macronuclear development. This section describes developmental landmarks during conjugation that are visible with DNA nuclear stains (Cole et al., 1997; Cole and Soelter, 1997).

Once two *Tetrahymena* cells have formed mating pairs (Fig. 1.3B) the micronucleus moves away from the macronucleus and elongates increasing its length by over 50-fold. This micronuclear 'crescent' stretches the entire length of the cell and curves around the macronucleus (Fig. 1.3C) (Wolfe et al., 1976). Following crescent formation chromosomes condense (Fig. 1.3D) and undergo two rounds of meiosis (Fig. 1.3E and G). After the first round of meiosis one of the nuclei will move to the anterior portion of the cell (Fig 1.3E). Chromosomes then diffuse into nuclear spheres and the posterior micronucleus moves to the anterior portion of the cell where it joins the other micronucleus (Fig. 1.3F). Then both nuclei will undergo meiosis II (Fig. 1.3G). Anaphase will again deliver five chromosomes to the posterior portion of the cell and chromosome will defuse for a second time forming four micronuclei (Fig. 1.3H) (Cole et al., 1997).

Following both rounds of meiosis the four micronuclei will undergo a process termed 'nuclear selection' where one of the micronuclei moves to the anterior portion of

the cell and the other three degenerate in the posterior of the cell (Fig. 1.3H) (Gaertig and Fleury, 1992). The selected micronucleus then undergoes mitosis forming two genetically identical pronuclei (Fig. 1.3I). One of these pronuclei will migrate to the fusion point of the mating pair and be transferred to the mating partner (Fig. 1.3J) while the other pronucleus remains in the cell of origin (Kaczanowski, et al., 1991). After pronuclear exchange the migratory and stationary pronucleus will both migrate to the anterior of the cell (Fig. 1.3K). At this point the two pronuclei will fuse to form the fertilized micronucleus (Fig. 1.3L).

This new fertilized micronucleus serves as the progenitor for both the new micro- and macronucleus of the developing cell. After nuclear fusion, the new micronucleus undergoes the first of two postzygotic rounds of mitosis causing one of the nuclei to move to the posterior of the cell (Fig. 1.3M) (Kaczanowski, et al., 1991). Once this occurs, the nuclei undergo the second round of mitosis (Fig. 1.3N). Following mitosis the chromatin of the nuclei remaining in the anterior decondenses and begin to form new macronuclei (Fig. 1.3O) (Martindale et al., 1982). This is the first stage of macronuclear development. During the second stage of macronuclear development the two micronuclei and developing macronuclei move to the center of the cell (Fig. 1.3P). The mating pair then separates (Fig. 1.3Q). After mating pair separation the old macronucleus moves to the posterior of the cell where it undergoes condensation and apoptotic DNA fragmentaion (Mpoke and Wolfe, 1996). At this point one of the micronuclei will also be degraded (Fig. 1.3R). Finally the remaining micronucleus will undergo mitosis and the separated cells will each divide by binary fission to produce four cells which are the progeny of the mating pair.

During the above mentioned macronuclear differentiation developmentally programmed DNA rearrangements occur. One such process involves the site specific fragmentation of the five micronuclear chromosomes into ~280 minichromosomes that range in size from ~1,000 base pairs to a few megabases (Yao, 1982; Yao et al., 1987). During this developmental program the excised macronuclear chromosomes undergo *de novo* telomere addition and are amplified to ~45 copies (Fig 1.4A) (Fan and Yao, 1996). Subsequently these chromosomes are replicated only once per cell cycle during vegetative cell divisions (Prescott, 1994).

Another programmed DNA rearrangement that occurs during macronuclear development is rDNA minichromosome rearrangement and amplification. During this process the single copy of the *Tetrahymena* gene for the 35S ribosomal RNA precursor is excised from the micronuclear genome (Fig. 1.4B). This 10.5 kilobase fragment then undergoes head-to-head palindrome formation resulting in a 21 kilobase inverted repeat to which telomers are added *de novo* (Gall, 1974; Yasuda and Yao, 1991). The rDNA is amplified to 10,000 copies in the developing macronucleus (Yao and Gorovsky, 1974). As for the previously described chromosomes rDNA amplification occurs during a single S phase and the resulting minichromosomes are maintained at this level during subsequent vegetative division (Karrer and Blackburn, 1989).

Transformation methods in Tetrahymena: *biolistic transformation*

In my dissertation research on the TIF1 protein, I used homologous gene targeting to knockout expression of the TIF1 gene. This was accomplished by targeting a linear DNA fragment containing the neomycin drug resistence gene. The neocassette was flanked by sequences homologous to the 5' and 3' ends of the TIF1 gene in the

transformation vectors. These linear ends are recombinagenic and will be specifically targeted to the TIF1 locus (Cassidy-Handley et al., 1997). I used biolistic transformation to introduce DNA into *Tetrahymena*.

Biolistic transformation can be used to transform the micro- or macronucleus. This method uses helium pressure to accelerate DNA-coated gold particles into target cells. Targeting of the micronucleus can be accomplished by transforming matings during early conjugation, ~3.5 hours after mating. Developing macronuclei can be targeted later in development (~8.5 h). Alternatively the vegetative macronucleus can be transformed in starved non-mating cells (Cassidy-Handley et al., 1997). In this dissertation I utilized the methods of micronuclear transformation and vegetative macronuclear transformation for gene replacement.

Transformation of the micronucleus is advantageous for studying gene disruptions or replacements by incorporating the target sequence into the germline of cells. The effects of transformed DNA can then be observed as cells undergo conjugation and subsequent development. Furthermore, a mutation can be propagated from one generation to the next since the mutation resides in the germline. In contrast, macronuclear transformation can only be used to study the effects of a mutation on vegetatively cycling cells as the micronucleus of transformed cells remains wild-type. Both methods of transformation have been found to be useful in genetic studies (Cassidy-Handley et al., 1997).

After micronuclear transformation, the resulting transformants are heterozygous in the micronucleus and macronucleus for the transformed DNA due to homologous recombination at the targeted locus. In the case of a gene replacement or knockout, these

transformed micronuclear heterozygotes can be induced to generate micronuclei that are homozygous for the mutation of interest but contain a wild-type macronucleus by utilizing an abortive developmental program called genomic exclusion (Fig. 1.5). Progeny from such a mating remain sexually mature, due to the retention of the parental macronucleus, and when mated again give rise to progeny that are genetically homozygous at all loci. These two consecutive rounds of conjugation have been termed Round 1 and Round 2 genomic exclusion (Allen, 1967b).

Round 1 genomic exclusion occurs when wild-type cells are mated to cells with a defective micronucleus, (referred to as star strains, (Allen, 1966). In these matings the micronucleus of the star strain is degraded and contributes no genetic information to progeny. Instead the wild-type mating partner will undergo unidirectional exchange of one pronucleus to the star strain. After exchange the haploid pronucleus becomes diploid through the process of endoreplication (Allen, 1966; Doerder and Shabatura, 1980). At this point, instead of completing conjugation and generating a new macronucleus, cells separate prematurely. This results in progeny that are heterokaryons and harbor a new micronucleus but retain the parent macronucleus (Gaertig and Kaczanowski, 1987).

Considering that both heterokaryons resulting from Round 1 conjugation each contain a diploid micronucleus derived from the wild-type mating partner, Round 2 conjugation proceeds with no abnormalities. However, the resulting progeny of this mating will all be homozygous for the original wild-type strain as all genetic information in star strain heterokaryons is derived from the exchanged wild-type pronucleus. Progeny are sexually immature as they have generated a new macronucleus and destroyed their parental macronucleus.

In order to generate a homozygous macronuclear knockout a heterozygous micronuclear knockout strain is mated to star strains of two different mating types, such as A* III and A*V. These cells are allowed to undergo Round I conjugation to generate heterokaryons that have either the parental macronucleus of the A*III or A*V strain. The micronuclei of these progeny will be homozygous for either the untransformed wild-type allele or the introduced mutant allele.

At this point, if the knockout disruption encodes a drug resistance marker, the Round 1 heterokaryons are screened for drug sensitivity (Bruns and Brussard, 1974). This allows for the identification of cells that retained the A* macronucleus. PCR is then used to genotype the micronucleus. Homologous germline knockouts of different mating types are then mate to one another to allow for Round 2 conjugation and the formation of progeny that are homozygous for the transformed allele in the macronucleus. If the null state is not lethal, viable progeny will express the drug resistance allele in their new macronucleus.

An alternate approach can be used to titrate the wild-type copies of a gene in the amitotic macronucleus. This process, termed phenotypic assortment, involves transformation of the macronucleus with a gene disruption construct (Sonneborn, 1974). Since the macronucleus undergoes amitotic division, wild-type and mutant alleles segregate randomly at the end of each cell division. This results in an unequal distribution of alleles to each daughter cell. If the transforming allele contains a drug resistance marker this process may be exploited by successively passaging cells in increasing concentrations of the drug. This technique will select for cells that contain more copies of the drug resistance marker. By gradually increasing the drug concentration it is possible to select

for cells that are homozygous or near homozygous for the allele of interest (Shen et al., 1995). However, if the transformed allele is deleterious for cell growth it may not be possible to completely assort away the wild-type allele (Liu et al., 1996; Hai and Gorovsky, 1997).

In this dissertation I use gene replacement strategies to disrupt the TIF1 gene so as to examine the role of this protein in chromosomal processes such as DNA replication. I employ methods such as genetic analysis, immunofluorescence techniques, flow cytometry and 2D gel analysis in order to determine the affect of decreased amounts of TIF1 protein in *Tetrahymena*. My studies revealed a role for TIF1 in DNA replication and the DNA damage response and indicate that this protein is essential for micronuclear genome stability.

CHAPTER II

TIF1 REPRESSES rDNA REPLICATION INITIATION, BUT PROMOTES NORMAL S PHASE PROGRESSION AND CHROMOSOME TRANSMISSION IN *Tetrahymena*

OVERVIEW

The non-ORC protein, TIF1, recognizes sequences in the *Tetrahymena thermophila* ribosomal DNA (rDNA) minichromosome that are required for origin activation. We show here that TIF1 represses rDNA origin firing, but is required for proper macronuclear S phase progression and division. TIF1 mutants exhibit an elongated macronuclear S phase and diminished rate of DNA replication. Despite this, replication of the rDNA minichromosome initiates precociously. Since rDNA copy number is unaffected in the polyploid macronucleus, mechanisms that prevent re-initiation appear intact. Although mutants exit macronuclear S with a wild-type DNA content, division of the amitotic macronucleus is both delayed and abnormal. Nuclear defects are also observed in the diploid mitotic micronucleus, as TIF1 mutants lose a significant fraction of their micronuclear DNA. Hence, TIF1 is required for the propagation and subsequent transmission of germline chromosomes. The broad phenotypes associated with a TIF1-deficiency suggest that this origin binding protein is required globally for the proper execution and/or monitoring of key chromosomal events during S phase and possibly at later stages of the cell cycle. We propose that micro-and macronuclear defects result from exiting the respective nuclear S phases with physically compromised chromosomes.

INTRODUCTION

The initiation of eukaryotic DNA replication is regulated by protein-DNA interactions that occur within defined chromosomal domains, termed replicators or replicons. Eukaryotic replicators are modular and contain binding sites for the conserved six-subunit origin recognition complex (ORC) (Bell and Stillman, 1992) and non-ORC DNA binding proteins (Marahrens and Stillman, 1992). ORC plays a central role, recruiting proteins involved in replication initiation and elongation to form the pre-replicative complex (pre-RC). These proteins include a replicative helicase -- the minichromosome maintenance (MCM) complex (Ishimi, 1997; Labib et al., 2000) and factors that regulate origin activation, such as Cdc6 and Cdt1 (Nishitani et al., 2000; Oehlmann et al., 2004).

While ORC binding sites are plentiful in the *S. cerevisiae* (Sc) genome, only a fraction are routinely engaged in replication initiation (Theis and Newlon, 1993; Wyrick et al., 2001). Genetic studies indicate that ScORC binding is necessary, but not sufficient to confer replicator status to a chromosomal domain. Whereas ScORC binds DNA in a sequence-specific manner, metazoan ORCs exhibit no obvious sequence-specificity, displaying a preference for degenerate, asymmetric A:T-rich sequences *in vitro* (Austin et al., 1999; Chesnokov et al., 2001; Vashee et al., 2001) and *in vivo* (Kong et al., 2003; Vashee et al., 2003). This relaxed specificity is similar to *Schizosaccharomyces pombe* (Sp) ORC, which binds DNA via an unusual A-T hook domain in its Orc4 subunit (Chuang and Kelly, 1999; Kong and DePamphilis, 2001). While metazoan ORCs lack AT hooks, they still associate with specific replicator domains *in vivo* (Austin et al., 1999; Abdurashidova et al., 2004).

The contribution of non-ORC DNA binding proteins to replication initiation is less well understood. Several proteins have been shown to impart replicator status to a given chromosomal site. For example, *S. cerevisiae* ABF1 appears to function primarily as a physical barrier that prevents nucleosomes from invading the adjacent ORC binding site (Vendetti et al., 1994; Lipford and Bell, 2001). By comparison, localization of DmORC to the chromosome 3 chorion gene locus may be facilitated by interactions with a sequence-specific DNA binding complex (Beall et al., 2002). Genetic experiments indicate that this myb-containing complex facilitates chorion gene amplification, but represses replication at sites in the genome in terminally differentiated follicle cells (Beall et al., 2004). Consequently, the selective activation of chorion gene replicons involves additional layers of regulation.

Similar to metazoan replicators, the *Tetrahymena thermophila* ribosomal DNA (rDNA) minichomosome contains dispersed cis-acting replicator elements, including essential determinants that either co-localize to replication initiation sites or act at a distance. The 1.9 kb 5' non-transcribed spacer (5' NTS) is necessary and sufficient for developmentally-programmed amplification and cell cycle-regulated replication of the macronuclear rDNA minichromosome (reviewed in Kapler et al., 1996). Origin-proximal and distal type I elements act in concert to control replication which initiates within two nucleosome-free regions (Fig. 1A) (Larson et al., 1996; Blomberg et al., 1997; Zhang et al., 1997; Gallagher and Blackburn, 1998; Reischmann et al., 1999). In addition to their role in replication initiation, type I elements induce the pausing of replication forks at adjacent pause site elements (PSEs) (MacAlpine et al., 1997). Promoter-proximal type I elements are also required for ribosomal RNA transcription (Gallagher and Blackburn,

1998). Separation-of-function alleles support a model in which replication and transcription are regulated by different type I element binding factors. Whether these factors compete or cooperate to regulate origin firing is unknown.

Type I elements are recognized by four distinct DNA binding activities *in vitro*, designated **T**ype **I** **F**actors (TIF1, TIF2, TIF3 and TIF4) (Umthun et al., 1994; Mohammad et al., 2000). In contrast to ORC and non-ORC replicator proteins in other systems, all four TIFs associate exclusively with single-stranded DNA *in vitro* (Mohammad et al., 2000; Saha and Kapler, 2000, Mohammad et al., 2003). Consistent with an *in vivo* role for these sequence-specific single-stranded DNA binding proteins (ss-SSBs), *in vivo* footprinting studies indicate that the *Tetrahymena* rDNA origin and promoter regions are naturally unwound in native chromosomes (Saha et al., 2001). The multi-subunit TIF4 complex (MW ~550 kDa) shares several biochemical properties with ScORC (Mohammad et al., 2003). Like ScORC, TIF4 recognizes a single known target that co-localizes with the site of replication initiation. TIF4 binding to the type I element T-rich strand, is both ATP-dependent and sequence-specific. TIF4 contains an Orc2-related subunit, Tt-p69, which cross-reacts with antibodies that recognize yeast, *Drosophila* and human Orc2 proteins. Tt-p69 associates with DNA in a cell cycle-regulated manner, similar to the metazoan Orc1and Orc2 subunits (Kreitz et al., 2001; Fugita et al., 2002; Li and DePamphilis, 2002). Tt-p69 re-localizes from the cytoplasm to macronucleus during vegetative S phase (Mohammad et al., 2003). Furthermore, Tt-p69 is restricted to replicating nuclear compartments during development and has been implicated in rDNA gene amplification.

The TIF1 homotetramer (subunit MW 21 kDa) exhibits a more relaxed sequence specificity than TIF4, binding to the A-rich or T-rich strand of type I elements or adjacent PSEs, but not to the respective duplex DNA substrates. PSEs map to the 5' border of the nucleosome-free origin regions (MacAlpine et al., 1997) and are required for replication of the rDNA minichromosome (Saha et al., 2001). Footprinting studies revealed that TIF1 modulates the occupancy of origin- and promoter-proximal PSE and type I elements *in vivo* (Saha et al., 2001). Remarkably, TIF1 affects the footprint on the A-rich strand at the origin and T-rich strand at the promoter. Consequently, TIF1 might facilitate TIF4 binding to the T-rich origin strand, or function at a later step to regulate origin firing.

An important event in the *Tetrahymena* life cycle is the programmed amplification of rDNA minichromosomes. The rDNA minichromosome is formed as part of a genetic program that transforms a transcriptionally-silent, diploid (germline) micronucleus into a transcriptionally-active polyploid (somatic) macronucleus. The single copy 10.3 kb ribosomal RNA gene is excised from its parental chromosome by site-specific fragmentation, rearranged into a palindromic 21 kb minichromosome and amplified to ~9,000 copies in a single S phase (Yao et al., 1974). The remainder of the five germline chromosomes are fragmented into ~280 segments that attain a copy number of 45. During subsequent vegetative growth, macronuclear chromosomes replicate once (on average) per cell cycle. Although these chromosomes lack centromeres, genic balance is somehow maintained in the macronucleus (Doerder, 1979; Preer and Preer, 1979; Pan and Blackburn, 1995). In contrast, micronuclear chromosomes segregate by conventional mitosis and meiosis.

Here we assess the role of the non-ORC rDNA origin binding protein, TIF1, in DNA replication. We demonstrate that TIF1 is a negative regulator of rDNA replication. TIF1-deficient cells replicate the rDNA minichromosome precociously, but exhibit a delay in macronuclear S phase progression that is associated with a diminished rate of DNA replication. TIF1 mutants also undergo aberrant macronuclear division, despite the fact that they exit S phase with a normal DNA content. TIF1's role is not restricted to macronuclear functions, as the mutant also fails to faithfully propagate chromosomes in the mitotic, diploid germline micronucleus. Thus, TIF1 plays an important role in the replication and transmission of chromosomes in these two distinct nuclear compartments.

MATERIALS AND METHODS

Tetrahymena strains and DNA transformation

Tetrahymena thermophila strains were cultured as previously described (Kapler and Blackburn, 1994). The wild-type strain CU428 was used for comparative studies with TIF1 gene replacements. The TIF1 gene disruption plasmid (pTIF1::neo) was generated by replacing the TIF1 coding region with a -tubulin promoter-driven neomycin phosphotransferase (neo) gene. The plasmid insert was released from the vector backbone by restriction digestion for homologous targeting in the micro- or macronucleus.

Mating cultures (strains CU427 x B2086) were transformed by biolistic bombardment at 3.5 h after mixing to generate germline transformants that underwent targeted homologous recombination in the micronucleus (Cassidy-Hanley et al., 1997). Cells that expressed the transforming neo gene were selected for resistance to 150 μg/ml paromomycin (pm). Heterozygous germline transformants were subsequently cultured in

the absence of drug selection. Gene replacements were initially verified by PCR using TIF1 and neo-coding region primers. The genotypes of heterozygous germline transformants (TXh102 and TXh106) and additional strains were verified by PCR, Southern blot analysis and/or mating to wild-type testers as previously described (Kapler et al., 1994).

TIF1:neo heterokaryons were generated by mating sexually mature heterozygous TIF1::neo micronuclear transformants with the functionally-amicronucleate A* strains (mating type III or V). Since A* strains contribute no genetic information to progeny, a process termed Round 1 genomic exclusion generates progeny that are homozygous at all loci in the micronucleus, but retain the macronucleus from the A* or transformant parent (Allen, 1967a). Pm-sensitive progeny that expressed the mating type of the A* parent and contained the TIF1::neo allele in the micronucleus were isolated. A single homozygous null TIF1 strain, TXk202, was subsequently generated by mating homozygous TIF1 germline knockout heterokaryons (TXa28 and TXa42) to one another and selecting for pm-resistant progeny. Micronuclear genome stability of homozygous TIF1:neo heterokaryons (TXa28 and TXa42) and the homozygous TIF1 knockout (TXk202) was assessed by mating these mutants with heterokaryon strains (CU354, CU357, CU361 and CU371) that are nullisomic for micronuclear chromosome 2, 3, 4 or 5, respectively, but contain a wild-type macronucleus.

Transformation of the vegetative macronucleus was achieved by biolistic transformation of starved CU428 cells. Transformant strains TXh48 and TXh29 were cultured in increasing concentrations of pm (150-4500 μg/ml) to select for cells with decreasing amounts of the wild-type TIF1 gene in the macronucleus. Random assortment

of amitotic macronuclear chromosomes (~45C) was exploited in an attempt to generate complete macronuclear gene replacements (reviewed in Turkewitz et al., 2002).

Molecular biology techniques

Standard molecular biology techniques, including Southern blotting, northern blotting, electrophoretic mobility shift assays (EMSA) and RT-PCR were performed as described (Mohammad et al., 2000; Saha et al., 2001). DNA and RNA hybridization signal were quantified on a Biorad Molecular Imager FX PhosphoImager. Quantitation of rDNA chromosome copy number was achieved by determining the ratio of the rDNA hybridization signal to two non-rDNA probes derived from either a large (>1000 Kb) and small (50 Kb) macronuclear chromosome in wild-type and TIF1-deficient strains. RNA samples were prepared using an RNAeasy mini-kit (Qiagen) according to the manufacturer's recommendations.

Cell cycle synchronization

Cell cycle synchronization was achieved by starvation for at least 8 h and refeeding, or by modification of a stationary phase synchronization protocol in which saturated cultures are placed in starvation medium for 8 h prior to dilution into growth medium at a density of 0.6×10^5 cells/ml (Mohammad et al., 2003). Cells were collected at each time point and incubated with 100 μg/ml bromo-deoxyuridine (BrdU) (Sigma Chemical) for 15 min to assess micro- and macronuclear DNA synthesis by immunofluorescence microscopy (see below). Alternatively, cells were radiolabeled for 15 min at 30 min intervals with tritiated thymidine (Perkin Elmer, 79Ci/mmol) at a final

concentration of 5 μCi/ml. Thymidine incorporation was measured by liquid scintillation counting of trichloroacetic acid precipitates.

Two-dimensional gel electrophoresis of rDNA replication intermediates

DNA samples were prepared from re-fed stationary phase synchronized cultures harvested at 30 min intervals. For each time point, twenty micrograms of *Hind*III-digested total genomic DNA was resolved by two-dimensional gel electrophoresis and hybridized to an rDNA 5' NTS probe (Zhang et al., 1997).

Immunofluorescence studies

For mating experiments, wild type strains (CU427, CU428 and B2086) were distinguished from TIF1 knockout (TXk202) and knockdown (TXh48, TXh29) strains by incorporation of Mitotracker Green FM or Red-CMXRos dyes (Molecular Probes) during overnight starvation of pre-mating (single strain) cultures. Reciprocal labeling experiments revealed that these dyes do not alter the phenotypes described in the results section. Cell preparation and fluorescence microscopy were performed essentially as previously described (Marsh et al., 2004). 1 ml mating cultures were harvested at selected developmental time points, washed sequentially with 1 ml of distilled water, 50% methanol, 70% methanol, and 70% methanol/15% acetic acid fixative. Cells were resuspended in 100 μl of methanol/acetic acid fixative, dropped onto microscope slides from a height of 30-60 cm and air-dried. Slides were sequentially dipped in 95% ethanol (15 sec), 0.1 μg/ml 4', 6'-diamidino-2-phenylidole (DAPI, Sigma Chemical) in 70% ethanol /300 mM NaCl) (1 min), 70% ethanol (15 sec), 35% ethanol (15 sec), and

examined by fluorescence microscopy. Live cells were stained with Apofluor (0.001% acridine orange and 5 μg/ml Hoescht 33342/ml) and examined by fluorescence microscopy.

For cell cycle analysis of DNA replication, BrdU labeled cells (15 ml cultures) were harvested by centrifugation, washed with 10 mM Tris (pH 7.4), and incubated in 2 ml of PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) + 0.5% Triton X-100 for 3 min. Cells were re-centrifuged, fixed in 1 ml PHEM + 3% paraformaldehyde for 30-60 min at 4^0C, and washed 3 times with phosphate buffered saline (PBS) (Marsh et al., 2004). Fixed cells were then placed in PBT blocking buffer (PBS + 3% BSA/0.1% Tween 20) for >1 h at 4^0 C. Cells were sequentially incubated at room temperature (RT) for 20 min with PBT + 5% normal donkey serum (Jackson ImmunoResearch Laboratories), 1 h with anti-bromo-deoxyuridine antibody (mouse monoclonal, Amersham-Pharmacia Biotech) according to the manufacturer's specifications and 1 h with rhodamine-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, 1:100 dilution). Cells were resuspended in 0.1 μg/ml DAPI (Sigma) for 10 min and washed twice with PBS, and mounted onto slides in glycerol:PBS (9:1) for microscopy examination. The percentage of BrdU-labeled nuclei was determined for 300-500 cells per time point.

Flow cytometry

Vegetative wild type (CU428) and TIF1 KO (TXk202) cultures were starved overnight and refed at a density of $1.0x10^5$ cells/ml. Cells were harvested at 1h intervals, resuspended in 0.5 ml PBS + 4.5 ml of 70% ethanol and incubated for 2 h at 4^0C. Samples were washed at

RT with 0.5 ml PBS followed by staining for 30 min in PBS + 0.1 % Triton X-100, 0.02 mg/ml propidium iodide, 0.2 mg/ml RNAseA (Darzynkiewicz et al., 2003). Cell fluorescence was measured on a Becton Dickenson (FACScalibur) flow cytometer.

RESULTS

TIF1 binds to dispersed, genetically-defined replicator sequence elements *in vitro* (Fig. 1A, type I elements and PSEs) and modulates the *in vivo* footprint of the rDNA replication origins and promoter in native chromosomes (Saha et al., 2001). To better understand the biological role of TIF1, we examined vegetative cell division and development in strains carrying partial or complete replacements of the wild-type TIF1 gene.

TIF1 DNA binding activity and mRNA levels are cell cycle regulated

The ability of TIF1 to alter the *in vivo* occupancy of the rDNA origin suggests a role for this protein in the initiation of DNA replication. Since many S phase-specific genes are subjected to cell cycle regulation at the RNA or protein level, we examined TIF1 steady state mRNA and protein (*in vitro* DNA binding activity) levels in synchronized vegetative cultures. Affinity-purified TIF1 generates three distinct DNA:protein complexes that can be resolved by electrophoretic mobility shift analysis (EMSA) in a Tris:glycine buffer (Saha et al., 2001). The EMSA profile of extracts prepared from starved/refed vegetative cultures revealed that TIF1 DNA binding activity is cell cycle regulated (Fig. 1B), producing a profile similar to that obtained for the TIF4 origin binding complex (Mohammad et al., 2003).

Northern blotting was performed to monitor TIF1 gene expression. This analysis revealed that TIF1 mRNA levels are also regulated across the cell cycle (Fig. 1C). Maximal signals were obtained at the 90 –180 min interval, which includes the distinct periods for macro- and micronuclear DNA replication (Mohammad et al., 2003). A pronounced decline in TIF1 mRNA abundance was evident prior to cytokinesis (240 min). While tritiated thymidine labeling revealed experimental variation in the lag period before S phase (data not shown), TIF1 mRNA levels reproducibly rose prior to the onset of macronuclear DNA synthesis. The maximal TIF1 mRNA signal was 4-7-fold greater than that observed in pre-S phase cells (Fig. 1C, graph).

Northern blotting was also used to monitor TIF1 mRNA levels during development. Pre- and post-meiotic micronuclear replication precedes the formation of a new macronucleus in progeny cells (3-8 h), with the Orc2-related TIF4 subunit specifically localizing to nuclei that are actively engaged in DNA replication (Mohammad et al., 2003). Selective amplification of the rDNA occurs between 10-20 h. In contrast to cycling vegetative cells, TIF1 mRNA levels were relatively constant throughout development. Signal intensities were comparable to that seen in starved cell cultures (Fig. 1D, 0-24 h), and much lower than S phase vegetative cells (Fig. 1D, right lane: 150 min). Despite the low level of TIF1 mRNA, previous EMSA experiments showed an increase in TIF1 DNA binding activity in cells undergoing macronuclear development (Mohammad et al., 2000). The basis for this difference is unknown.

Partial and complete macronuclear replacement of the wild-type TIF1 gene

Partial inhibition of TIF1 mRNA translation using an antisense ribosome strategy previously revealed that TIF1 modulates the occupancy of PSE and type I elements *in vivo,* and differentially marks the rDNA origin and promoter regions (Saha et al., 2001). To further investigate the role of TIF1 in rDNA replication and assess whether TIF1's role is restricted to macronuclear rDNA functions, we generated strains that were partially depleted for TIF1 or that lacked the TIF1 gene entirely (Cassidy-Hanley et al., 1997).

Since macronuclear chromosomes segregate randomly, TIF1:neo transformants that retain more copies of the disruption allele can be obtained by pm-selection (reviewed in Turkewitz et al., 2002). Transformation of the vegetative macronucleus with a TIF1 disruption construct (Fig. 2A, left panel) produced strains TXh48 and TXh29. Phenotypic assortment of the wild-type TIF1 gene was used to titrate TIF1 to a rate limiting dosage. Primary transformants were sequentially cultured in increasing concentrations of pm (from 100 to 4500 μg/ml) to select for cells that harbored a higher percentage of the TIF1::neo disruption allele in the polyploid amitotic macronucleus. Cells that were resistant to intermediate and high levels of pm grew more slowly than wild-type (data not shown), suggesting that selection against the TIF1 gene was being counteracted by a slower rate of cell division. Southern blot analysis of two macronuclear transformants, resistant to 4500 μg/ml pm, revealed that the wild-type TIF1 gene had not been completely replaced (Fig. 2A, middle panel, TXh48 and TXh29). PhosphorImager quantitation indicated that the copy number of the intact TIF1 gene in lines 48 and 29 was reduced to ~25% and 50% of wild-type, respectively (following normalization to the -tubulin loci, btu1 and btu2).

Northern blotting revealed a comparable decrease in TIF1 mRNA abundance (data not shown). The inability to completely replace the wild-type TIF1 gene in the macronucleus argues that there is strong selective pressure to retain a threshold level of TIF1 in the cell.

Germline transformation was employed to determine if TIF1 is essential for cell viability. Biolistic transformation of pre-meiotic mating cells was used to generate heterozygous germline knockout strains, in which the micronuclear TIF1 coding region was replaced with the neomycin phosphotransferase gene (neo). Primary transformants were heterozygous for the disruption in the transcriptionally-silent micronucleus and contain approximately equivalent amounts of the wild-type and disrupted TIF1 alleles in the new progeny macronucleus (Fig. 2A: TXh102 (HET); TXh106, data not shown).

Heterozygous transformants were grown to sexual maturity (~80-100 fissions) in the absence of selection for the disrupted allele to minimize potentially deleterious effects of depleting TIF1. Heterokaryon strains were subsequently generated by Round 1 genomic exclusion in crosses with functionally-amicronucleate strains, A* mating type III or A* mating type V (Allen, 1967a). 25% of the progeny derived from this abortive developmental program should be homozygous for the TIF1::neo disruption allele in the micronucleus and contain a wild-type macronucleus derived from the A* parental strain. Clonal isolates were genotyped to identify progeny that met these criteria. These strains were then mated to one another to generate homozygous TIF1 null progeny that completely lack the TIF1 gene in the transcriptionally-active macronucleus. A single homozygous null line, TXk202, was obtained (Fig. 2A, right panel, KO). While there is strong selective pressure to retain the TIF1 gene in the macronucleus (Fig. 2A, middle

panel), our ability to isolate a homozygous null line indicates that TIF1 is not absolutely required for vegetative (macronuclear) functions.

TIF1 is required for micronuclear genome stability

Although we were able to generate a homozygous null line, TXk202 exhibited defects in macronuclear DNA replication and division during vegetative propagation (see below), and eventually senesced. Repeated attempts to generate new homozygous null mutants failed, even when freshly derived heterozygous germline knockout transformants were mated immediately upon reaching sexual maturity. These observations raised the possibility that TIF1 might be required for long-term vegetative propagation of the micronucleus.

To determine if TIF1 is required for micronuclear genome stability, the homozygous null strain (TXk202) and two homozygous TIF1::neo heterokaryons (TXa28 and TXa42) were mated with nullisomic heterokaryon strains that lacked one of the five micronuclear chromosomes. In contrast to wild-type controls, all of the TIF1 mutants failed to generate viable progeny in these crosses, suggesting that the chromosomal composition of the micronucleus was compromised during vegetative cell divisions. Similar results were obtained when these mutants were mated to wild-type tester strains.

To assess whether a TIF1-deficiency led to degeneration of the micronuclear genome, we visualized nuclei in asynchronous vegetative cultures with DAPI. Partial (TXh48) and complete (TXk202) TIF1-deficient mutants exhibited two consistent differences from wild-type cells at early stages of the cell cycle (in cells containing one micronucleus and one macronucleus). First, the mutant macronucleus was somewhat

enlarged, producing fainter, punctate DAPI staining (Fig. 2B, large arrows). Second, the micronucleus was reproducibly smaller than wild-type (Fig. 2B, small arrows). Cell-to-cell variation in the size of mutant micronuclei was observed, along with an overall diminution in DAPI staining intensity over time.

More revealing information on the micronucleus was obtained from sexually mature mating cells (after 80+ fissions). Prior to mating, cells were pre-labeled with fluorescent mitochondrial dyes to identify the wild-type (green) and mutant partner (red) in each mating pair. During the developmental stage that precedes meiosis I, the micronucleus detaches from the macronucleus and elongates into a crescent (reviewed in Karrer, 2000). Control crosses between two wild-type strains (CU427 and CU428) produced mating partners with crescent nuclei of comparable length and DAPI staining intensity (Fig. 2C, upper left panel). In contrast, the intensity of the micronuclear DAPI crescent was markedly diminished in the homozygous TIF1 knockout (TXk202) (Fig. 2C, upper right panel). Crescent formation was not simply delayed in the mutant, since the staining intensity did not increase at later time points (data not shown). Similar results were obtained with heterozygous TIF1:neo germline transformants that contained partial macronuclear gene replacements (Fig. 2C, TXh48: lower left panel; TXh29: data not shown) and homozygous TIF1 knockout heterokaryons (data not shown). Although the mutant macronucleus is replaced with a wild-type (TIF1+) macronucleus in heterokaryons prior to conjugation, diminished micronuclear crescent staining was still observed. Consequently, the apparent loss of micronuclear DNA must occur during vegetative cell divisions.

Precocious replication of the rDNA minichromosome in TIF1-depleted cells

Since TIF1 recognizes rDNA replication determinants (type I elements and PSEs) *in vitro* and contributes to the *in vivo* footprint at these sites, replication of the rDNA minichromosome was examined in TIF1-deficient cells. Cells were synchronized to examine the timing of bulk macronuclear DNA replication and rDNA origin firing. A stationary phase/starvation/refeeding protocol was used to synchronize cells (Mohammad et al., 2003) and BrdU pulse-labeling was employed to monitor cell cycle progression. Wild-type (CU428) and TIF1-deficient strains (TXh48) entered S phase with similar kinetics; however, the mutant reproducibly exhibited an elongated macronuclear S phase (2.5 h vs 2.0 h) (Fig. 3A, TXh48, solid line; CU428, dashed line). BrdU labeling was first detected 1.5-2 h after refeeding, with a modestly higher percentage of BrdU positive mutant cells (2-3%) being observed throughout the first cell cycle.

2D gel analysis was performed on DNA samples prepared from time points in the first cell cycle to assess origin utilization, replication fork arrest at PSE elements and the timing of rDNA replication. Total genomic DNA was digested with Hind*III* to generate a palindromic fragment containing both (inverted) copies of the 5' NTS (Fig. 3B). Wild-type and mutant strains produced identical (bubble-to-Y) replication arc profiles with an rDNA 5' NTS probe, indicating that the mutant initiated replication exclusively from 5' NTS origins (Fig. 3C). Furthermore, the patterns of stalled intermediates were indistinguishable, indicating that replication fork pausing at PSE elements was not perturbed in the mutant. However, rDNA replication intermediates were detected earlier in TIF1-deficient cells. Extremely faint signals, corresponding to accumulated replication intermediates that had stalled at PSE elements, were detected at 1.0 h in the mutant and 1.5 h in wild-type cells.

Clear replication intermediate arcs were detected at 1.5 h in the mutant and at 2.0-2.5 h in wild-type cells. We conclude that TIF1 functions in a negative regulatory fashion to repress initiation at rDNA replication origins.

Quantitative Southern blotting was used to assess whether the rDNA was over-replicated in TIF1-deficient cells. No significant change in the abundance of macronuclear rDNA was detected relative to two non-rDNA chromosomes of differing length (Fig. 3D; Chr 9A: 50 kb, -tubulin: ~1000 kb). Thus, it seems unlikely that TIF1 regulates rDNA copy number control in the amitotic macronucleus (Pan and Blackburn, 1995).

TIF1 is required for normal S phase progression and cytokinesis

Similar to the TIF1 knockdown strain, TXh48 (Fig. 3A), the TIF1 null mutant, TXk202, exhibited a reduced growth rate compared to wild-type cells (Fig. 4A). Microscopic analysis revealed a statistically significant increase in the percentage of mutant cells undergoing cytokinesis, suggesting that the null mutation perturbs a late step in cell division (Fig. 4B). Cells were synchronized to evaluate the temporal relationship between S phase progression and cytokinesis. The TIF1 null strain reproducibly exhibited an elongated macronuclear S phase, indistinguishable from that observed for the TIF1 knockdown mutant (Fig. 4C, dashed black line: wild-type (CU428) and solid black line: mutant (TXk202); see Fig. 3A for comparison). Upon exiting S phase, the TIF1 null strain showed a further delay in cytokinesis (Fig. 4C, dashed grey line: wild-type and solid grey line: mutant). While the peak for macronuclear BrdU labeling typically occurred 30 min later than wild-type, the peaks for cytokinesis was delayed an additional 30 min. Furthermore, whereas wild-type cells divided with good synchrony, the mutant division

profile was extremely broad. We conclude that TIF1 is required, either directly or indirectly, for the normal temporal progression of at least two cell cycle-regulated processes, DNA replication and cytokinesis.

Macronuclear division and cytokinesis are temporally uncoupled in TIF1 mutants

Asynchronous wild-type and TIF1 knockout cultures were stained with DAPI to investigate whether the delay in cytokinesis was associated with a defect in macronuclear division. Macronuclear division reproducibly occured well before cytokinesis in wild-type controls (Fig. 5A, upper panels). Consistent with previous cytological studies, a trailing signal of DAPI staining material is associated with dividing macronuclei, and daughter nuclei are well separated prior to extensive constriction at the cleavage furrow in 90-95% of dividing cells. In contrast, TIF1 knockout cells (TXk202) often contained residual DNA at the cleavage furrow at late stages in cell division (Fig. 5A, lower left panel). The frequency of dividing cells that were simultaneously undergoing macronuclear division and cytokinesis was 4 to 8-fold higher in the mutant, fluctuating between 30-50% in asynchronous log phase cultures. Identical results were obtained with the partial macronuclear replacement strain, TXh48 (Fig. 5A, lower right panel). In the vast majority of cases, aberrantly dividing macronuclei were symmetrically positioned across the cleavage furrow, suggesting that TIF1 does not play a significant role in nuclear migration. Amacronucleate cells or cells with two macronuclei were observed less frequently in the mutant (Fig. 5B; ~1% of aberrant cell divisions). Asymmetric cell division was also observed (Fig. 5B, lower two panels). Although rare, the incidence of these events was elevated in the TIF1 mutant; no such cells were seen in a comparable sampling of wild-type cells (n=>5,000).

The global macronuclear defects associated with the loss of TIF1- prolonged S phase and delayed nuclear division raised two possibilities: that dividing macronuclei had not completed S phase, or that macronuclei exited S, but were unable to undergo normal nuclear division. To distinguish between these possibilities, log phase cultures were briefly pulse-labeled with BrdU and examined by immunofluorescence with a BrdU-specific antibody. DAPI analysis was simultaneously performed to identify cells undergoing aberrant macronuclear division. Both dividing and pre-divisional cells were examined. Pre-divisional wild-type and mutant cells (cells with a single macronucleus) incorporated BrdU into their micronucleus (Fig. 5C, left panels) or macronucleus (Fig. 5C, right panels), but not both. Thus, the relative timing of macro- and micronuclear replication was not altered in the mutant. BrdU labeling was restricted to the micronucleus in dividing wild-type cells, whose daughter macronuclei were well separated (Fig. 5C, third panel). Similarly, only the micronucleus was labeled in aberrantly dividing TIF1 mutants (Fig. 5C, right panel); no BrdU was detected in daughter macronuclei or residual nuclear material at the cleavage furrow. Thus, TIF1-deficient cells that undergo abnormal macronuclear division exit macronuclear S phase, albeit later than normal.

DNA replication occurs at a slower rate in TIF1-deficient cells

Sytox staining of log phase vegetative cultures detected a significant population of pre-divisional mutant cells with a single prominent macronucleus and extranuclear vesicles that contained DNA (Fig. 6A, lower left panel (TIF1 homozygous knockout, TXk202); frequency ~25%). While these vesicles could be ‘chromatin extrusion bodies’ (CEBs) that form when macronuclear DNA content exceeds an upper limit (Bodenbender et al., 1992),

the loss of micronuclear DNA during vegetative propagation (Figs. 2B and 2C) is inconsistent with a simple over-replication model. Alternatively, these structures could be generated by mechanical shearing of undivided macronuclei during cytokinesis. Extranuclear sytox staining was not detected in starved or pre-divisional (post-S phase) cells, suggesting that these DNA-containing vesicles either fuse with the macronucleus or are degraded.

Flow cytometry was used to assess whether the prolonged macronuclear S phase in TIF1-deficient cells is associated with a diminished rate of DNA synthesis or over-replication of the macronuclear genome. DNA content and cell cycle progression were evaluated by fluorescent activated cell sorting (FACS) of propidium iodide-stained cells. Synchronized cultures were assayed at 1 h intervals over an 8 h period. To facilitate comparisons, the FACS profiles for wild-type (purple) and mutant (pink) cells were overlaid. Magenta areas correspond to overlapping cell populations with the same DNA content. The DNA content (peak widths) of wild-type and mutant strains were in good agreement over the first 4 h (Fig. 6B, 60-240 min), and were consistent with BrdU labeling experiments presented above (Fig. 4C) which showed that wild-type and mutant cells enter S phase with similar kinetics. However, the wild-type DNA peak shifted to the right first, suggesting a slower rate of DNA replication (initiation and/or elongation) in the mutant.

The difference in wild type and mutant replication rates was more apparent in the 5-8 h interval (Fig. 6B, 300-480 min). Whereas wild-type cells generated a symmetric peak at 300 min with a 2N DNA content, the mutant peak was not only asymmetric, but contained a predominance of cells with lower DNA content. At the 360 min time point, two peaks were detected in both strains. The 1N peak in wild-type cells corresponds to

cells that have divided and entered the second cell cycle. Cells in the mutant 2N peak had not achieved the maximal DNA content of wild-type cells at this time. However, the 2N DNA content was achieved at 420 min in the mutant, and a 1N peak appeared at the appropriate position 60 min later. Within the limits of resolution, the mutant appears to have replicated its entire macronuclear genome. Importantly, there was no evidence for gross over- or under-replication.

DISCUSSION

TIF1 was previously shown to bind to essential replication determinants in the *T. thermophila* rDNA minichromosome (Umthun et al., 1994; Saha and Kapler, 2000) and generate *in vivo* marks that distinguish the sites for replication and transcription initiation (Saha et al., 2001). In the work presented here we provide evidence that TIF1 regulates rDNA origin firing. We also show that TIF1 serves a more global role during macronuclear S phase. A deficiency in TIF1 produces opposite effects on replication of the rDNA minichromosome and bulk macronuclear DNA. Mutant cells precociously activate rDNA replication origins, but require additional time to replicate the remainder of their macronuclear genome. Consequently, TIF1 delays rDNA replication, but promotes S phase progression. We uncovered additional cellular processes that were perturbed in the TIF1 mutant. Cytokinesis was delayed and frequently associated with aberrant macronuclear division. Furthermore, TIF1 mutants failed to faithfully propagate their micronuclear genome during vegetative cell divisions and consequently, were sterile. Since micronuclear chromosomes contain centromeres and macronuclear chromosomes do not, it seems unlikely that TIF1 regulates a common pathway for chromosome segregation.

Regulation of the *Tetrahymena thermophila* rDNA replicon

TIF1 is one of four single-stranded DNA binding activities that specifically recognize type I elements *in vitro* (Mohammad et al., 2000; Mohammad et al., 2003). As such, these distinct biochemical entities might compete or cooperate *in vivo* for binding to these reiterated, essential replication determinants. While TIF1 shares homology with a sequences-specific single-stranded DNA binding protein that functions as a transcription factor, sequence and structural similarity is confined to a segment required for homo-tetramerization (Desveaux et al., 2000; Saha et al., 2001; Desveaux et al., 2002; 3DPossum analysis, GMK, data not shown). Although TIF1 lacks motifs found in transcriptional activator proteins, we cannot rule out a role in transcription. Directly or indirectly, TIF1 serves an important function during DNA replication and subsequent transmission of chromosomes.

We show here that TIF1 regulates the initiation of DNA replication, specifically affecting the timing of rDNA origin firing. Although TIF1-deficient cells exhibit a prolonged macronuclear S phase (TIF1 mutant- 2.5 h; wild-type- 2.0 h), rDNA origins begin firing ~30-60 min earlier than wild-type cells. Our unexpected discovery that TIF1 depletion accelerates the timing of rDNA origin activation rather than delaying or eliminating it indicates that TIF1 is a negative regulator of rDNA replication. Epigenetic mechanisms have been shown to influence the temporal pattern of origin firing in other eukaryotes (reviewed in McNairn and Gilbert, 2003). A link between histone acetylation and origin activation has been recently demonstrated (Pasero et al., 2002; Aggarwal and Calvi, 2004, Aparicio et al., 2004; Kemp et al., 2005). Since we have not detected

substantive change in histone H3 acetylation in the rDNA 5' NTS of TIF1 mutants (JSY and GMK, unpublished results), TIF1 probably regulates rDNA replication timing by a different mechanism.

We previously showed that TIF1 contributes to the type I element A-rich strand footprint at the rDNA origin (Saha et al., 2001). Consequently, a potential target for TIF1 regulation is the T strand-specific, type I element binding factor TIF4. Cytological studies have implicated the TIF4 Orc2-related subunit, Tt-p69, in global DNA replication in the micro- and macronucleus, as well as selective amplification of the rDNA minichromosome (Mohammad et al., 2003). The biochemical properties of TIF4 raise the possibility that this multi-protein complex is *Tetrahymena* ORC. Our recent discovery of Orc and MCM subunit orthologs in the *T. thermophila* genome sequence database (http://www.tigr.org; GMK, unpublished results) not only indicates that the fundamental constituents of the pre-replicative complex are conserved, but provides new avenues for exploring the relationship between TIF4 and ORC.

Global roles for TIF1 in macro- and micronuclear chromosomal processes

To a first approximation TIF1 mutants fully replicate their macronuclear genome during S phase, albeit more slowly than wild-type. Mutants exit macronuclear S, but display additional defects later in the cell cycle. Macronuclear division and cytokinesis are significantly delayed, often producing a ‘cut phenotype’ in which nuclear division and cell division are concurrent. Several non-exclusive scenarios can account for the effect of TIF1 depletion on macronuclear division and cytokinesis.

In the first model, TIF1 functions during and after S phase. While the temporal peak in TIF1 rDNA binding activity and mRNA abundance suggest that TIF1's primary role occurs during S phase, TIF1 might associate with other nuclear or cytoplasmic targets at later stages in the cell cycle. By analogy, the *Drosophila* and human Orc6 subunit regulates critical cellular processes during mitosis. DmOrc6 associates with peanut, a septin family protein involved in cytokinesis (Chesnokov et al., 2003). Mutations that disrupt this interaction lead to the formation of multinucleate cells, by disrupting cytokinesis or nuclear positioning at the cleavage furrow. Similarly, silencing of the human Orc6 gene generates an array of M phase defects, including multipolar spindles, aberrant mitosis and the formation of multinucleate cells (Prasanth et al., 2002). TIF1, by contrast, appears to have a minor role in cytoplasmic events associated with cell division. Although more frequent than in wild-type cells, defects in macronuclear migration and/or cytokinesis were observed in a small fraction of aberrant cell divisions. Moreover, in contrast to the paclitaxel-hypersensitive β-tubulin mutant, btu1-1 (Smith et al., 2004a), the vast majority of aberrant nuclear divisions involved macronuclei that were properly localized to the cleavage furrow. Amacronucleate cells or cells with two macronuclei were rarely observed in the TIF1 mutant background. Furthermore, TIF1 mutants did not exhibit wide fluctuations in DNA content that are characteristic of *btu1-1* cells.

In the second model, the role of TIF1 is restricted to S phase. We propose that the macronuclear and micronuclear phenotypes documented here (diminished rate of macronuclear DNA replication, aberrant macronuclear division, micronuclear chromosome loss) arise from a common defect: the inability to activate the S phase checkpoint or repair DNA damage at stalled replication forks. Preliminary experiments suggest a role for TIF1

at the replication fork in both the micro-and macronucleus, as TIF1 mutants are hypersensitive to DNA damaging agents and activate repair pathways in the absence of exogenous mutagens (TLM and GMK, unpublished results). By analogy, the metazoan checkpoint proteins, ATR and ATM, which arrest replication forks in response to DNA damage, were recently shown to regulate replication initiation and elongation in unperturbed cell cycles (Schechter et al., 2004). Like the TIF1 deficiency, inactivation of ATM and ATR results in precocious (global) origin firing. Additional *Tetrahymena* origins are needed to determine if TIF1 is a global regulator of origin activation. Since TIF1 bears no obvious sequence similarity to ATM or ATR, and depletion of TIF1 has an opposite effect on the rate of DNA synthesis than ATM/ATR, we predict that TIF1 would act through different regulatory targets.

The differential sensitivity of the micro- and macronucleus to TIF1 depletion may reflect fundamental differences in how chromosomes are segregated during nuclear division. Micronuclei undergo conventional mitosis, whereas macronuclear chromosomes segregate by a poorly understood amitotic mechanism. *Tetrahymena* has evolved several ways to compensate for genic imbalances associated with amitotic macronuclear division. Excess macronuclear DNA is eliminated through the formation of chromatin extrusion bodies, while endo-replication occurs when macronuclear DNA content falls below a minimal threshold (Cleffmann, 1968). Since the DNA content of the macronucleus was maintained in the TIF1 mutant, these compensatory mechanisms appear intact. By contrast, the diploid mitotic micronucleus lacks these pathways. The diminished micronuclear DNA content and sterility observed in the TIF1 mutant indicates that TIF1 is essential for the long-term propagation of micronuclear chromosomes. Whereas a TIF1-

like deficiency would be lethal in organisms that contain a single diploid nucleus, the ability of the ‘somatic’ macronucleus to remain functional allows for the propagation of cells with a severely compromised micronuclear genome. Consequently, we are positioned to study chromosomal events that go awry in a dispensable, but otherwise conventional mitotic nucleus.

CHAPTER III

TIF1 IS REQUIRED FOR MICRONUCLEAR GENOME STABILITY AND THE PREVENTION OF DNA DAMAGE IN *Tetrahymena*

OVERVIEW

TIF1 is a non-ORC protein that recognizes cis-acting sequences in the *Tetrahymena thermophila* ribosomal DNA (rDNA) minichromosome that are required for origin and promoter activation. TIF1 suppresses origin firing at the rDNA replicon, but is necessary for proper macronuclear S phase progression and division. We demonstrate that TIF1 is essential for micronuclear genome stability and the prevention of DNA damage. TIF1 mutants exhibit fewer micronuclear chromosomes and are unable to pass on genetic information to progeny during conjugation. This is a progressive defect as clonal mutant lines exhibit micronuclear instability during vegetative cell cycling which could be the result of the accumulation of DNA damage. TIF1 mutant cells show hypersensitivity to the DNA damaging agent MMS suggesting that these cells may have defects in the DNA damage response pathway. Additionally, micronuclei of unperturbed cells exhibit H2AX phosphorylation which is a marker for DNA damage. This suggests that TIF1-deficient cells are incurring DNA damage with no exogenous damaging agents. We propose that the accumulation of DNA damage in both the macro- and micronuclei of cells may be the cause for the observed defects in TIF1 mutants and that TIF1 may play a role in the prevention, recognition or repair of DNA damage.

INTRODUCTION

The type I element binding protein TIF1 is a non-ORC DNA binding protein that binds to essential cis-acting replication determinants in the *Tetrahymena thermophila* ribosomal DNA (rDNA) minichromosome (Reischmann et al., 1999; Saha and Kapler, 2000; Larson et al., 1986; Umthun et al., 1994). Footprinting studies, using TIF1 knocked down cell lines, revealed that TIF1 affects the footprint on the A-rich strand at the origin and T-rich strand at the promoter suggesting that TIF1 may modulate binding of the origin- and promoter-proximal PSE and type I elements *in vivo* (Saha et al., 2001). In the previous chapter we used homologous gene replacement to study role of TIF1 in replication-based processes. These studies revealed that the loss of wild-type TIF1 levels result in precocious firing of the rDNA replicon, a slowed S phase progression followed by an elongated period for macronuclear division and cytokinesis, and decreased micronuclear genome stability. In this chapter I examine DNA damage and damage response pathways as they pertain to the propagation of micro- and micronuclear genomes.

TIF1 mutants exhibit early origin firing at the rDNA minichromosome locus. This suggests that TIF1 may play a negative regulatory role at this origin and may interact and regulate the activation of ORC. Thus far, our lab has identified an ORC-like complex, TIF4, that binds exclusively to the T-rich type I element strand. Considering that TIF1 selectively alters the A-rich strand footprint at the rDNA origin, it may play a role in the selective recruitment or inhibition of ORC binding at this locus. However, TIF1 must also play a global role as well since S phase defects and cell division defects are observed.

Cell cycle experiments using BrdU incorporation and immunofluorescence revealed that TIF1-deficient cells exhibit an elongated macronuclear S phase. Defects in

macronuclear S phase could result from delays in DNA replication initiation, elongation or fork stalling at natural pausing sites or sites of DNA damage. Slowed S-phase progression could also arise from the activation of DNA damage intra S checkpoints resulting from errors in replication (Larner et al., 1999; Falck et al., 2002). Flow cytometry experiments reveal that TIF1 mutant cells achieve an approximate 2N genome composition prior to cell division, suggesting that these mutants complete S phase albeit at a slower rate. In addition to defects in macronuclear S phase, TIF1 mutants are further delayed in cytokinesis showing a higher percentage of cells in the late stage of cytokinesis. In contrast to wild-type cells, macronuclear division is not complete in a major percentage of dividing cells. Macronuclear division in *Tetrahymena* is a poorly understood process that does not involve traditional spindle formation and segregation of chromosomes. Instead the macronucleus elongates and constricts, and the randomly segregated genetic material is partitioned to the daughter cells (Orias, 1991). In wild-type cells macronuclear division occurs during early cytokinesis when the cells begin to elongate. During this stage the macronucleus stretches between both poles of the cell and undergoes an amitotic nuclear division which is completed before the initiation of a cellular cleavage furrow evident of advanced stages of cytokinesis (Fig. 3.1 left panel). However, the late stages of cytokinesis in TIF1 mutant cells often contain macronuclei that span the cleavage furrow and have not divided completely and partitioned to daughter cells (Fig 3.1 right panel). If macronuclear chromosomes are not replicated properly and/or the DNA is damaged, this could affect macronuclear chromosome segregation and nuclear division resulting in the abnormal cell division defect.

Preliminary studies revealed a role for TIF1 in the micronucleus. TIF1-deficient cells exhibit less robust DAPI staining compared to wild-type cells. Additionally, genetic studies showed that homozygous micronuclear TIF1 knockout strains are unable to generate viable progeny when mated to strains that lack one of the five micronuclear chromosomes (Fig. 2.2 and previous chapter). This finding suggests that the micronuclear chromosome composition becomes compromised when TIF1 is rate-limiting and that the micronucleus does not contain a full complement of genetic material. Micronuclear genome instability could result from incomplete replication of the micronuclear genome or the accumulation of damage during DNA replication. Either mechanism could result in abnormal mitosis or meiosis. Alternatively, TIF1 might be required to execute a step in mitosis or meiosis, similar to the ORC6 subunit in humans and *Drosophila* (Prasanth et al., 2002; Chesnokov et al., 2003).

In this section I use genetic analysis and immunofluorescence of mating cells to further examine the effects of decreased levels of TIF1 on *T. thermophila* micronuclear and macronuclear chromosomes. In an effort to determine if both macronuclear and micronuclear defects result from a common defect, the accumulation of DNA damage, I examined the DNA damage response in wild-type and mutant backgrounds. These studies revealed that the macronucleus in TIF1 mutants is hypersensitive to a DNA damaging agent suggesting that the macronucleus is accumulating DNA damage. The micronucleus in TIF1 mutant cells revealed activation of DNA damage response pathways during normal vegetative division. Additionally, micronuclear studies revealed that the TIF1 mutant micronucleus is aneuploid and undergoes delayed and/or aberrant meiosis. We conclude

that TIF1 is required to prevent or repair DNA damage in the micronucleus and macronucleus.

MATERIALS AND METHODS

Tetrahymena thermophila strains and culture methods

The wild-type strains CU427 and CU428 were used for comparative analysis with TIF1-deficient strains and A* heterokaryons. The TIF1 null (TXk202), TIF1 knockdown strain (TXh48) and TIF1 heterozygotes (TXh102 and TXh106) were generated using biolistic transformation as described in the previous chapter. The ten TIF1 null clonal lines (TXk202-C1 - C10) were isolated by making serial dilutions of the TXk202 cell line. Cells were plated in 96 well dishes at densities of 1, 0.3 and 0.03 cells per well. All clonal lines selected were chosen from the 0.03 plates which showed cell growth in 5 or less wells in each 96 well dish. The A* heterokaryons mating types III and V were also used for comparative analysis.

Cultures were grown at 30° C with gentle shaking in 2% PPYS (2% proteose peptone, 0.2% yeast extract, 0.003% sequestrine) to which 250 μg/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B had been added (Orias and Bruns, 1975).

Genetic analysis of micronuclear genome stability

Micronuclear genome instability was assayed three ways. In the first approach, heterozygous TIF1:neo/TIF1^{+} germline transformants were mated to one another. Mating cultures were re-fed at 6 h and serially diluted to establish clonal lines. Forty eight clonal progeny lines were tested for resistance to paromomycin (pm) and for sexual immaturity.

For fully fertile strains, 75% of progeny should be pm-resistant. Furthermore, progeny that underwent normal macronuclear development should not form mating pairs until they have been propagated for 70-100 fissions. Significantly deviations from the norm are consistent with the activation of an abortive developmental program associated with 'functionally-amicronucleate' stars strains. In such instances, the parent with a compromised micronucleus fails to transmit a pronucleus to its partner during genetic exchange. The parental macronuclear is retained in both progeny of this non-reciprocal exchange event, and hence the macronuclear phenotypes of the parents are expressed in the resulting heterokaryon strain.

In the second approach for assessing micronuclear fertility, 40 clonal lines were established from two independent heterozygous TIF1:neo/TIF1^{+} germline transformants. Small scale matings (~500 cells/mating partner) were initiated with the tester strain, SB210, and progeny were selected for resistance to 2-deoxygalactose, encoded in the SB210 micronucleus. Progeny were then screened for pm-resistance, encoded by the TIF1::neo disruption. Since multiple progeny are generated in these crosses, all of the matings should generate pm-resistant progeny if the micronuclear genome of the TIF1:neo/TIF1^{+} parent is intact.

In the final approach 82 heterokaryon lines were established by mating TIF1:neo/TIF1^{+} germline transformants with A* mating type III or A* mating type V strains. These lines were then mated to strain SB1969 and progeny were selected for resistance to cycloheximide (encoded in the SB1969 micronucleus). Progeny were subsequently screened for pm-resistance. Since half of the pm-sensitive heterokaryons

should be homozygous for the TIF1::neo gene in the micronucleus, 50% of the cycloheximide resistant test cross progeny should be pm-resistant as well.

Immunofluorescence of mating cells

For mating experiments, wild type strains (CU427 and CU428) were distinguished from TIF1 knockout (TXk202), knockdown (TXh48), or A* (A*III and A*V) strains by incorporation of Mitotracker Green FM or Red-CMXRos dyes (Molecular Probes) during overnight starvation of pre-mating (single strain) cultures. Reciprocal labeling experiments revealed that these dyes do not alter the phenotypes described in the results section. Cells were grown up to 2.5 x105 cells/ml, washed and resuspended in 10 mM Tris (pH 7.4). Cells were starved overnight and 0.1 μg of Mitotracker Green FM or Mitotracker Red-CMXRos (Molecular Probes) was added to the cultures in order to distinguish cells by immunofluorescence. Cells incorporate these dyes into their mitochondria during the overnight starvation period. Mitotracker Green FM was primarily used for the wild-type CU428 strain while Mitotracker Red-CMXRos was primarily used for all other strains. Following starvation, cells were mated at a concentration of $2.0x10^5$ cells/ml. 1 ml aliquots of mating cells were harvested each hour for up to 8 h and washed sequentially with 1 ml of distilled water, 50% methanol, 70% methanol, and 70% methanol/15% acetic acid fixative. Cells were resuspended in 100 μl of methanol/acetic acid fixative and dropped onto microscope slides from a height of 30-60 cm and air dried. Slides were then dipped sequentially in 95% ethanol (15 sec), 0.1 μg/ml 4',6'-diamidino-2-phenylidole (DAPI, Sigma Chemical) in 70% ethanol/300 mM $NaCl_2$ (1 min), 70% ethanol (15 sec), and 35% ethanol (15 sec) then allowed to air dry. Slides were prepared for

fluorescence microscopy by adding a drop of 35% ethanol to the center of the slide and adding a coverslip.

BrdU labeling with MMS and immunofluorescence

For studies involving MMS, wild-type (CU428) or TIF1 knockdown (TXh48) cultures were grown to a concentration of 1-2x10^5 cells/ml. 30 ml of each culture was then aliquoted into separate flasks to which MMS was added to the specified concentrations (Tables 3.3-3.5). Cells were grown at 30^{o}C for 1 h prior to the addition of bromo-deoxyuridine (BrdU) (Sigma Chemical) and incubated for an additional 30 min. 15 ml samples were then harvested by centrifugation, washed with 10 mM Tris (pH 7.4), and incubated in 2 ml of PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) + 0.5% Triton X-100 for 3 min. Cells were recentrifuged and fixed in 1 ml PHEM + 3% paraformaldehyde for 30-60 min at 4^{0}C with gentle rocking. After fixation cells were washed 3 times with phosphate buffered saline (PBS). Fixed cells were then incubated at RT in PBT blocking buffer (PBS + 3% BSA/0.1% Tween 20) + 5% normal donkey serum (Jackson ImmunoResearch Laboratories) for 20 min followed by incubation with primary monoclonal anti-bromo-deoxyuridine (Amersham/Pharmacia Biotech) for 1 h. Following three washes with PBT, cells were incubated for 1 h at RT with rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; 1:100 dillution in PBT). Cells were washed once with PBT and suspended in 0.1 μg/ml DAPI (Sigma) for 10 min and washed twice with PBS. Cells were mounted onto slides in glycerol:PBS (9:1) and examined by microscopy. The percent BrdU labeled micro and macronuclei was determined by counting 300-400 cells per time point.

Immunofluorescence using γ-H2AX with MMS-treated cells

Cells were treated with MMS as above, except no BrdU was added to the vegetative cultures. After harvesting, fixation, and pretreatment with 5% normal donkey serum as previously described, cells were incubated for 1 h at RT with a monoclonal γ-H2AX primary antibody (Upstate Biotech; 1:250 dilution in PBT).
Following three washes with PBT, cells were incubated as before for 1 h at RTwith rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; 1:100 dillution in PBT). Cells were washed once with PBT and suspended in 0.1 μg/ml DAPI (Sigma) for 10 min and washed twice with PBS. Cells were mounted onto slides in glycerol:PBS (9:1) and examined by microscopy. The percent BrdU labeled micro and micronuclei was determined by counting 300-400 cells per time point.

RESULTS

Chromosome transmission in TIF1 mutant strains

TIF1 deficient strains are functionally amicronucleate

Initial matings between the TIF1 knockout (TXk202) or TIF1 heterokaryons (TXa42 and TXa28) and nullisomic strains which lacked one of the five micronuclear chromosomes failed to generate progeny (previous chapter). This result suggested that the micronuclear genome of TIF1 mutant cells was compromised, as it is unable to compensate for the missing chromosome in the nullisomic mating partner. Inviability could result from the loss of a single essential gene or the entire chromosome that was absent in the nullisomic mating partner. The fact that the TIF1 heterokaryon strains, which

contain a wild-type macronucleus, were unable to generate viable progeny reveals that restoring TIF1 to wild-type levels in the macronucleus was not sufficient to compensate for the low levels of TIF1 during prolonged vegetative propagation. Additionally, new homozygous TIF1 knockout strains were not obtained even when freshly generated heterozygous mutants were mated immediately upon reaching sexual maturity. This suggests that partial loss of TIF1 expression is sufficient to cause the micronuclear defects.

To further investigate the severity of TIF1 depletion on micronuclear genome instability we performed three sets of crosses. In the first cross we mated TIF1 heterozygotes to one another to determine if these cells were able to contribute genetic information to progeny during conjugation. In the second cross we mated TIF1 heterozygotes to a wild-type tester strain to determine if TIF1 deficient cells underwent an abortive developmental program that results in retention of the parental macronucleus. In the third cross we used TIF1 heterokaryons to examine whether restoring TIF1 expression levels to wild-type in the macronucleus could rescue the micronuclear transmission defects.

In the first cross we mated two freshly generated germline heterozygous knockout strains (TIF1::neo/TIF1+) to one another after passaging to sexual maturity (~70 fissions). Clonal lines were then established by isolating mating pairs ~6 h after initiating pair formation and progeny were refed at 24 h in the absence of paramomycin (pm). Progeny were replica transferred into media that contained or lacked pm. If reciprocal exchange occurred, but each mating partner lacked a common segment of the genome, the resulting progeny would be inviable. However, 48 viable clonal lines were generated and examined for pm resistance. Considering that these lines, TXh102 and TXh106, were heterozygous

for the neomycin disruption cassette it was expected that they would generate progeny with a 3:1 ratio of paramomycin resistant to sensitive progeny. However, all of the clonal lines from this mating were paramomycin resistant (pm-r) (Table 3.1, cross 1). This significant deviation from the expected result suggests that the progeny had undergone an abortive developmental program where the parental macronucleus is retained (Allen, 1967b).

To determine if macronuclear retention had occurred, the derived clonal lines were mated to a wild-type tester at the earliest possible time point, ~20 fissions after the F1 cross. If the clonal lines failed to generate a new macronucleus, then they would be sexually immature and unable to form mating pairs at this time. All of the clonal progeny lines formed mating pairs with the wild-type tester strain indicating that the old macronucleus was retained (Table 3.1, cross 1). This feature is characteristic of functionally amicronucleate strains that fail to generate a viable pronucleus for genetic exchange (Allen, 1967b; Gaertig and Kaczanowski, 1987). These results however did not reveal whether one or both parents in cross 1 was behaving as a micronuclear star strain.

A feature of cells that are “functionally amicronucleate” is that they undergo uniparental genetic exchange when mated to wild-type tester strains. A second set of test crosses were used to address whether one or both heterozygous TIF1 transformants, TXh102 or TXh106, were functionally amicronucleate. These two strains were mated individually with a tester strain that encodes 2-deoxygalactose (2dgal) resistance in its micronucleus but contains a 2dgal-sensitive macronucleus. 40 clonal lines derived from the two TIF1 heterozygous transformants, TXh102 and TXh106, were mated to this wild-type tester (SB210). Small scale matings were performed with ~500 cells of each strain, and progeny were initially selected for 2dgal resistance. Surviving progeny were then

selected for pm resistance, which is encoded in the TIF1::neo micronuclear genome. None of these progeny were resistant to paramomycin (Table 3.1, cross 2a-2b). These results indicate that genetic exchange in these matings was unidirectional, neither of the TIF1::neo/TIF1^{+} heterozygotes contributed genetic information to the progeny.

The defective germline chromosome transmission could be due to a requirement for the TIF1 protein during meiosis or instability of the micronucleus prior to conjugation. To distinguish between these possibilities we initiated a third cross where we asked whether the replacement of the TIF1 mutant macronucleus with a wild-type macronucleus could rescue the defect in micronuclear transmission. We first established 82 heterokaryon strains by mating the TIF1 heterozygous lines TXh102 or TXh106 to the functionally amicronucleate A* strain (Allen, 1967b). Under these conditions, Round 1 genomic exclusion occurs in which the parental macronucleus is retained (A*pm^{s} or TIF1 pm^{r}) and the haploid pronucleus from the non-A* strain undergoes endoreplication to generate homozygous diploid micronuclei. Progeny that contained a wild-type macronucleus were isolated based on their sensitivity to pm. Half of the F1 progeny should be homozygous for the TIF1::neo disruption in the micronucleus. In the subsequent F2 test-cross TIF1 heterokaryons were mated with a wild-type strain (SB1969), which contains cycloheximide (cycl) resistance in the micronucleus and cycl-sensitivity in the macronucleus. None of the cycl-resistance progeny were resistant to pm indicating that unidirectional transfer of genetic material occurred in the F2 mating. We concluded that the TIF1 deficient micronucleus contributes no genetic information even when the TIF1 protein is present in the macronucleus (Table 3.1, cross 3).

TIF1 deficient and A strains exhibit common cytological defects during meiosis*

The genetic analysis revealed that homozygous and heterozygous TIF1 knockout strains are functionally amicronucleate. In the previous chapter we showed that the micronucleus appeared to be smaller and exhibit less DNA staining in vegetative cells deficient in TIF1 when compared to wild-type (Chapter 2, Fig 2.2B). TIF1 mutant cells show less DNA staining during the premeiotic crescent stage of conjugation as well (Chapter 2, Fig 2.2C). However, the composition of the micronuclear chromosomes was still unknown.

Diminished chromosome number and aberrant meiosis in TIF1 mutants

To look more closely at the micronuclear chromosomes in TIF1-deficient lines, TIF1 knockout (TXk202) and knockdown strains (TXh48 and TXh29) were mated to a wild-type strain (CU428) to examine the chromosome composition at various stages of conjugation ranging from early pre-crescent formation to post anaphase II. These studies revealed that TIF1 deficient cells contained fewer micronuclear chromosomes when compared to wild-type. This micronuclear genome instability is progressive as clonal lines revealed micronuclear heterozygosity after only a few passages. Additionally, anaphase structures were difficult to detect in these cells and appeared to be abnormal. TIF1 mutant cells also exhibited a developmental delay during conjugation that is analogous to functionally amicronucleate A* strains.

TIF1 deficient cells were mated to wild-type and prepared for immunofluorescence at hourly interval to examine the micronucleus at various stages of development during conjugation by DAPI staining. To identify the genotype of cells in each mating pair, the parental strains were pre-labeled with fluorescent mitochondrial dyes that stain wild-type

cells green and mutant cells red. Matings with the TIF1 knockout to wild-type cells showed less DNA staining during all stages of conjugation from the pre-crescent (Fig 3.2A) to post anaphase II (Fig 3.2B) in all observed mating pairs when compared to its wild-type mating partner (n=210). Most significantly all of the TIF1-deficient cells showed fewer meiotic chromosomes compared to their wild-type mating partner (n=80) (Fig. 3.2C&D). In these matings, 80% of conjugating cells involving the TIF1 knockout that showed condensed meiotic chromosomes revealed only one small unelongated chromosomal staining body (Fig. 3.2C). However, a single meiotic staining body was only seen in 30% of the TIF1 knockdown lines (Fig 3.2D). This observation, that the micronuclear defect was more advanced in the TIF1 knockout strain, suggests that chromosome loss may be progressive rather than the product of a single stochastic event. Thus the cytological data support the genetic analysis of TIF1 mutants noted above and in Chapter II.

In order to further examine whether chromosomal loss was continually happening in TIF1 mutant cells or if this was the result of a single early event, 10 clonal lines were established from the TIF1 knockout (TXk202-C1 - TXk202-C10) and were subsequently mated to a wild-type strain to assess the meiotic chromosome composition within the population. If the micronuclear DNA loss was due to a single event or series of early events, then there should be no heterogeneity within a newly established clonal line. However, if micronuclear genome instability was progressive, then heterogeneity should be observed within individual clonal lines. Clonal lines exhibited heterogeneity in chromosome number in crosses to wild-type testers. As seen in the clonal line TXk202-C2, some TIF1 mutant cells contained more than one condensed micronuclear staining

body while other siblings contained only one meiotic staining body (Fig. 3.3A&B). This heterogeneity, where some mutant cells showed more than one meiotic staining body and others showed only one, was observed in 3 of the 10 clonal lines. The other 7 clonal lines showed only a single staining body, however the staining intensity of the condensed DNA varied, as seen in TXk202-C3 (Fig 3.3C&D). This supports the hypothesis that the micronuclear instability is not the result of a single or several early events. Instead this phenotype is progressive as the micronuclear meiotic chromosome number and DNA content decreased during prolonged vegetative propagation.

Considering that the TIF1 mutants show a progressive micronuclear genome instability, I attempted to observe anaphase in TIF1 mutant cells to determine if the TIF1-deficient cells underwent abnormal anaphase. This could result in the loss of micronuclear DNA if chromosomes are compromised and are unable to undergo proper segregation. Mitotic anaphase occurs very rapidly and is not easily observed in vegetatively growing cells. However, it was possible to isolate a few instances of cells undergoing meiotic anaphase in conjugating wild-type and mutant cells. In each case the TIF1 mutant appeared to be experiencing difficulty progressing through anaphase (Fig 3.4A-C).

In Fig. 3.4A, the upper wild-type cell is undergoing anaphase I and shows meiotic chromosomes being pulled to either end of the cell. The TIF1 mutant, lower cell, does not exhibit these characteristics. Instead the mutant contains a single micronuclear staining mass which is in the correct position for anaphase I, but no chromosomes are undergoing segregation. In Fig. 3.4B, the lower wild-type cell is undergoing anaphase I and the upper mutant cell appears to be attempting to segregate chromosomes. However, these chromosomes are not properly condensed or undergoing symmetric segregation. In Fig.

3.4C, the left wild-type cell is undergoing anaphase II while the mutant cell again seems to be having difficulties during anaphase. Only a few chromosomes are present in the mutant cell and segregation again appears to be abnormal.

Developmental delay in TIF1 mutants

Since we were able to visualize mutant cells that had completed anaphase I and II (Fig. 3.3B) and considering that TIF1 mutant cells do not completely lose their micronucleus during vegetative growth, it can be inferred that these cells do not arrest in anaphase, but do attempt to segregate their micronuclear chromosomes. It is not clear if abnormal anaphases are the direct cause of the micronuclear DNA loss, but the observation that TIF1-deficient cells have difficulties separating their remaining chromosomes indicates that the mutant chromosomes are prone to undergoing aberrant separation.

The TIF1 knockout to wild-type matings also revealed developmental asynchrony 20% (n=210) in conjugating cells. In these mating pairs the TIF1 mutant micronucleus was at an earlier stage of development compared to its wild-type mating partner (Fig. 3.5B,D,F). This was not observed in wild-type to wild-type matings (n=300) (Fig. 3.5A,C,E). It is unclear whether this phenotype is due to the arrest of development in the TIF1 mutant cell or a developmental lag. However, the mutant micronucleus was never more than one or two developmental stages behind the wild-type so if the TIF1 mutant was arrested, the wild-type partner micronucleus must have also been arrested. Additionally there was no build up of asynchronous cells as mating progressed. Instead abnormal pairs were seen throughout the conjugation with the wild-type mating partner showing the appropriate developmental timing as compared to mating pairs that were not abnormal.

This developmental delay in the TIF1 mutant could be due to the loss of TIF1 expression or the loss of micronuclear DNA. To determine if this phenotype was unique to TIF1 mutants or a characteristic of functionally amicronucleate cells matings were performed between known functionally-amicronucleate strains (A* mating types III or V) and wild-type (CU428). These matings showed developmental asynchrony between the A* strain and wild-type mating partner (Fig. 3.6B&D), similar to that seen in the TIF1 knockout (Fig. 3.6A&C). The frequency (22%) was comparable to that observed in the TIF1 mutant x wild-type mating (n=100). This observation suggests that the asynchrony seen in the TIF1-deficient cells may result from the loss of micronuclear DNA rather than a direct requirement for TIF1 during development.

In summary, the cytological studies indicate that TIF1 must be expressed at wild-type or close to wild-type levels in order to assure the faithful transmission of the germline micronuclear chromosomes. All TIF1 knockout lines contained less micronuclear DNA compared to wild-type strains. DNA loss was progressive and associated with a diminished chromosome number. Additionally, TIF1 mutant cells exhibit developmental asynchrony when compared to a wild-type mating partner, but this phenotype appears to be a result of micronuclear genome instability as functionally amicronucleate strains showed similar charcteristics.

Accumulation of DNA damage in TIF1 knockdown mutants

TIF1-deficient cells are functionally amicronucleate and are unable to transmit genetic information to progeny. This appears to result from the fact that TIF1 mutants have lost micronuclear DNA and contain fewer chromosomes than wild-type cells. This is

a progressive defect that becomes more advanced as cells are propagated and may result from difficulties during micronuclear DNA replication that lead to an abnormal anaphase. TIF1 mutant cells also show an elongated macronuclear S phase suggesting that these cells may have problems replicating their DNA. Taken together both of these observations could be the result of cells accruing DNA damage during replication.

Macronuclear DNA damage analysis

To begin to address this possibility the DNA-damaging agent methyl methanesulfonate (MMS), which is commonly used to induce S phase DNA damage checkpoint responses, was used to determine if TIF1 mutant cells are more susceptible to DNA damage. MMS is a DNA alkylating agent which is reported to induce phosphorylation of Chk1, a downstream effector kinase of ATR in *Xenopus* (Lupardus, et al., 2002) and Rad53 in yeast (Chang et al., 2002) both of which induce S phase arrest. Studies in *S. cerevisiae* have shown that MMS also causes significant reduction in the rate of replication fork progression (Tercero, et al., 2001). If TIF1 mutants are having difficulties replicating or repairing DNA they may exhibit hypersensitivity in response to the DNA damaging agent MMS by activating DNA damage response pathways earlier than wild-type cells. Additionally, if TIF1 is playing a role in the detection of DNA damage, cells may also exhibit hypersensitivity to MMS as they are not able to sense the damage and direct repair mechanisms to the DNA lesion. This could result in an accumulation of damaged DNA which could in turn block replication fork progression.

In order to examine these possibilities we used BrdU incorporation to monitor DNA replication in vegetatively growing wild-type or TIF1 mutant cells. A mouse

monoclonal antibody raised against BrdU was utilized to detect BrdU incorporation. To first determine the concentration of MMS that induces DNA damage and replication arrest in vegetative wild-type cells, strain CU428 was incubated for 1 h with increasing concentrations of MMS (0.06-0.72%). Then cells were pulse labeled with BrdU for an additional 30 min (Lui, et al., 2003). Cells were fixed and assayed using immunofluorescence to assess BrdU incorporation (Table 3.3).

As anticipated increasing concentrations of MMS resulted in decreasing BrdU incorporation into macronuclear DNA. This correlates with the induction of DNA damage resulting in stalled replication forks preventing the incorporation of BrdU or activation of S phase checkpoints. BrdU incorporation was eliminated at 0.24% MMS. Higher concentrations (0.72%) led to lysis of most cells. In surviving cells both the mac and micronuclei had incorporated BrdU (Fig. 3.7A&B). This is not observed in the normal cell cycle, as these discrete nuclear compartments replicate at different times in vegetatively growing cells. BrdU staining was also detected in dividing macronuclei of cells undergoing cytokinesis (Fig. 3.7C&D). This observation was unexpected because DNA replication in unperturbed wild-type and TIF1 mutant cells is completed prior to macronuclear division. The fact that both nuclei stained completely maybe be indicative of a massive DNA repair response. Additionally since BrdU was incorporated into dividing macronuclei the S phase checkpoint response must be rendered nonfunctional. Since my goal was to examine the initial DNA damage response in wild-type and TIF1 mutant strains, MMS concentrations this high were not used in subsequent experiments.

Having established the MMS concentration needed to block BrdU incorporation in wild-type cells, a narrower MMS titration curve was generated for the wild-type (CU428)

and TIF1 knockdown strain (TXh48). This experiment was carried out using the same methods with smaller increments of MMS to look more closely at the effects of MMS on wild-type and TIF1 mutant strains. Samples were pulse labeled with BrdU and prepared for immunofluorescence with the BrdU antibody. Cells were assayed as before using immunofluorescence to determine the concentration of cells incorporating BrdU. Table 3.4 shows the results for the BrdU incorporation.

These results indicate the TIF1-deficient line, TXh48, is more sensitive to MMS than the wild-type strain. The TIF1 mutant showed a marked decrease in BrdU incorporation at 0.06% of MMS whereas the same decrease in the wild-type strain was not evident until the MMS concentration was increased to 0.18%. The MMS hypersensitivity of the TIF1 mutant could result from the accumulation of DNA damage in unperturbed cell cycles. Additionally the incorporation of BrdU in TIF1 mutant cells was completely blocked at 0.18% of MMS whereas wild-type cells were able to incorporate BrdU until the MMS concentration reaches o.24%. This observation indicates that replication of the TIF1 knockdown line is halted at lower concentrations of MMS than the wild-type. This may be the result of the accumulation of more DNA damage in the TIF1 mutant strain as stalled replication forks due to DNA damage would result in the cessation of BrdU incorporation. It is also possible that TIF1 may be required for the activation of the DNA damage response pathway as a delay in the damage response pathway or inability to repair damaged DNA could lead to the accumulation of DNA damage.

Micronuclear DNA damage analysis

Unfortunately BrdU cannot be easily used to monitor DNA replication in the micronucleus due to the high magnification needed to visualize BrdU labeling of micronuclei and the close physical association of the mic with the macronucleus. The much smaller micronucleus of the TIF1-deficient cells further complicates this process. Consequently, I used a commercial γ-H2AX antibody as a probe for activation of the micronuclear DNA damage response pathway.

MMS has been shown to induce the phosphorylation of histone H2AX (termed γ-H2AX when phosphorylated), which is an isoform of histone H2A that plays an important role in connecting DNA damage sensors and effectors (Lui, et al., 2003). The H2AX histone variant is found in eukaryotes from animals to protists and is incorporated randomly into core nucleosomes. Phosphorylation of γ-H2AX is an early signal for DNA damage occurring within 1 min of γ-radiation producing nuclear foci that can be detected with γ-H2AX specific antibodies (Rogakou, et al., 1999). Additionally, H2AX is phosphorylated early in apoptosis. H2AX is distributed randomly across the genome and contains a longer carboxy-terminal region with an invariant SQ motif at a fixed position. This motif is always followed by acidic and hydrophobic residues. The serine of the SQ motif is rapidly phosphorylated in cells with damaged DNA or replication stress in *Xenopus, Drosophila,* and *S. cerevisiae* (Redon et al., 2002; Ward and Chen, 2001). *Tetrahymena* contains three H2A genes, one of which encodes an H2AX homologue that contains the SQ carboxy-terminal motif (Table 3.2) (Redon et al., 2002).

To examine the DNA damage response in micronuclei, I used a monoclonal commercial antibody that was raised specifically against the phosphorylated human H2AX

carboxy-terminus and cross-reacts with H2AX from many species. As in the previously described macronuclear BrdU experiment, wild-type and TIF1 mutant cells were incubated with increasing concentrations of MMS (0.03-0.24%) for 1 h. Cells were then prepared for immunofluorescence with the γ-H2AX antibody. Cells were assayed as before using immunofluorescence to determine the concentration of cells exhibiting phosphorylated H2AX using a commercial γ-H2AX antibody. Table 3.5 shows the results for the corresponding γ-H2AX staining.

These results indicate that TIF1-deficient cells exhibit γ-H2AX staining in the micronuclei of cells with no DNA damaging agent added. This is in contrast to the wild-type cells which show no γ-H2AX staining at all, indicating that TIF1 mutant cells accumulate DNA damage in unperturbed cycling cells. TIF1-deficient cells also show hypersensitivity to MMS in the micronucleus similar to that seen in the BrdU macronuclear data (Table 3.4). As in the BrdU labeling experiment, TIF1 mutant cells show a sharp increase in DNA damage at 0.06% MMS, whereas very little damage is seen in the wild-type cells. This may indicate the earlier activation of DNA damage response pathways in the TIF1 knockdown cell line. These results correlate with the previous findings revealing that the micronuclei of TIF1-deficient strains are both progressively losing micronuclear DNA and have difficulties completing anaphase. Additionally, almost all γ-H2AX staining in both the wild-type and TIF1 mutant lines was predominantly in the micronuclei (Fig 3.8A&B) supporting the argument that the *Tetrahymena* micronucleus does have traditional DNA checkpoints and DNA repair pathways in the mitotic micronucleus.

Only a few cells were observed in TIF1 mutants that contained macronuclear H2AX staining. The *Tetrahymena* macronucleus is capable of employing a process called endoreplication in which additional rounds of replication are initiated when DNA is lost. Considering that macronuclear chromatin contains the H2AX histone variant this low level of staining may indicate that the MMS concentrations used were not high enough to elicit a DNA damage response. Alternatively it is also possible that the macronucleus may use a different method for identifying DNA damage. Since macronuclear staining was only seen at higher concentrations of MMS (0.18% - 0.24%) it may also be an indication of cells undergoing apoptosis, as H2AX is phosphorylated early in apoptosis (Ward and Chen, 2001).

These results indicate that both nuclei accumulate DNA damage in TIF1 mutants. Damaged chromosomes may form the underlying basis for prolonged macronuclear S phase and aberrant macronuclear division, as well as, micronuclear instability.

DISCUSSION

TIF1 was previously found to interact with essential cis-acting replication determinants in the *Tetrahymena thermophila* rDNA replicon (Umthun et al., 1994; Saha and Kapler, 2000). Further studies revealed that TIF1 affects the *in vivo* footprint at both the rDNA origin and promoter region, binding to alternate strands at each location. This suggests that TIF1 may play a role distinguishing the sites for replication and transcription initiation (Saha etal., 2001). Additionally, studies with TIF1 depleted cells revealed that the loss of TIF1 results in early firing at the rDNA origin, an elongation of S phase and cytokinesis, and decreased genomic stability in the micronucleus (see previous chapter).

In this section I provide evidence that TIF1 mutants exhibit massive genome instability in the micronucleus and show that TIF1 prevents DNA damage in both the micro- and macronuclear compartments. TIF1-deficient cells are functionally amicronucleate and are unable to transfer any genomic information to progeny during conjugation indicating that TIF1 is required for the maintenance of genome stability in the micronucleus. Cytological studies revealed that TIF1-deficient cells contain fewer germline chromosomes than wild-type cells. Micronuclear genome instability appears to be progressive as clonal lines show heterogeneity after vegetative passaging. TIF1 mutants also exhibited hypersensitivity to the DNA damaging agent MMS in both the macro- and micronucleus. Additionally, the histone variant H2AX is phosphorylated in unperturbed cycling cells, revealing that TIF1 mutants activate damage response pathways in the absence of exogenous stimuli.

DNA damage occurs routinely during normal cell cycles as a result of errors in DNA replication, leading to the stalling or collapse of replication forks (reviewed in Ward and Chen, 2004). Consequently, cells have developed numerous cross-acting pathways to recognize damage and to allow for the repair of compromised DNA, including G1, intra S and G2 checkpoints (Bartek et al., 2001b; Larner, et al., 1999; Falck, et al., 2002; Xu, et al., 2002). These cell cycle checkpoint pathways are activated upon recognition of DNA damage. They can either arrest or slow the cell cycle to allow for DNA repair or initiate cellular apoptosis if damage is too severe.

Checkpoint pathways were first uncovered in yeast mutants that were hypersensitive to DNA damaging agents (Weinert and Hartwell, 1988; Hoyt et al., 1991). Three general classes of mutants were uncovered. The first class failed to recognize DNA

damage. The second class recognized the damage but was unable to initiate checkpoint signaling pathways that block cell cycle progression. The third class arrested the cell cycle but could not repair the DNA damage. My observation that TIF1 mutant cells exhibit an increase in the accumulation of DNA damage indicates that the integrity of chromosomes is severely compromised when TIF1 is rate limiting. Thus, TIF1 is not restricted to regulating rDNA origin activation. Instead it serves a more global function in the micro- and macronucleus. The accumulation of DNA damage may provide a common basis for the micronuclear and macronuclear defects that are evident in TIF1 mutants.

Potential roles for TIF1 in the prevention of DNA damage

TIF1 mutants exhibit several characteristics common to identified checkpoint mutants, including prolonged S phase, chromosome loss, and hypersensitivity to DNA damaging agents. In *S. cerevisiae* the loss of DNA damage checkpoint pathways and activation of damage repair pathways result in an increased rate of chromosome loss (Klein, 2001). This phenotype is similar to what is observed in the micronucleus of TIF1-deficient cells. Alternatively, TIF1 may act to increase the fidelity of DNA replication. It could act at specific sites such as secondary structures or DNA sequences that induce replication fork pausing or function throughout the elongation process.

TIF1 could prevent the accumulation DNA damage by acting to help identify damage. If damaged DNA is not recognized, cell cycle checkpoints are not activated and DNA is not repaired leading to an increase of damaged DNA. TIF1 mutant cells do not exhibit cell cycle arrest, suggesting that these cells may have difficulty recognizing damaged DNA. However, TIF1-deficient cells do exhibit H2AX phosphorylation in the

micronucleus indicating that they are able to identify sites of damage. The fact that H2AX phosphorylation is not witnessed in the macronucleus may indicate that DNA damage is recognized by a different mechanism in the macronucleus. This relaxed stringency for damage response pathway activation could be due to the observation that the macronucleus divides amitotically and is able to undergo additional rounds of replication to maintain gene copy number. This observation, coupled with the fact that the macronucleus contains multiple copies of chromosomes, may result in the need for higher levels of damage to induce checkpoint responses in the macronucleus. However, the result that H2AX phosphorylation is found in the micronucleus suggests that TIF1 mutants are able to recognize DNA damage, at least in the micronucleus.

Another potential role for TIF1 in response to DNA damage is the activation of cell cycle checkpoints. TIF1-deficient cells do not exhibit cell cycle arrest suggesting that the activation of checkpoints in response to DNA damage may be compromised. The findings that TIF1 mutants are hypersensitive to DNA damage and accumulate damage at lower concentrations of MMS, and that both the micro- and macronuclei show defects in DNA replication suggests that TIF1 may play a role in the DNA damage response or S phase checkpoint pathways similar to the metazoan checkpoint proteins ATM and ATR. Similar phenotypes have been observed in *Xenopus* egg extracts where these proteins have been inactivated (Schecter, et al., 2004). ATM is a kinase whose primary role is to activate cellular responses to DNA double strand breaks in all phases of the cell cycle (Shiloh, 2001). ATR is a DNA binding protein that preferentially binds UV damaged DNA and forms foci at stalled replication forks in response to arrested DNA replication (Unsal-Kacmaz, et al., 2002; Tibbetts, et al., 2000). In addition to their involvement in arresting

replication forks in response to DNA damage, these proteins have been implicated in the regulation of replication initiation and elongation in normal cell cycles (Schecter, et al., 2004). It is possible that TIF1 is playing a similar role to these proteins or is acting in the pathways activated by ATM or ATR. The later scenario is more likely as TIF1 does not display significant sequence similarity to ATM or ATR.

TIF1 mutants exhibit an elongated macronuclear S phase which could indicate an activation of intra S checkpoints which could slow the cell cycle during S phase allowing for the repair of DNA damage. This would suggest that checkpoint pathways in TIF1 mutants are active and that defects seen in TIF1-deficient cells may be the result of defects in other DNA damage responses such as DNA repair. However, the underlying basis for elongated S phase is unclear at this time. The observed delay in S phase progression could simply result from the slowing of replication forks due to lesions in the DNA or a decrease in the efficiency of non-rDNA origin firing due to TIF1 loss.

A third potential role for TIF1 in the prevention of DNA damage accumulation could be at the level of DNA repair. The main strategies employed by cells in response to DNA damage are the direct reversal of lesions, excision of damaged DNA and the rejoining of DNA double-strand breaks (reviewed in Ward and Chen, 2004). Cells that are unable to repair damaged DNA due to defects in any of these mechanisms would accumulate DNA damage in unperturbed cell cycles, as is witnessed in TIF1 mutants. Rad51 is a protein that is important for both genetic recombination and DNA damage repair by homologous recombination (Basile et al., 1992). Rad51 has homologues in species ranging from *S. cerevisiae* and *S. pombe* to *Drosophila* and humans [Basile et al., 1992; Muris, et al., 1993; Akaboshi, et al., 1994; Shinohara et al., 1993), and is a

downstream target of the ATM DNA damage response pathway (Chen et al., 1999). A *Tetrahymena thermophila* homologue for Rad51 has been identified (Campbell and Romero, 1998) and null mutations in this gene exhibit phenotypes similar to those seen in TIF1 null mutants. These phenotypes include a slower growth rate by ~25% that is comparable to the slow growth phenotype exhibited by TIF1 null mutants (Fig. 2.4 A), hyper-sensitivity to MMS as seen in the TIF1 knockdown (Tables 3.4 and 3.5), and a star strain-like phenotype where cells are unable to pass genetic information to progeny similar to TIF1-deficent strains (Table 3.1). Rad51 mutants also exhibit rapid micronuclear chromosome loss during vegetative cell cycling (Marsh et al., 2000).

Tetrahymena Rad51 is cell cycle regulated with mRNA levels increasing during macronuclear S phase, similar to TIF1, as well as during development (Marsh et al., 2000). Rad51 levels are transcriptionally controlled and three cis-acting elements have been identified as essential for this regulation with one element being specific for up regulation of this gene in response to DNA damage and meiosis (Smith et al., 2004b). Considering the similarities of Rad51 and TIF1 mutant phenotypes, TIF1 may play a role in the regulation of Rad51 levels in response to DNA damage by acting in DNA damage response pathways.

In summary, the experiments described in this chapter reveal that TIF1 is essential for the propagation of the micronuclear genome. Additionally, TIF1 is necessary for the prevention of DNA damage in both the macro- and micronucleus. In addition to its role in the regulation of rDNA replication, TIF1 prevents the accumulation of DNA damage.

CHAPTER IV

SUMMARY AND DISCUSSION

SUMMARY

Future studies on the role of TIF1 in *Tetrahymena* chomosome biology

DNA replication in eukaryotic cells from yeast to mammals is a highly regulated process to ensure that the genome is duplicate only once per cell cycle. This process is regulated by both cis-acting replication determinants within the DNA, as well as trans-acting factors that interact with these DNA sequences. Previous studies of the *Tetrahymena thermophila* TIF1 protein revealed that it interacts *in vivo* with cis-acting replication determinants in the rDNA minichromosome (Saha et al. 2000; Saha and Kapler 2001). One such determinant, the type I element, is essential for replication initiation, fork pausing at specific sites, and transcription of the rRNA gene. Previous studies of the TIF1 protein revealed that TIF1 forms a homotetramer *in vivo* and binds single-stranded DNA (Hou et al., 1995; Saha and Kapler, 2000). Additionally, TIF1 may modulate the binding of other trans-acting proteins to the origin and promoter-proximal type I elements (Saha et al., 2001). Once potential interacting protein is TIF4, which has been proposed to be the *Tetrahymena* ORC and binds the T-strand at the origin region (Mohammad et al., 2003). These findings suggest that TIF1 plays a role in regulating replication and transcription.

In my dissertation research I used homologous gene disruption in an effort to elucidate the role of TIF1. Initial observations described in Chapter 2 revealed that TIF1 does play a role in replication, as cells deficient in TIF1 exhibit early firing of the rDNA replication origin. Additionally, TIF1 seems to have a more global role for replication

regulation, as TIF1 mutants undergo an elongated macronuclear S phase followed by a delay in cytokinesis. Further studies, described in Chapter 3, revealed that TIF1 is essential for genome stability in both the macro- and micronucleus. TIF1 mutant cells accrued DNA damage in both nuclei. This could solely result from defects in replication because if cells do not properly duplicate their genomes they will incur DNA damage. However, TIF1 could be playing additional roles in the detection, prevention or repair of damage as well. Further experimentation will be necessary to determine which of these possibilities is the case.

In order to begin to answer these questions I have constructed a plasmid containing the TIF1 gene with a his-myc tag inserted into the amino terminus. Preliminary studies using this transformed construct revealed that the tagged TIF1 protein is expressed in *Tetrahymena* cells. Additionally, I have also used site-directed mutagenesis on a plasmid containing the TIF1 cDNA to generate TIF1 deletion constructs. Unlike most systems studied, the traditional stop codons, UAA and UAG, encode glutamine in *Tetrahymena* (Horowitz and Gorovsky, 1985; Hanyu et al., 1986). The TIF1 gene contains seven such glutamine codons. I was able to take advantage of this fact by using stepwise site-directed mutagenesis to sequentially change these glutamine codons to sequences that will encode for glutamine in *Tetrahymena*, as well as, other systems such as *E. coli*. This resulted in four deletion constructs that when expressed in *E. coli* will result in truncations of the TIF1 protein as translation of the protein will stop at the unchanged *Tetrahymena* glutamine codons. These deletion constructs and the TIF1 tagged cell lines will be useful to help us further our understanding of the function of TIF1.

DNA recognition

Previous studies in our lab have shown that TIF1 binds *in vivo* to both the type I and PSE elements (Saha and Kapler, 2000). However, the precise timing and action of these interaction is still unknown. We will begin to answer these questions by first determining when TIF1 binds rDNA replication determinants *in vivo* by utilizing the TIF1-tagged lines for chromatin immunoprecipitation (ChIP). ChIP can be performed across the cell cycle utilizing starved and synchronized cultures. Using this technique we will be able to determine if TIF1 binds the origin and promoter regions of the rDNA before, during, or after S phase. By ascertaining when TIF1 localizes to the rDNA origin region we can begin to deduce what role it plays in replication. For example, our studies have shown that TIF1-deficient cells exhibit early firing of the rDNA origin. This may indicate that TIF1 is acting as a negative regulator for DNA replication by potentially inhibiting the binding of pre-RC proteins. Conversely, TIF1 may be acting to aid in replication initiation by interacting with other replication initiation proteins to ensure the proper timing of origin firing. Both of these scenarios would require TIF1 to bind the rDNA origin prior to the beginning of replication. Alternatively, TIF1 may affect replication after pre-RC formation or the initiation of DNA replication. In this case TIF1 may only interact with the rDNA origin during S phase. ChIP analysis using the tagged-TIF1 lines will allow us to differentiate between these two nonexclusive possibilities.

In addition to determining the timing of TIF1 rDNA binding, it is also necessary to examine how this protein binds DNA by mapping the DNA binding domain. The carboxy terminus of TIF1 is remarkably similar to that of the plant transcription factor, p24. Both of these proteins bind DNA as a homotetramer, and recognize single-stranded DNA.

However, TIF1 lacks the corresponding amino terminal transcription activation domain (Saha and Kapler, 2000; Desveaux et al., 2000). This suggests that the DNA binding domain of TIF1 may lie within the amino terminus of the protein. Previously described deletion constructs that can be expressed in *E. coli* will allow us to determine what portion of TIF1 is essential for origin binding. Truncated TIF1 proteins can be assayed *in vitro* by gel shift analysis with type I element oligos to ascertain what portion of the protein is necessary for DNA binding. Once the TIF1 DNA binding domain has been identified, tagged deletion constructs can be introduced into *Tetrahymena* and assayed for *in vivo* origin binding using ChIP analysis.

The *S. cerevisiae* transcription factor Abf1 has been shown to play an important role in the activation of yeast replication origins (Diffley and Stillman, 1988). Mutations that disrupt Abf1 binding result in a decrease in the efficiency of ARS1 origin firing (Marahrens and Stillman, 1992). Once we have isolated the necessary amino acids required for DNA binding, site-directed mutagenesis may be used to construct TIF1 transformation vectors that encode a mutant form of TIF1 that is unable to bind DNA. The resulting TIF1 mutant lines could then be examined to determine if TIF1 origin binding is necessary to prevent abnormal phenotypes as seen in TIF1 deficient cells such as replication defects and genome instability. The role for TIF1 may extend beyond its origin binding activity and TIF1 mutants deficient in DNA binding will allow the opportunity to explore this possibility.

It will also be possible to examine whether TIF1 DNA binding alone is sufficient for its action. As mentioned above, TIF1 forms a homotetramer *in vivo*, but it is not known if this oligomerization is necessary for TIF1 function. Transformation with TIF1

constructs that lack the proposed carboxy terminal oligomerization domain will allow us the chance to determine if a single TIF1 molecule is able to bind origins *in vivo* using ChIP analysis. Additionally, TIF1 oligomerization mutants may be assayed for TIF1-deficient-like phenotypes. We would also be able to determine if TIF1 DNA binding is sufficient to rescue TIF1 mutant cells by introducing the TIF1 DNA binding domain into TIF1-deficient cells and assaying for a loss of early rDNA origin firing and a return to proper S phase progression and cell division. These experiments will allow us the opportunity for further our understanding of TIF1 function as it relates to its DNA binding activity.

Cell cycle regulation and localization

Previous studies, as discussed in Chapter II, revealed that TIF1 mRNA is cell cycle regulated with a peak during macro- and micronuclear S phase followed by a decrease in abundance prior to cytokinesis. This suggested that TIF1 acts primarily during S phase. However, mRNA studies are an indirect way of observing protein expression as the protein may still be present even after mRNA is degraded. To further examine the cell cycle regulation of TIF1 it is necessary to monitor the protein itself. Current preliminary studies in the lab using western blot analysis with TIF1-tagged lines have revealed that TIF1 is only present immediately before and during S phase. These results suggest that the TIF1 is primarily playing a role before and during S phase. However, these results are limited to western blot sensitivity, and a small amount of TIF1 may still be present in the cell to act in other cellular processes such as cytokinesis.

Recent studies have indicated that factors essential for replication may also play additional roles in other cellular processes. In *Drosophila* Orc6 has been found to localize

to the cell membrane and cleavage furrow during cell division. Mutations preventing this membrane localization resulted in multinucleated cells indicating that dmOrc6 has an essential role in cytokinesis in addition to replication (Chesnokov et al., 2003). Additionally, human Orc2 has been shown to localize to centromeres, centrosomes and heterochromatin during late S, G2 and M phases. Depletion of human Orc2 by siRNA resulted in cells with multiple centrosomes, failed chromosome congression and abnormally condensed chromosomes. These results suggest that Orc2 is necessary for proper chromosome inheritance as well as its previously described roles in replication initiation (Prasanth et al., 2004).

It is possible that small amounts of TIF1 may remain in the cell after S phase to affect other cellular processes similar to the replication factors Orc6 and Orc2. In fact, TIF1 may play a role in the progression of cytokinesis as TIF1-deficient cells exhibit an elongated period for cell division. The TIF1-tagged lines will allow us to monitor TIF1 cellular localization using immunofluorescence. In this way we will be able to assay whether TIF1 is localizing to the cleavage furrow, cell membrane, or if it remains in the nucleus. Additionally, previous studies have shown that the rDNA is localized to nucleoli in vegetatively growing cells and to two distinct foci during amplification (Ward et al., 1997). Immunofluorescence techniques using the tagged-TIF1 transformants will allow us to monitor the nuclear localization of TIF1 during S phase to determine if TIF1 only co-localizes with the rDNA or if it is dispersed throughout the nucleus. Considering that TIF1 appears to play a global role in S phase progression and genome stability it is expected that TIF1 would bind multiple sites throughout the genome. This experiment will allow us to begin to answer this question.

Roles for TIF1 at other replication loci

Studies of TIF1 thus far have centered on the rDNA origin where TIF1 prevents early origin firing. As mentioned above TIF1 mutants exhibit a prolonged macronuclear S phase with a diminished rate of macronuclear DNA replication and micronuclear genome instability indicating that TIF1 may play a global role in DNA replication. Our lab has recently identified another *Tetrahymena* origin of replication, TtARS1. This replicon does not contain the type I element. Additionally, 16 other chromosomes ranging from 37-100 kb have also been sequenced as a result of the recent *Tetrahymena* genome project. These non-rDNA chromosomes will allow us to determine if TIF1 directly interacts with other origins of replication by either *in vitro* gel shift analysis or ChIP. Using gel shift analysis we will be able to utilize sequences within the TtARS1 origin region to determine if TIF1 binds these sequences *in vitro*. Alternatively, ChIP may be used with primer sets corresponding to the TtARS1 replicon to assay if TIF1 binds this DNA region *in vivo*. Additionally, as more *Tetrahymena* replication origins are identified, we will be able to determine if TIF1 does act at other regions in the genome.

Delays in macronuclear S phase in TIF1 mutants may result from either a decrease in origin firing activity or slowing of the replication fork during elongation. This suggests that TIF1 may play a role in DNA elongation, potentially acting at the replication fork. In order to test this hypothesis, ChIP experiments may be utilized to determine if TIF1 migrates with the replication fork as seen with *S. cerevisiae* MCM proteins (Aparicio et al., 1997). If TIF1 does interact with TtARS1 ChIP may be used on synchronized cell cultures using primers to assay for origins binding, as well as, binds to sequences distal to the

replication origin as S phase progresses. If TIF1 does migrate with the replication fork its presence would be most evident at non-origin sequences. Alternatively, if TIF1 does not interact with the TtARS1 replicon, we may be able to perform this assay using the rDNA locus. The smaller size of the rDNA minichromosome would make this assay more difficult as there is only ~10 kb of DNA sequence between the origin and the end of the chromosome. However, previous studies in yeast using this method have been able to detect the movement of MCM proteins as little as 8 kb from the origin of replication (Aparicio et al., 1997).

Chromatin regulation

Tetrahymena rDNA origins localize to two domains within the 5' non-transcribed spacer of the rDNA minichromosome. These domains reside within two of three nucleosome-free regions that are bracketed by positioned nucleosomes. The third nucleosome-free region encompasses the distal rDNA promoter. TIF1 has been shown to bind to cis-acting determinants in each of these regions including the PSE elements, which map to the 5' border of the nucleosome free regions (Palen and Cech, 1984; Saha et al., 2001). This observation is similar to the *S. cerevisiae* Abf1 transcription factor that binds DNA at the 5' border of the ARS1 nucleosome-free region. Abf1 has been found, in conjunction with ScORC, to be necessary for the establishment of a specific chromatin structure at the ARS1 origin (Lipford and Bell, 2001). It is possible that the binding of TIF1 to elements adjacent to the positioned nucleosomes at the rDNA origin may serve a similar function in establishing chromatin structure.

It will be possible to address this possibility by mapping the nucleosome free region of TIF1-deficient cells in order to determine if nucleosome positioning is maintained. The chromatin structure of the rDNA origin may be mapped by isolating nuclei and digesting the chromatin with limiting amounts of micrococcal nuclease (MNase) followed by labeled primer extension. In this method DNA bound by positioned nucleosomes is protected from MNase digestion. Conversely, nucleosome-free DNA regions are hypersensitive to MNase digestion. By using a radiolabeled primer corresponding to the *Tetrahymena* origin or promoter region we will be able to identify what DNA is protected from MNase digestion after primer extension as it would not be cleaved by MNase. If the loss of TIF1 results in the invasion of nucleosomes into the nucleosome-free origin or promoter regions MNase hypersensitivity regions would be lost or diminished due to nucleosome protection.

Interacting proteins

TIF1 is one of four distinct binding activities found to interact with the *Tetrahymena* type I element *in vitro* (Mohammad et al., 2000; Mohammad et al., 2003). We will be able to determine if TIF1 interacts with any of these other type I element binding proteins, such as TIF4, by utilizing TIF1 tagged lines for immunoprecipitation and assaying the precipitates for the other three TIFs. TIF4 is an ORC-like complex that is proposed to be the *Tetrahymena* ORC and binds the opposite strand at the origin than TIF1 (Mohammad et al., 2003). This observation suggests that TIF1 may interact directly with TIF4 or bind the origin region at the same time. Considering that rDNA origins fire earlier in TIF1 mutants, TIF1 may play a role in blocking or specifying TIF4 origin

binding. Alternatively, if these proteins do bind the origin at the same time, TIF1 may act to prevent origin activation until it is removed or is post-translationally modified. In order to test these theories ChIP may be utilized at different times during the cell cycle to assay the occupancy of the rDNA origin for TIF1 and TIF4. Additionally, it will be possible to uncover other potential interacting proteins with either immunoprecipitation or ChIP techniques. Once isolated, tandem mass spectrometry may be used to obtain peptide sequences of the interacting protein. These peptide sequences may then be used in conjunction with the *Tetrahymena* gene prediction database to identify the gene for the interacting protein.

Recent studies in human cells have uncovered a new member of the MCM family, MCM8 (Gozuacik et al., 2001). This protein displays helicase activity and only binds to chromatin after DNA replication has been initiated. It has been proposed to function as a replicative helicase. The loss of MCM8 results in a decreased rate of DNA synthesis. However, it is still unknown how this protein is recruited to replication forks (Gozuacik et al., 2003; Maiorano et al., 2005). TIF1 has been shown to co-purify with an unrelated protein that exhibits intrinsic DNA helicase activity (Drena Dobbs, personal communication, Associate Professor, Iowa State University, Ames). Considering that TIF1 mutants show a decreased rate of DNA synthesis and a higher incidence of DNA damage, similar to MCM8 mutants, TIF1 may play a role in the recruitment of an MCM8-like protein. The loss of DNA replicative helicase localization to replication forks could result in fork collapse and the formation of double-strand DNA breaks which may account for phenotypes witnessed in TIF1 mutants. By assaying co-immunoprecipitation or ChIP

precipitates for helicase activity we may determine whether TIF1 directly interacts with this helicase and if this interaction takes place at the rDNA origin.

DNA damage response

In this work we have also proposed that TIF1 may play a role in the identification or repair of DNA damage. We will begin to test this theory using the TIF1-tagged transformant lines by examining if TIF1 mRNA and protein levels increase in response to MMS-induced DNA damage. If TIF1 plays a direct role in the DNA damage response its levels may increase upon the induction of damage. Preliminary studies in our lab indicate that Rad51 mRNA levels are elevated in unperturbed TIF1 knockdown lines. Additionally, Rad51 mRNA levels appear to be further elevated when compared to wild-type cells after MMS induced DNA damage. *Tetrahymena* Rad51 mutants exhibit similar phenotypes to TIF1 mutants including rapid chromosome loss during vegetative cell cycling (Marsh et al., 2000). Rad51 levels also are found to increase during S phase in a similar manner to TIF1 and it is possible that TIF1 may interact directly with Rad51. If TIF1 is playing a role helping to recruit Rad51 to sites of damage, this could explain the similarities in phenotypes between Rad51 and TIF1 gene disruption mutants. A combination of immunofluorescence and immunoprecipitation techniques may assist in answering these questions.

Recent studies have indicated that a commercial antibody raised against recombinant Rad51 protein is able to recognize *Tetrahymena* Rad51 by immunofluorescence (Loidl and Scherthan, 2004). We will be able to use this antibody to assay for Rad51 expression and localization in TIF1 tagged lines that have been induced

for DNA damage by MMS. Subsequently, antibodies against the tagged TIF1 protein may be used to determine if TIF1 and Rad51 co-localize using immunofluorescence techniques. Additionally, TIF1-deficient lines may also be assayed by immunofluorescence with the Rad51 antibody to determine if Rad51 is able to localize to DNA damage foci in TIF1 mutant cells. The existence of a direct interaction between TIF1 and Rad51 may also be tested by performing co-immunoprecipitation on TIF1 tagged cell lines that have been induced for DNA damage. These immunoprecipitation experiments may be done using an antibody for either the tagged TIF1 protein or the commercial Rad51 antibody and assaying for the precipitation of the opposite protein.

Micronuclear genome instability

As previous discussed, TIF1 mutants exhibit micronuclear genome instability resulting in the loss of micronuclear chromosomes. The observation that the macronucleus is able to compensate for this deficiency allowing cells to remain viable affords us the opportunity to study a defect that would be lethal in typical eukaryotic cells which contain a single mitotic nucleus. In order to more closely study the loss of micronuclear chromosomes it is necessary to generate a cell line where the expression of TIF1 may be controlled. In this way we can propagate cells in the presence of TIF1 to maintain micronuclear integrity and subsequently turn off TIF1 expression in order to witness early effects of TIF1 depletion on the micronucleus. Previous studies have only allowed us to examine TIF1 mutant cells after several rounds of passaging necessary for selection of mutant transformants. Additionally, we have not be able to determine if TIF1 plays a role in macronuclear rDNA gene amplification and development, as isolated TIF1 germline

mutants become sterile rapidly in TIF1 heterozygous strains. However, we will be able to explore each of these possibilities by placing the TIF1 gene under the control of the inducible *MTT1* promoter.

The *Tetrahymena MTT1* promoter can be selectively induced in proportion to $CdCl_2$ concentration. This promoter can be turned on and off rapidly with the addition or removal of $CdCl_2$ (Shang et al., 2002). By replacing the endogenous TIF1 gene with a copy of the TIF1 gene under the control of the *MTT1* promoter we will be able to generate TIF1 conditional mutants. After germline transformation of the *MTT1*/TIF1 construct cells may be propagated in media containing $CdCl_2$ to ensure that TIF1 is expressed. The *MTT1*/TIF1 construct may be co-transformed with another vector currently being used in the lab that is also under the control of the *MTT1* promoter and confers paramomycin resistance to aid in the selection of transformants. Once *MMT1*/TIF1 heterozygotes are isolated, cell homozygous for *MTT1*/TIF1 may be generated by either directly mating the *MTT1*/TIF1 heterozygotes or by genomic exclusion as described in Chapter 2 for the TIF1 knockout.

The resulting TIF1 conditional mutants may be used to assay the immediate affects of TIF1 depletion by the removal of $CdCl_2$ from the growth media. In this manner we will be able to examine early events in micronuclear chromosome loss., We will also be able to determine if TIF1 is necessary for either rDNA amplification in the macronucleus or macronuclear development by mating TIF1 conditional mutants in the absence of $CdCl_2$. Additionally, Rad51 mutants, which show similar vegetative phenotypes to TIF1 mutants (Marsh et al., 2000), are able to undergo conjugation and generate progeny. However, progeny from this mating are unable to initiate the first vegetative cell division following

conjugation (Marsh et al., 2001). By utilizing the above described techniques we will be able to determine if this is also the case for TIF1 mutants.

The studies presented here are just the beginning steps for determining what role TIF1 plays in *Tetrahymena*. In this work I have shown that TIF1 plays a role in regulating DNA replication at the rDNA origin. TIF1 also exhibits a more global affect on S phase progression. My dissertation research has also demonstrated that TIF1 is essential for genome stability in both the macronucleus and micronucleus. This is evident by the observation that DNA damage occurs in both nuclear compartments when TIF1 is rate limiting. Thus TIF1 plays an important role in the maintenance of the *Tetrahymena* genome. Well developed reverse genetic approaches will provide new opportunities to gain more mechanistic insight into the role(s) of TIF1 in chromosome biology (Turkewitz et al., 2002).

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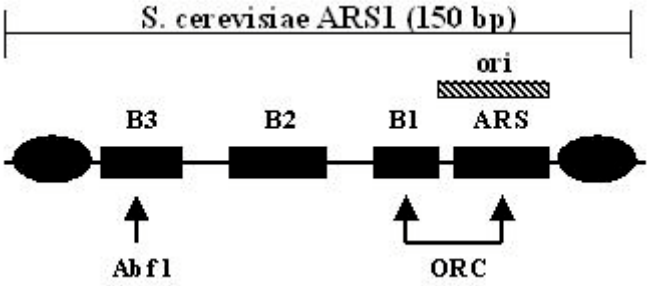
APPENDIX A

FIGURES

A.



B.

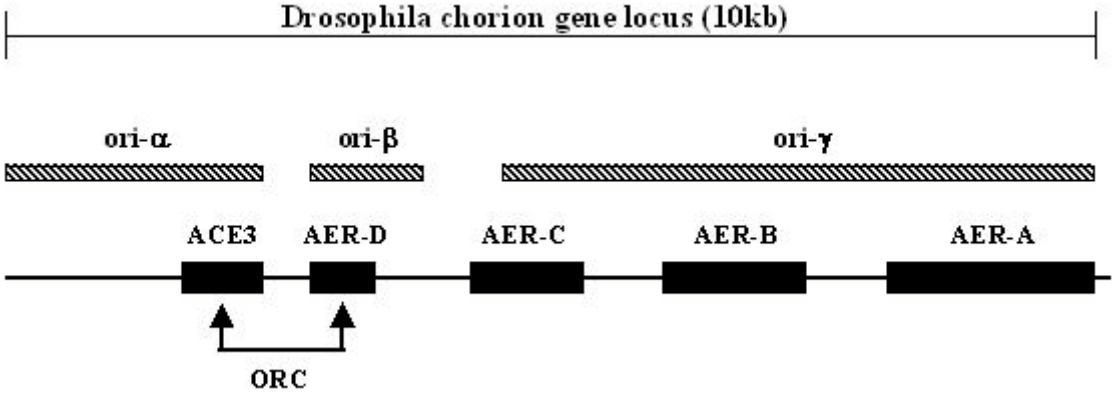


Figure 1.1 Eukaryotic replicons. (A) *S. cerevisiae* ARS1 replicon. Black rectangles represent known cis-acting relication determinants and the hatched box indicates the known site for replication initiation. Black ovals represent positioned nucleosomes. Arrows indicate sites for known DNA binding proteins. (B) *Drosophila* chorion gene replicon. Black rectangles represent cis-acting replication determinants and hatched boxes indicate known sites for replication initiation. Arrows show sites for dsORC binding.

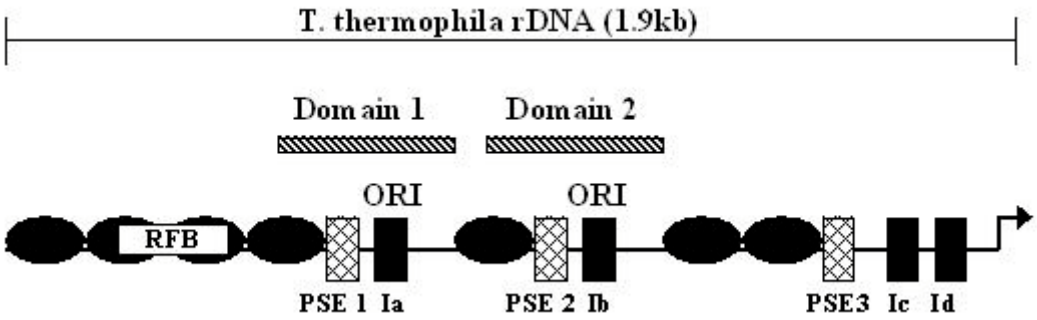


Figure 1.2 The rDNA minichromosome replicon. Black rectangular boxes represent type I elements and diamond boxes represent pause site elements. Black ovals signify positioned nucleosomes.

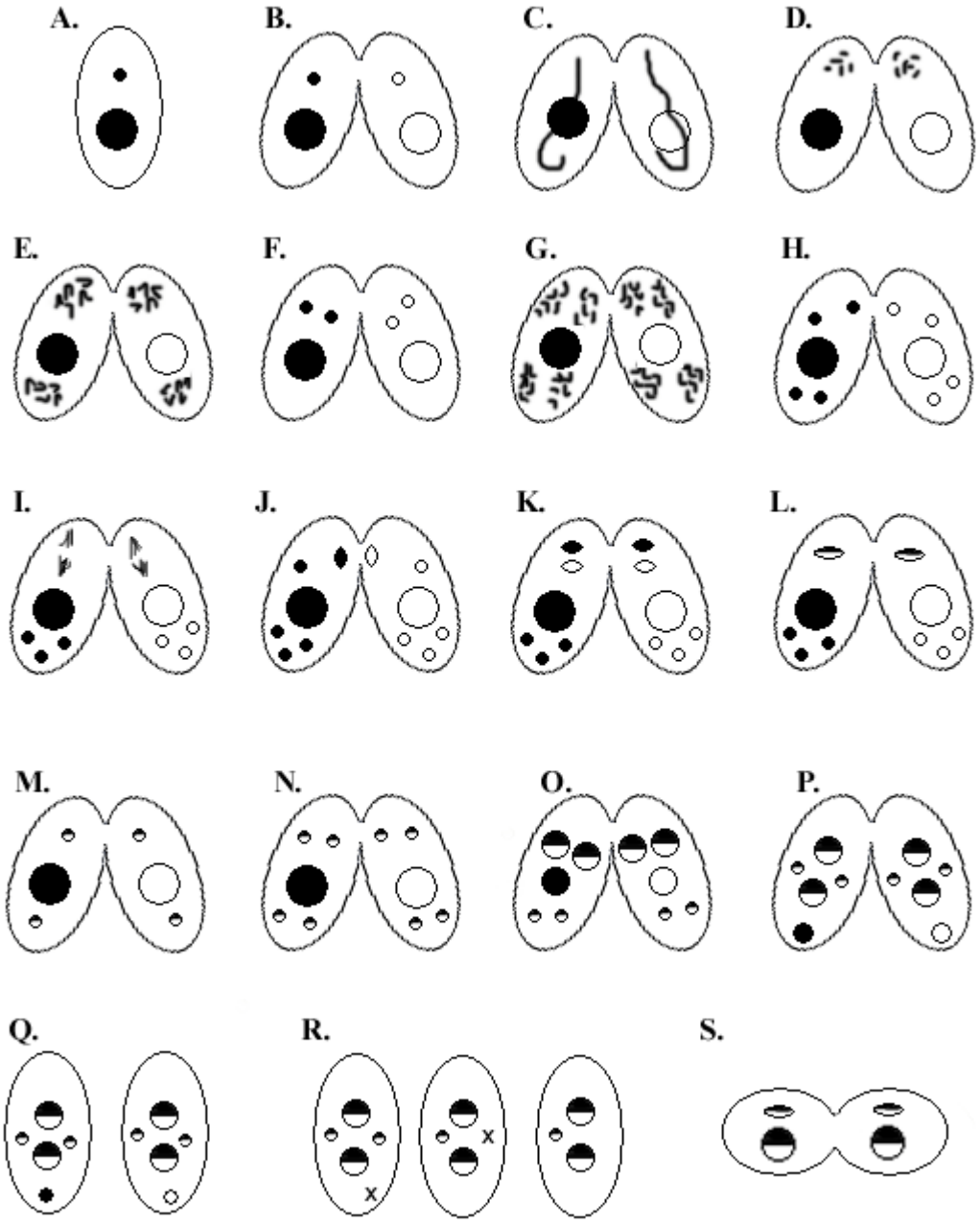
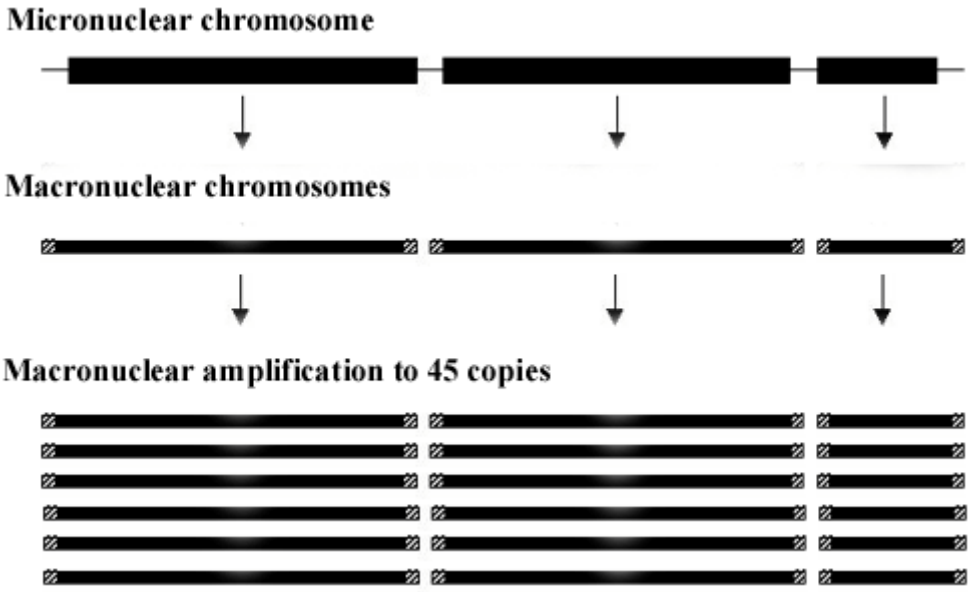


Figure 1.3 Nuclear events during conjugation in *Tetrahymena*. (A) Vegetative cell (B) Pair formation (C) Crescent micronucleus (D) Chromosome condensation (E) Meiosis I (F) Meiosis I complete (G) Meiosis II (H) Micronuclear selection (I) Prezygotic mitosis (J) Pronuclear differentiation (K) Pronuclear exchange (L) Pronuclear fusion (M) 1st postzygotic mitosis (N) 2nd postzygotic mitosis (O) Macronuclear anlagen I (P) Macronuclear anlagen II (Q) Pair separation (R) Macronuclear apoptosis and micronuclear degradation (S) Micronuclear mitosis and cell division. Adapted from Fig. 1 of Cole et. al., 1997.

A.



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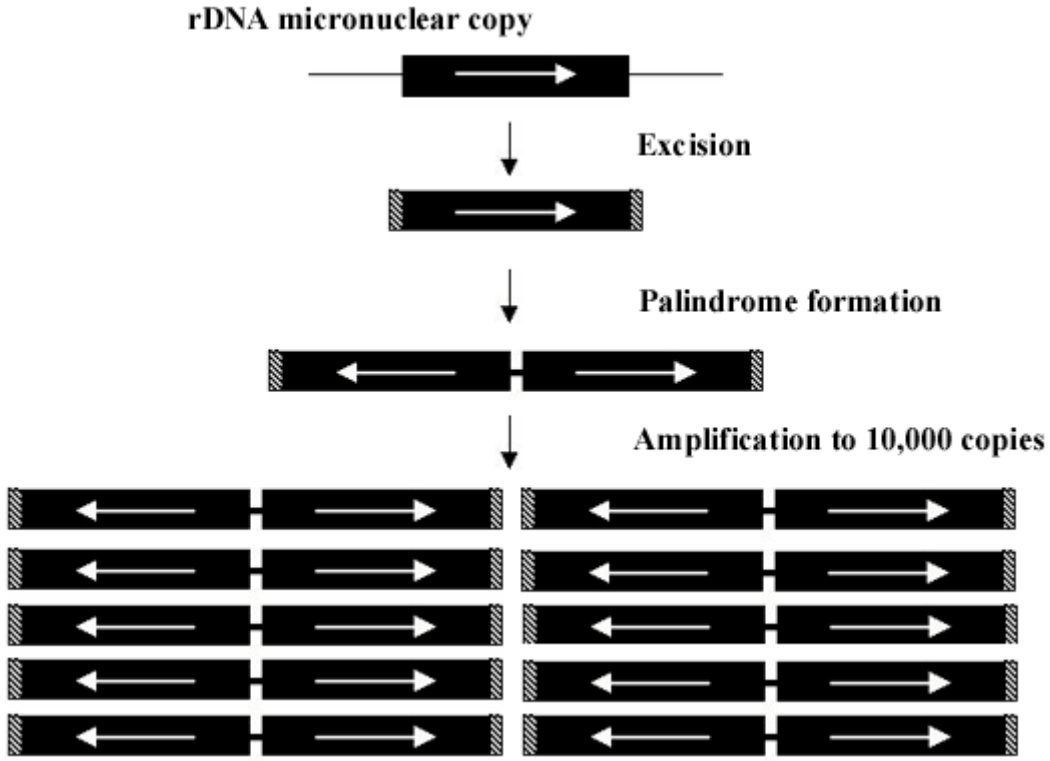


Figure 1.4 DNA rearrangements during macronuclear development Hatched boxes represent telomeres. (A) Micronuclear chromosomes are site specifically fragmented to form macronuclear minichromosomes which are amplified to ~45 copies. (B) Micronuclear rDNA gene is excised from the chromosome followed by head-to-head palindrome formation. The rDNA minichromosome is amplified to ~10,000 copies.

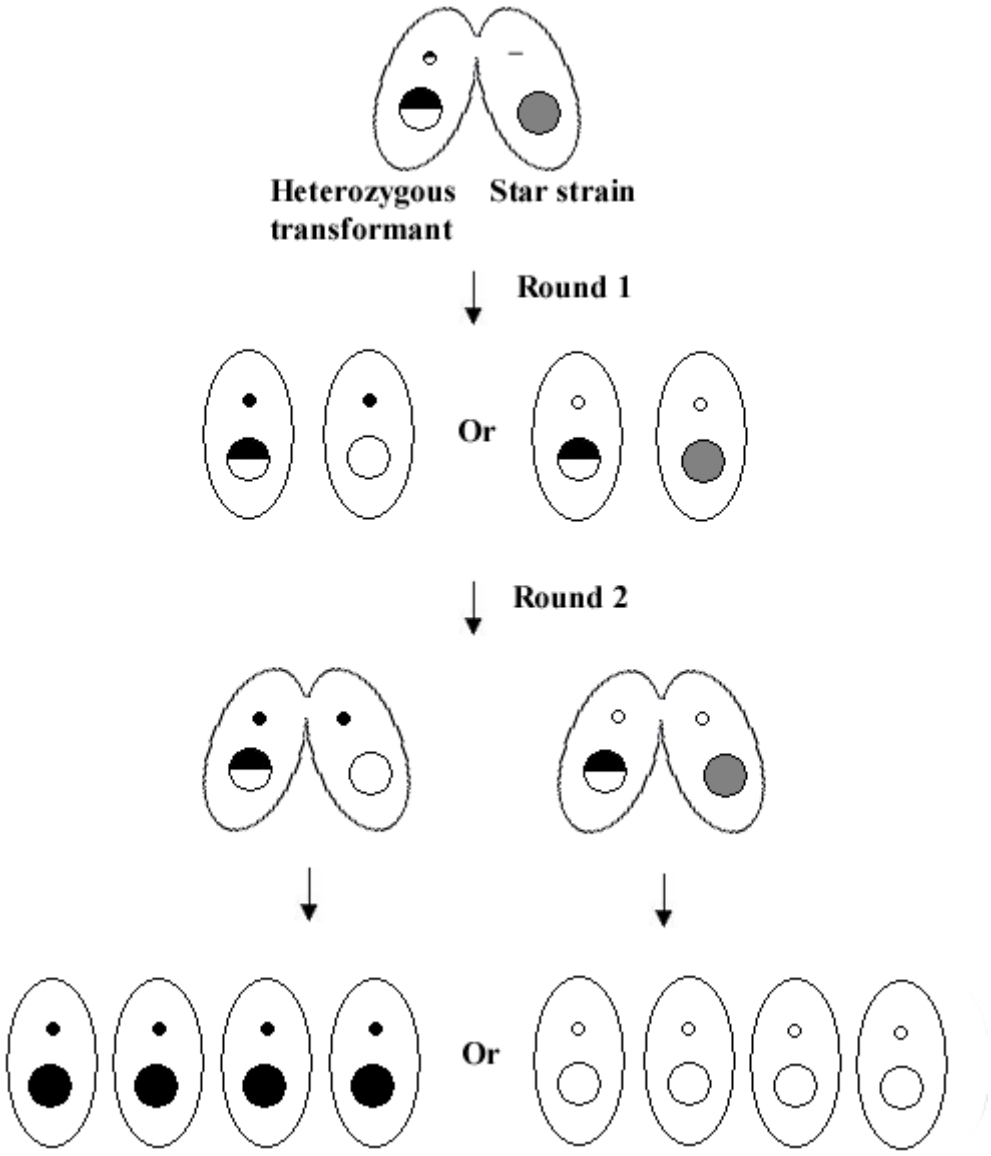


Figure 1.5 Genomic exclusion in *Tetrahymena*. Matings between a heterozygous strain and an A* strain result in progeny that are homozygous at all loci after two rounds of mating. Grey circles indicate the star strain macronucleus and black and white circles indicate the wild-type micro-or micronucleus. Adapted Fig. 6 Karrer, 2000.

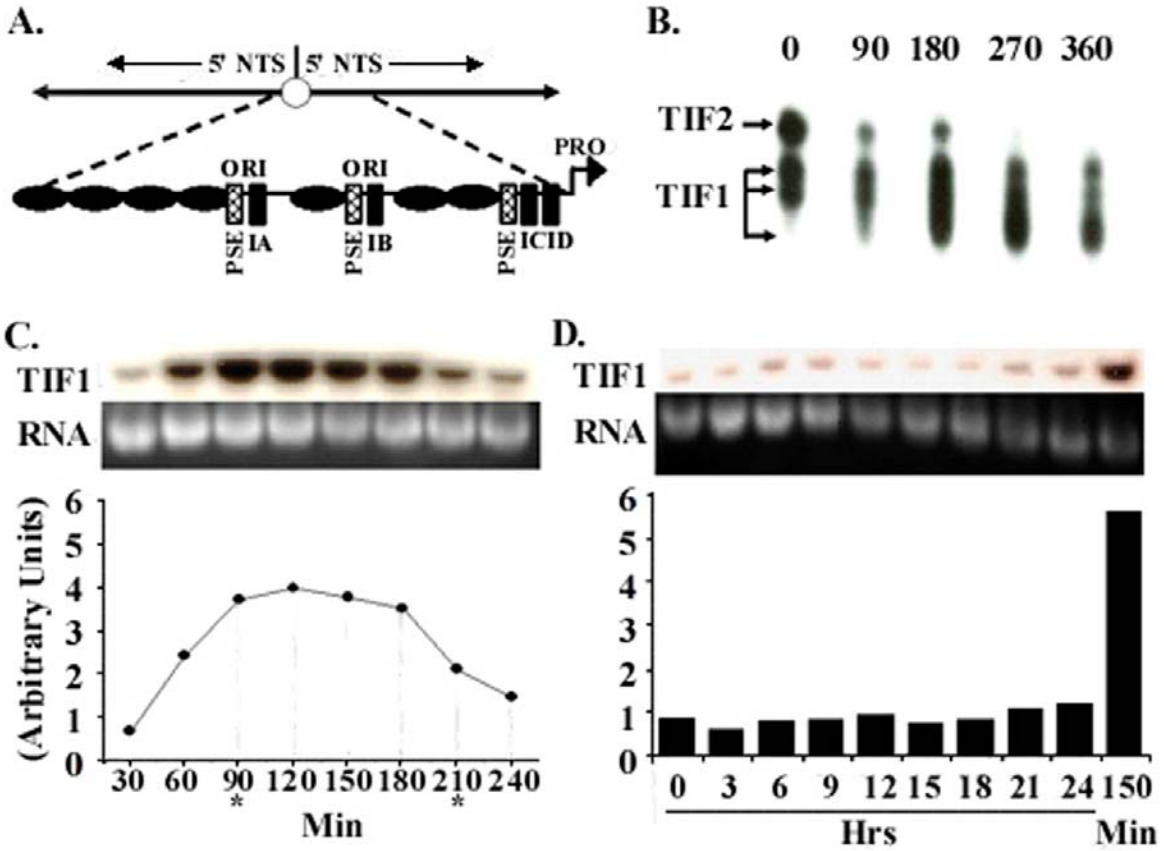


Figure 2.1 TIF1 DNA binding activity and mRNA levels peak in S phase. (A) Schematic of the 21 kb palindromic rDNA minichromosome and 1.9 kb 5' nontranscribed spacer (5'NTS; expanded diagram), including TIF1 binding sites (PSE and type I elements) and positioned nucleosomes (black ovals), and replication origins (ori) which reside in the 230 bp nucleosome-free regions that are part of an imperfect 430 bp tandem duplication. (B) TIF1 DNA binding activity is cell cycle regulated. Gel shift analysis of extracts prepared from vegetative cultures synchronized by starvation and refeeding. The probe, ssA37, corresponds to the A-rich strand of the type IB element (Saha and Kapler, 2000). (C) TIF1 mRNA levels are cell cycle regulated. Northern blot analysis with an intron-spanning TIF1 coding region probe on mRNA prepared from cells synchronized by starvation and refeeding (Mohammad et al., 2000). Hybridization signals were normalized to ethidium bromide staining of the ribosomal RNA, and plotted as a function of time. Asterisks demarcate the approximate beginning and end of macronuclear S phase. (D) TIF1 mRNA levels are constant throughout development. TIF1 northern blot analysis on mRNA prepared from cells at various time points during conjugation. Developmental landmarks: 3 h: pre-meiotic S, 7-8 h: post-zygotic S, 10-24 h: macronuclear development, including rDNA gene amplification. T=150 min: peak TIF1 mRNA level in synchronized vegetative cell cultures.

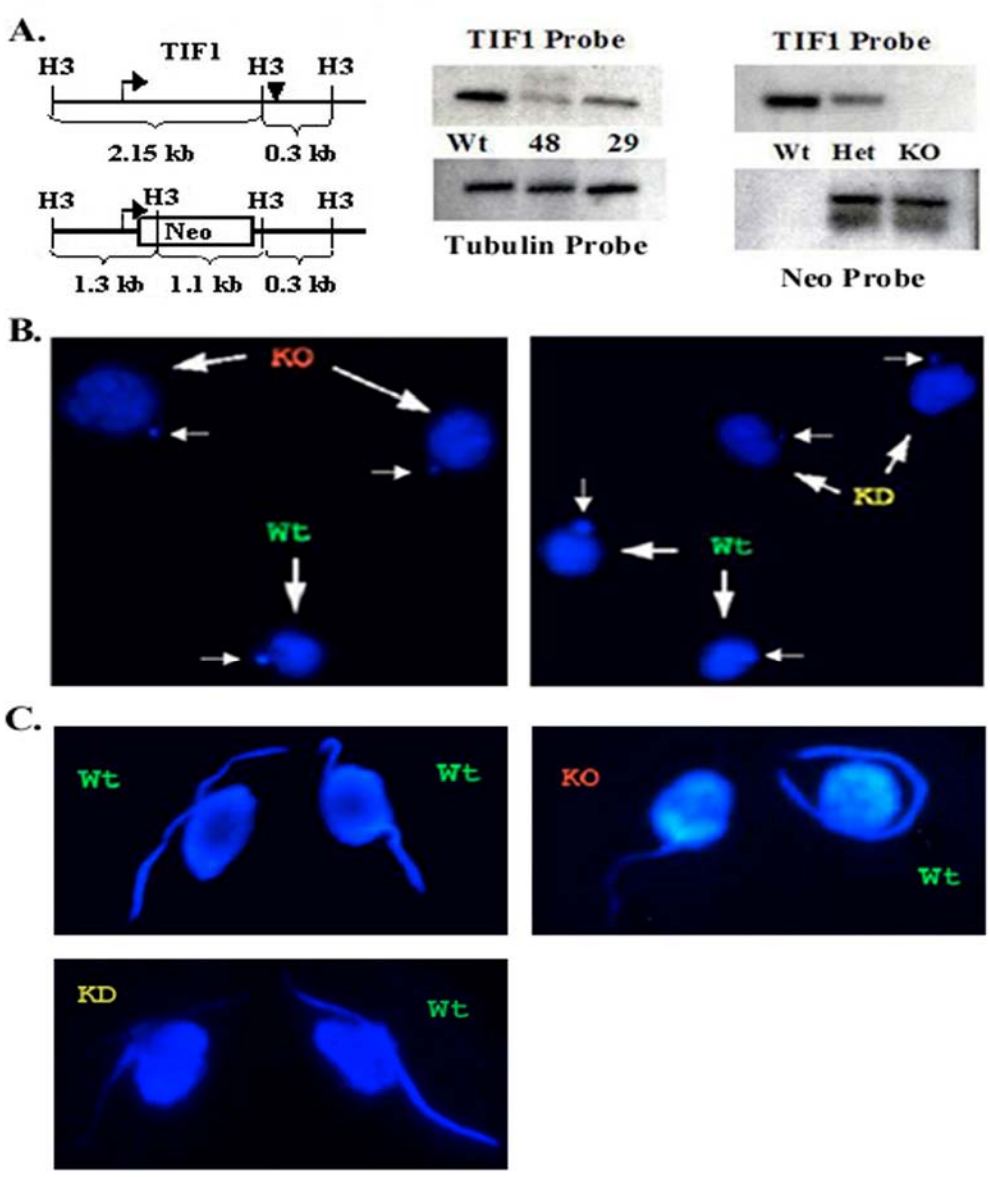


Figure 2.2 Molecular and cytological analysis of TIF1 knockout and knockdown strains. (A) Restriction map of the wild-type TIF1 gene and TIF1::neo replacement allele (Neo). (Bent arrow, TIF1 initiator methionine; filled triangle, TIF1 stop codon). The TIF1 coding region probe recognizes an ~2.15 kb *Hind*III fragment, while the neo probe hybridizes to 1.3 and 1.1 kb fragments. Middle panel: Southern blot analysis of a macronuclear TIF1::neo transformants (TXh48 and TXh29: resistant to 4500 μg /ml paromomycin) and the wild-type strain, CU428. The TIF1 signal was normalized to β-tubulin to estimate the macronuclear TIF1 gene copy number. Right panel: Southern blot analysis of micronuclear (germline) TIF1::neo transformants. Het: heterozygous germline TIF1::neo replacement, TXh102. KO: homozygous TIF1::neo gene replacement, TXk202 (knockout). (B) DAPI staining of vegetative wild-type (Wt), TIF1::neo germline knockout (KO, TXk202) and macronuclear knockdown (KD, TXh48) strains. Wild-type and mutant cells were pre-labeled with different mitochondrial dyes and mixed prior to DAPI staining for comparative analysis. Macronuclei (large arrows), micronuclei (small arrows). (C) Diminished DAPI staining in pre-meiotic micronuclear crescents in TIF1-deficient cells. DAPI (nuclear) staining of mating pairs in wild-type (CU427 x CU428) and wild-type (CU428) x mutant (KO, TXk202; KD, TXh48) crosses. Prior to meiotic S phase, the micronucleus elongates into a characteristic crescent shape. The mitochondrial dyes used to identify individual cells in mating pairs had no effect on DAPI staining intensity.

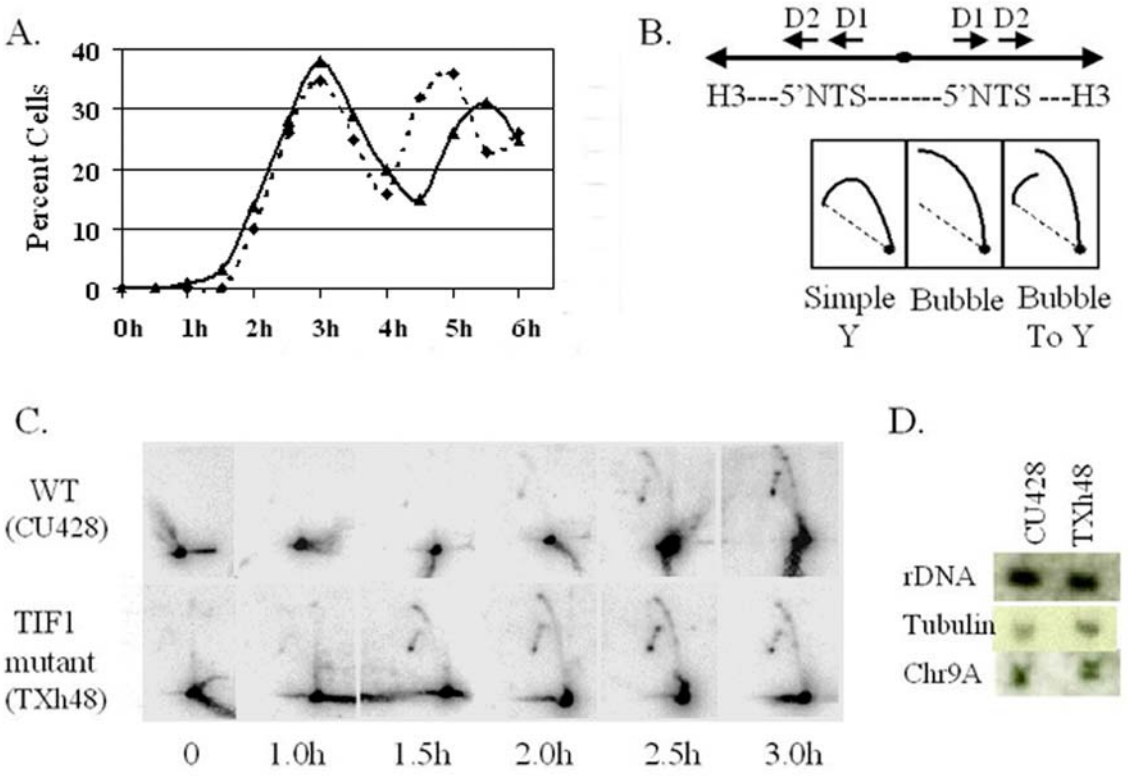


Figure 2.3 Precocious rDNA replication in TIF1-deficient cells. (A) BrdU labeling profiles of cells synchronized by a stationary phase/starvation protocol. The graph depicts the percent BrdU-positive cells in TIF1-deficient (TXh48, solid line) and wild-type (CU428) cultures examined at 30 min intervals after refeeding. (B) Upper panel: restriction map of the 4.2 kb *Hind*III fragment that spans the two inverted copies of the rDNA 5' NTS. Replication origins were previously localized within the tandem 430 bp duplications, designated Domains 1 and 2 (D1, D2; (Zhang et al., 1997). Lower panel: expected 2D gel arc profiles for passive replication of the rDNA 5' NTS (simple Y), replication from a centrally-positioned origin (bubble), and replication from an asymmetrically-position origin (D1 or D2) (bubble-to-Y). (C) 2D gel analysis of Hind*III*-digested DNA from samples from the 0-3 h refeeding interval depicted in panel A. ~20 μg of total genomic DNA was loaded in each lane. (D) Quantitation of rDNA and non-rDNA chromosome abundance in wild-type (CU428) and TIF1-deficient (TXh48) cells. Southern blot analysis of total genomic DNA with probes specific for the rDNA 5' NTS, -tubulin genes (BTU1 and BTU2), and the 51 kb macronuclear chromosome, Chr9A (TIGR sequence scaffold 1172176).

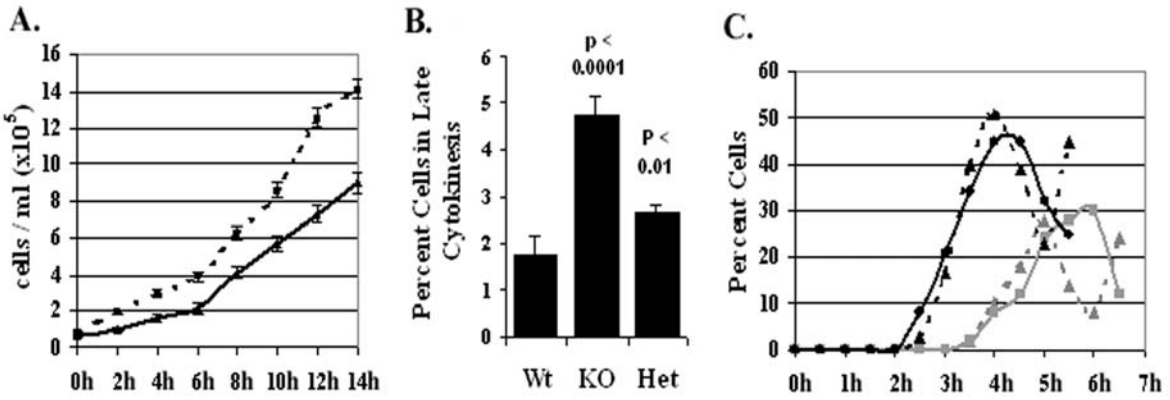


Figure 2.4 Cell cycle defects in the TIF1 knockout mutant. (A) Growth curves for the wild-type (CU428, dashed line) and homozygous TIF1 knockout (TXk202, solid line) mutant were generated by averaging six hemocytometer cell counts per time point (n=5 experiments). (B) Delayed cell division in the TIF1 germline knockout. Asynchronous vegetative cultures were visually examined for dividing cells (presence of a cleavage furrow). Knockout: homozygous TIF1::neo germline replacement (TXk202). Heterozygote: heterozygous TIF1::neo germline replacement (TXh102). The percentage of cells in late cytokinesis was determined by averaging the results from 6-7 independent experimental analyses. (C) Elongated macronuclear S phase and delayed cytokinesis in TIF1-deficient cells. Wild-type and homozygous TIF1 knockout strains were synchronized with a stationary/starvation/refeeding protocol (Mohammad et al., 2003). Refed cultures were pulse labeled with BrdU for a 15 min at 30 min intervals. Indirect immunoflourescence was used to quantify the percentage of BrdU positive cells (dashed black line: wild-type (CU428); solid black line: TIF1 knockout (TXk202). Cytokinesis (dashed gray line: wild-type; solid gray line: TIF1 knockout) was scored by microscopic detection of a cleavage furrow.

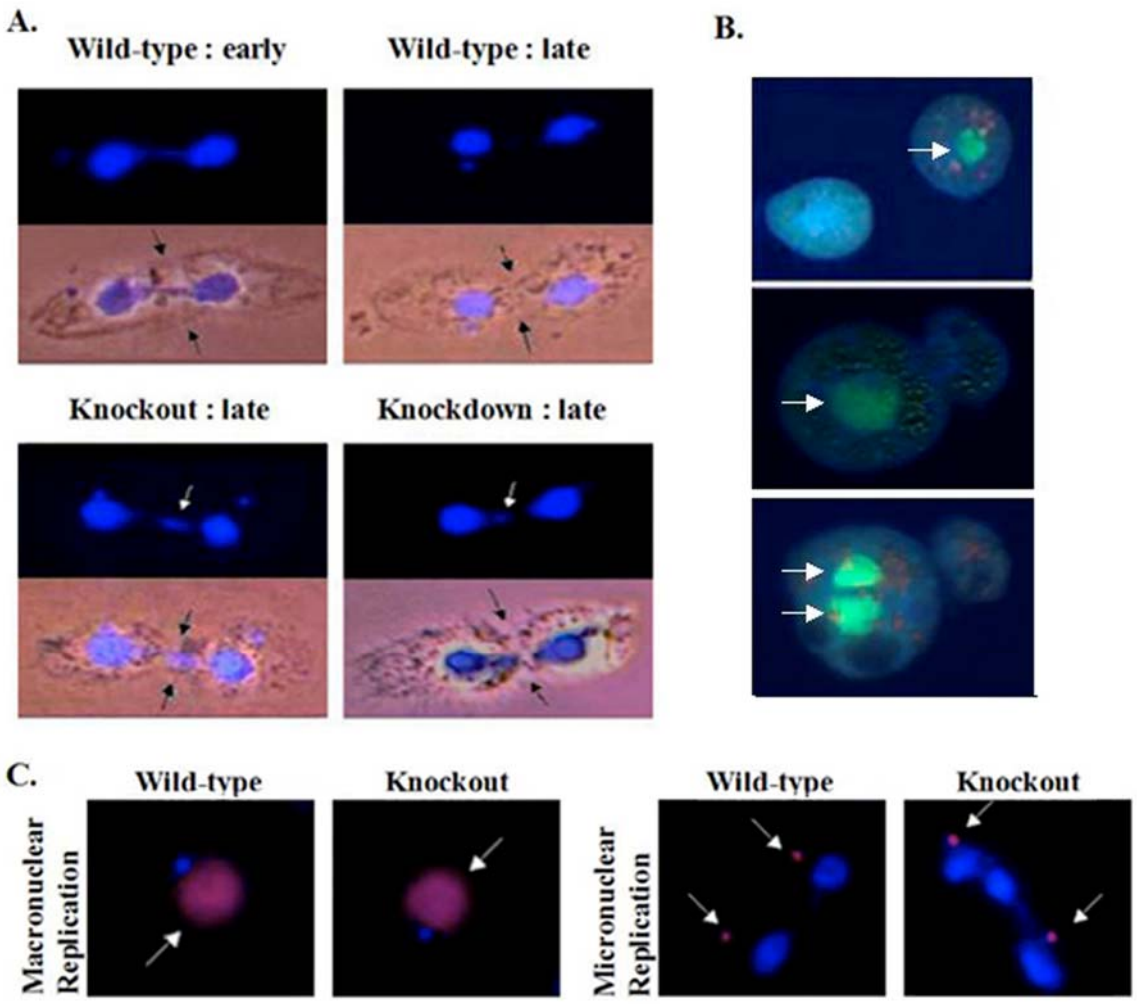


Figure 2.5 Aberrant macronuclear division and cytokinesis in TIF1-deficient strains. (A) Nuclear division and cytokinesis were examined in asynchronous wild-type (CU428), homozygous TIF1 knockout (TXk202), and macronuclear TIF1 knockdown (TXh48) strains following fixation of asynchronous log phase cultures. Light images of representative pre-divisional wild-type cells show the typical relationship between the extent of cleavage furrow invagination (black arrows) and position of daughter macronuclei (DAPI) at early and late stages of cytokinesis. Residual macronuclear DNA at the cleave furrow in mutant cells (white arrow, late stage cytokinesis) was observed in 30-50% of mutant cell divisions. (B) Less frequent cell division phenotypes in TIF1-deficient cells. Apoflour stained live cells were photographed immediately after cell division. Upper micrograph: macronuclear division failure associated with normal cytokinesis (TXh48; arrow: macronucleus). Center micrograph: macronuclear division failure associated with asymmetric cytokinesis. Lower micrograph: macronuclear segregation failure associated with asymmetric cytokinesis. The daughter cell on the left has two macronuclei and the one on the right has none. (C) BrdU pulse labeling of wild-type (CU428) and homozygous TIF1 knockout (TXk202) strains. Cells were pulse labeled for 15 min with BrdU, fixed and stained with DAPI (blue stain) and anti-BrdU antibodies (red stain, white arrow) to examine macronuclear and micronuclear DNA replication. BrdU labeling of the macronucleus was not observed in TIF1-deficient cells undergoing aberrant macronuclear division (right micrograph).

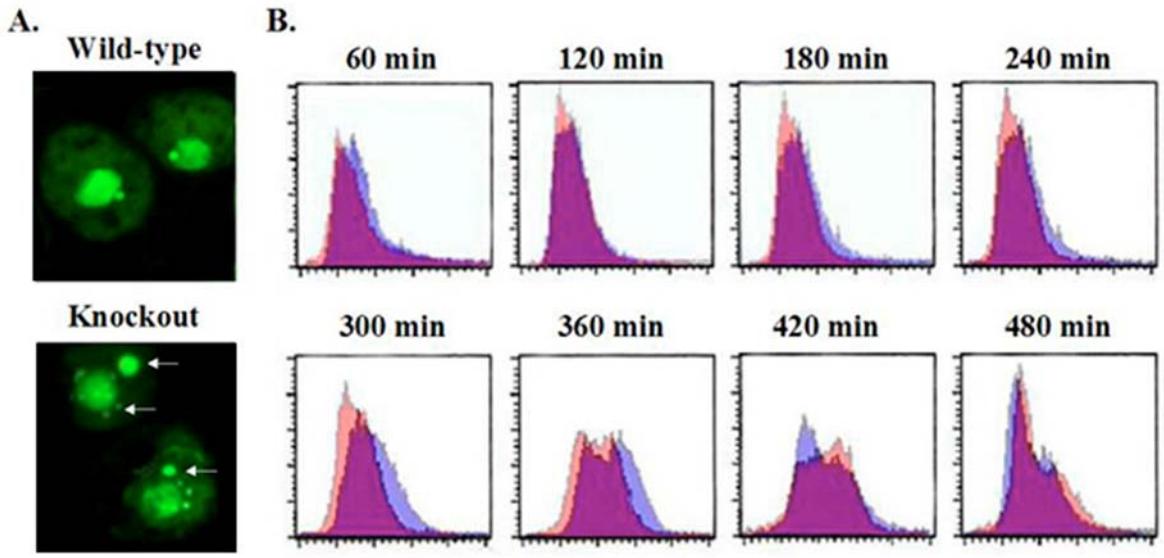


Figure 2.6 TIF1-deficient cells synthesize DNA more slowly than wild-type, but maintain a normal macronuclear DNA content. (A) Sytox (DNA) staining of log phase vegetative cultures of wild-type (CU428) and homozygous TIF1::neo knockout (TXk202) strains. White arrows point to extranuclear DNA-staining bodies in TIF1-deficient cells. These structures were absent in cells transferred into starvation media (data not shown). (B) Flow cytometry of synchronized wild-type and TIF1 knockout strains reveals a diminished rate of DNA synthesis in TIF1-deficient cells. Stationary phase/starved cultures were refed and stained with propidium iodide at 1 h intervals for FACS analysis. The FACS profiles for synchronized wild-type (CU428, purple) and TIF1 knockout (TXk202, pink) cultures were overlaid, the area of overlap appearing as magenta.

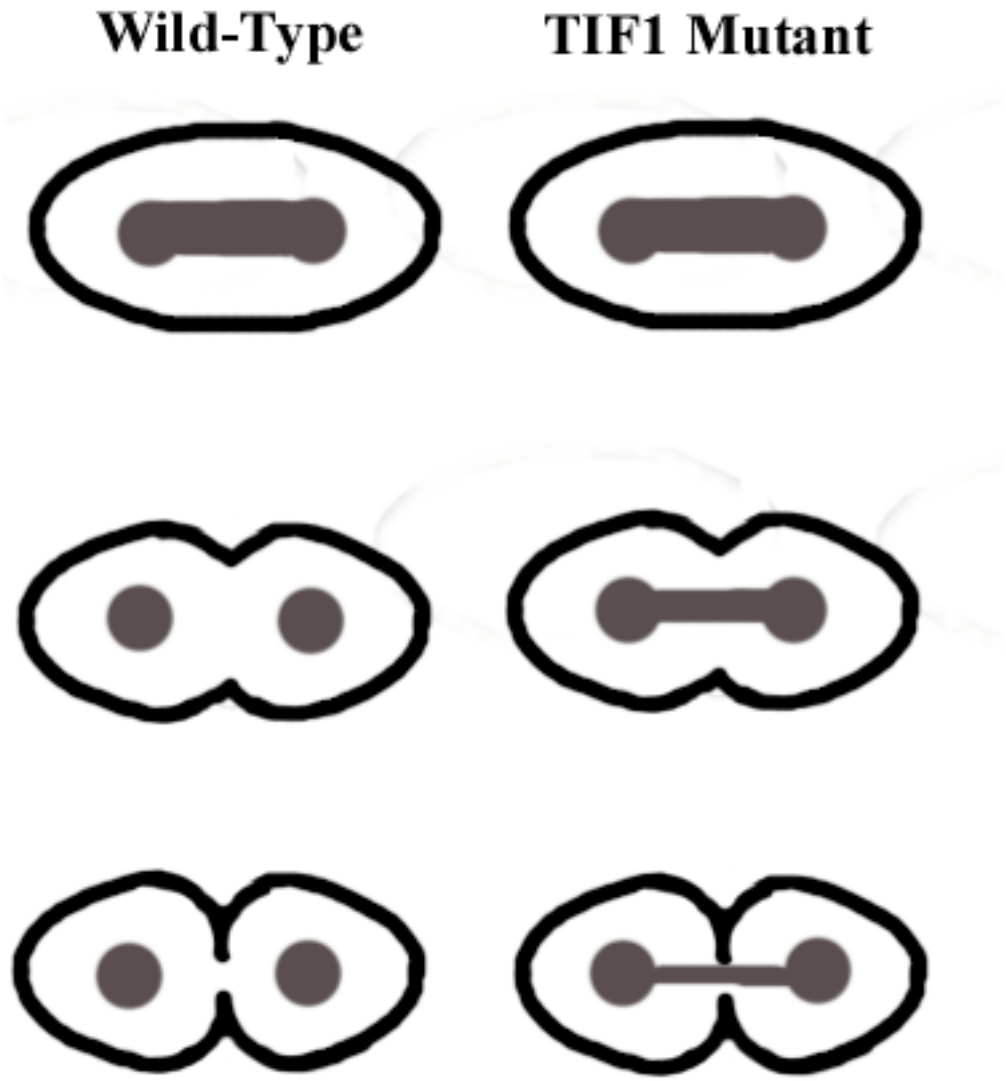


Figure 3.1 Macronuclear division is delayed in TIF1 mutants. Schematic representation of macronuclear division and cytokinesis in wild-type (left panel) and TIF1 mutant cells (right panel).

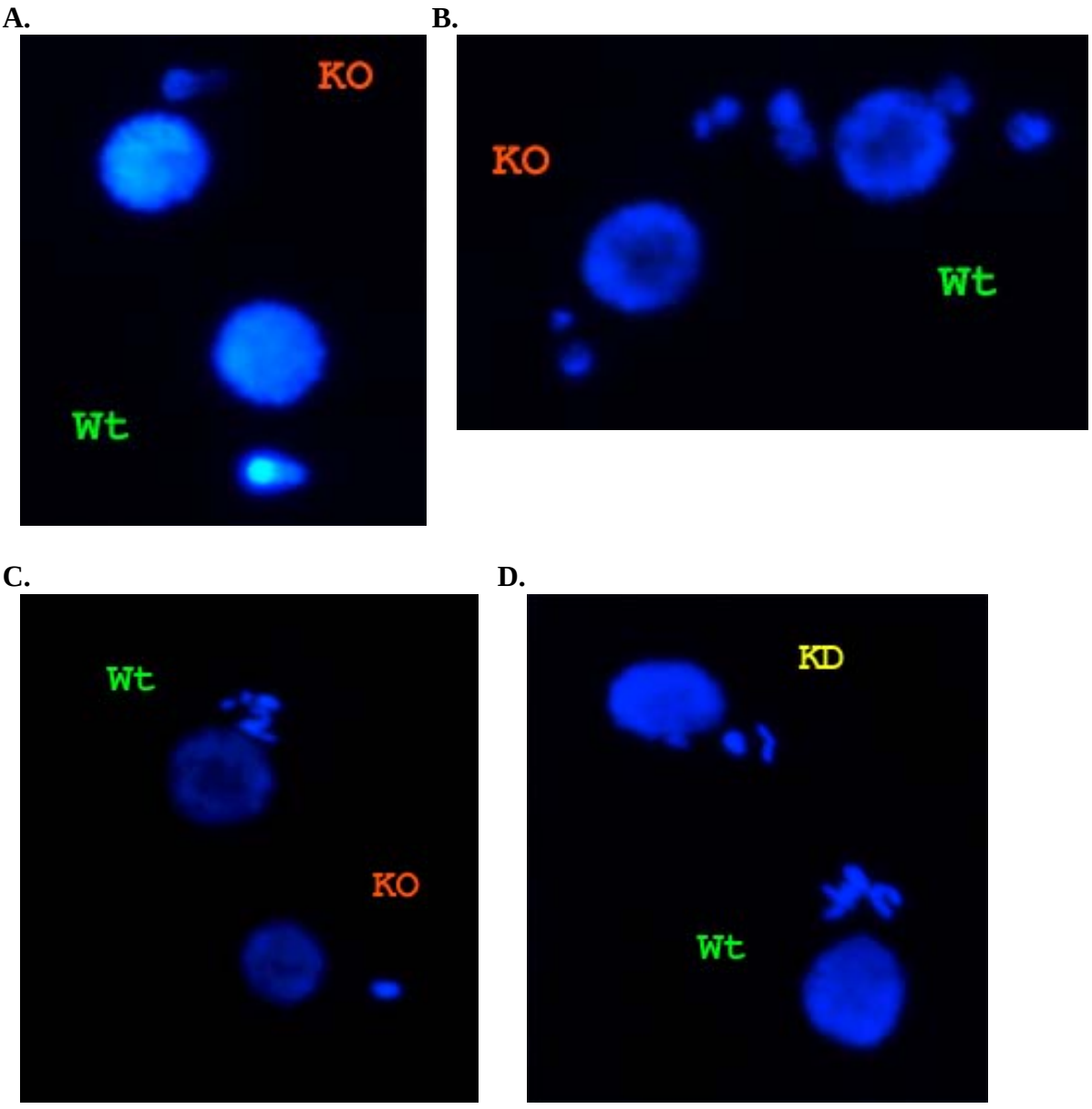


Figure 3.2 Less mic DNA staining in TIF1 mutant cells throughout conjugation. Immunofluorescence of matings between wild-type and TIF1 mutant strains are visualized with DAPI nuclear stain. Cell lines were starved overnight, mated and aliquots were fixed at 1h intervals to observe conjugation. (A) Wild-type (CU428) x TIF1 null (TXk202) showing less DNA staining in the pre-crescent micronucleus of the TIF1 null. (B) Wild-type (CU428) x TIF1 null (TXk202) showing less staining of the mutant mic after Anaphase II (C) Wild-type (CU428) x TIF1 null (TXk202) showing a single condensed mic chromosome in the TIF1 cell before Anaphase I. (D) Wild-type (CU428) x TIF1 knockdown (TXh48) showing fewer condensed chromosomes in the TIF1 knockdown cell before Anaphase I.

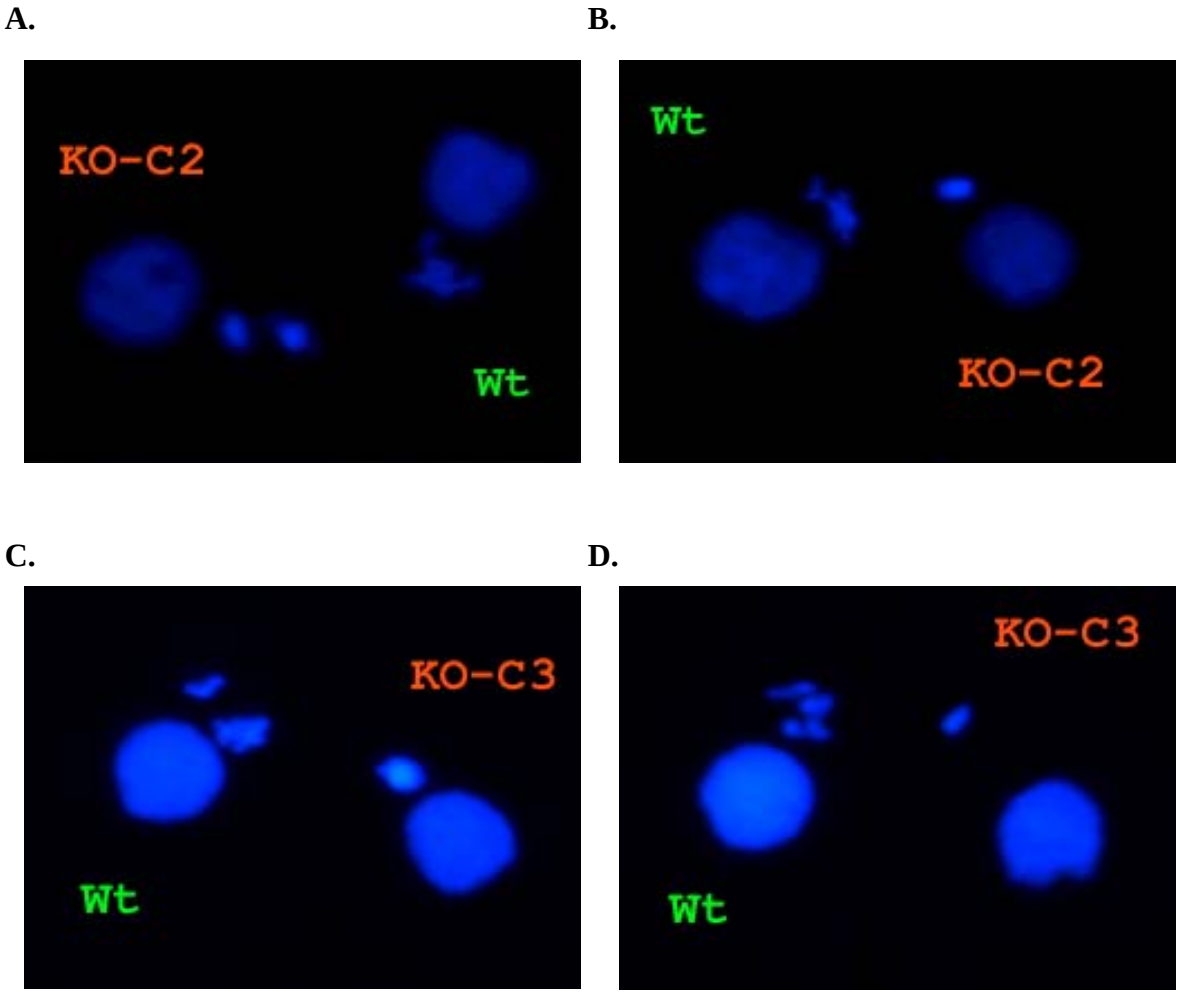
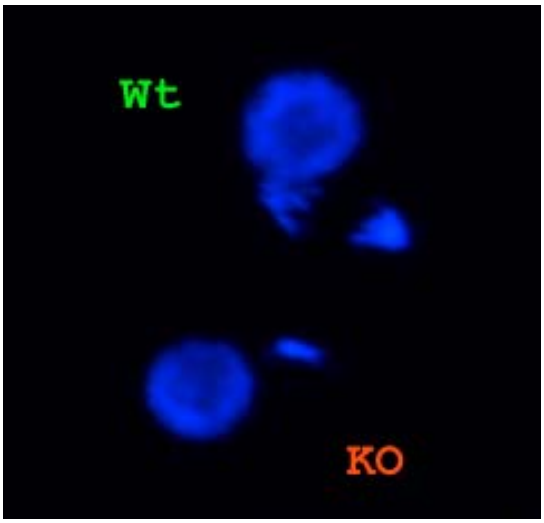
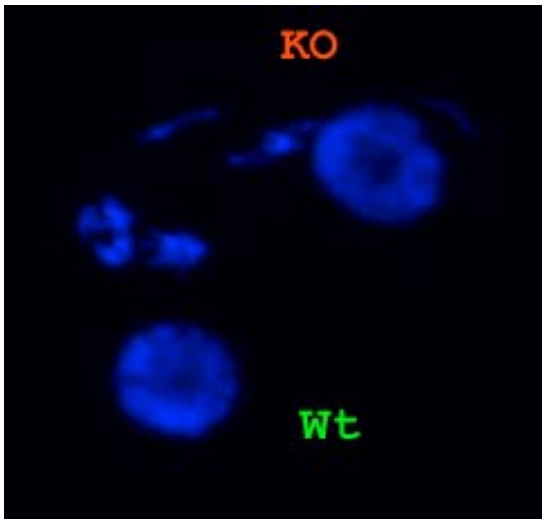


Figure 3.3 Mic genome instability with clonal TIF1 mutant lineages. Immunofluorescence of matings between wild-type and TIF1 knockout clonal lines (TXk202-C3 & TXk202-C2) derived from TXk202 are visualized with DAPI nuclear stain. Cell lines were starved overnight, mated and aliquots were fixed at 1h intervals to observe conjugation. (A) Wild-type (CU428) x TIF1 null clonal line (TXk202-C3) showing 2 condensed mic staining masses in the TIF1 null clonal line. (B) Wild-type (CU428) x TIF1 null clonal line (TXk202-C3) showing 1 condensed mic staining mass in the TIF1 null clonal line. (C) Wild-type (CU428) x TIF1 null clonal line (TXk202-C2) showing 1 large condensed mic staining mass in the TIF1 null clonal line. (D) Wild-type (CU428) x TIF1 null clonal line (TXk202-C2) showing 1 small condensed mic staining mass in the TIF1 null clonal line.

A.



B.



C.

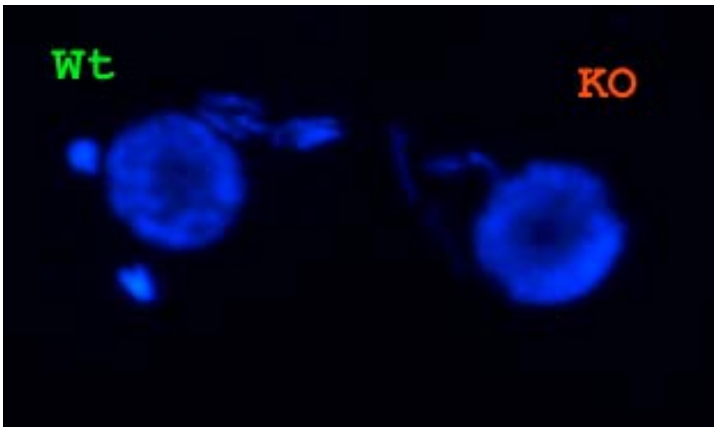


Figure 3.4 Abnormal anaphase in TIF1 mutants. Immunofluorescence using DAPI nuclear stain. Cell lines were starved over night, mated and aliquots were fixed at 1 hour intervals. (A) Wild-type (CU428) x TIF1 null (TXk202) showing the wt cell in Anaphase I and the mut condensed chromosome staining mass. (B) Wild-type (CU428) x TIF1 null (TXk202) showing Anaphase I in the wt cell and potential Anaphase I in the mut cell. (C) Wild-type (CU428) x TIF1 null (TXk202) showing Anaphase II in the wt cell and potential Anaphase I in the mut cell.

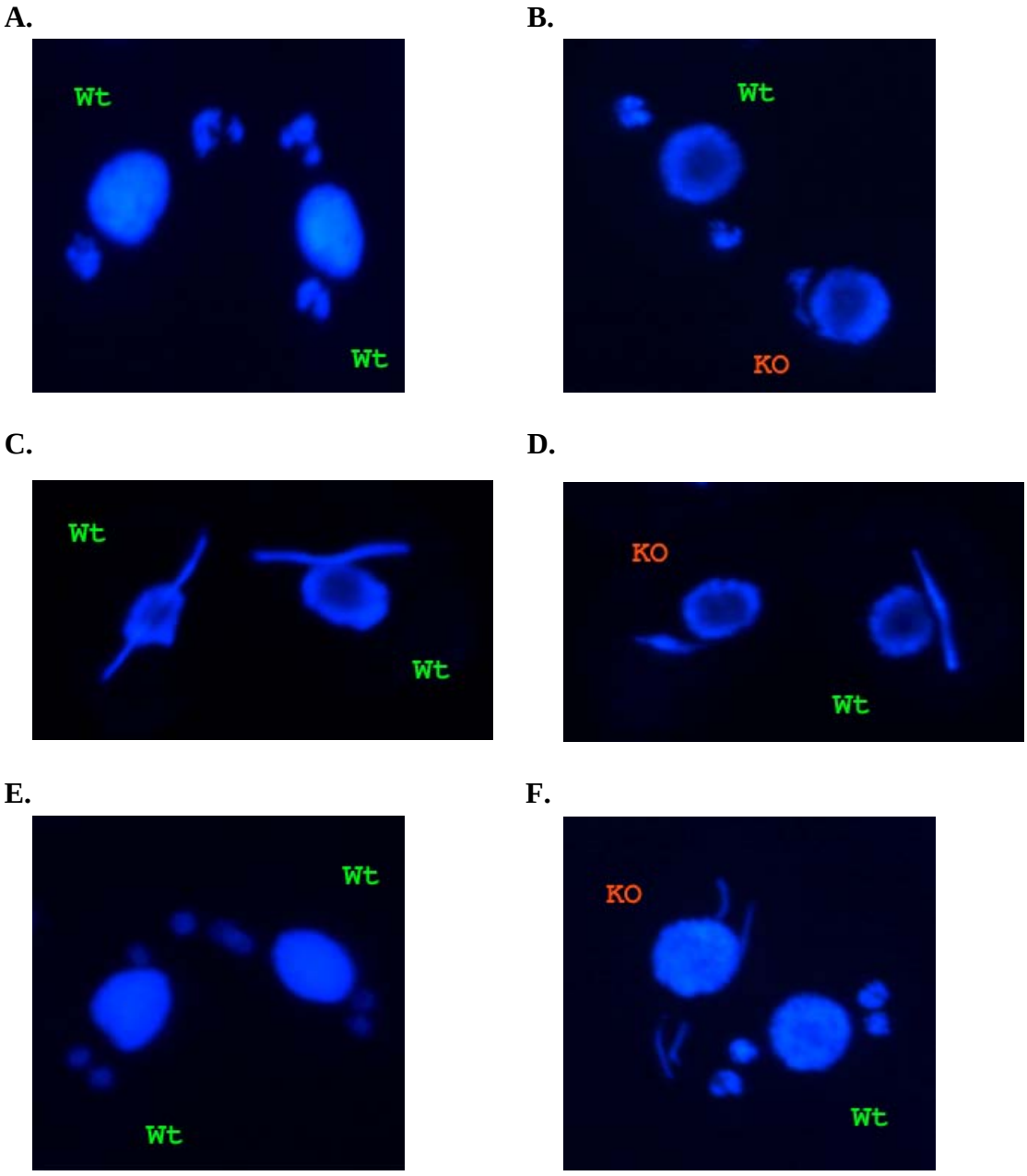


Figure 3.5 Developmental delay in TIF1 mutants. Immunofluorescence using DAPI nuclear stain. Cell lines were starved overnight, mated and aliquots were fixed at 1h intervals to observe conjugation. (A) Wild-type (CU428) x wild-type (CU427) showing synchronized chromosome condensation in the mics preceding Anaphase II. (B) Wild-type (CU428) x TIF1 null (TXk202) showing condensed chromosomes in the wt mic preceding Anaphase II and condensed chromosomes in the mut mic preceding Anaphase I. (C) Wild-type (CU428) x wild-type (CU427) showing synchronized mic crescent formation. (D) Wild-type (CU428) x TIF1 null (TXk202) showing asynchronous crescent formation with the mut cell in an earlier stage than the wild-type mating partner. (E) Wild-type (CU428) x TIF1 null (TXk202) showing synchronized decondensing mic chromosomes post Anaphase II. (F) Wild-type (CU428) x TIF1 null (TXk202) showing decondensing mic chromosomes post Anaphase II in the wt cell and late stage crescent formation in the mut cell.

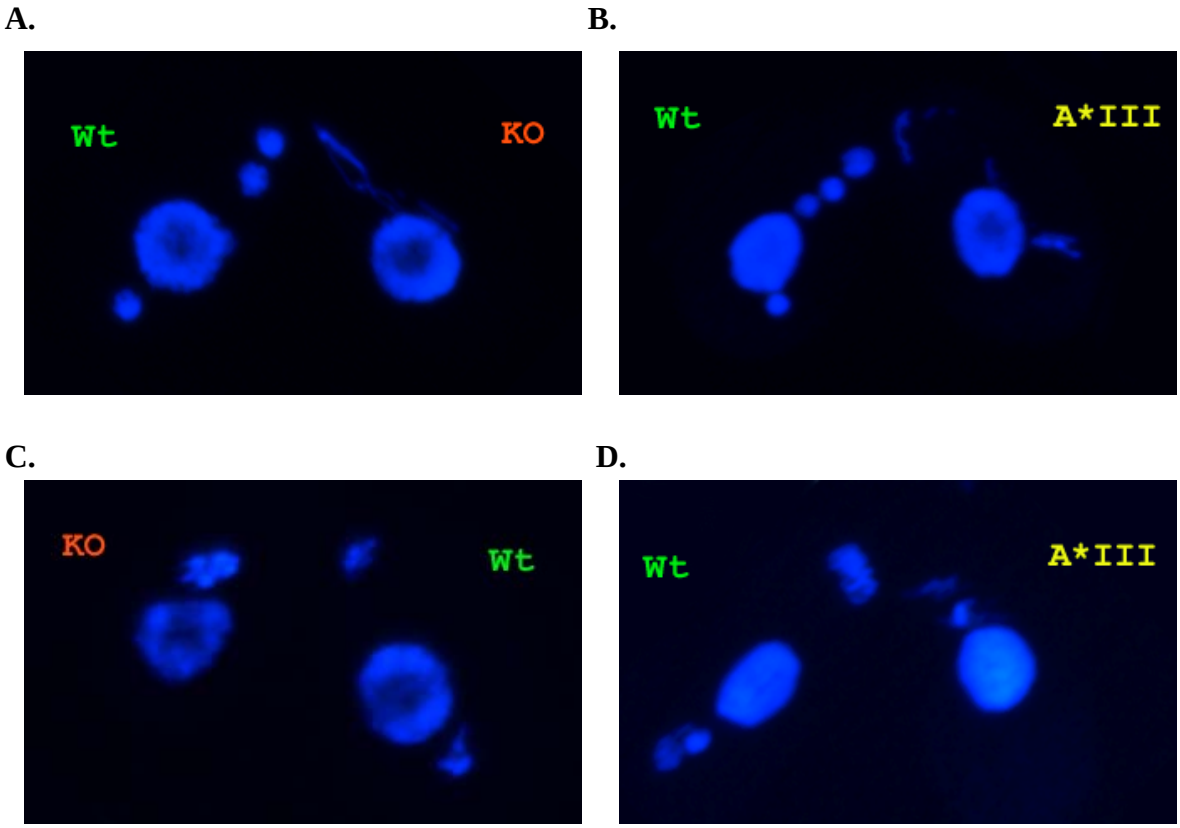


Figure 3.6 Developmental delay in TIF1 mutants and A* amicronucleate cells. Developmental asynchrony between TIF1-deficient and A* cells and wild-type. Immunofluorescence using DAPI nuclear stain. Cell lines were starved over night, mated and aliquots were fixed at 1 hour intervals. (A) Wild-type (CU428) x TIF1 null (TXk202) showing decondensing post Anaphase II chromosomes in the wt and late stage crescent formation in the mut. (B) Wild-type (CU428) x A*III heterokaryon showing decondensing post Anaphase II chromosomes in the wt and late stage crescent formation in the A* cell. (C) Wild-type (CU428) x TIF1 null (TXk202) showing condensed chromosomes in the wt mic preceding Anaphase II and condensed chromosomes in the mut mic preceding Anaphase I. (D) Wild-type (CU428) x A*III heterokaryon showing condensed chromosomes in the wt mic preceding Anaphase II and condensed chromosomes in the A* mic preceding Anaphase I.

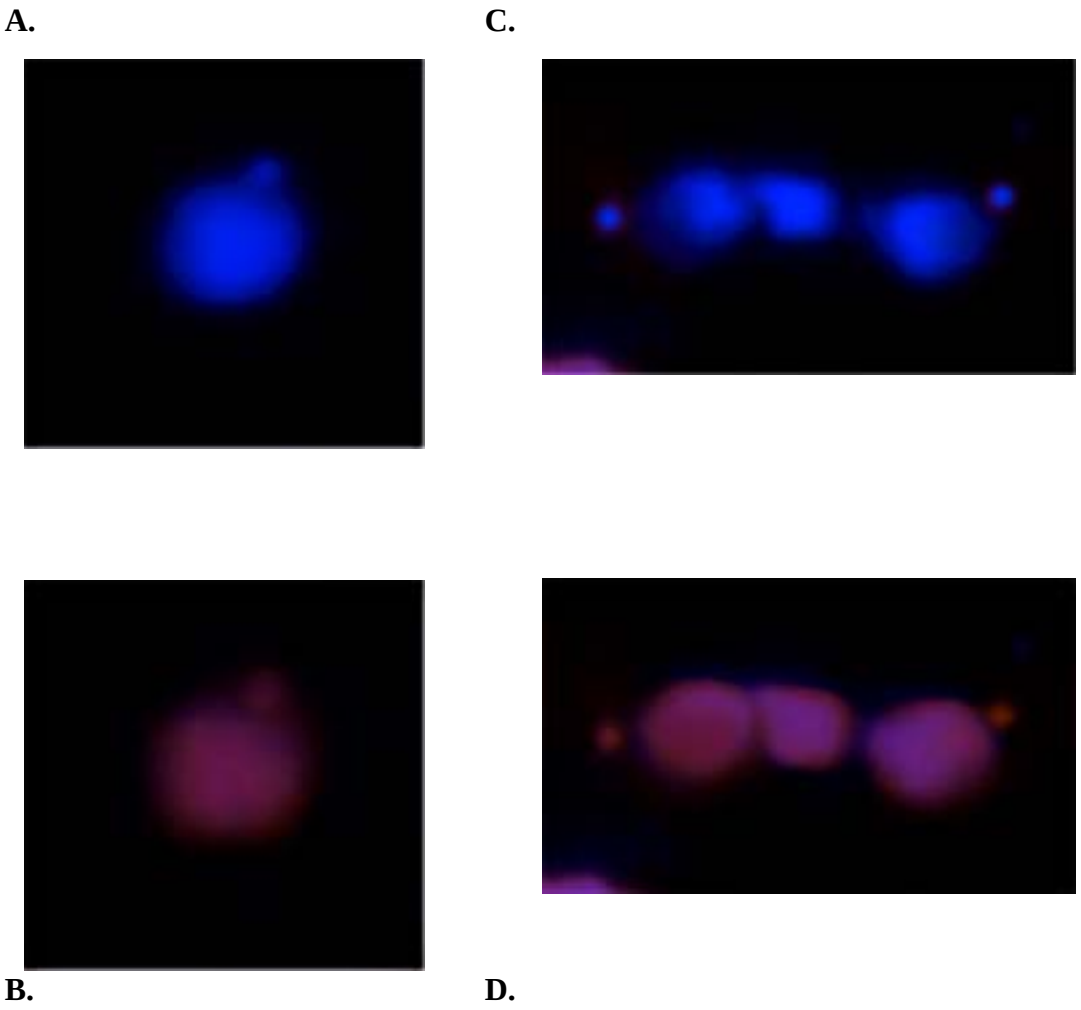
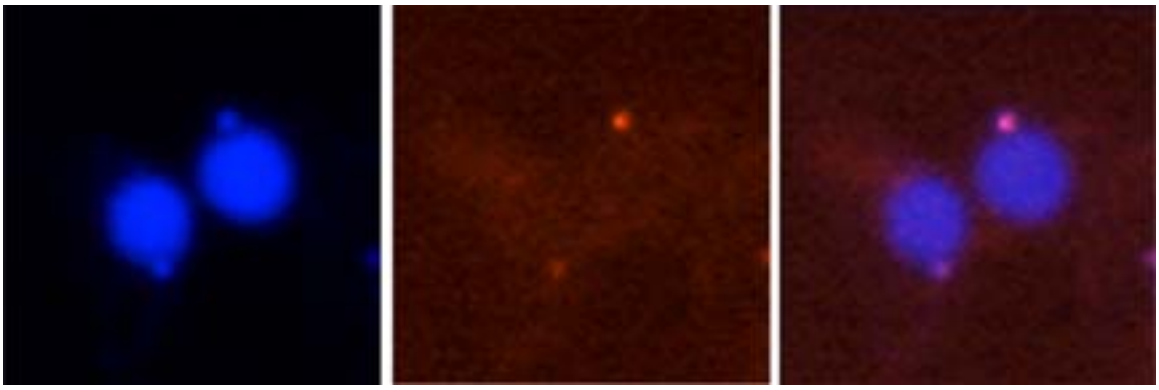


Figure 3.7 Concurrent BrdU labeling of wild-type mics and macs in high concentrations of MMS. Immunofluorescence of BrdU staining in wild-type cells incubated with 0.72% MMS. (A) DAPI staining of a single wild-type cell showing both the mac and micronucleus. (B) BrdU staining of the same cell in A. showing BrdU staining of both the mic and macronucleus. (C) DAPI staining of a wild-type cell undergoing macronuclear division. (D) BrdU staining of the same cell as in C. showing BrdU labeling in both the micronuclei and the dividing macronucleus.

A.



B.

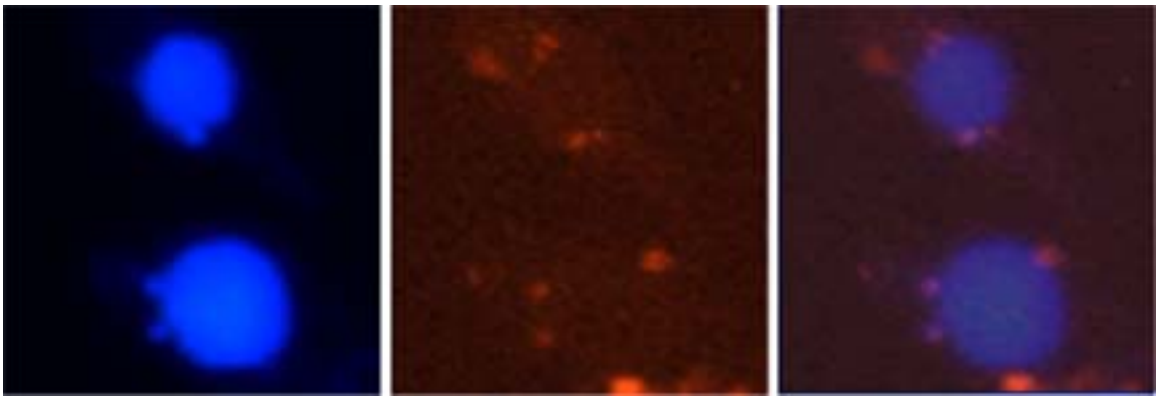


Figure 3.8 Mic staining of γ-H2AX in wild-type and TIF1 mutants treated with MMS. γ-H2AX staining of wild-type and TIF1-deficient cells. (A) Left panel shows DNA DAPI nuclear staining of wild-type, (CU428) nuclei. Center panel shows H2AX staining of micronuclei. Right panel is and overlay of the left and right panels showing both the DAPI and H2AX staining. (B) Left panel shows DNA DAPI nuclear staining of TIF1-deficient, (TXh48) nuclei. Center panel shows H2AX staining of micronuclei. Right panel is and overlay of the left and right panels showing both the DAPI and H2AX staining.

APPENDIX B

TABLES

Table 3.1 Genetic crosses of TIF1 heterozygotes and heterokaryons

Cross	Strains	Phenotype	Expected	Observed	
1	TXh102 x TXh106	pm-r clonal progeny	75%	100%	n=48
		precocious sexual maturity	0%	100%	
2a	TXh102 x SB210	2dgal-r/pm-r progeny	50%	0%	n=40
2b	TXh106 x SB210	2dgal-r/pm-r progeny	50%	0%	n=40
3	Round 1 Heterokaryons x SB1969	cycl-r/pm-r progeny	50%	0%	n=82

Resistance to 2-deoxygalactose (2dgal-r), cycloheximide (cycl-r) or paromomycin (pm-r) is encoded in the micronucleus of SB210, SB1969, or TIF1:neo heterozygotes and heterokaryons, respectively. For cross 1, n= the number of clonal progeny tested for pm-resistance. For crosses 2 and 3, n= the number of small scale matings in which the non-clonal progeny pool was tested for pm-resistance.

Table 3.2 H2A sequences containing the S(-4)Q motif

Species	Classification	
Human	(mammal)	LGGV**TI**AQ**GGV**LPNIQAVLLPKKTSATVGPKAPSGGKKA**TQ**A**S** **Q**EY*
Mouse	(mammal)	LGGV**TI**AQ**GGV**LPNIQAVLLPKKSSATVGPKSPAVGKKA**SQ**A**S** **Q**EY*
Drosophila	(insect)	IKA-**TI**AG**GGV**IPHIHKSLIGKK-(4)EETVQDPQRKGNVIL**SQ**EF*
Arabidopsis	(plant)	LGVS**TI**AN**GGV**LPNIHQTLLPSK-(8)----VGKNKGDIGSA**SQ**EF*
Tet. Py.	(protozoa)	MANT**TI**AD**GGV**LPNINPMLLPSK-(9)-----SKKTESRGQA**SQ**DI*
Tet. Th.	(protozoa)	MANT**TI**AD**GGV**LPNINPMLLPSK-(9)-----SKKTESRGQA**SQ**DL*
Aspergillus	(yeast)	LGHV**TI**AQ**GVV**LPNIHQNLLPKK-(12)-------TPKAGKG**SQ**KL*
S. cerevisiae	(yeast)	LGNV**TI**AQ**GGV**LPNIHQNLLPKK-(12)-------SAKATKA**SQ**KL*
S. pombe	(yeast)	LGHV**TI**AQ**GGV**VPNINAHLLPKT-(12)-------SGGTGKP**SQ**EL*
Giardia	(protist)	FANV**TI**RE**GGV**ARSAKEGREGKG-(16)----------SHR**SQ**DL*

H2A sequences with an S($^{-4}$)Q motif were obtained from GenBank® and aligned manually. The S($^{-4}$)Q motif including the second SQ or TQ motif in mammalian H2AX, the TI, and the GGV conserved motifs are shown in bold, all other residues are in normal font. [Figure from Redon (2002)]

Table 3.3 Macronuclear BrdU incorporation in MMS treated wild-type cells

MMS (w/v)	BrdU positive macronuclei (n=2100)
0 %	40 %
0.06 %	37 %
0.12 %	32%
0.24 %	0 %
0.48 %	11 %
0.72 %	85 %

Table 3.4 Macronuclear BrdU incorporation in MMS-treated cells

MMS (w/v)	BrdU positive macronuclei	
	Wild-type (n=1900)	TIF1 mutant (n=1850)
0 %	40 %	39 %
0.03 %	38 %	36 %
0.06 %	36 %	26 %
0.12 %	32 %	23 %
0.18 %	23 %	0 %
0.24 %	0 %	0 %

Table 3.5 Micronuclear H2AX staining in MMS-treated cells

MMS (w/v)	H2AX positive micronuclei	
	Wild-type (n= 1870)	TIF1 mutant (n=1790)
0 %	0 %	4 %
0.03 %	3 %	21 %
0.06 %	15 %	48 %
0.12 %	61 %	55 %
0.18 %	63 %	59 %
0.24 %	67 %	61 %

VITA

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