PNAS COMMENTARY

How ciliates got their nuclei

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Biologists who spend time observing environmental samples under the microscope are used to the incredible range of shapes, sizes, and behaviors displayed by eukaryotic microorganisms, which rivals or exceeds that of animals, just on a smaller scale. These are lumped together as "protists" and generally relegated to the skippable second chapter of Zoology textbooks, but they actually account for most of the diversity of eukaryotes. A few have occasionally and ever so slightly emerged from this comparative anonymity, and many of these "stars" belong to one lineage: ciliates. Due to their impressive size, ubiquity, and—for lack of a better word—elegance, ciliates are sometimes used as stand-ins for protists as a whole. Certain genera like *Paramecium* and *Tetrahymena* are staples of teaching labs and even models used in cell and molecular biology. But a few models cannot do justice to the richness of ciliate diversity (let alone protists). Ciliates include the majestic trumpet-shaped *Stentor*, able to regenerate itself from the tiniest cell fragment; *Euplotes*, which walks on leg-like bundles of coordinated cilia; *Loxodes*, which senses gravity using a subcellular organelle, not unlike a vertebrate inner ear; *Didinium*, which can paralyze and engulf prey far larger than itself in one "bite"; and *Blepharisma*, one of the few microorganisms that can easily be recognized without a microscope by its purple tinge, and the subject of the two papers in this issue from Seah, Singh, Swart, and colleagues (1, 2).

In these papers, the authors describe the two nuclear genomes of *Blepharisma*. Why two? It turns out that above and beyond the immense morphological and ecological diversity of ciliates, their most bizarre features are those hidden in the molecular realm. Ciliates have dozens of "alternative" genetic codes, some of which use context-dependent rules rather than a rigid relationship between codons and amino acids, as recently shown by some of the same authors (3). Ciliates have sex, but not sexual reproduction, and variably determined "mating types": in some species only two (as our biases lead us to expect) but in others over 100. But the oddity most fundamental to ciliate biology is nuclear dimorphism, the existence in the same cell of two types of nuclei. Besides forcing ciliate molecular biologists to deal with two nuclear genomes per species (the sequencing and assembly of each not an easy feat to begin with), nuclear dimorphism results in a trait that many biologists probably assume to be exclusive to multicellular organisms: the separation of germline and soma.

"Micronuclei" are the germline, the repositories of the complete genome, which is transmitted to the offspring at every cell division. Micronuclei are also filled with noncoding DNA and disrupted genes that are never expressed. The functional forms of the genes are expressed by a large somatic "macronucleus," which is destroyed during each sexual exchange, its genetic information lost instead of passed on to the next generation. New macronuclei (usually) develop from postmating micronuclei that undergo a radical transformation. Among other things, all the disruptions that make micronuclear genes nonfunctional, the so-called "internal eliminated sequences" (IESs), are precisely removed in a complex, multistep process using information from the old, degrading macronucleus as a template. This information is transmitted epigenetically, and some somatic variations can be epigenetically inherited by the macronucleus of progeny cells without ever entering the germline.

Unfortunately, while ciliates are very diverse, virtually all the data on this amazing nuclear transformation comes from two very closely related genera, *Paramecium* and *Tetrahymena*, and a third more distant cousin, *Oxytricha*. Expecting these models to adequately represent the process would be like expecting to understand animals by looking only at humans, rats, and fruit flies. This is why the newly obtained micronuclear and macronuclear genomes are so important: because *Blepharisma* is so distantly related to the model species that their last common ancestor is also the last common ancestor of all ciliates. Knowledge about *Blepharisma* therefore greatly clarifies our overall picture of ciliate evolution, in particular for ancient and highly variable traits like macronuclear differentiation and IES removal. And indeed, the authors describe a new category of IESs (1) and new mechanistic insights into the ancient origin of IES removal based on the PiggyBac transposase (2).

Since the initial discovery of macronuclear development, it has been challenging to imagine how such a unique process evolved, or, even more vexingly, why it evolved. As far as we can tell, IES removal is an expensive, convoluted, and potentially dangerous way to return nonfunctional genes to something close to their ancestral state, a goal that most organisms achieve by simply not messing up their genes in the first place. We do not have all the answers yet, but we can say how IES removal did not evolve: it was not assembled piece by piece by natural selection in order to "solve the problem" of IESs. While this and other adaptive explanations have been proposed, they do not stand up to much scrutiny. For IESs to have appeared first, selection would have to be so weak as to allow IESs to spread and disrupt scores of genes, not only failing to kill individual ciliates but also becoming fixed in the

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Author contributions: V.B. and P.J.K. wrote the paper.

The authors declare no competing interest.

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See companion articles, "MITE infestation accommodated by genome editing in the germline genome of the ciliate *Blepharisma*," 10.1073/pnas.2213985120 and "Origins of genome-editing excisases as illuminated by the somatic genome of the ciliate *Blepharisma*," 10.1073/pnas.2213887120.

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Published February 10, 2023.

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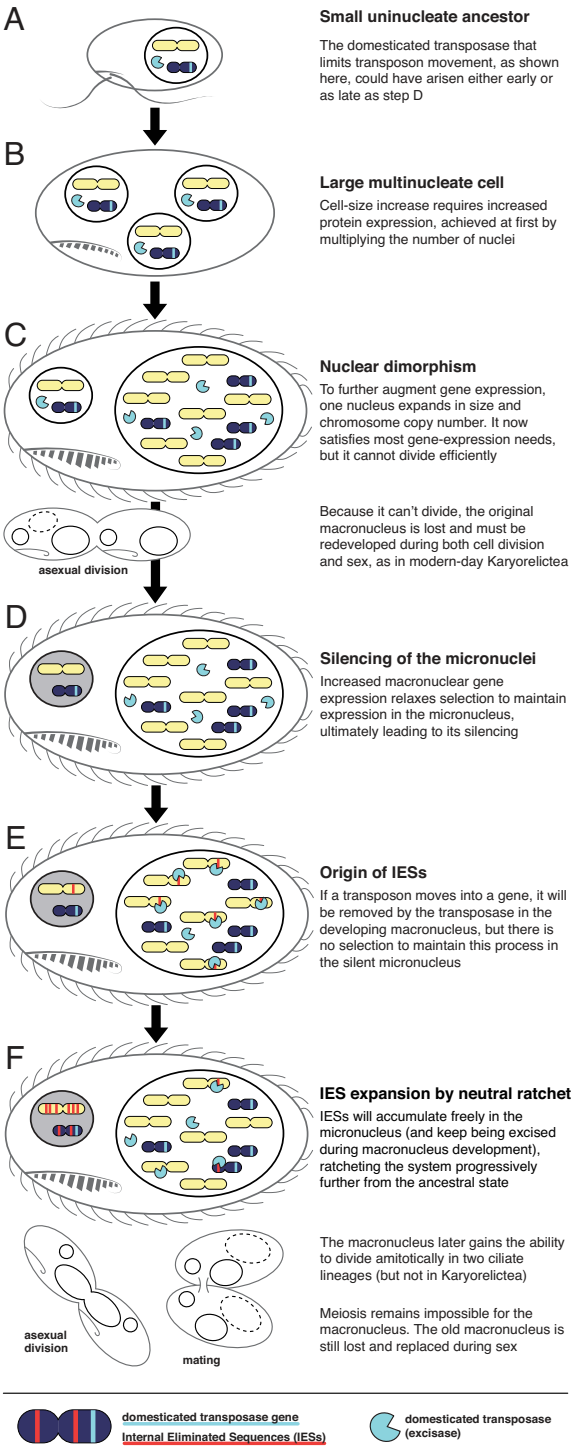

Fig. 1. Hypothesis for the evolution of ciliate nuclear dimorphism and IES spread (see text for details). (*A*) An ancient ancestor of ciliates would have been uninucleate. (*B*) Selection for increased cell size and the increased gene expression this requires was initially accommodated by maintaining multiple nuclei. (*C*) Subsequently, one nucleus specialized, becoming an enlarged "proto-macronucleus" with expanded chromosome numbers, but which cannot undergo mitosis or meiosis and cannot divide. (*D*) The remaining "proto-micronucleus" continues to undergo mitosis and meiosis, but is no longer required for gene expression, and eventually neutrally falls silent. (*E*–*F*) This functional specialization allows a runaway spread and inheritance of transposons (now referred to as IESs) in micronucleus because they are eliminated in the developing macronucleus by a domesticated transposase. All these steps occurred before the last common ancestor of ciliates. Subsequently two lineages independently evolved an imprecise amitotic division of the macronucleus, but no macronucleus can undergo meiosis, and the germline micronucleus is still required to reset the macronucleus during each sexual exchange.

population. Subsequently, while these nonfunctional pseudogenes were somehow not accumulating any other mutations over millennia, selective pressure became strong enough to evolve a complex and multistep process to rescue those very pseudogenes. Simply put, this places the cart before the horse (4). Instead, a rudimentary but functional IES removal system must have preceded the large-scale spread of IESs, its presence allowing this "infestation" to expand relatively harmlessly even to essential genes. In other words, the preexisting solution made the problem possible.

It is still conceivable that some of the evolutionary steps that led to the current system are adaptive in ways that currently elude us. But an alternative framework has also been suggested, which does not require positive natural selection: constructive neutral evolution (CNE) (4–6). Briefly, CNE is a model for the evolution of complexity in which the existence of redundant functions in molecular systems may incidentally turn a deleterious or lethal mutation into a neutral one, which then may be fixed in a population by genetic drift. In the case of ciliate IESs, a CNE explanation could begin with a class of transposases that can excise cognate transposons becoming constitutively expressed by the host, or "domesticated." This could initially be beneficial if selective pressure to limit the spread of transposons played a role, but it would also make any future insertion of the cognate transposons within genes far less deleterious, so long as transposon removal can occur before gene expression. Such uncoupling happens routinely in ciliates due to their nuclear dimorphism: micronuclear genes are silent and there is no selection to keep them free from transposons because the transposase eliminates such sequences during the development of the somatic macronucleus. At this point the most potent aspect of the CNE model kicks in: an evolutionary ratchet. Once a "presuppressor" (the solution to a problem) exists, the problem can multiply unabated. Whatever mess has appeared is not just allowed to persist, it is predicted to expand. The simplest way to visualize this is to realize that there are many more ways for a system to diverge from the original state than to return to it—the system will ratchet away from the ancestral state. As Seah et al. say about *Blepharisma*, "transposase domestication actually facilitates the accumulation of junk DNA" (1).

CNE would benefit from a formal mathematical theory to support it and more models amenable to empirical tests. The *Blepharisma* genomes help with the latter. A CNE explanation for IES removal was supported by the discovery that IESs are remnant transposons excised by domesticated transposases in *Paramecium* (7, 8), but not all evidence has aligned to neatly fit this interpretation. This is once again due to the huge diversity of ciliates. For example, IES excision is fundamentally different between *Paramecium* and *Oxytricha*, and even between *Paramecium* and *Tetrahymena*. This has obfuscated whether transposases were ancestral for IES removal, which transposases were used, and generally what the ancestral condition was like. Singh et al. (2) have now provided answers to some of these questions by simply investigating an organism with a useful position in the phylogenetic tree and found that the domesticated PiggyBac transposase in *Paramecium* and *Blepharisma* is the most likely ancestral state for all ciliates.

Despite these important advances, there is a larger mystery that goes beyond the existence, evolution, or function of IESs and indeed looms over the entire topic of strange

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ciliate biology: how did ciliates evolve nuclear dimorphism in the first place? This central and ancestral feature is a prerequisite for IESs and probably other unusual aspects of ciliate genomics, but how it arose is unknown. This is seldom even the subject of speculation, so, hoping to spur further ideas, we will stick out our necks and propose a scenario that at least accounts for what we currently know (Fig. 1). The first step involves a transition from one to multiple nuclei in a distant ancestor (Fig. 1 *A* and *B*). Ciliates are big, and large cells require proportionate expression of RNA and proteins. There are several ways to achieve this, but evolving multiple nuclei by uncoupling nuclear and cellular division is not uncommon and is seen in other particularly large cells (e.g., some parabasalians and amoebozoans). Another route to increasing expression is polyploidization, and we propose that one (or more) of the multiple nuclei subsequently underwent an expansion in size and chromosome copy number (Fig. 1*C*). The enlarged nucleus would provide more gene expression, but at the expense of being able to properly sort its numerous chromosomes and divide. This means that the ancestral "proto-micronuclei" would need to be maintained and that initially a new "proto-macronucleus" would have to develop at each cellular division from a micronucleus, which is still the case for karyorelicteans (the sister lineage to *Blepharisma* and its close relatives). The domesticated transposase that Singh et al. (2) show was ancestral to all extant ciliates that could have appeared at any time up to the next step, the only additional element necessary to initiate the CNE ratchet: silencing expression in the micronucleus (Fig. 1*D*). This too could be nondeleterious, since most gene expression is already carried out by the macronucleus, it would involve massive changes to nuclear targeting, and it would eliminate selection for transposase activity in the micronucleus (Fig. 1*D*).

With a now-silent germline and domesticated transposases in the soma, transposons inserted into essential genes would only be excised in the macronucleus and not the micronucleus (where they are invisible to selection) (Fig. 1*E*). Micronuclear transposons are now IESs, and their spread through the genome can proceed in an unabated and neutral ratchet-like way (Fig. 1*F*). And indeed the whole process is also a ratchet in that even one such insertion will make it very difficult to revert to the ancestral state of a single functional nucleus. This would be the situation in the last common ancestor of modern ciliates; sometime later (and at least twice according to the phylogeny), the macronucleus acquired the ability to divide amitotically and probably somewhat imprecisely. This would lead to unbalanced chromosome copy numbers over many such divisions; however, this potential problem is circumvented by "resetting" the macronucleus during each sexual cycle. The macronucleus has never crossed the "meiosis barrier," probably for two reasons. First, accurate pairing of chromosomes would be problematic for such a highly fragmented and unevenly amplified genome. And second, without the development of a new macronucleus after meiosis, the toll of imprecise macronuclear division would eventually become unsustainable, which can be seen in monoclonal lab strains, where the cumulative asymmetry of chromosome copies leads to senescence.

"It turns out that above and beyond the immense morphological and ecological diversity of ciliates, their most bizarre features are those hidden in the molecular realm."

All these steps required a single, ancient adaptive push—the need to increase the copy number of genes to accompany an increase in cell size—that initiated a cascade of events that did not require additional "reasons," but that ultimately resulted in complex, inefficient machinery that could never revert to a more manageable state. This is inelegant, perhaps, but probably the cause for much biological complexity not only in ciliates but in other lineages as well.

1. B. K. B. Seah *et al.*, MITE infestation accommodated by genome editing in the germline genome of the ciliate *Blepharisma*. *Proc. Natl. Acad. Sci. U.S.A.* **4**, e2213985120 (2023).
2. M. Singh *et al.*, Origins of genome-editing excisases as illuminated by the somatic genome of the ciliate *Blepharisma*. *Proc. Natl. Acad. Sci. U.S.A.* **4**, e2213887120 (2023).
3. B. K. B. Seah, A. Singh, E. C. Swart, Karyorelict ciliates use an ambiguous genetic code with context-dependent stop/sense codons. *PCI Genomics* **2**, e42 (2022).
4. M. W. Gray, J. Lukeš, J. M. Archibald, P. J. Keeling, F. W. Doolittle, Irremediable complexity? *Science* **330**, 920–921 (2010).
5. P. S. Covello, M. W. Gray, On the evolution of RNA editing. *Trends Genet.* **9**, 265–268 (1993).
6. A. Stoltzfus, Constructive neutral evolution: Exploring evolutionary theory's curious disconnect. *Biol. Direct* **7**, 35 (2012).
7. C. Baudry *et al.*, PiggyMac, a domesticated piggyBac transposase involved in programmed genome rearrangements in the ciliate *Paramecium tetraurelia*. *Genes Dev.* **23**, 2478–2483 (2009).
8. O. Arnaiz *et al.*, The *Paramecium* germline genome provides a niche for intragenic parasitic DNA: Evolutionary dynamics of internal eliminated sequences. *PLoS Biol.* **8**, e1002984 (2012).

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