
Review

Programmed nuclear death and its relation to apoptosis and autophagy during sexual reproduction in *Tetrahymena thermophila*

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SUMMARY

The ciliated protozoan *Tetrahymena thermophila* has evolved remarkable nuclear dualism that involves spatial segregation of the polyploid somatic macro- and canonical diploid germinal micro-nucleus in a single cytoplasm. Programmed nuclear death (PND), also referred to as nuclear apoptosis, is a remarkable catabolic process that occurs during conjugation to finish the lifespan of parental soma, in which only the parental macronucleus is eliminated from the cytoplasm, but other co-existing nuclei are unaffected. We found that PND involves unique aspects of autophagy, which differ from mammalian or yeast macroautophagy. When PND starts, the envelope of the parental macronucleus changes its nature as if it is an autophagic membrane, without the accumulation of other membranous structures from the cytoplasm. The alteration of the parental macronuclear membrane involves exposing certain sugars and phosphatidylserine on the envelope, which are members of a class of “eat-me” signals found on the surface of apoptotic cells, that are not found on other types of nuclei. Subsequently, small autophagic vesicles that contain mitochondria and lysosomes fuse with the nuclear envelope stepwise and release their contents into the nucleus at distinct stages. Mitochondria of *Tetrahymena* contain apoptosis-inducing factor (AIF) and endonuclease G-like DNase, which are responsible for the nuclear condensation and kb-sized DNA fragmentation, corresponding to the early stage of the nuclear apoptosis. On the other hand, acidic lysosomal enzymes are responsible for final resorption of the nucleus. These elaborate mechanisms, unique to ciliates, ultimately achieve specific macronuclear elimination.

Key words: Nuclear dimorphism, Nuclear degradation, Caspase, Mitochondria, AIF, EndoG, Autophagosome, Lysosome, Death commitment, Nuclear differentiation

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INTRODUCTION

Ciliates are large unicellular protozoans (up to ~ 400 μm in length), which belong to alveolates. The alveolates consist of three different phyla; dinoflagellates, apicomplexans, and ciliates. The most remarkable and virtually unique feature of ciliates is that they maintain stably differentiated germ-line and somatic nuclear genomes within a single cytoplasm (Orias et al., 2011), which is called nuclear dualism or nuclear dimorphism. The canonical diploid germ-line genome is housed in the micronucleus, while the polyploid somatic genome is housed in the macronucleus. The micronuclear genome, which is transcriptionally silent in the vegetative, asexual stage, is the store of genetic information for sexual progeny. The macronuclear genome is the primary source of gene transcripts in the cell, and its active working maintains the life of the cell and provides the phenotype. The micro- and macronuclei have distinct nucleoporins on their surface, which characterize their functional and morphological features (Iwamoto et al., 2009; Iwamoto, 2011).

T. thermophila (hereafter referred to as *Tetrahymena*) is an exceptional model ciliate that has been involved in two studies recognized as the Nobel Prize, on catalytic RNA and ribozymes (Cech, 1990) and telomeres and telomerase (Greider and Blackburn, 1985). The macronuclear genome containing ~ 25,000 known or predicted protein coding genes, a number comparable to that of the human genome, has been sequenced (Eisen et al., 2006; Coyne et al., 2008). The microarray gene expression profile (Miao et al., 2009), some gene networks (Xiong et al., 2011) and some organelle proteomics have also been analyzed (Smith et al., 2005; Jacobs et al., 2006; Smith et al., 2007; Kilburn et al., 2007; Cole et al., 2008; Gould et al., 2011). Gene expression analysis using RNA seq (Xiong et al., 2012) has also been reported and the micronuclear sequence is also available (<http://www.broadinstitute.org/annotation/genome/>

[Tetrahymena/MultiHome.html](http://www.broadinstitute.org/annotation/genome/Tetrahymena/MultiHome.html)).

Sexual reproduction of ciliates is called “conjugation”. Unlike other eukaryotes, the alteration of generations of ciliates is performed between the two kinds of nuclei through the process without fusion or disintegration of the cytoplasm. In *Tetrahymena*, the progression of conjugation has been clearly illustrated by Cole and Sugai (2012). Briefly, conjugation is initiated by cell-to-cell interaction between different mating types, followed by meiosis in the micronucleus, exchange of the haploid meiotic products, and formation of a diploid zygotic micronucleus, which corresponds to fertilization in metazoans. The zygotic micronucleus divides mitotically twice (postzygotic divisions), resulting in four micronuclei. Two of these at the anterior region of the cell differentiate into new macronuclei, while the other two at the posterior region of the cell remain as micronuclei. The new macronuclear development involves large scale genome rearrangement and amplification that is accomplished by a mechanism involving RNAi-mediated, heterochromatin formation (Mochizuki and Gorovsky, 2004; Yao et al., 2007; Noto et al., 2010; Chalker and Yao, 2011). During the process, at least 6,000 distinct sequences, likely remnants of transposable elements, called Internal Eliminated Sequences (IES), are deleted from the micronuclear genome in a site-specific manner. Concomitant with new macronuclear differentiation, the parental macronucleus starts to degrade and eventually disappears from the cytoplasm. This process is called Programmed Nuclear Death (PND), in which only the parental macronucleus is eliminated while other co-existing nuclei, such as new micro- and macronuclei are unaffected (Davis et al., 1992; Endoh and Kobayashi, 2006).

PND plays a critical role in the lifecycle of ciliates because it obligates the parental macronucleus to complete the lifespan and to reset the developmental time of the cell to zero. This is similar to a death program that almost all eukaryotes are obligated to for completion of the lifespan of so-

ma. While much has been learned about the mechanisms for new macronuclear development, the mechanisms for PND are still largely unexplored. In this decade, we have been challenged to improve the study of PND and have shown that PND is a remarkable catabolic process that involves multiple apoptotic and autophagic markers. In this review, we discuss the process of PND in ciliates with emphasis on *Tetrahymena* from perspectives of apoptosis and autophagy. We also discuss its relation to new macronuclear differentiation and development for a better understanding of the novel lifecycle observed in ciliates.

INVOLVMENT OF MULTIPLE APOPTOTIC MARKERS

When PND starts, the parental macronucleus begins to condense and reduce in size. In this period, nuclear DNA is degraded into high molecular weight fragments of more than 30 kb involving a Ca^{2+} -independent, Zn^{2+} -insensitive nuclease activity (Mpoke and Wolfe, 1997). Subsequently, an oligonucleosome-sized ladder appears (Kobayashi and Endoh, 2003). These phenomena are similar to the apoptotic events in Programmed Cell Death (PCD) in multicellular organisms, so PND is sometimes referred to as nuclear apoptosis. Lysosomes cluster around and in close proximity to the parental macronucleus in the later stage of PND, by which time the nucleus becomes acidic and finally disappears from the cytoplasm (Lu and Wolfe, 2001; Kobayashi and Endoh, 2005). Mpoke and Wolfe (1996) suggested that acidic enzymes similar to DNase II, which is involved in apoptosis in Chinese hamster ovary cells, or DNase β and γ , both of which are involved in apoptosis, may be involved in nuclear death in *Tetrahymena*.

Caspases

When animal cells undergo apoptosis, the morphological features are nuclear condensation and formation of apoptotic bodies without a break-

down of the plasma membrane (Kerr et al., 1972). In addition to morphological changes, apoptosis is also characterized by DNA fragmentation and activation of the caspase enzyme family (Nicholson and Thornberry, 1997). Caspase is a family of cysteine proteases that play essential roles in apoptosis. Initiator caspases, such as caspase 9, cleave the inactive pro-form of effector caspases (e.g., caspase 3, 6, and 7) to activate them. Sequentially, cleaved (activated) effector caspases then cleave downstream protein substrates, such as poly(ADP-ribose) polymerase (PARP), triggering the apoptotic process (Lamkanfi et al., 2007).

The involvement of caspase-like activities in *Tetrahymena* PND have been identified by *in vitro* enzymatic assays showing that competitive inhibitors for caspase-8 (Ac-IETD-CHO) or caspase-9 (Ac-LEHD-CHO) inhibited caspase-8 and -9 like activities in *Tetrahymena* extracts. Caspase-3 like activity was not detected (Kobayashi and Endoh, 2003). Both caspase-8 and -9-like activities significantly increased during the final resorption process. Similarly, a specific inhibitor against caspase -1 (Ac-YVAD-CHO) and a general caspase inhibitor (z-VAD-fmk) blocked PND *in vivo*, but localization of the activity in the degenerating macronucleus was not detected (Ejercito and Wolfe, 2003), suggesting its role in PND is indirect. After these two reports were published in 2003, the complete macronuclear genome sequence of *Tetrahymena* became available (Eisen et al., 2006) and no caspase genes were found. Caspase-dependent pathways for apoptosis appear to have been established in independent lineages of animals later in eukaryotic evolution given that fungi, plants, and protists commonly lack caspase homologues. Taken together, organisms including ciliates may have independently evolved proteases that may play roles in each of apoptosis or PCD pathways. In fact, arginine/lysine-specific proteases called metacaspase are found in plants, fungi, and some protists, but not in slime mold, animals and ciliates. In an analogous manner to caspases, metacaspases

induce PCD in these organisms (Madeo et al., 2002; Bidle and Falkowski, 2004; Bozhkov et al., 2005).

Mitochondria

Mitochondria contain multiple cell death-associated factors, which are known to play a major role in plant and animal PCD processes (Kroemer, 1998). Cytochrome c, Smac/DIABLO, and Omi/HtrA2 are mitochondrial factors involved in caspase activation. Furthermore, some caspase-independent pathways involve either apoptosis-inducing factor (AIF) or endonuclease G (EndoG), both of which are direct effectors of nuclear condensation and DNA fragmentation (Lorenzo et al., 1999; Low, 2003). These proteins are released from mitochondria and translocate into the nucleus owing to breakdown of the mitochondrial membrane potential when the cell responds to apoptosis signals. Kobayashi and Endoh (2005) confirmed a relationship between mitochondria and the nucleus during PND using DePsipher, which stains mitochondria depending upon their membrane potential. During PND, many mitochondria that have lost their membrane potential migrate toward only the parental macronucleus and seem to cluster in or in close proximity to the nucleus. The timing of mitochondrial and macronuclear co-localization suggests that some dead or broken mitochondria are taken into the nucleus, resulting in the execution of nuclear apoptosis.

Kobayashi and Endoh (2005) have also identified that mitochondria of *Tetrahymena* have a DNase activity. This has an optimal pH at nearly neutral, requires divalent cations, and is strongly inhibited by Zn^{2+} , a strong inhibitor of most DNases. These characteristics are reminiscent of those of mammalian EndoG, which mediates the caspase-independent pathway in apoptosis (Widlak et al., 2001), suggesting the possibility that the putative mitochondrial DNase is responsible for DNA fragmentation during PND. This possibility has been strengthened by incubation of isolated mitochon-

dria with isolated macronuclei in a cell-free system, which generated 150-400 bp DNA fragments, roughly corresponding in size to the monomeric and dimeric units of the DNA ladder (Kobayashi and Endoh, 2005).

Apoptosis-inducing factor (AIF)

Akematsu and Endoh (2010) further analyzed PND and its relation to mitochondria, and demonstrated that PND shared highly conserved elements with the caspase-independent apoptosis pathway, an AIF-mediated type of apoptosis pathway in multicellular eukaryotes. AIF is a nuclear-encoded mitochondrial flavoprotein that possesses NADH oxidase activity in its C-terminal region. The primary sequence of AIF is highly similar to those of oxidoreductases from animals, fungi, plants, eubacteria, and archaeobacteria (Koonin and Aravind, 2002), whereas components for a caspase-cascade have been identified in only animals. Therefore, AIF-mediated cell death is thought to be an ancient type of apoptosis. Upon induction of apoptosis, AIF is released from mitochondrial inter membrane space into the nucleus in a caspase-independent manner, which causes nuclear condensation and large scale DNA fragmentation (Lorenzo et al., 1999).

Observation with a strain expressing *Tetrahymena* AIF-GFP showed that the signal appeared to penetrate into the inside of the parental macronucleus, suggesting that AIF is released from mitochondria and differentially translocated into the parental macronucleus (Akematsu and Endoh, 2010) (Fig. 1). Somatic knockout of *AIF* causes delay of large scale DNA fragmentation and nuclear condensation in the early stage of PND (Akematsu and Endoh, 2010). In *Caenorhabditis elegans*, *Wah-1/AIF* cooperates with *Cps-6/EndoG* to promote DNA degradation *in vitro* (Wang et al., 2002). In addition, *Wah-1* (RNAi) strains and *Cps-6* mutants display similar defects in cell death and DNA degradation, and both *Wah-1* and *Cps-6* are localized to mitochondria (Wang et al., 2002).

Similar to the observation in *C. elegans*, EndoG-like activity in mitochondria of *Tetrahymena* is also affected by AIF. When a plasmid cleavage assay is performed using mitochondrial proteins from wild-type and *AIF* deficient strains, absence of AIF is responsible for a drastic decrease of the activity *in vitro* (Akematsu and Endoh, 2010). These findings suggest that *Tetrahymena* utilizes an AIF-mediated apoptosis system, by which the suicide process of the parental macronucleus may be facilitated at almost same time with beginning new macronuclear differentiation. However, knocking out of the *AIF* gene in the parental macronucleus only slows the early stages of PND by up to 4 h, including nuclear condensation and kilobase-size DNA fragmentation, and does not completely inhibit the progression of PND (Akematsu and Endoh, 2010). Indeed, by the end of conjugation, the *AIF*-deficient cells are delayed only approximately 1 h, as compared with the wild-type controls. Hence, there must be a mechanism that compensates for the deficiency of AIF, thereby allowing the appropriate execution of the death program. We have examined several other DNase activities in mitochondria, which can slightly degrade DNA without AIF (Osada, E., unpublished data). These activities may rescue the deficiency in AIF despite slow progression of the early stage. It would also be reasonable to assume that DNase II-like activities found in lysosomes (Mpoke and Wolfe, 1996) could assist the digestion of the DNA into small fragments allowing the cells to catch up at later stages of the process.

AUTOPHAGIC/LYSOSOMAL PROCESS

In PND, the parental macronucleus must trigger signaling pathways to activate the engulfing machinery of a membranous structure to protect other nuclei and cytoplasm from the harm of digestive proteins, such as AIF, EndoG-like DNase and lysosomal enzymes, when commitment is made to the death program. In the catabolic system

for degenerating disused organelles represented by macroautophagy (sometimes referred to as just autophagy), formation of a double membranous structure is crucial for sequestering targeted components from the remaining cytoplasm. This is called autophagosome formation (Yang and Klionsky, 2010; Mizushima et al., 2011). Although the origin of the autophagosome is still controversial, the autophagosome fuses with lysosomes to form an autolysosome, and the sequestered components are degraded by the lysosomal enzymes and then recycled. However, no wrapping process of the parental macronucleus with other membranous structures has been observed during conjugation (Weiske-Benner and Eckert, 1987). Thus, it has been unclear whether PND involves typical membrane dynamics like mammalian and yeast macroautophagy. Moreover, it has also been unclear why the PND machinery recognizes the parental macronucleus while the other nuclei are present at the same time in the same cytoplasm. Such timely and selective elimination of the disused nucleus is essential for ciliates to protect progeny genotype from the deleterious degradative proteins.

Formation of an autophagosomal property on the parental macronucleus

To resolve the problem, Akematsu et al. (2010) tried to observe autophagic/lysosomal processes during PND using a combination of a fluorescent autophagosome indicator, monodancylcadaverine (MDC), and a lysosome indicator LysoTracker Red (LTR). MDC is a fluorescent compound, which can accumulate in the autophagosome but does not accumulate in endoplasmic reticulum or in the endosome. This has therefore been used as a useful tracer for the autophagosome (Biederbick et al., 1995). Concomitant with new macronuclear differentiation and subsequent condensation of the parental macronucleus, a haze of the MDC fluorescence is exclusively hanging over the parental macronucleus, and the periphery of the nucleus becomes uniformly MDC-positive but

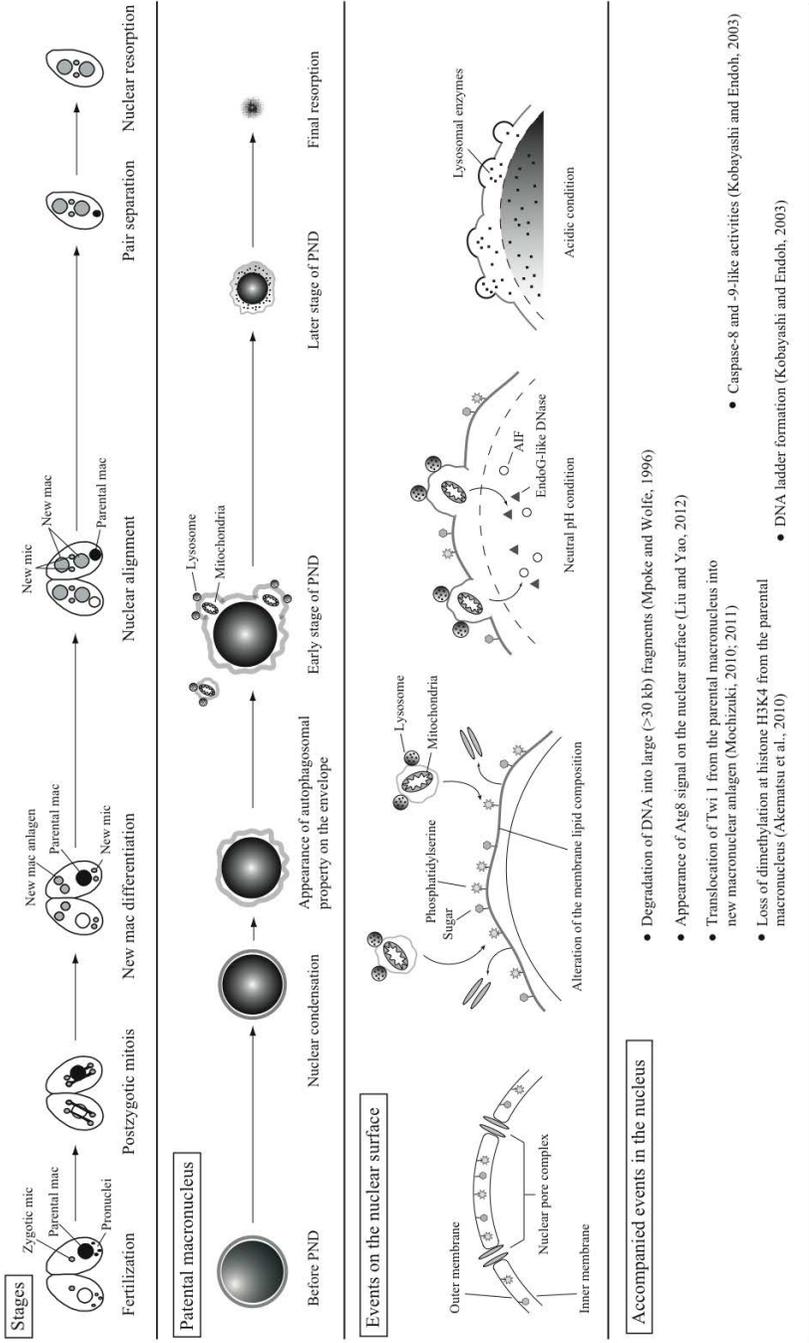


Fig. 1. Schematic representation of PND in *Tetrahymena thermophila*, based on information described in Akematsu and Endoh (2010) and Akematsu et al. (2010). Once PND starts, the parental macronucleus begins to condense and the envelope of the parental macronucleus becomes an autophagosomal membrane together with exposing its intraluminal sugar and lipid on the surface, and disappearance of the nuclear pore complex from the envelope. Meantime, Atg8 appears around the nucleus, and Twi1-scRNA complex translocates from the parental macronucleus into the developing new macronuclear anlagen. During this time, a methyl mark at histone H3K4 disappears from the parental macronucleus while it turns up in the anlagen. The exposed sugars and lipid on the parental macronuclear surface are responsible for attraction of mitochondria and lysosomes from the cytoplasm. At the early stage of PND, only AIF and EndoG-like DNase are released from mitochondria into the nucleus, by which large-scale DNA fragmentation and further nuclear condensation is facilitated at neutral pH. EndoG-like DNase is responsible for DNA ladder formation. Lysosomes fuse with the envelope and release their contents including DNase II into the nucleus at the later stage, by which final resorption of the degenerating macronucleus is performed in an acidic environment. Caspase-8 and -9-like activities are involved in this stage, although no caspase homologues have been identified in this organism.

LTR-negative without migration or accumulation of other membranous structures or vesicles from the cytoplasm (Akematsu et al., 2010). Since specificity of the MDC-staining is derived from interaction with lipid molecules found in the autophagosome (Biederbick et al., 1995), a double membrane autophagosomal structure may be formed around the degenerating parental macronucleus. However, such a structure could not be found around the nucleus and only the nuclear membrane was observed throughout PND under electron microscopic observation (Akematsu et al., 2010).

In mammalian and yeast macroautophagy, more than 30 Autophagy (Atg)-related proteins have been characterized, which are required for autophagosome formation (Mizushima et al., 2011). Among them, Atg8 has been proposed as a useful tracer for an autophagosome precursor. Recently, a *Tetrahymena* homologue similar to yeast *ATG8* was identified and its subcellular localization and function were analyzed (Liu and Yao, 2012). The signal of both EGFP-Atg8 and mCherry-Atg8 appears in the parental macronucleus at the beginning of PND, but not in other nuclei. Similar to the situation with the appearance of the MDC stainability on the parental macronucleus at an early stage of PND, it is unlikely generated by random aggregations or other causes. Knocking-out of the genes causes delay of the final resorption of the parental macronucleus by prevention of the nuclear acidification (Liu and Yao, 2012). These observations strongly suggest that membrane lipid composition on the parental macronucleus may be modified at an early stage of PND, by which the envelope appears to directly change into an autophagosomal structure unlike the case of general macroautophagy (Fig. 1). In addition, under electron microscopy, it has been observed that the early stage of PND involves loss of nuclear pore complexes, attached ribosomes associated with the outer membrane, and continuities with the endoplasmic reticulum (Weiske-Benner and Eckert, 1987). This may be a part of the process to

ensure the spatial and functional sequestration of the nucleus from the cytoplasm without other membrane dynamics like general macroautophagy.

Degradation of the parental macronucleus in a stepwise fashion

Akematsu et al. (2010) observed that many small MDC-positive vesicles and lysosomes migrate to the nuclear envelope once the autophagosomal property appears on the envelope of the parental macronucleus. The vesicles most likely contain mitochondria that have undergone loss of membrane potential (Fig. 1). This migration causes acidification of the periphery, whereas the inside of the nucleus remains at neutral pH until the final stage (Fig. 1). This observation is not contradicted by the observations of Akematsu and Endoh (2010), who demonstrated the collaboration of AIF and EndoG-like DNase acts under neutral pH, suggesting that only the vesicles containing mitochondria may fuse with the macronuclear envelope in the early stage of PND, but the lysosomes seem to remain at the periphery and release their contents into the nucleus in the final stage.

Genetic analysis of PND (Davis et al., 1992) using a *Tetrahymena* mutant pair nullisomic for chromosome 3 of the micronucleus (NULLI 3) supports the model that PND appears to occur in a stepwise fashion. When NULLI 3 cells are mated, the exconjugants failed to establish progeny because the developing macronuclear anlagen lacked the germinal chromosome 3. In these conjugants, chromatin condensation and DNA fragmentation in the parental macronucleus occurred normally, but failed in resorption of the parental macronucleus, because of a lack of zygotic gene expression from the nullisomic macronucleus. This information suggests that the collaboration of AIF and EndoG-like DNase functions as a suicide factor in the parental macronucleus, and that the early stage of suicide and the following final resorptive stage by lysosomes are genetically distinct.

Possible “attack-me” signals on the macronuclear membrane

Early assumptions were that macroautophagy was a random process. During the last decade, however, many examples of selective macroautophagy, including mitophagy (for mitochondria) (Okamoto et al., 2009), ribophagy (for ribosomes) (Kraft et al., 2008), and pexophagy (for peroxisomes) (Sakai et al., 2006) have been increasing. In mitophagy, for example, the loss of membrane potential on aged or damaged mitochondria are specifically targeted by the autophagosome (Kim et al., 2007). By analogy, in mammalian cells, cells in apoptosis are known to expose some molecules on the cell surface as “eat-me” signals, toward activated macrophages. The most common molecules as eat-me signal are phosphatidylerine (PS) and a variety of glycolyx (glycoprotein) compounds, both of which are restricted to the inner leaflet of the bilayer plasma membrane by active transport from the outer to the inner leaflet (Depraetere, 2000; Eda et al., 2004; Savill and Gregory, 2007). When apoptosis is induced, they are exposed on the outer surface by randomizing of their asymmetric distributions. Relying on the signals, receptors on the membrane of macrophages can distinguish the dying cells from normal cells for phagocytosis. The engulfed cells are finally degraded by phagocytes with activation of the lysosomal enzymes. Considering the information, we can expect appearance of some signals on the macronuclear membrane. Relying on the signals, autophagic/lysosomal processes during PND may selectively recognize the parental macronucleus destined to die. No such signal molecules have as yet been identified.

In PND, the digestive vesicle complexes seem to approach the parental macronucleus by targeting MDC-positive signals of the nuclear envelope, comparable to a pinpoint attack by guided missiles. We propose to call such a signal an “attack-me” signal. Akematsu et al. (2010) have observed specific appearance of PS and glycolyx

on the parental macronuclear membrane using FITC-labeled annexin-V and lectin binding specificities as well as being MDC-stainable. Among lectins, wheat germ agglutinin that recognizes N-acetyl-D-glucosamine and sialic acid has much more binding specificity than the others. These marks turn up once the parental macronucleus enters PND, and the stainabilities against FITC are specifically increased when the nucleus begins to condense. Similar to the situation with PS exposition in apoptosis, it is most likely that the glycolyx are not newly synthesized during PND, usually restricted in the inner leaflet of the macronuclear membrane, and exposed to the outer surface of the envelope with beginning of PND (Fig. 1), because no significant difference is observed by a lectin western-blot analysis using proteins extracted from the normal- and degrading parental macronucleus (Akematsu et al., 2010). These molecules could be candidates for the “attack-me” signals in *Tetrahymena* by the autophagic/lysosomal processes.

DEATH COMMITMENT AND DEVELOPMENTAL PROGRAMMING

PND appears to occur at almost the same time as the initiation of new macronuclear differentiation in anlagen. Once this happens, transcriptional activity of the parental macronucleus is replaced by the newly formed macronucleus. For instance, immunofluorescence against RNA-polymerase II, which is a central enzyme for gene transcription, showed that the signal disappears from the parental macronucleus while it turns up in the developing new macronuclei (Mochizuki and Gorovsky, 2004). Immunofluorescence observation of dimethylation of histone H3 at lysine 4 (H3K4), which is an epigenetic mark for active transcription, also demonstrated that the chromatin in the parental macronucleus becomes heterochromatic while in the new macronuclei becomes euchromatic (Akematsu et al., 2010). Indeed, much

evidence indicates that macronuclear development is achieved by the transport of small RNAs (scnRNAs) together with a *Tetrahymena* argonaute protein Twi1, both of which are kept in the parental macronucleus until PND is initiated and are essential for removal of Internal Eliminated Sequences (IES) from the new macronuclear genome (Mochizuki, 2010, 2011), suggesting that the two programs perform in close co-operation.

Observations indicating an inextricable link between the degradation of the parental macronucleus and successful macronuclear differentiation have been made not only in *Tetrahymena* but also in other ciliates including *Paramecium*. Failure of the new macronucleus to differentiate can be artificially induced in *Tetrahymena* by conjugating cells with different micronuclei (genomic exclusion) (Allen, 1967; Allen et al., 1967) or amiconucleate mutants (Kaney, 1985), allowing cells to mate at 40°C (Scholnick and Bruns, 1982), or UV-B irradiation at meiotic prophase (Kobayashi and Endoh, 1998), as well as by mating of NULLI 3 mutants (Davis et al., 1992; Ward et al., 1995). In all of these cases, the parental macronucleus is retained after conjugation, suggesting an inextricable link between execution of PND and programming of the new macronucleus, which triggers expression of the zygotic genes.

The behavior of the new macronucleus and the parental macronuclear fragments of *Paramecium caudatum* are somewhat different from those observed in *Tetrahymena*. In *P. caudatum*, completion of the new macronucleus does not occur immediately after differentiation but, instead, it occurs in the fourth cell cycle after conjugation. In the intervening period, the parental macronucleus persists in the form of multiple fragments and continues to express its genes (Kimura and Mikami, 2003; Kimura et al., 2004). Once developmental programming occurs at the fifth cell cycle, just after completion of the new macronucleus, the parental macronuclear DNA is destined to degrade and is eliminated within the next few cell cycles

(Kimura et al., 2004). The death commitment of the cell coincides temporally with completion of its new macronucleus in the fourth cell cycle after conjugation. Dimethylation of H3K4 reaches its maximum level at this stage (Kimura et al., 2006), as in *Tetrahymena*. These observations strongly suggest that gene expression from the parental macronucleus commits to trigger both its death program and new macronuclear differentiation, and final resorption of the parental macronucleus is integrated into the developmental program of the new generation.

This elaborate mechanism for differential determination of the fates of the two distinct macronuclei may ensure survival of parental clones in the case of failure to develop the new macronucleus, which could arise from a genetic defect of the germinal micronucleus. Furthermore, the clones that succeed in recovering a parental macronucleus after conjugation will have another chance to produce progeny, by mating with other strains that have different genetic backgrounds.

UNANSWERED QUESTIONS

Much remains unknown about commitment to the death program and what intracellular signaling is activated. If both PND and differentiation of new macronuclei are commonly regulated, phosphatidylinositol 3-kinase (PI3K) might be a possible candidate for controlling this. The PI3K pathway has been linked to diverse groups of signal transduction processes in eukaryotic organisms such as cellular growth, cell survival (anti-apoptotic), cell-cycle progression, glucose metabolism, and autophagy (Fruman et al., 1998; Naumann, 2011; Mizushima et al., 2011). Potential PI3K activities have been detected in the asexual stage of *Tetrahymena* with PI3K-inhibitors such as wortmannin and LY294002 (Kovács and Pállinger, 2003; Leondaritis et al., 2005; Deli et al., 2008). These reports suggest that PI3K is an important factor for regulation of proper phagocytotic activi-

ty and vesicular trafficking in the asexual stage of *Tetrahymena*. It can therefore be postulated that conserved PI3K pathways are utilized in conjugation. Yakisich and Kapler (2004) have provided data on the effect of PI3K-inhibitors on conjugating *Tetrahymena*. These authors report that the treatment blocks final resorption of several types of nuclei such as meiotic products (pronuclei) and the parental macronucleus, resulting in the accumulation of extra nuclei in progeny cells. The remaining parental macronuclei during PND show no acidification of the nucleoplasm and actively synthesize DNA, implying that the treatment disturbs the process of proper alteration of generation within a single cell. It appears that a potentially disturbed pathway during conjugation with the PI3K-inhibitors is an autophagic pathway involved in degradation of the pronuclei and in PND. In mammalian and yeast macroautophagy, it is known that activation of PI3K complexes is essential for nucleation of the autophagosome (Chen and Klionsky, 2011). Two ubiquitin-like Atg proteins, Atg8 and Atg12, downstream of the nucleation process, are involved in vesicle expansion and completion of the autophagosome formation (Chen and Klionsky, 2011). As discussed previously, knocking-out of *Tetrahymena ATG8* causes delay of the final resorption of the parental macronucleus by prevention of the nuclear acidification (Liu and Yao, 2012), suggesting the involvement of PI3K expression for execution of the autophagic/lysosomal events during the nuclear degradation. Moreover, Yakisich and Kapler (2004) also reported that in the presence of PI3K inhibitors, a small fraction of the remaining pronuclei could undergo a single endo-replication cycle to generate a new micro- or macronucleus-like form without fertilization. These findings strongly suggest that PI3K plays a central role that relates to control of both PND and differentiation of new macronuclei.

Another unanswered question is if the parental macronucleus affects the later stage of conjugation even after PND is started. Unlike the case of

Paramecium, the parental macronucleus starts to lose hallmarks for active transcription (H3K4) as soon as the degeneration is initiated, while the developing new macronuclei begin to gain the hallmarks (Mochizuki and Gorovsky, 2004; Mochizuki, 2010, 2011; Akematsu et al., 2010). If these observations substantially reflect actual transcription, the new macronuclei would serve all transcription to carry out the later stage of conjugation once the parental macronucleus becomes transcriptionally silent. However, IES elimination from the new macronuclei occurs at the very last stage of conjugation (Mochizuki, 2010, 2011). If the IES elimination were critical for gene expression, there would be a time lag of transcription from both the parental- and new macronuclei until genome re-arrangement was completed in the new macronuclei. On the other hand, with NULLI 3 mutants (Davis et al., 1992; Ward et al., 1995), final resorption of the parental macronucleus is strictly controlled by gene expression from the new macronuclei. These data suggest that the new macronuclei could transcribe some genes that participate in the later stages of PND irrespective of the presence of IES in their genome. Alternatively, some mRNAs transcribed from the parental macronucleus before PND could be stable until the IES removal stage, and their translated proteins might make up for the limited transcription in the developing new macronuclei with IES. To insure proper regulation of PND, the amount of transcription from the parental macronucleus and its timing might be controlled by upstream signaling of PND.

CONCLUSION

In unicellular ciliates, the parental cell-derived cytoplasm is taken over by the progeny macronucleus after conjugation. Therefore, evolution of a mechanism for elimination of the parental macronucleus might have been inevitable when the first ciliate ancestors established a spatial differentiation between their soma and germ-line nuclei.

Ciliate PND is uncoupled from the plasma membrane events associated with PCD, such as Fas ligand-Fas receptor binding, but it utilizes the similar down stream effectors. In addition, an autophagic/lysosomal system ensures that PND occurs only in a limited area of the cytoplasm. Thus, ciliates have developed a novel mechanism for implementing PND that, nonetheless, utilizes some parts of the PCD and autophagy machinery, implying that part of the intrinsic machinery for PCD and autophagy might have been diverted for use in PND as a specialized form of apoptosis and autophagy. These features of PND make it an excellent new model system to indentify and highlight the conserved core mechanisms for timely cell death and its relation to differentiation (development), and selective catabolism.

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