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An evolutionary balance: conservation vs innovation in ciliate membrane trafficking

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As most of eukaryotic diversity lies in single-celled protists, they represent unique opportunities to ask questions about the balance of conservation and innovation in cell biological features. Among free-living protists the ciliates offer ease of culturing, a rich array of experimental approaches, and versatile molecular tools, particularly in *Tetrahymena thermophila* and *Paramecium tetraurelia*. These attributes have been exploited by researchers to analyze a wealth of cellular structures in these large and complex cells. This mini-review focuses on 3 aspects of ciliate membrane dynamics, all linked with endolysosomal trafficking. First is nutrition based on phagocytosis and maturation of food vacuoles. Secondly, we discuss regulated exocytosis from vesicles that have features of both dense core secretory granules but also lysosome-related organelles. The third topic is the targeting, breakdown and resorption of parental nuclei in mating partners. For all 3 phenomena, it is clear that elements of the canonical membrane-trafficking system have been retained and in some cases repurposed. In addition, there is evidence that recently evolved, lineage-specific proteins provide determinants in these pathways.

KEYWORDS

Alveolate, autophagy, calcium signaling, nucleophagy, Rab GTPase, SNARE, vesicles

1 INTRODUCTION

Ciliates have been marveled at since the dawn of light microscopy. Named after the abundant cilia that cover some or all of the cell surface,[1] ciliates are a highly diverse and ecologically important group of protists. With few exceptions, the thousands of known ciliate species are unicellular and all are heterotrophic, though some also host algal endosymbionts or chloroplasts obtained from algal prey. They are large and often highly active cells and there is a richly detailed historical literature dedicated to their morphologies and behaviors.[2,3]

The unique cellular features of ciliates have prompted research that has yielded signal contributions to several areas of cell biology. One powerful ongoing application of these cells is to study structure and function of cilia, including the formation and patterning of ciliary basal bodies.[4–8] Similarly, the near-universal nuclear dimorphism (of a germ-line micronucleus and a transcriptionally active polyploid macronucleus) has presented unique research opportunities, leading to prize-winning discoveries.[9–17] In the course of these efforts, many tools were developed that turned several ciliates into versatile model organisms.[18,19]

The membrane-trafficking system of ciliates likewise shows excitingly unusual aspects, as well as clear homology with the standard eukaryotic organelle set. Like all eukaryotes, ciliates have a cytoplasm that is partitioned into membrane-bounded organelles that facilitate both secretion and uptake of macromolecules. Many of these membrane-bounded organelles are familiar eukaryotic features (eg, endoplasmic reticulum, endosomes and Golgi). Other membrane-trafficking organelles are rather unusual, including the functionally distinct nuclear envelopes for the micro- and macronuclei, the calcium-storing alveoli (an array of adjoining membranous

sacs that underlie the plasma membrane, and place the ciliates in the higher taxonomic group, the "Alveolata"), and the water-pumping contractile vacuole.[20–23] The best-described membrane-trafficking pathways are those involved in various aspects of the endocytic system, such as food vacuole formation, secretory granule deployment and autophagy. A key issue is to clarify the relationship of these organelles and pathways to those that are so well-described in model cell biological systems such as animals.

The question of relatedness is particularly pertinent because ciliates and animals belong to 2 different, and evolutionarily separated major groups of eukaryotes, the Alveolata and Opisthokonta respectively, with a last common ancestor roughly 1.5 billion years ago.[24,25] Thus, a mechanistic understanding of membrane trafficking in ciliates, to complement the body of research primarily in animal and fungal (also Opisthokonta) cells, would allow one to compare different cell biological outcomes achieved over enormous evolutionary intervals. This is true for protists in general since unicellular organisms represent most of the diversity in Eukarya.[26–28] However, ciliates have an advantage in that, for several species, there are already well-developed experimental toolkits facilitating a wide range of approaches for functional studies. These tools are most advanced for *Tetrahymena thermophila* and *Paramecium tetraurelia*, but molecular studies are also feasible in *Oxytricha trifallax* and *Stentor coereleus* (Figure 1).[18,19,29] Another significant point is that most other protist model organisms (eg, the Apicomplexans *Toxoplasma* and *Plasmodium*, and Trypanosomes) were chosen due to their medical relevance as parasites, so ciliates in contrast offer a rare window into the evolution of free living protists.

Importantly, the well-developed toolkits in *Tetrahymena* and *Paramecium* have been put to good use for investigating the molecular machinery underpinning membrane trafficking. From extensive studies in animals and fungi, several major protein superfamilies have been identified as critical to vesicle formation, cargo specificity and delivery to membrane-trafficking organelles. These include the Rab GTPases that serve as protein hubs for regulators and effectors that determine the specificity of vesicle fusion, and the 4 families of SNARE proteins (Qa, Qb, Qc and R) that come together to encode specificity and also provide force to fuse the incoming vesicle with the target organelle[30] (SNARE = soluble NEM-sensitive attachment protein receptors; NEM = N-ethylmaleimide). Comparative genomic surveys have shown these families to be pan-eukaryotic,[31] and set up a reasonable expectation that these proteins and others implicated in membrane trafficking act in ciliates as they do in so many other eukaryotes.

Below we focus on 3 variants of endocytic pathways that have been examined in ciliates. Rather than provide comprehensive reviews of this work, we hope to point out opportunities to gain novel insights from studies using these organisms.

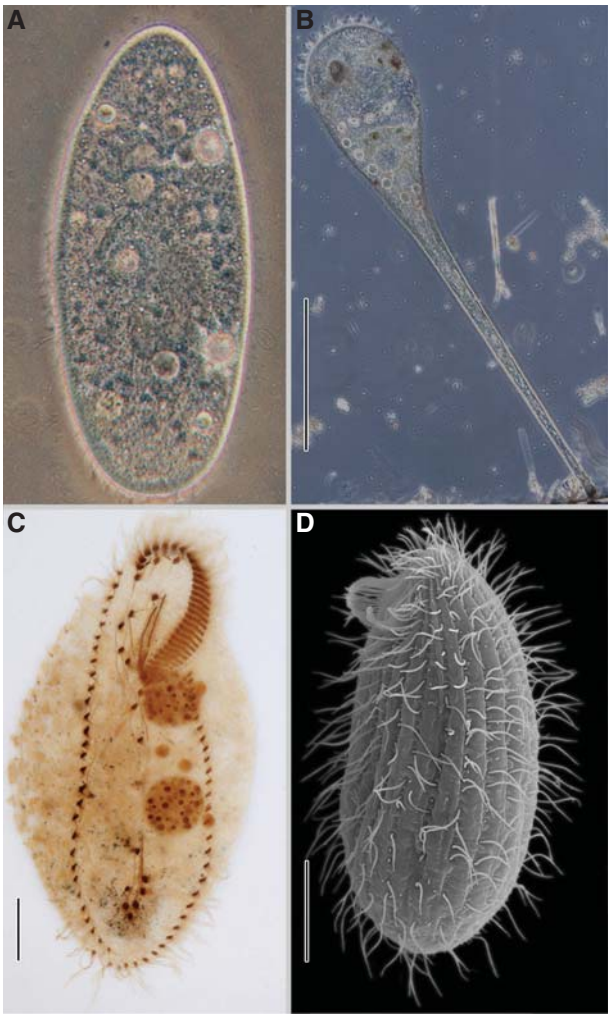

FIGURE 1 Ciliated protists (Ciliophora) currently studied as model organisms. A, *Paramecium* sp. from the *aurelia* "complex," which consists of a group of morphologically indistinguishable sister species with cell length of ~150 μm. *P. tetraurelia* is the chief experimental organism. Phase contrast image. (B, *Stentor coeruleus*, perhaps the largest single celled microbial eukaryotic model organism (length up to 2000 μm). Brightfield image. C, *Oxytricha* sp. from the *fallax* complex, of which *O. trifallax* is the chief experimental organism. Image showing ciliature, following protargol impregnation. D, *Tetrahymena thermophila*. Scanning electron micrograph. Scale bars: (B) 500 μm; (C) 25 μm; (D) 10 μm. Credits: (A) *Wikipedia, The Free Encyclopedia*. Retrieved from https://en.wikipedia.org/w/index.php?title=Paramecium_aurelia&oldid=732190666; (B and C) courtesy William Bourland; (D) courtesy Aswati Subramanian.

2 | FOOD UPTAKE AND THE PHAGOCYTIC PATHWAY

Many ciliates are voracious eaters, with striking adaptations for capturing and ingesting other organisms. The ciliate literature is rich in microscopy-based studies of remarkable predators like *Tokophrya infusionum*, which first harpoons its prey and then harvests the cytoplasm through a microtubule-ringed straw,[32] or *Tetrahymena vorax*, which can dramatically remodel its "mouth" depending on the type of prey that is available.[33] However, molecular data are available almost exclusively from studies in *P. tetraurelia* and *T. thermophila*. These organisms both possess an oral apparatus comprising a tubular or conical cleft leading to the base where the food vacuoles are formed. Particulate prey like bacteria are swept to the base of the cleft by a current generated by coordinated ciliary beating, which can produce extraordinarily effective prey capture. However, since there are viable

Tetrahymena mutants that lack an oral apparatus and cannot ingest bacteria, feeding by particle uptake is not the only way that ciliates can obtain nutrients.[34] This parallel nutrient uptake may involve clathrin-mediated endocytosis of small vesicles, which occurs at regularly spaced sites over the plasma membrane and which in *T. thermophila* requires a ciliate-specific dynamin-family protein.[35]

Broadly similar overall pathways of food uptake and digestion exist in *Paramecium* and *Tetrahymena*, reviewed in Refs 36–38 and outlined in Figure 2. At the base of an oral apparatus, formation of large vesicles termed food vacuoles transports the particulate material into the cell. A newly formed vacuole rapidly fuses with much smaller heterogeneous vesicles derived from endocytic or endolysosomal trafficking pathways. As a result, the food vacuole contents are acidified and exposed to hydrolytic enzymes, thereby promoting efficient digestion of the internalized material. Beginning immediately after their formation and during the subsequent maturation, which involves both membrane retrieval and lumenal neutralization,[39] food vacuoles can move in the cytoplasm by some combination of molecular motors (including an unconventional myosin[40]) and cytoplasmic streaming. At the end of the process, the food vacuoles undergo fusion with the plasma membrane at a specific site called the cytoproct.[41] This fusion allows egestion of any remaining undigested vacuole contents. All of these steps bear obvious similarity to phagocytosis and phagosome formation in mammalian cells such as macrophages.[42] Also like phagosome-based digestion in mammalian cells, some microbes have mechanisms to subvert the usual pathway in ciliates and establish long-term residence, following ingestion, in the cytoplasm (eg, algal endosymbionts in *Paramecium bursaria*) or re-emerge unscathed.[43–45] The latter phenomenon may have medical consequences, since some bacteria appear to increase in pathogenicity during passage through the ciliate phagocytic pathway.[46]

Many detailed features of this pathway were established by Allen, Fok, and their colleagues in exquisite studies using *Paramecium multimicronucleatum*, in which they combined light and particularly electron microscopy with a variety of probes (reviewed in Refs 36,38) (images accessible at www5.pbrc.hawaii.edu/allen/). For some of the features revealed in these studies, there are now molecular data, primarily from studies in *P. tetraurelia* and *T. thermophila*. For example, freeze fracture microscopy of phagosome membranes at different stages argued that the cohort of membrane proteins changed during maturation.[47] Molecular studies subsequently revealed that specific R-SNAREs in *P. tetraurelia* are associated with just a subset of the food vacuoles[48] and the same is true for specific Rab GTPases in *T. thermophila*.[49]

At the first step in the pathway in *P. multimicronucleatum*, the membrane supply for a newly-forming food vacuole does not appear to come from the plasma membrane but instead is provided by recruitment of cytoplasmic discoidal vesicles (Figure 2). These vesicles originate, in part, at the site where an old food vacuole undergoes fusion at the cytoproct, and are subsequently transported across the cell to the oral apparatus.[50] Thus a transcytotic pathway appears to recycle the phagosome membrane. The discoidal vesicles in *P. tetraurelia* are labeled by the Qa-SNAREs PtSyx1, which is also found at multiple other sites, and PtSyx4 which may be preferentially localized to these vesicles.[48] TtRabD3 labels what appear to be equivalent vesicles in *T. thermophila*.[49]

Other studies in *Paramecium micronucleatum* examined the identities of the vesicles that fuse with food vacuoles.[36] These appear to comprise multiple classes of endosomes that deliver proton ATPases (vATPases) as well as digestive enzymes. vATPases are hetero-oligomers in which the transmembrane V_o complex contains 6 subunits. The gene encoding 1 subunit has undergone a remarkable expansion to 17 paralogs in *P. tetraurelia*, and the vATPase delivered by acidosomes specifically contain 1 of these 17, a striking example of paralog-specific localization in membrane trafficking factors.[51] The vesicles delivering vATPase may be recruited in a Rab7-dependent fashion, judging by results obtained in a sibling species, *Paramecium octaurelia*, in which 2 Rab7 paralogs are expressed.[52] One of these localizes to the phagosome membrane, while the second localizes near the oral apparatus and appears to serve a different function.

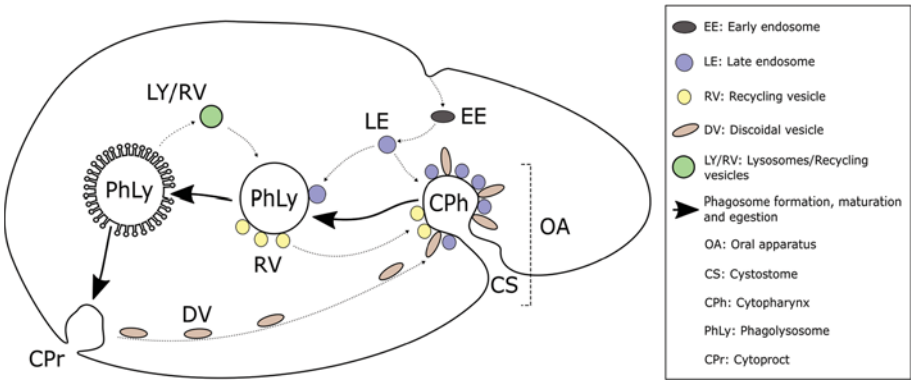

FIGURE 2 A cartoon of vesicle trafficking involved in phagocytosis. At the oral apparatus, phagosomes are formed in the depth of the cytostome (CS), called the cytopharynx (CPh). Here, recycling vesicles originating from the cytoproct (CPr) and from maturing phagolysosomes (PhLy) coalesce to form a nascent phagosome. Acidosomes are late endosomes (LE) that fuse with the newly-forming phagosomes to deliver vATPases, acidifying the lumen. Phagosomes gain their digestive enzymes via fusion with lysosomes (LY), thus becoming phagolysosomes (PhLy). Phagolysosomes also pinch off recycling vesicles (RV) that fuse with the cytopharynx (CPh). Lysosomal material (eg, hydrolytic enzymes) can be recycled by vesicle budding from tubular extensions from mature phagolysosomes (left-most PhLy), generating lysosomes/recycling vesicles (LY/RV). Undigested phagolysosome contents are released by exocytosis at the cytoproct (CPr). The scheme is conceived according to the work by Allen and Fok, as cited.

In *P. tetraurelia*, another size class of vesicles that fuse with newly formed food vacuoles is marked by specific R-SNAREs (eg, PtSyb6-1, 8-1 and 9-2).[53] Yet a third cohort may correspond to vesicles documented in EM tracer studies using *P. multimicronucleatum*, in which some of the vesicles that fuse with food vacuoles appear to be early endosomes.[54] This idea is consistent with the localization of PtSyb11-1 in *P. tetraurelia*, which is found on early endosomes and occasionally on single food vacuoles.[53]

Although still far from comprehensive, these results point to a remarkably complex set of interactions between different classes of vesicles, which occur in a short time period during and immediately after food vacuole formation, and illustrated in Figure 2. The Rabs and SNAREs, as in other organisms, must provide some of the specificity. Actin and actin assembly co-factors have been shown in many studies to be required for food vacuole formation, and also for subsequent fusion with vesicles delivering vATPases.[51,55–57] As in other systems, actin is likely to be providing force to move membranes. But actin may also potentially provide specificity determinants, particularly since distinct actin isoforms are present at different stages of food vacuole maturation in *P. tetraurelia*.[55] A significant open question is the extent to which ciliates utilize lipid-based specificity determinants such as phosphoinositides (PIs) in this pathway.[58] One hint is that wortmannin, which inhibits the formation of PI 3-phosphate, affects secretion of hydrolytic enzymes in *T. thermophila*, and the effect may be at the level of phagosome maturation.[59] This phenomenon is ripe for analysis, since *Tetrahymena* appear to synthesize most but not all of the phosphorylated PI derivatives made in mammalian cells, and the PI interconverting enzymes have been identified.[60–63] Thus, ciliates appear to offer promising systems to illuminate evolutionary aspects of lipid-based protein targeting, particularly with regard to the phagosomal pathway.

The studies outlined above have relied almost exclusively on candidate gene (or gene family) approaches to identify factors involved in phagolysosomal pathways. One exception is the isolation of phagocytosis mutants following nitrosoguanidine mutagenesis.[34] Such forward-genetic approaches in ciliates can now be followed up with whole genome sequencing, to identify precise genetic lesions.[15,64,65] A second non-biased approach that has been exploited is proteomic, taking advantage of the ability to use density gradient centrifugation to isolate highly purified *T. thermophila* phagosomes from cells fed small polystyrene beads.[66] Mass spectrometry of this preparation identified ~30 proteins whose homologs in other organisms have been linked with phagocytosis. The precise roles of many such proteins are poorly understood, and *Tetrahymena* may be an advantageous system for their future mechanistic analysis. The *Tetrahymena* phagosome proteome defined in this study also included another ~40 proteins, which are either novel or not previously associated with phagocytosis, and more work will be required to determine what fraction of these are *bona fide* phagosome constituents. To date, a small set of the novel proteins have been validated as phagosome-associated based on GFP-tagging. One protein, a vacuolar protein sorting 13 (*VPS13*) homolog, was further shown to be required for efficient phagosome formation and content digestion.[67]

How is phagosome formation and maturation in ciliates related, mechanistically and evolutionarily, to the process in animal cells? Both rely on dynamic actin, but there is not yet sufficient information in ciliates to draw detailed comparisons. Unfortunately, few of the implicated Rabs or SNAREs as yet provide much information about evolutionary or mechanistic relatedness, since many Rabs and SNAREs in ciliates are products of lineage-restricted expansions within those gene families. Moreover, robust relationships may be difficult to recognize or establish due to evolutionary distance. Where clearly homologous proteins are involved in mammals and ciliates, for example, Rab5 in *T. thermophila*,[66] Rab7 in *P. octaurelia*,[52] or phagosome-associated Rab11 in the distantly-related ciliate *Euplotes octocarinatus*,[68] more detailed studies will be needed to determine whether homology extends to upstream and downstream factors. In particular, the functional analysis of factors identified via proteomics, and identification of protein binding partners, could illuminate these questions. Fortunately, many of these approaches are facilitated by the high culture densities to which some ciliates can be rapidly grown.

3 A PATHWAY OF REGULATED EXOCYTOSIS

In all cells, the release of proteins and other molecules can modify the environment and shape interactions with other cells. The roles of ciliates in complex natural and man-made ecosystems are increasingly appreciated,[69–71] but precisely how secretion contributes to ciliate physiology and interactions is still largely unexplored. For example, growing *Tetrahymena* copiously secrete a wide array of hydrolytic enzymes, probably involving secretory lysosomes, but whether these enzymes have physiologically-important activities after being dispersed in the extracellular non-acidic environment is unknown.[72] Moreover, in addition to release via secretory lysosomes, *Tetrahymena* releases some newly synthesized proteins by rapid constitutive secretion,[32,73,74] and stores yet another set of proteins to be released upon stimulation.[75] There is also evidence for other modes of secretion, including exosome-mediated release.[73] The egestion of digested food vacuole contents also constitutes a mode of secretion.

Of all these, the ability to secrete upon demand (eg, in response to some extracellular event) has been best studied. The phenomenon is present in many ciliates, and involves the exocytic fusion of vesicles that are docked at the PM.[76] For example, many predatory ciliates use such secretion as a hunting strategy, releasing compounds that immobilize prey, which are often other ciliates.[77] Prey ciliates in turn use compensatory secretion to blunt the attack, sometimes successfully.[78,79] The mechanistic bases for immobilization and defense, and in most cases the molecules themselves, are only beginning to be explored.[79,80] In contrast, the formation of such secretory vesicles is now understood in some detail, primarily in *T. thermophila*, where they are called mucocysts, and in *P. tetraurelia*, where they are called trichocysts. All evidence indicates that mucocysts and trichocysts in these 2 species share biosynthetic mechanisms, and should be considered homologous organelles, that is, evolved from a common origin.

Mucocysts and trichocysts are elongated vesicles that are surrounded by a single lipid bilayer and filled with a dense crystalline

core of proteins, and thus resemble dense core secretory granules in animal cells.[75] Current evidence, particularly immunoelectron microscopy in *Paramecium*, indicates that major core proteins are delivered to the ciliate granules via the classical eukaryotic secretory pathway.[81,82] Consistent with this, *Paramecium* induced to synthesize a cohort of granules (as a consequence of releasing their store of docked granules) upregulate a large set of genes that encode widely conserved trafficking machinery in the endoplasmic reticulum and Golgi.[83]

One basic question is how the granule proteins in ciliates are routed from the general secretory pathway to accumulate in specific vesicles. In animal cells, targeting of soluble proteins to granules relies on their tendency to form aggregates within the secretory pathway.[84] Such aggregation is also a marked characteristic of the most abundant ciliate granule proteins, although there is no discernible evolutionary relatedness between the ciliate proteins and those in metazoans.[85] A second aspect of protein targeting to animal granules is selective cargo retention during granule maturation, a process that involves the removal of a subset of immature granule contents by vesicle budding, at the same time that the granule contents are being remodeled by proteolytic processing. Remarkably, similar proteolytic remodeling occurs in the ciliate granules, and is also required for efficient retention.[86–88] However, the known proteolytic maturases, discovered via transcriptome profiling in *T. thermophila*, are not related to their functional analogs in metazoan granules.[89,90] Thus similar mechanistic programs appear to be accomplished by unrelated molecules in animals and ciliates. A similar situation may pertain for some mechanisms facilitating granule exocytosis, which has been studied in *P. tetraurelia*. There, trichocyst discharge depends not only on genes in conserved families, for example, SNAREs[91] but also on a set of genes that are both quite heterogeneous in architecture and apparently ciliate-restricted.[92,93]

However, some soluble proteins in *Tetrahymena* mucocysts do not aggregate.[85] Instead, their delivery depends upon a receptor encoded by a ciliate-specific paralog within the pan-eukaryotic *VPS10*/sortilin family,[94] called Sor4p, with which they show strong physical interactions consistent with receptor-ligand binding.[95] The *SOR4* results thus show the unanticipated requirement for a sorting receptor in mucocyst biogenesis.

Sor4p localizes to endosomal compartments. In the budding yeast *S. cerevisiae*, in human cells and in the Apicomplexan parasite *Toxoplasma gondii*, *VPS10*-family receptors are involved in directing proteins to lysosome-related organelles (LROs).[96,97] Taken together, the results suggest that mucocyst formation likely involves trafficking from one or more endo-lysosomal compartments. This idea is supported by recent experiments, in which disruption of other *Tetrahymena* genes associated with endolysosomal trafficking, also resulted in defective mucocyst formation (Kaur and Turkewitz, unpublished).

LROs constitute a strikingly heterogeneous collection of organelles in animals, whose formation often involves trafficking from both the trans-Golgi and from endosomes.[98] The current data for *Paramecium* and *Tetrahymena* raise the possibility that 2 major classes of luminal trichocyst/mucocyst proteins arrive via 2 distinct routes, for example, the aggregated proteins from the trans-Golgi, and the receptor-bound proteins via endosomal intermediates. A divergent R-SNARE, PtSyb5, is associated with trichocyst biosynthetic intermediates in *P. tetraurelia*, and could provide a valuable molecular marker to explore the 2-pathway model.[53] Interestingly, electron microscopic images of a third, more distantly related ciliate, *Pseudomicrothorax dubius*, show beautifully that 2 populations of vesicles with different luminal contents contribute to trichocyst formation in that species.[99] Taken together, the data suggest that ciliate granules are a sub-class of LROs whose biogenesis depends on LRO-associated machinery. At the same time, biogenesis also depends on mechanisms more closely associated with metazoan secretory granules. Excitingly, many mutants have been characterized with defects in mucocyst or trichocyst secretion, affecting many different steps during biogenesis.[86,100–102] The ability to now identify the molecular lesions in these mutants by whole-genome sequencing offers a powerful approach to revealing both conserved and novel proteins deployed by ciliates in this pathway.[64]

4 PROGRAMMED NUCLEAR DEATH

Ciliates possess 2 distinct nuclei: a germline, diploid Mic and a polyploid Mac.[103,104] In conjugating *T. thermophila* cells, the Mic undergoes both meiotic and mitotic division yielding 2 haploid Mic products. One of these products is stationary; the other is mobile and exchanges with the equivalent product from the mating partner. The mobile and stationary products then fuse to create a new diploid Mic, which undergoes several rounds of mitosis to produce both new Mics and Macs.[105,106] Concomitant with new Mac development, the parental Mac is destroyed by nucleophagy. Programmed nuclear death (PND) is the name given to the process by which ciliates selectively degrade the parental Macs following conjugation (Figure 3). The mechanisms involved have been best studied in *T. thermophila*.[107,108] PND involves the fragmentation of parental macronuclear DNA and nuclear condensation, in a process that shares similarities with animal cell apoptosis, and the resorption of the macronucleus is a process that can be broadly described as autophagic.[109,110] The apoptotic features of ciliate PND have recently been reviewed,[108] and we here focus on autophagic aspects of this pathway.

Autophagy is a process by which eukaryotic cells engulf and then recycle cellular material primarily during periods of starvation.[111,112] The engulfing membranes, which are derived from many sources within the cell, take the form of a double bilayer. The initial phagophore membrane is expanded through membrane fusion with other organelles, such as endosomes, allowing it to engulf additional cytoplasmic material. Subsequent fusion with the lysosome facilitates the breakdown of contents for recycling.

Many variants of autophagy exist in mammalian and other cells. For example, autophagic machinery can be repurposed for selective degradation of protein aggregates, specific organelles, or bacteria.[113] Autophagic mechanisms also contribute to organellar protein targeting in yeast and to phagocytosis.[114,115] Striking variation can exist within autophagic phenomena that have the same goal. Nucleophagy refers to the targeting of nuclear material for degradation by autophagosomes. Studies of nucleophagy in many organisms, including

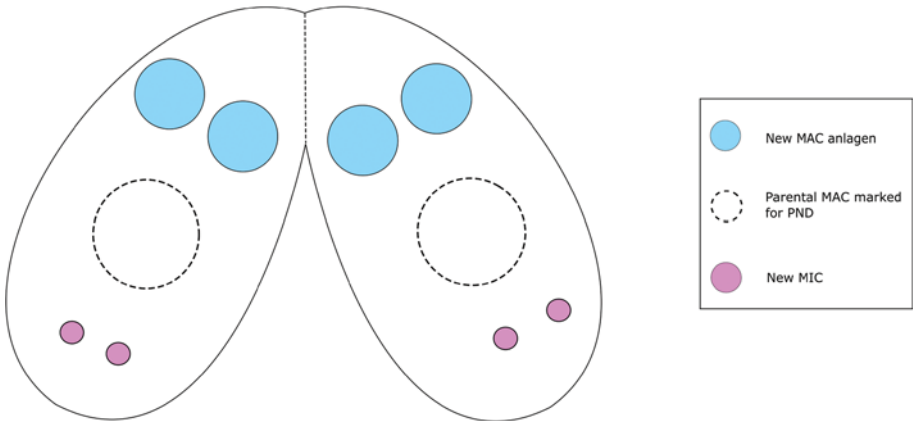

FIGURE 3 A cartoon summarizing the types and fates of nuclei present in conjugating *T. thermophila*. At the stage shown, each cell within the pair has 5 nuclei. The parental macronucleus in each cell (dashed line) is uniquely destined for nucleophagic degradation, or PND, involving mechanisms described in the next. In the same cytoplasm, the new macronuclei will develop from the nuclei positioned near the conjugation junction (called MAC anlagen, light blue), while 2 other nuclei in the cell posterior (pink) are destined to become micronuclei. Following pair separation and a round of cell division, each daughter will have 1 micronucleus and one macronucleus.

yeast, *Aspergillus* species and humans, have revealed a diversity of mechanisms and morphologies.[116] Nucleophagy in *Tetrahymena* is still very incompletely understood, but appears to represent yet another variation.

Notwithstanding their diversity, autophagy-related pathways rely on a common set of genes. A set of 19 are referred to as core autophagy genes.[111,117] Key genes include *ATG1*/ULK genes, which are involved in transducing upstream signals (eg, starvation, mTOR inactivation), *VPS34* complex genes involved in autophagosome nucleation, and a complex of genes (*ATG12–ATG5–ATG16L1*) that regulate, via lipidation, the *ATG8* family proteins involved in autophagosome expansion. Combinations of the core autophagy genes along with additional factors function in non-canonical and autophagy-like pathways.[114,115,118,119]

Ciliates almost certainly rely on multiple autophagy-related pathways, but the only one which has been investigated in molecular detail is nucleophagy in *T. thermophila*. To understand the relationship between these steps and known autophagic pathways in other organisms, one can first ask whether autophagy-related genes are present in the *T. thermophila* genome, and in particular whether they are expressed during the appropriate interval in conjugation. In fact, many of the core autophagy genes are present and expressed during conjugation, with some notable absences[119,120] Conjugating *Tetrahymena* also express homologs of several genes associated with non-canonical autophagy pathways. These genes include *ATG20* and *ATG24*, discussed further below, and a homolog (but not necessarily ortholog) of Rubicon. In mammalian cells, Rubicon is a key determinant of LC3/ATG8-associated phagocytosis.[121] These observations indicate that nucleophagy in *Tetrahymena* relies on autophagic mechanisms, many of which are present in other organisms, but which may be deployed in novel combinations in ciliates.

A small subset of the autophagy-related genes in *Tetrahymena* have been analyzed in functional studies. In mammals, ATG8/LC3 plays a role in expansion and cargo targeting of autophagosomes.[113] *Tetrahymena* expresses 2 ATG8 paralogs. TtAtg8 initially localizes to vesicle-like structures during the early phases of conjugation, but shifts to forming a peripheral ring around the parental Mac just prior to the initiation of PND.[120] Disruption of either of the 2 *ATG8* paralogs disrupted Mac degradation, and in particular blocked the fusion of lysosomes with the targeted parental Mac, but did not block DNA fragmentation.

In yeast and humans, autophagosome formation is regulated by the activation of class III PI3K, VPS34.[117] Current evidence suggests the PI3K family may play a similar role in *Tetrahymena*. First, suppression of PI3K by small molecule inhibitors led to accumulation of nuclei during conjugation, suggesting defects in nucleophagy.[122] More recently, the *Tetrahymena* VPS34 homolog was studied by gene disruption. *VPS34* deletion strains showed defects in Mac degradation, including lysosome fusion with the parental Mac.[123] Unlike the *ATG8* deletion strains, the loss of *VPS34* led to decreased DNA fragmentation. The idea that DNA fragmentation is regulated independently of autophagy-related resorption is also consistent with other data. In particular, the loss of Mic chromosome 3 resulted in impaired resorption without affecting DNA fragmentation,[107] while disruption of *TMN1*, a mitochondrial nuclease that translocates to the parental Mac, impaired DNA fragmentation but had no effect on lysosomal fusion.[124]

Autophagosomes are targeted to parental Macs, sparing the new Macs and Mics that are present in the same cytoplasm. The *TtVPS34* knockout phenotype described above suggests that phosphoinositide 3-phosphate (PI3P) may be one determinant for this targeting. Interestingly, a pathway in budding yeast in which autophagosomes transfer specific cytoplasmic cargo to the vacuole, requires 2 proteins, Atg20 and Atg24, which interact directly with PI3P.[125] Moreover, the deletion of these genes also caused defects in targeted autophagic digestion of mitochondria (mitophagy) and perixosomes (pexophagy).[126,127] Atg20 and Atg24 bind to PI3P via PX-BAR (phox-homology-bin-amphiphysin-RVS) domains.[125] Importantly, the defects seen in the gene knockouts were likely to be due to the targeting of Atg20 and Atg24 by PI3P, since the defects could be phenocopied by mutations within the PX-BAR domains of either protein. The PX-BAR domains are therefore likely to play a critical and conserved role in *ATG20* and *ATG24* by facilitating phosphoinositide binding. Intriguingly, *Tetrahymena* has homologs of *ATG20* and *ATG24*, and the proteins are highly expressed during conjugation. Moreover, TtAtg24 specifically localizes to degrading Macs (Guerrier, unpublished).

The mechanism by which Atg20 and Atg24 promote selective targeting is not clear, but these proteins could potentially promote the expansion of autophagosomes.[113] *ATG20* and *ATG24* are members of the sorting nexin family. Human ATG24, also known as SNX4, regulates endosomal trafficking and membrane tubulation.[128] While the role of SNX4 in any form of selective autophagy has not been reported in mammals, recent work showed that the related SNX18 contributes to autophagosome expansion by tubulating endosomes to serve as a source of autophagosomal membranes.[129] In *Tetrahymena*, the endoplasmic reticulum, which represents the most well characterized source of autophagosomal membranes, is cleared from the zone surrounding the parental Mac prior to its degradation.[109,130] It would therefore be interesting to determine whether endosomes contribute to autophagosome expansion in *Tetrahymena*, and whether this depends on a SNX-BAR protein. A small family of SNX-BAR domain proteins in *T. thermophila* are highly diverged in sequence from those in animals, and therefore not efficiently detected by BLAST-based searches.[94] However, they are confidently identified using structure-prediction algorithms, for example, hhpred (Guerrier, unpublished), similar to results for other homologous but sequence-diverged genes, for example Ref 131.

PI3P is not the only molecular candidate to potentially mark the parental Mac for degradation. The parental Mac envelope appears to become both lectin-reactive and annexin V-positive just before autophagosomes localize to it.[130] These results suggest that sugar-modified proteins and/or phosphatidylserine are exposed on the surface of the parental Mac, and could serve as signals for targeting of cytoplasmic proteins. In fact, carbohydrate binding provides an important determinant in some autophagic targeting pathways.[132,133] However, *Tetrahymena* proteins that recognize these potential signals have yet to be identified. Another class of potentially important cues are molecules released from the parental Mac. In particular, during the period when the rough ER is cleared from the zone of the parental Mac, the Mac envelope loses visible nuclear pore complexes.[109] The deterioration of these complexes might lead to increased permeability of the nuclear envelope, as has been shown in studies of nematodes and mammalian cells.[134] The mechanism of nuclear pore degradation, and the idea that leakage of nucleoplasm functions as a targeting signal for autophagosomes remains to be explored, but no leakage of material from the parental Mac has been visualized to date in *Tetrahymena*. Interestingly, chromatin extrusion from the Mac has been visualized in the ciliate *Colpoda cucullus*.[135] This takes place when cells are undergoing encystment, as part of a process of degradation/resorption that results in Mac reduction. The relationship between such processes and PND remains to be explored.

5 LOOKING AHEAD

Although the molecular analysis of membrane trafficking in ciliates is still at an early stage, the picture emerging is that elements of the canonical membrane-trafficking system have been retained and in some cases repurposed, while additional lineage-specific versions of membrane-trafficking proteins have arisen more recently that help support the pathways. This is true both for pathways that appear, judging by morphology and function, to be clearly homologous to those in animals (eg, phagocytosis), and for pathways that are more obviously ciliate-specific (eg, targeting of the Mac in a developmental program).

The challenge moving forward will be to extend the identification of key proteins to a broader range of ciliate structures and pathways, while at the same time working to understand the mechanisms by which they act. For example, alveoli are an array of adjoining membranous sacs that underlie the plasma membrane.[136] In *Paramecium*, alveoli can store millimolar calcium, which in response to upstream signals is mobilized to the cytoplasm where it triggers downstream events.[22,23] Alveoli bear comparison with specialized calcium stores in animals, such as the sarcoplasmic reticulum in muscle, and the membrane proteins involved in calcium transport are indeed related.[137] But whether alveoli are related to the endoplasmic reticulum, or share more general structural or functional features, has not yet been thoroughly investigated. Similarly, there are remarkably few molecular data to aid in understanding the membrane trafficking aspects of the contractile vacuole or its evolutionary origins, though some compartment-specific SNAREs and Rabs have been identified.[49,138] The contractile vacuole is a highly dynamic organelle that shows markedly different organization even between *Tetrahymena* and *Paramecium*, whose water-pumping activity is essential for ciliates and other fresh water protists lacking cell walls. Importantly, the rich history of ultrastructural and physiological studies can provide, as for some pathways discussed in this paper, a critical framework for interpreting molecular studies.[20] In addition, the ability to harness forward genetic approaches in several ciliate species now facilitates unbiased approaches to gene discovery, which can complement candidate gene approaches based on findings in animal, fungal or other lineages.

Ciliates may be particularly well suited for addressing some broad issues in evolutionary cell biology. During the evolution of the complex endomembrane network in eukaryotes, new compartments may have arisen when paralogous gene duplication, with subsequent mutations that ensure co-evolution between interacting components, generated new specificity determinants.[31] Testing this idea requires understanding the roles of paralogous genes within key gene families, and ciliates may be ideal for this effort. Ciliates are large complex cells with gene-rich genomes; for example, estimated gene number is higher in *T. thermophila* than in humans.[139] Importantly, the catalogs of *Tetrahymena* or *Paramecium* genes include many paralogous expansions within gene families involved in membrane trafficking.[140,141] The presence of multiple paralogs is potentially challenging for functional studies, since paralogs may have similar roles. In practice, many individual *Tetrahymena* paralogs appear to have unique functions, based on analysis of single gene knockouts in numerous studies. *P. tetraurelia* has larger numbers of closely-related paralogs, due to several notably recent whole genome duplications.[141] As a result, the 2 ciliates offer opportunities to study the role of paralog diversification in shaping new pathways over different time intervals. One particularly interesting class of paralogous expansions in *Paramecium* are gene families involved in calcium sequestration and mobilization, phenomena that are central to signaling pathways and to regulation of membrane dynamics.[142] Many of the proteins show exquisite compartment-specific localization,

suggesting that ciliates may offer exceptional opportunities to investigate the integration of signaling and membrane-trafficking machineries, from both mechanistic and evolutionary perspectives.

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