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Neutral evolution of cellular phenotypes

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Eukaryotes exhibit a great diversity of cellular and subcellular morphologies, but their basic underlying architecture is fairly constant. All have a nucleus, Golgi, cytoskeleton, plasma membrane, vesicles, ribosomes, and all known lineages but one have mitochondrion-related organelles. Moreover, most eukaryotes undergo processes such as mitosis, meiosis, DNA recombination, and often perform feats such as phagocytosis, and amoeboid and flagellar movement. With all of these commonalities, it is obvious that eukaryotes evolved from a common ancestor, but it is not obvious how eukaryotes came to have their diverse structural phenotypes. Are these phenotypes adaptations to particular niches, their evolution dominated by positive natural selection? Or is eukaryotic cellular diversity substantially the product of neutral evolutionary processes, with adaptation either illusory or a secondary consequence? In this paper, we outline how a hierarchical view of phenotype can be used to articulate a neutral theory of phenotypic evolution, involving processes such as gene loss, gene replacement by homologues or analogues, gene duplication followed by subfunctionalization, and constructive neutral evolution. We suggest that neutral iterations of these processes followed by entrenchment of their products can explain much of the diversity of cellular, developmental, and biochemical phenotypes of unicellular eukaryotes and should be explored in addition to adaptive explanations.

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> Evolutionary adaptation is a special and onerous concept that should not be used unnecessarily. – GC Williams 1966 p. v [1]

Introduction

All of life goes through a single-cell stage; and the vast majority of the historical and extant diversity of life is unicellular. Investigation of cellular evolution is therefore essential to fully understand the evolutionary mechanisms of phenotypic change. In this review, we consider the importance of neutral evolutionary mechanisms for generating eukaryotic cellular phenotypes.

The vast majority of eukaryotes retain features traceable to the Last Eukaryote Common Ancestor (LECA) including a nucleus, mitochondrion, flagellum, and complex endomembrane and cytoskeletal systems [2,3]. Additionally, most share the capacity to undergo mitosis and meiosis and recombine through sexual reproduction [4], and to produce pseudopodia for movement and phagocytosis of prey [5]. Even though these basic features and many underlying genes are conserved across eukaryotes, intracellular and organismal morphologies can vary drastically, especially among unicellular forms [6]. Currently, our in-depth knowledge about eukaryotic cell biology derives from a very limited number of well-developed model organisms, of which all but yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) are multicellular. Some single-celled protozoan/algal models have been employed for many years (e.g. *Trypanosoma brucei*, *Dictyostelium discoideum*, *Chlamydomonas reinhardtii*), but only recently have they neared the tractability of the yeast systems. Even so, most cell biological studies investigate the function of either highly conserved or very derived lineage-specific features. Few studies focus on explaining how these features evolved and diversified. Therefore, it is at this point usually impossible to know what genomic changes are responsible for the cell morphological differences observed between major eukaryotic groups. However, the new field of evolutionary cell biology is emerging [7], bringing with it new approaches and new model organisms. As we move forward with these novel approaches, it will be useful to have the broadest possible explanatory toolkit. We therefore believe it is desirable to develop a broadly applicable neutral theory of phenotypic evolution, if for no other reason than to serve as an alternative hypothesis against which 'onerous' claims for adaptation might be evaluated.

Here, we introduce a hierarchical approach to understanding phenotypic evolution as suggested previously [8••]. Phenotype can be defined at numerous hierarchical levels, ranging from survival and reproduction at the highest level to purely molecular phenotypes at the lowest. Two consequences fall out of this approach. First, all genotypic changes cause phenotypic change at at least one hierarchical level. Second, neutral phenotypic changes are permitted at any level in the phenotypic hierarchy, so long as they do not impinge on a level upon which selection is acting. In cases where multiple lower-level phenotypes can satisfy higher-level requirements, there is the possibility of neutral change. We begin with a brief review of the neutral theory of molecular evolution, showing how it can be expanded into a neutral theory of phenotypic evolution. We then discuss mechanisms by which mutations can neutrally affect phenotype at molecular and cellular levels. Paying explicit attention to non-adaptive explanations will better equip us to design experiments to distinguish cellular traits that have neutral histories from those with selective ones.

Box 1 Recasting the neutral theory of molecular evolution as a neutral theory of phenotypic evolution

A neutral theory of phenotypic evolution can be derived from the neutral theory of molecular evolution. The current form of the neutral theory of molecular evolution states (simplified from [56•] but see [57•]):

1 Most mutations in the genome do not affect fitness and are predominantly subject to drift.
2 Most mutations that affect fitness are deleterious and are purged by purifying selection.
3 Population dynamics change and have major effects on genomic population diversity.

Adapted to make reference to phenotypes:

1 Most mutations in the genome do not appreciably affect phenotypes under selection and are therefore subject to drift (i.e. most phenotypic change is invisible to selection).
2 Most mutations that affect phenotypes under selection, negatively affect fitness and are purged by purifying selection (i.e. neutral phenotypic evolution occurs only where phenotypes are not under selection).
3 Changes in population dynamics can have major effects on phenotypic trajectories (i.e. drift can cause not only fixation of deleterious genotypes, but also of deleterious phenotypes).

Toward a neutral theory of phenotypic evolution

Last year marked the 50th anniversary of Kimura's proposal of the neutral theory of molecular evolution in population genetics and was celebrated by a special issue of *Molecular Biology and Evolution*. In simple terms, the neutral theory states that very few mutations are beneficial; rather, most mutations are either deleterious and quickly purged by purifying selection, or (nearly) neutral such that they might be fixed by drift [9–12]. Although Kimura himself eschewed the possibility of a neutral theory of phenotype evolution, others have shown that neutral evolution of phenotypes is possible and indeed probable [8••,13–15]. Analogous to the currently accepted version of the neutral theory of molecular evolution, we can describe a neutral theory of phenotypic evolution (Box 1).

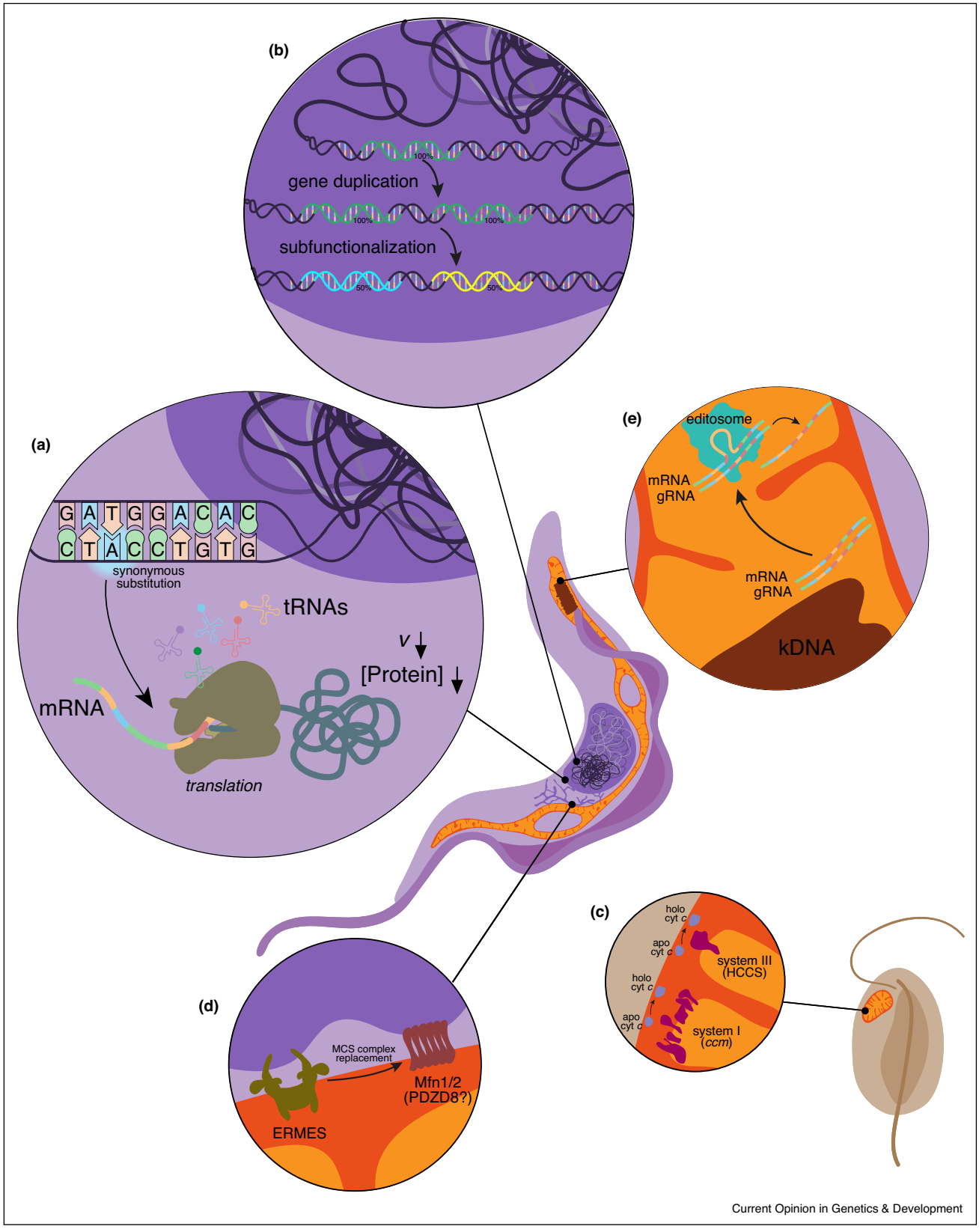
A neutral theory of phenotypic evolution requires a hierarchical approach to understanding phenotype, as suggested by Ref. [8••]. At the highest, most general level are survival and reproduction—phenotypes that directly track and indeed might be taken to define, fitness. At the lowest level are molecular phenotypes, including restriction fragment length polymorphisms (RFLPs), protein isoelectric points, redundant gene duplications, and even novel retrotransposon expansions (Figure 1a). All genetic changes involve phenotypic change at this low level. Between these simple molecular phenotypes and the level of survival and reproduction are intermediate levels, including metabolite concentrations, gene expression, protein folding, antibiotic sensitivities, chromatin state, cellular morphology, developmental stages, and behavior. When selection operates at a particular level, there may be multiple lower-level phenotypes that generate the phenotype under selection (e.g. distinct amino acid sequences may yield functionally equivalent proteins). This is captured by the concept of multiple realizability, in which multiple lower level phenomena can independently 'realize' the same higher-level phenomenon [16,17]. In other words, natural selection only sees the solution—it is blind to the route taken.

The neutral theory of evolution in its classic formulation focuses on neutral evolution at lower, molecular levels, where multiple realizability is more easily imagined. However, neutral evolution need not be absent from higher phenotypic levels. This is the basis for a neutral theory of phenotypic evolution (Box 1). For a phenotype to be under selection, its effects must reach the highest level (survival and reproduction). So, any change occurring at a level to which selection is blind will be neutral, as long as it does not affect the higher level (Figure 1). This kind of reasoning is used to explain how eukaryotic genomes can become bloated with 'junk DNA' like repetitive sequence and transposable elements [18,19]. To date, much of the focus on neutral phenotypic evolution has been on intraspecific neutral evolution (e.g. Refs. [8••,20••]). Our aim here is to question whether neutral processes can explain phenotypic differences interspecifically and across even greater macroevolutionary distances.

Iterative contingency and entrenchment – neutral ratchets in protein evolution

Sequence divergence of homologous proteins from a common ancestor either within a genome (paralogues) or between species (orthologues) can be the result of either selected or neutral events. Mere comparison of distantly related lineages is insufficient to determine

Figure 1


(b)
gene duplication
100%
100%
100%
subfunctionalization
50%
50%
(a)
G A T G G A C A C
C T A C C T G T G
synonymous substitution
tRNAs
v
[Protein]
mRNA
translation
(e)
editosome
mRNA gRNA
mRNA gRNA
kDNA
(c)
holo cyt c
apo cyt c
system III (HCCS)
holo cyt c
apo cyt c
system I (ccm)
(d)
MCS complex replacement
Mfn1/2 (PDZD8?)
ERMES
Current Opinion in Genetics & Development

which non-synonymous changes in particular protein sequences were fixed by natural selection and which were fixed by drift. Substitutions fixed by drift can later become entrenched due to epistatic interactions with other neutral substitutions, with the result that reversion to the ancestral state is impossible [17,21]. In this way, as a protein evolves, the gene and protein sequence can change drastically without higher-level phenotypic change. Because most commonly used evolutionary models assume site-independent evolution, they are likely to overpredict adaptive evolution in such cases [21].

One way to study neutral protein evolution is by functional investigations of ancestral reconstructions. Hsp90 is a chaperone protein whose cellular role is conserved across all eukaryotes, as demonstrated by the fact that human and other Hsp90s can complement yeast Hsp90 [22,23], even though these organisms are separated by at least a billion years of evolution. Similar examples exist whereby distant orthologues can complement across major eukaryotic lineages (e.g. Ref. [24]). This highlights the multiple realizability of the function of these proteins: many different sequence states can perform the necessary functions.

A recent study [25••] from the Thornton group found that, although replacement is possible, most reversions to Hsp90 ancestral states lead to minor fitness defects if introduced individually. There are two possible explanations for these results. One possibility is that the yeast-specific substitutions are adaptive and were selected for a particular reason within that lineage, and it is just good luck that homologues can complement the yeast protein. Alternatively, it may be that neutrally diverged sites are restricted in extant lineages due to other neutral changes in the same molecule that caused their entrenchment. Using deep-sequencing–based bulk fitness assays to measure selection coefficients, Starr *et al.* [25••] demonstrated that many ancestral forms of Hsp90 had near (>0.96–0.99x) contemporary levels of fitness in *S. cerevisiae*, which was much higher compared to the cumulative effects predicted from introducing each individual reversion. This means that many of the deleterious effects are context-dependent and are alleviated by epistatic interactions with other reversions, favoring the second explanation.

Extending this rationale from the case of yeast to all eukaryotic lineages, evolutionary diversification in sequences of Hsp90 across eukaryotes may be largely due to neutral, rather than adaptive, mechanisms. This would mean that adaptation is not the sole cause of molecular divergence, suggesting that even highly divergent proteins in distantly related taxa may have accumulated many or even most of their distinct fixed sites by drift. Drastic divergence is allowed as long as function is conserved and higher phenotypic levels remain unaffected [26]. Each mutation in each lineage not only opened up new possible neutral mutations, but also closed off previously neutral possibilities. In what follows, we show how this kind of thinking can be applied to higher levels of biological organization, such as gene duplications, metabolic pathways, and even gross cellular morphology through processes of 'iterative contingency and entrenchment' [25••].

Neutral gene duplications, subfunctionalization, and constructive neutral evolution

Evolution by gene duplication was first defended strongly by Ohno [27]. In his model, positive selection on one of two duplicates was how novel proteins emerge and how new functions originate. After gene duplication, without selection pressure on maintaining the redundancy, one or the other copy will inevitably be lost. In 1999, Force *et al.* [28] and Stoltzfus [29] both presented mechanisms by which gene duplications can persist without positive selection (Figure 1b). Perhaps the most easily described mechanism by which this can occur is duplication followed by complementary partial loss-of-function mutations (subfunctionalization), so that products of both genes are now required to perform at a level previously accomplished with one. When this occurs, both copies of the gene must be retained. In eukaryotes, gene duplications followed by subfunctionalization can also explain paralogous expansions of ancestral monodimers into heterodimers, as documented recently in *S. cerevisiae* (See Ref. [30••]).

A hierarchical understanding of phenotype. **(a)** Any genetic change results in phenotypic change at least one hierarchical level. At the very lowest level, a change in a nucleotide changes the structure of the DNA which could, but does not necessarily affect higher levels. It could be non-coding DNA and could now recruit a transcription factor. Or it could be a synonymous change that affects the translation of a protein because of codon bias. Alternatively, a non-synonymous change at the DNA level directly affects the primary, and possibly secondary, tertiary, and quaternary structure of a protein. **(b)** Gene duplication followed by subfunctionalization is one route to increased complexity. Gene duplications not only alter the amount and structure of DNA, but can also increase the amount of protein in a cell. Subfunctionalization renders both proteins necessary for function without evolutionary 'progress'. **(c)**. Neutral replacement of analogous systems. In the evolutionary history of eukaryotes, the ancestral bacterium-derived cytochrome *c* maturation system I (ccm) has been repeatedly replaced by the eukaryote-derived system III (HCCS). Simple modules with low connectivity can be easily replaced by other systems. **(d)** Neutral replacements can underlie phenotypic stability. Mitochondria-ER contact sites exist across eukaryotes, but the underlying components are not conserved. Iterative replacement by both homologues and analogues has maintained the likely ancestral phenotype and function although the underlying genes and mechanisms for maintaining the phenotype have changed. **(e)** Constructive neutral evolution of an organismal phenotype. Through iterative processes of neutral replacement, gene duplication followed by subfunctionalization, and constructive neutral evolution, the bizarre characteristics of the kinetoplast emerged.

Subfunctionalization of monodimers into heterodimers is in fact one of the simplest examples of constructive neutral evolution (CNE) – a process by which complexity increases without adaptation [29,31,32]. But, in CNE, the interacting proteins need not be related either by descent or by function. In a simple example, two proteins happen to physically interact by chance in a lineage. This interaction is non-functional and does not contribute to fitness, but its existence permits a mutation in one of the proteins that renders the interaction necessary (and therefore functional). As with Hsp90 evolution, further permissible mutations may further entrench the novel interaction such that any reversal to the ancestral state is highly improbable: two evolutionarily unrelated components are now necessary to perform a task where one was once sufficient. Such iterative contingency and entrenchment forms the basis of all constructive neutral evolution.

Neutral replacement: multiple realizability at the molecular level

A single function can be carried out by two different components. In some cases, like in a gene duplication event, the components are homologues and have a shared evolutionary history (i.e. redundancy). However, components with the same function may also be analogues and, despite their independent evolutionary histories, have the same biochemical outcome (i.e. degeneracy). When two analogous systems find themselves within the same organism, just like the case of gene duplication, two outcomes are possible. Either of the components can be lost, or subfunctionalization can occur resulting in both components being retained.

Horizontal gene transfer (HGT) is perhaps the most common mechanism (other than gene duplication) by which two genes with the same function can occur in a single organism. Once two ways to carry out the same function appear in a single genome, a path to complexity is opened [33]. But in most cases, one of the copies is lost, leading to patchy distributions of genes with identical functions (e.g. Ref. [34]). Resulting replacements often involve one for one, like for like replacements of genes that have low connectivity with other components [35]. During endosymbioses, many opportunities for neutral replacement occur which can lead to complex dependencies (e.g. Ref. [36]). Below, we present a somewhat complex case involving analogous cytochrome *c* maturation systems in order to convince readers of the power of neutral explanations.

In bacteria and eukaryotes, an enzymatic maturation pathway is necessary to incorporate cytochrome *c* into the electron transport chains [37]. The majority of eukaryotic lineages contain a single nucleus-encoded enzyme that functions in this way in the mitochondrial intermembrane space (Figure 1c). However, several lineages retain the multi-component bacterium-derived cytochrome *c* maturation machinery at least in part encoded in their mitochondrial genomes [38]. These include both protistan sister groups to animals and fungi, most plants, some cryptists, some red algae, ciliates, and many excavates [3]. One species, *Ancoracysta twista*, which belongs to an orphan lineage unrelated to any other major eukaryotic group, is unique in containing both (1) the bacterium-derived and mitochondrion-encoded, and (2) the eukaryote-derived nucleus-encoded systems [39•]. Two hypotheses can accommodate the observed distribution of these systems. First, both systems may have been present in LECA and, after persisting for ~1 billion years in every eukaryotic lineage, have been differentially lost in every lineage except that containing *A. twista*. Second, the multipartite bacterial system was present in LECA from the mitochondrial endosymbiont, whereas the eukaryotic system evolved independently in a single eukaryotic lineage. This eukaryotic novelty was subsequently horizontally transferred independently to many eukaryotic lineages. If we assume the second explanation is true, then for the exceptional case of *A. twista*, the presence of both systems can also be explained in two ways. First, the HGT could be very recent and there has not been enough time for the loss of either system to occur. Or, as in subfunctionalization after gene duplication, partial loss of function mutations could have been incurred by each maturation system such that neither system can be lost outright.

The replacement of the cytochrome *c* maturation pathway is not unique. Similar replacements have been observed in the cell cycle network of fungi [40], the mitochondrial RNA polymerase of most eukaryotes [41], the histones of dinoflagellates [42], and possibly mitochondrial membrane contact sites ([43] and Figure 1d). Whether or not these are neutral replacements, however, is unclear.

Neutral evolution of an organismal phenotype

So far, we have discussed basic neutral mechanisms that lead to slight increases in complexity (gene duplication and subfunctionalization) and replacements (by homologues or analogues) that do not impinge upon gross morphological characters. Here, we wish to highlight an example where these mechanisms could have neutrally produced the defining morphological character in kinetoplastids.

Kinetoplastids are extremely divergent protists [44]. Ancestrally, eukaryotes bore bacterium-like circular mitochondrial genome architectures and transcriptional mechanisms [45]. Instead of this, kinetoplastids have a complex mitochondrial genome architecture with thousands of excess base pairs and require complex RNA editing to produce translatable transcripts [46,47]. These features together are responsible for the mitochondrial genome being a densely packed and (upon staining for DNA) easily visible cellular feature termed the kinetoplast. One

can imagine that RNA editing evolved by a neutrally occurring haphazard transcript (the first guide RNA) allowing a deletion or point mutation to persist in an essential mitochondrial gene. Editing could then complexify through a process of iterative contingency and entrenchment whereby new deletions and point mutations were suppressed by an ever-growing array of guide RNAs. Over time, the rising complexity of the ancestral kinetoplastid mitochondrial genome resulted in a large amount of mitochondrial DNA per cell such that, unlike most eukaryotes, the mitochondrial DNA is an easily visible characteristic upon staining (Figure 1e).

The genomic structure and RNA editing of kinetoplastids are locked in place by strong purifying selection against loss, as no reversion is possible. However, it is intuitive that a simpler ancestral-like mitochondrial genome architecture and translation mechanism would not hinder survival. In this vein, one popular hypothesis is that the kinetoplast evolved via constructive neutral evolution [29,31,46,48] (but see Ref. [49]). Technologically, we are not at a point where we can test this hypothesis, but perhaps as tools for investigating the cell biology of *T. brucei* are developed, we will soon be able to directly investigate this hypothesis. Several other examples in which neutral evolutionary processes may have been responsible for major eukaryotic cellular phenotypes include mitotic mechanisms [50], cristae architectures [51,52], and Golgi morphologies [53]. However, recent investigations suggest that cellular phenotypes in yeasts are under strong selection pressure [20••], once again leading to suspicion of the possibility of neutral evolution of organismal phenotypes.

Moving forward: is a neutral theory of phenotypic evolution testable?

It is obviously simpler to test the neutrality of ancestral changes in Hsp90s or cytochrome *c* maturation pathway replacements across eukaryotes by heterologous expression than it is to test the hypothesis that the kinetoplast evolved neutrally. However, this should not dissuade us from intermediate challenges. With the advent of CRISPR technologies more cellular phenomena can be investigated in a growing number of alternative model organisms [54]. Heterologous expression of more complex modules in established model systems like *S. cerevisiae* has become a much simpler task such that we may be able to answer just which cellular modules can undergo functional replacement, in which lineages, and why. This will be a major line of research in the burgeoning discipline of evolutionary cell biology.

Conclusions

The above examples illustrate that neutral molecular diversification can, at least in principle, have effects on biochemical, cellular, and even organismal phenotypes. A hierarchical understanding of phenotype makes it clear that any change below the level upon which selection is acting can evolve neutrally. We therefore contend that much of the diversity of unicellular eukaryotes may be the product of neutral processes such as loss [55], gene replacement, subfunctionalization, and constructive neutral evolution. Thus, much of eukaryotic diversity may have evolved much the same way that Hsp90 has diversified in eukaryotes. The morphological diversity of eukaryotic cells has likely been shaped by iterative contingency and entrenchment. The multiple realizability of many cellular processes brings forth the possibility that much of the most bizarre and interesting features of eukaryotes may have arisen by neutral rather than adaptive pathways.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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In response to Kern and Hahn57•] (see below), Jensen *et al*. defend the neutral theory and review its contributions. Of particular importance to non-specialists, they succinctly present a modern account of the neutral theory of molecular evolution.

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This review is extremely critical of the neutral theory and suggests that, although the theory has been useful historically, it has effectively been refuted, and selection-based theories should be generated in its stead.