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6. EVOLUTION OF CELLULAR COMPLEXITY

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Having gained an appreciation for how various population-genetic forces interact to define the accessibility of alternative evolutionary pathways, we now turn to more specific issues relevant to the diversification of cellular features. The idea that natural selection provides a powerful mechanism for advancing adaptive mutations is well-established, so there is no need to belabor that issue further. Likely less familiar and/or less fathomable is the idea that the nonadaptive forces of mutation and drift can often dictate the paths down which phenotypic evolution is most likely to travel, sometimes with minimal involvement from selection. In certain settings, the net result can be a gradual, passive increase in cellular complexity, with little (if any) increase in organismal fitness throughout the process.

The goal here is to instill an appreciation for the riskiness of rigid adherence to the assumption that natural selection is a process in relentless pursuit of biological complexity. This first pass focuses on general issues regarding the evolution of complex features with minimal involvement of adaptive forces, with details specific to a range of cellular structures and functions unfolding in subsequent chapters. Unlike the preceding chapter, to maximize the accessibility of the key issues, a distinctly nonmathematical sojourn will be taken, which is not to say that the mathematical details are irrelevant.

Before proceeding, a brief recap of the population-genetic issues relevant to phenotypic divergence is in order. First, the classes of mutations available to selection depend on the effective population size (N_e), the inverse of which defines the power of random genetic drift. Although there may be no such thing as a truly neutral mutation (with absolutely no selective consequences), selection will be ineffective if the randomizing potential of genetic drift is sufficiently strong. Small populations can only advance beneficial mutations with relatively large effects and cannot prevent the accumulation of deleterious mutations with small effects. Large populations are more capable of evolutionary fine tuning.

Second, owing to the granularity and directional biases of mutations, phenotypic optima will be only occasionally, if at all, attained for cellular traits. Large-N_e species are expected to evolve higher levels of efficiency and accuracy of molecular attributes. However, small N_e enables populations to move into domains that can dramatically shift the course of evolution by natural selection, with mutation playing a powerful role in directing the paths open for exploration. As these fundamental evolutionary principles are unavoidable consequences of the nature of life's genetic material, they must be kept in mind in any attempt to explain cellular diversification.

Illusions of Grandeur

A peculiarly common view is that biological complexity represents the crown jewel of the awesome power of natural selection, with metazoans (humans in particular) representing the pinnacle of what can be achieved. Yet, there is no evidence that increases in complexity are intrinsically advantageous. Nor is there any evidence that biology's metabolic, morphological, and behavioral features have reached a maximum level of refinement or ever will. To think that a mammal is superior to a bacterium is about as meaningful as proclaiming that an Olympic athlete is superior to an an award-winning pianist. In the evolutionary arena, ecological context is paramount, and the currency of natural selection (relative fitness) is only exchangeable for members of the same gene pool. Bacteria can outperform vertebrates in a myriad of ways with respect to metabolism and environmental sensing. Whereas the brain is often quite useful, is there any objective basis for concluding that the streamlined signal-transduction systems of prokaryotes are fundamentally inferior to the baroque and error-prone nervous systems of animals?

Although there are mathematical indices for quantifying complexity in physical systems, things are not so straight-forward in living systems, and the term is used loosely here to simply reflect differences in the numbers of unique parts and interactions within organisms. Even these measures are not always easily enumerated, rendering comparisons among closely related organisms difficult. However, aspects of cellular complexity that most capture the interest of biologists are features such as large protein complexes, the emergence of the eukaryotic cell plan from a prokaryotic ancestor, and the transition from unicellularity to multicellularity. In these cases, there is no disagreement on where things lie on the complexity gradient.

Is the fact that prokaryotes, for the most part, have not evolved internal cell structure or complex multicellularity a sign of evolutionary inferiority, i.e., of innate inability to generate increased morphological complexity despite the benefits that could be reaped? Given their enormous population sizes, their ability to recombine, and their presence on the planet for ~ 4 billion years, the supply of variation is certainly not limiting for microbes. Moreover, as noted in Chapters 2 and 3, aspects of intracellular complexity and even multicellularity have emerged in some prokaryotes. Perhaps then morphological complexity is actively selected against in the prokaryotic world. And if that is the case, what is the evidence that increased complexity is universally advantageous in eukaryotes?

The evolution of root systems and support tissues enabled land plants to occupy ecological niches unavailable to microbes, and the evolution of predatory capacity in animals opened up new ways of living. Surely, such transitions were promoted by natural selection, although other modes of living were left behind, new survivorship challenges were encountered, and rapid rates of reproduction were relinquished. Moreover, the question remains as to whether all of the accompanying underlying genetic and cellular changes in such organisms were the necessary antecedents to such adaptation, as opposed to being inadvertent by-products of such changes. For example, relative to their unicellular ancestors, in just a few hundred million years, the genomes of metazoans and land plants independently became bloated with nonfunctional, energetically costly, and mutationally hazardous DNAs such as mobile-genetic elements and large introns (Lynch 2007). Were all such embellishments essential tickets to the evolution of organismal complexity, somehow maintained in anticipation of future benefits? No credible mechanisms exist for such evolutionary

prescience. More likely, many aspects of increased genome complexity simply reflect the reduced efficiency of natural selection in larger organisms with reduced effective population sizes.

There are at least three reasons why cellular / organismal complexity can be suppressed in certain lineages, while passively increasing in others. First, more complex features inevitably impose greater bioenergetic costs for construction and maintenance. For small cells with relatively low total energy budgets and large effective population sizes, even minor additions to the cellular repertoire can be efficiently opposed by selection unless there are immediate benefits. In contrast, larger cells with higher total energy budgets typically reside in populations with smaller effective population sizes. In this case, a given genomic addition will comprise a smaller fraction of the total energy budget. With a higher power of random genetic drift, moderate-sized cellular additions will then be less visible to the eyes of natural selection, and subject to fixation in an effectively neutral fashion. These issues will be addressed more formally in Chapter 17, the main point here being that cell size alone can dictate the degree to which initially unnecessary (and sometimes weakly harmful) embellishments can become established in a population.

Second, virtually all gene-structural embellishments increase the vulnerability of genes to inactivating mutations (Lynch 2007). Typically, the increased mutational susceptibility is relatively small (on the order of the product of the mutation rate per nucleotide site, u, and the number of key nucleotide sites for proper gene function imposed by the embellishment, n). As an example, the latter number is on the order of 25 for proper intron splicing, and u is in the range of 10^{-10} to 10^{-8}. If nu is smaller than the power of drift ($1/N_e$ for a haploid), the mutational excess associated with such a gene addition cannot be countered by purifying selection. Again, the key point is that weak selection of this sort will only be effective in populations with very large effective sizes.

Finally, all other things being equal, the drift-barrier hypothesis implies that organisms with lower N_e will also evolve to have less refined capacities for their individual enzymes and structural features. The negative correlation of the mutation rate with decreased N_e (Chapter 4) provides a case in point, and other examples will be encountered in subsequent chapters. In some cases, the reduced fucntionality of a system can open up opportunities for the establishment of additional layers of complexity, which can in turn lead to further relaxation of selection on previously established mechanisms (Chapter 20). Taken together, these arguments highlight the fact that N_e limitations, driven by fundamental constraints associated with ecology and the genetic machinery, play a central role in encouraging particular lineages to ascend up the hierarchy of complexity even though simpler (more streamlined) modes of adaptation may be possible.

The key point is that certain population-genetic environments are conducive to the passive operation of a complexity ratchet, with small incremental changes accruing on short time scales cumulatively leading a lineage to a new location in phenotypic space. One might expect that in moving up the ladder of cellular organization – from nucleotide sequences to translated protein products to higher-order structural and biochemical features, there will be a diminishing probability of effectively neutral evolution. However, as will be seen below, the organization of genome and cell biology facilitates the emergence of neutral evolutionary pathways. Just as

the third positions of codons for amino-acids with four-fold redundancy in the genetic code renders nucleotide substitutions effectively neutral, many aspects of cell biology are structured in ways that there are multiple degrees of freedom to make molecular shifts with minor fitness consequences. Thus, the evolution of increased complexity need not imply increased superiority in any sense of the word, and evolution driven by nonadaptive mechanisms (mutation, recombination, and random genetic drift) need not imply a descent towards maladaptive change.

Constructive Neutral Evolution

A verbal model presented by Stoltzfus (1999) and colleagues (Gray et al. 2010; Lukeš et al. 2011) suggests ways in which seemingly gratuitous cellular complexity might grow in the absence of direct selection for such features. The process they call constructive neutral evolution (CNE) has some antecedents in early verbal models of Woese (1971) and Zuckerkandl (1997).

Consider an ancestral cellular function carried out by the product of a single gene (A) (Figure 6.1). Suppose a fortuitous interaction then develops with another protein B, which although binding to A has negligible effects on either's functionality and on cell fitness. By binding to part of A's surface, B may suppress the effects of future mutations arising at the A interface that would be destabilizing if exposed (Chapter 13). Over time, this permissive interfacial environment could lead to enough mutational buildup that A would no longer be functional without B. In principle, this evolved functional dependence of A on B could be followed by a similar scenario involving a third protein, C, and so on.

Under this scenario, the intricate inter-dependencies of the components of molecular complexes need not be intrinsically essential for a high level of functionality. Rather, they are simply the result of a series of effectively neutral coevolutionary steps accompanied by relaxed selection against previously forbidden mutations.

Although this verbal model provides a plausible argument for the passive origin of complexity, two key assumptions underlie the CNE hypothesis. Foremost is the idea that biological systems often harbor excess capacity, as the process requires that the evolutionary diversion of B molecules to A has negligible effects on any preexisting benefits of B, at least to the extent that could be opposed by natural selection. As excess capacity implies a superfluous energetic drain on the cell, why would such conditions exist? As discussed in the following section, although redundancy is unlikely to be promoted on its own merits, recurrent gene duplication may lead to a sort of quasi-equilibrium level of redundancy at the population level, although the genes involved at any particular time will vary. In addition, transient conditions may exist in which a change in environment may render the prior function of B detrimental or obsolete.

The second issue is that the evolution of A's dependency on B requires that the genetic background allowing the fortuitous A-B interaction survives for a long enough period for A to acquire the conditionally harmful mutations essential to the development of dependency on B. This returns us to the kinds of scenarios outlined in Chapter 5 whereby a small number of mutations are required for a transition to an alternative semi-stable state. However, a key difference from the theory presented

in Chapter 5 is that under CNE the transition to complexity may be essentially a one-way street – once the complex is established, the acucmulation of conditionally lethal mutations eliminates the possibility of an evolutionary reversion to the simpler condition.

The population-genetic requirements for the operation of CNE have not been formally evaluated except in the case of evolution by gene duplication covered in the following section. However, based on the theory outlined in the previous chapters, one can at least envision conditions in the process is most likely to proceed. All of these involve a relaxation in the efficiency of selection, in particular an initial A:B state that no worse than very weakly deleterious, combined with a sufficiently small effective population size to render the initial transition effectively neutral. In potential support of the CNE model, numerous examples exist in which molecular complexes with universally conserved functions have larger numbers of subunits in eukaryotes than in prokaryotes.

Consider, for example, oxidative phosphorylation. Carried out in the mitochondria of eukaryotes, this energy-generating mechanism involves multiple complexes with conserved functions throughout the Tree of Life. Well over 100 subunits are distributed among the multiple electron-transport chain (ETC) complexes in eukaryotes, more than double the number found in bacteria (Hirst 2011; Huynen et al. 2013), and although most of these changes occurred prior to LECA, there have been numerous subsequent lineage-specific additions. Nearly all of the accessory proteins are encoded in the nuclear genome, with the favored explanation for their existence being their essential roles in maintaining structural stability of the complexes. The larger eukaryotic complexes are not more stable than those in bacteria, but they achieve their stability in a more complex manner. It has been argued that the subunit additions evolved as structural compensation for evolved defects in the mitochondrially encoded components (resulting from deleterious-mutation accumulation in organelle genomes; Chapter 23) (Angerer et al. 2011; Hirst 2011; van der Sluis et al. 2015). However, a CNE scenario in which structural dependency is a consequence rather than a cause of subunit recruitment has not been ruled out.

A second potential example of the gratuitous evolution of complexity involves the ribozyme RNase P, a complex of proteins surrounding a single catalytic RNA molecule that processes precursor transfer RNAs to their mature form. Although the RNA subunit is similar in all organisms, bacterial RNase P contains just a single protein, whereas the archaeal and eukaryotic complexes contain five to ten proteins. This is a seemingly substantial investment in complexity for an enzyme whose sole role is to cleave a single phosphodiester bond. Again, the primary function of these additional proteins appears to be in stabilizing the overall complex (Lan et al. 2008). Moreover, whereas the RNA core of the bacterial complex is internally stabilized by tertiary RNA-RNA interactions, these structural RNA features are reduced in archaeal and eukaryotic RNAs (Gopalan et al. 2018), as expected under the CNE model.

It has been argued that the evolution of higher-order RNase P complexes is a by-product of their having evolved additional cellular functions (Gopalan et al. 2018), but the possibility that any such functions could not also be carried out by less elaborate structures has not been ruled out. Notably, a few bacteria and eukaryotes have lost the RNA component of RNase P and carry out the usual function solely

with an enzymatic protein, showing that a simpler structure can indeed suffice. Complementation studies have shown that these RNA-free proteins will function with no apparent harmful effects when they are expressed in species that normally utilize RNA-containing RNase P (Weber et al. 2014; Lechner et al. 2015; Nickel et al. 2017).

Although a number of open questions remain, the simplest explanation for these observations on ETC complexes and RNaseP is that excess complexity has arisen within certain lineages by effectively neutral processes, maintaining ancestral functions in a highly conserved way, while increasing the bioenergetic cost to the organism. We now consider in more depth another potential example of CNE, in this case a very large ribonucleoprotein complex, the ribosome, whose function has again been highly conserved across the entire history of life. The following is an elaboration on earlier ideas of Stoltzfus (1999) and Lukeš et al. (2011).

Ribosomes. In all cells in all organisms, the ribosome has a singular, conserved role – it is the site of translation of messenger RNAs. The catalytic core of the ribosome consists of three to four ribosomal RNAs, which collectively operate as a complex ribozyme. However, no ribosome can operate unless assembled together with dozens of other proteins. The question of why a molecular machine of this sort would require such a large endowment of protein components is further motivated by the substantial phylogenetic variation in the set of ribosomal proteins utilized in different lineages.

Thirty-four ribosomal proteins appear to be universally deployed in all eukaryotes and prokaryotes (and hence are often referred to as the common core), but there are also at least 34 ribosomal proteins shared by eukaryotes and archaea but absent from bacteria, whereas bacteria share no ribosomal protein just with eukaryotes or just with archaea (Lecompte et al. 2002). Each of the three major domains also harbors unique ribosomal proteins not found in either of the other groups. This phylogenetic distribution is entirely consistent with the hypothesis that bacteria form an outgroup to archaea/eukaryotes (Chapter 3).

Not only do the protein constituents of ribosomes vary among the major domains of life, but the numbers of distinct proteins deployed varies as well, with about 21 and 33 being used in the small and large ribosomal subunits (denoted SSUs and LSUs, and respectively responsible for decoding mRNA information, and forming peptide bonds) in bacteria, but 33 and 46, respectively, in eukaryotes (Melnikov et al. 2012). Most of the proteins added to eukaryotic ribosomes have joined the external surfaces, like rings on an onion (Hsiao et al. 2009).

The two major ribosomal RNAs (rRNAs), deemed the small and large subunits, vary in size among organisms, with a clear expansion of both in eukaryotes relative to prokaryotes ($\sim$ 50% on average), and with weak coordination in size changes between the two subunits (Figure 6.2). As in the case of expansions in the ribosomal protein repertoire, rRNA enlargements generally occur by the addition of expansion segments that leave the common core undisturbed (Petrov et al. 2014).

On the other hand, the rRNAs coded and deployed within mitochondria are often reduced in size relative to those in bacteria. For example, the mammalian mitochondrial LSU rRNA contains less than a third the number of nucleotides as its counterpart in the cytosol ribosome (1559 vs. 5347) and only half that in typical

bacteria, although those in yeast and other protists can be comparable in size to those in bacteria.

Substantial modifications in the protein contribution to ribosomal structure have also been seen in mitochondria. Despite having to typically translate just a dozen or so mitochondrial membrane proteins, the protein repertoire of mitochondrial ribosomes is typically quite large. For example, the human mitochondrial LSU contains 48 proteins, all of which are encoded in the nuclear genome and 21 of which are mitochondrion specific (compared to 47 associated with the human cytoplasmic LSU) (Brown et al. 2014). Eleven of the mammalian mitochondrial-specific proteins are not found in the yeast mitochondrial ribosome LSU, which nevertheless contains 39 proteins (Amunts et al. 2014). Overall, mitochondrial ribosomes contain 10 to 20 proteins not found in their α-proteobacterial ancestors, with these again largely being distributed over the ribosome surface (Desmond et al. 2011).

The overall picture one gets from the above is that ribosome expansion likely followed the emergence of eukaryotes, with further gains and losses then occurring on individual lineages, and all such changes leaving the internal catalytic core intact. However, it is not just the structure of the ribosome, but also the pathways involved in ribosome biogenesis, that became more elaborate in eukaryotes (Strunk and Karbstein 2009). In bacteria, ribosome assembly involves no more than a handful of additional proteins, whereas on the order of 200 accessory proteins are essential for the development of mature eukaryotic ribosomes. The operation of many of these ribosome-biogenesis proteins requires hydrolysis of nucleotide triphosphates and hence is highly demanding energetically. Thus, given the expanded number of nucleotides and amino acids invested in eukaryotic ribosomal RNAs and proteins, it is clear that the overall energetic cost of the protein-production machinery in eukaryotes is substantially greater than that in prokaryotes.

It has been argued that such expansions and elaborations, seemingly related with organismal complexity, reflect a long-term pattern of adaptive divergence of ribosome architecture (Petrov et al. 2014, 2015). However, such a view is confronted with two fundamental problems: 1) the apparent inability of prokaryotes to achieve such changes despite having existed for longer periods of time and in much larger populations; and 2) the absence of evidence that either the expansion segments of rRNAs or the additional ribosomal proteins confer any intrinsic benefits or novel functions. The maximum rate of translation per ribosome (amino acids incorporated per second) in eukaryotes is estimated to be 17 in *Neurospora crassa* (Alberghina and Sturani 1975), 10 in *Saccharomyces cerevisiae* (Boehlke and Friesen 1975; Waldron and Lacroute 1975; Bonven and Gullov 1979), and 6 in mouse embryonic stem cells (Ingolia et al. 2011). Estimates in bacteria are 20 for *E. coli* (Forchhammer and Lindahl 1971; Dennis and Bremer 1974; Young and Bremer 1976), 16 for *Staphylococcus aureus* (Martin and Iandolo 1975), and 3 for *Streptomyces coelicolor* (Cox 2004). Although some of these estimates are likely more reliable than others, there is no indication of an elevated processing rate in larger eukaryotic ribosomes. Nor is there any indication that translation accuracy is improved in eukaryotes (Chapter 20).

Evolution by Gene Duplication

We now turn to a major route to the origin of complexity, for which an ample body of theory and empirical observation exists. Although much of the theory reviewed in the previous chapter focused on small incremental changes to individual genes, such as single-nucleotide substitutions, larger-scale changes are common. Duplications of entire genes or fragments thereof are of special interest because they generally contain fully functional domains tested under a prior history of selection. In this sense, novel gene functions do not have to be built from scratch, but more often than not arise as elaborations of pre-existing functions. The potential contribution of gene duplication to evolutionary innovation is substantial, as individual genes duplicate at rates that are comparable to or greater than the rates at which base-substitution mutations arise at individual nucleotide sites (Lynch and Conery 2000; Konrad et al. 2018).

The fates of duplicate genes depend on the mechanisms by which they arise and the population-genetic environments within which they reside. Owing to the random breakpoints of duplicated DNA spans, duplication events will not necessarily encompass the full regulatory and/or coding regions of parental genes, and hence may have divergent features at birth (Katju and Lynch 2006). At the other extreme, exceptional cases involve whole-genome duplication events in which all genes are simultaneously duplicated in entirety. Such events are known to have occurred in the ancestry of numerous eukaryotic lineages, including yeast (Wolfe and Shields 1997), ciliates (Aury et al. 2004; McGrath et al. 2014), vertebrates (Chain and Evans 2006; Putnam et al. 2008), arthropods (Kenny et al. 2016; Li et al. 2018), and land plants (Soltis and Soltis 2016).

Like all mutations, gene duplicates are initially present in just a single copy in a single individual. This will also be true for genes arising by other mechanisms, such as fortuitous *de novo* origin from preexisting noncoding sequence (Wissler et al. 2013; Bornberg-Bauer et al. 2015; McLysaght and Hurst 2016; Neme and Tautz 2016) or via horizontal transfer from exogenous sources (Keeling and Palmer 2008; Vos et al. 2015). Thus, all of the issues fundamental to the establishment of point mutations (Chapter 5), and more, apply to gene duplication. To be successful in the long term, a new gene must first drift towards fixation, and having arisen to high frequency, must then be preserved by sufficiently strong selective forces to prevent rapid loss by degenerative mutation.

The vast majority of duplicates arising by segmental duplications are lost from populations on time scales of less than a few million generations (Lynch and Conery 2000), most never even proceeding to fixation. Basic population-genetic principles (Chapter 5) indicate why. Letting N be the population size and assuming diploidy, in the absence of immediate positive (or negative) selection, a fraction $[1 - (1/2N)]$ of newly arisen gene duplicates will be lost by random genetic drift in an average of just $\sim 2\ln(2N)$ generations (Kimura and Ohta 1969), a flash on the evolutionary time scale. Moreover, the small remaining fraction, $1/(2N)$, that manages to drift to fixation is also expected to fall victim to silencing mutations relatively quickly unless a preservational mechanism is acquired. Letting μ_c denote the rate of appearance of gene-silencing mutations, the average time to gene inactivation is on the order of the mean waiting time for the appearance of a null mutation at one of the two loci, $\simeq 1/(2\mu_c)$ generations, which will generally be on the order of 10^6 generations (Watterson 1983; Lynch et al. 2001).

Although it is often argued that an increase in gene number is a sign of evolutionary success and superiority (e.g., Lane and Martin 2010), there is little support for this point of view. Indeed, the number of genes per genome is nearly decoupled from organismal complexity. For example, the genomes of the most behaviorally sophisticated animals contain fewer genes than found in many protists and only a few-fold more than in most bacteria. Only a few hundred genes are conserved across the entire Tree of Life (Tatusov et al. 2003; Koonin et al. 2004), and there can even be substantial differences in the numbers of genes among individuals within a species. This being said, the evidence is overwhelming that the repatterning of gene functions and gene locations by duplication events plays a central role in organismal diversification, although the connections often have little to do with adaptive processes.

The goals here are to summarize the ways in which gene duplication opens up novel pathways for evolutionary elaboration, provide insight into how the likelihoods of such processes are influenced by the population-genetic environment, and address some of the concerns with the more general model of constructive neutral evolution. More thorough reviews have appeared on the rates of origin, fates, and consequences of duplicate genes (e.g., Lynch 2007; Conant and Wolfe 2008; Innan and Kondrashov 2010; Katju 2012). The small minority of duplicates that are retained for long periods of time are thought to owe their preservation to one of four mechanisms, one of which will first be dispensed with.

The masking effect. Because all populations harbor low-frequency, suboptimal alleles due to the recurrent introduction of deleterious mutations, it is commonly thought that duplicate genes have an intrinsic selective advantage associated with their ability to mask the effects of deleterious mutations at the ancestral locus. However, the frequency at which a backup is useful is proportional to the incidence of deleterious genotypes at the opposite locus, which is on the order of the mutation rate to degenerative alleles. Thus, the selective advantage of a back-up gene is approximately equal to the rate of its own silencing by deleterious mutations. This leads to a miniscule selective advantage of the masking effect, generally smaller than the power of random genetic drift (Fisher 1935; Clark 1994; Lynch et al. 2001; Proulx and Phillips 2005).

The most serious challenge to the masking hypothesis for duplicate-gene retention is the general paucity of duplicate genes in haploid microbes despite their exceptionally high effective population sizes (which would maximize the efficiency of selection for weakly favorable redundancy). As will be discussed in Chapter 17, the energetic cost of a gene in bacterial species (relative to the total cellular energy budget) is generally sufficiently large for selection to efficiently remove redundant gene duplicates on this basis alone.

Neofunctionalization. Historically, the origin of a new gene function was thought to be the only preservational mechanism for the long-term survival of gene duplicates, with the usual fate being the mutational silencing of one copy by degenerative mutations (Haldane 1933; Muller 1940; Ohno 1970). The idea here is that gene duplication can free one copy for evolutionary exploration and eventual acquisition

of a new adaptive function. If the modifications underlying this new function are acquired at the expense of essential ancestral gene functions, the joint maintenance of both members of the pair will be enforced. A key issue here, of course, is that preservation by neofunctionalization requires a setting in which there is indeed a need for a new gene function.

Neofunctionalization is expected to be more common in populations with large N_e for at least three reasons (Lynch et al. 2001; Walsh 2003). First, the larger the population size, the greater the population-level rate of origin of a rare neofunctionalizing mutation, and hence the higher the probability of fixation of such a mutation prior to one or the other locus being silenced by a degenerative mutation. Second, in a sufficiently large population, even a duplicate gene initially destined to be lost by random genetic drift has a nontrivial chance of being rescued and propelled forward by a neofunctionalizing mutation. Third, in very large populations, the process need not depend on new neofunctionalizing mutations at all, as the requisite alleles may maintained at low frequency by selection-mutation balance in the base population (but incapable of spreading to fixation prior to duplication because individuals lacking the essential ancestral allele are inviable) or in rare cases by balancing selection (e.g., heterozygote superiority; Spofford 1969).

Subfunctionalization. With the emergence of genome-sequence data in a wide variety of lineages, it became clear that the levels of retention of duplicate genes following whole-genome duplication events are far too high to be consistent with a model in which most are preserved by evolving new functions. Given the mutation rate to degenerative mutations, they are also far too high to be fortuitous avoidances of gene silencing. Thus, something other than neofunctionalization must often be responsible for duplicate-gene retention. The fact that the vast majority of newly arising mutations are deleterious, combined with the emerging understanding of gene-structural complexity, suggested a mechanism by which duplicate-gene preservation can be completely driven by degenerative mutations. Under the DDC (duplication-degeneration-complementation) model, both members of a gene pair acquire complementary negative changes that necessitate joint preservation (Force et al. 1999; Lynch and Force 2000; Lynch et al. 2001).

In the case of multifunctional genes, subfunctionalization can involve the complete partitioning of independently mutable, essential gene functions, leading to specialized copies with nonoverlapping features (qualitative subfunctionalization; Figure 6.3). Subfunctionalization can also be instigated by partial reduction in the efficiencies of the same functions in both members of a pair down to the total level required in the single-copy state (quantitative subfunctionalization) (Lynch and Force 2000; Duarte et al. 2006; Gout and Lynch 2015; Thompson et al. 2016). In both cases, subfunctionalization eliminates the need for adaptive change in the gene preservational process, although this need not rule out the emergence of secondary, adaptive modifications, as noted in the following section.

Contrary to the situation with neofunctionalization, the probability of subfunctionalization is expected to diminish with increasing effective population sizes, for at least three reasons (Lynch et al. 2001; Walsh 2003). First, there are prices to be paid for a pair of subfunctionalized genes. With respect to the coding region, the system will be roughly twice as mutationally vulnerable as a single-copy gene,

imposing a selective disadvantage equivalent to the null mutation rate per gene; and as just noted, there will also be an energetic cost of duplicate-gene maintenance and operation. As both of these costs are relatively small, they will only be opposed by selection in large populations. Second, a subfunctionalized allele en route to fixation is vulnerable to acquiring secondary silencing mutations, and the likelihood of such an effect is magnified in a large-N_e setting owing to the longer time to drift to fixation. Finally, qualitative subfunctionalization requires the presence of independently mutable regulatory mechanisms or protein domains, and as discussed below the evolution of such modularity is reduced in large-N_e settings.

Adaptive-conflict resolution. Finally, the joint action of subfunctionalization and neofunctionalization may lead to gene copies that are not only largely distinct from each other but also have improved functionalities relative to the ancestral gene (Piatigorsky and Wistow 1991; Hughes 1994; Stoltzfus 1999). Consider, for example, a single-copy locus subject to a "jack-of-all-trades is a master-of-none" syndrome, i.e., with an adaptive conflict between its subfunctions. In such a situation, while initially ensuring preservation of the pair of sister genes, complementary loss-of-subfunction mutations may also alter the selective landscape experienced by the two pair members, enabling each copy to become more refined to its specific subset of tasks, potentially even opening up previously unavailable pathways to neofunctionalization. By this means, two of the most common forms of genomic upheaval, gene duplication and degenerative mutation, may provide a unique mechanism for the creation of novel evolutionary opportunities through the elimination of pleiotropic constraints. Again, however, whether such an adaptive-conflict resolution leads to a net selective advantage will depend on the degree to which the improvement(s) in gene functions exceed the cost of maintaining two genes.

A variant on the adaptive-conflict model is the IAD (innovation-amplification-divergence) model of Bergthorsson et al. (2007), which postulates that a common path to the origin of a new function in bacteria starts with the duplication of a gene with a promiscuous secondary function, which in times of extreme need might suffice to provide enough functional rescue to buy time for further evolutionary refinement. Additional duplications would increase the number of mutational targets for such improvements, with deletions of the excess copies after establishment of the neofunctionalized gene eliminating the cost of gene amplification. Näsvall et al. (2012) and Newton et al. (2017) demonstrated the operation of this mechanism in the bacterium *Salmonella,* with a bifunctional gene involved in histidine biosynthesis with weak promiscuous involvement in tryptophan biosynthesis. When placed on a genetic background lacking the primary tryptophan synthesis pathway, evolutionary rescue was accomplished as duplicates of the histidine gene arose, and in some cases became specialized to alternative pathways. Other examples of this sort will be discussed in Chapter 19, where it will be shown that adaptive-conflict resolution and gene duplication plays a major role in the evolutionary remodeling of metabolic pathways.

The Case for Subfunctionalization

Prior to the development of the DDC model, circumstantial evidence for duplicate-gene preservation via subfunctionalization was suggested by studies of polyploid fishes, which repeatedly demonstrated tissue-specific expression of duplicated enzyme loci (Ferris and Whitt 1977, 1979). Such observations have now been supplemented by a wide array of investigations in other ray-finned fish lineages, zebrafish in particular, all of which arose following a whole-genome duplication event (e.g., Pasquier et al. 2017). Without an outgroup, it is difficult to determine whether such specificity is an outcome of neofunctionalization vs. subfunctionalization. However, the evolutionary interpretations of such expression-pattern studies have been greatly facilitated by observations of orthologous single-copy genes in tetrapods (usually mouse or chicken). These lineages, which branched off prior to the fish-specific polyploidization event, generally reveal the presence of both gene subfunctions in their single-gene outgroup. Similar observations have been made in the tetraploid frog *Xenopus laevis* in comparison to its diploid relatives (Morin et al. 2006; Sémon and Wolfe 2008), as well as in land plants (Rutter et al. 2012). Indeed, there are now hundreds of examples of qualitative subfunctionalization of duplicated genes via the partitioning of tissue-specific expression in multicellular organisms.

Although this particular mechanism of duplicate-gene preservation is unavailable to unicellular species, there many other potential paths to subfunction partitioning. For example, gene products may become specialized for use in different subcellular localtions. Genes can be regulated in modular ways with respect to timing of expression during the cell cycle or in response to different environmental conditions. In addition, proteins that assemble as homomeric multimers raise the possibility of complementary interfacial changes after duplication enforcing assembly as heteromers between the duplicate-gene products (Diss et al. 2017).

Thus, although unicellular species often have large effective population sizes, which might be expected to reduce the incidence of subfunctionalization, the process is by no means restricted to multicellular species. Indeed, as outlined in subsequent chapters, key episodes of the process may have occurred during small-N_e phases in early eukaryotic history. A striking example of subfunctionalization deep in the eukaryotic phylogeny involves the dynamin family of proteins, which are used to pinch membranes. Phylogenetic analysis suggests the presence in LECA of a bifunctional dynamin with roles in vesicle scission from cell membranes and in mitochondrial division (Purkanti and Thattai 2015; Leger et al. 2015). Although this dual-function gene is retained in numerous eukaryotic lineages, following duplications in three independent lineages, the two copies became specialized to the two alternative ancestral functions.

Given the enormous amount of cell biological work done on yeast, and the whole-genome duplication that preceded the emergence of *Saccharomyces cerevisiae* (Wolfe and Shields 1997), much has been learned about the mechanisms preserving duplicate genes in this species. In particular, empirical studies in which *S. cerevisiae* duplicates have been swapped with the single-copy gene from a closely-related outgroup species have provided compelling evidence for subfunctionalization (van Hoof 2005). For example, Orc1 and Sir3 are sister genes in *S. cerevisiae*, with the former playing a role in chromosomal origins of replication and the latter being part of a nucleosome-binding complex involved in chromosome-silencing functions. In a related taxon that branched off prior to whole-genome duplication, *Kluyveromyces*

lactis, both functions are carried out by a single-copy gene (Hickman and Rusche 2010).

An example of subfunctionalization's role in adaptive-conflict resolution has also been revealed by molecular dissection in *S. cerevisiae,* where two sister genes are involved in galactose utilization, one (Gal3) playing a regulatory role in pathway induction and the other (Gal1) serving as a galactokinase (Hittinger and Carroll 2007). Again by reference to *K. lactis,* it was determined that the ancestral single-copy gene served both functions. Gene duplication then allowed the refinement of binding-site configurations that had previously been constrained in the ancestral gene, thereby enabling the emergence of a much more tightly regulated system (Figure 6.4).

A striking example of subfunctionalization based on structural alterations in yeast is provided by the hexameric membrane ring for the vacuolar ATP synthase pump (Figure 6.5). In metazoans and most other eukaryotes, the ring consists of five copies of one protein (Vma16) and one of another (Vma3), both of which arose from an ancient gene duplication. In fungi, a third duplicate (Vma11) that arose by duplication of Vma3 replaces one subunit of Vma16, specifically residing between Vma16 and Vma3. Experimental modifications of the subunit interfaces revealed that one side of Vma3 has lost the ability to bind to one side of Vma16, whereas the other side of Vma11 has lost the ability to bind to Vma3 (Finnigan et al. 2012). There is no evidence that this increase in the complexity of vacuolar ATP synthase has endowed yeast with increased fitness.

As noted above, it is unlikely that duplicate genes are selectively preserved on the basis of having backup features. However, observations from *S. cerevisiae* show that such properties can exist as an indirect consequence of overlapping gene functions retained after partial subfunctionalization. For example, two ancient yeast paralogs, Sir2 and Hst1, operate as histone deacetylases with rather different functions in the cell (Hickman and Rusche 2007). When one gene is absent, the other can partially compensate by engaging in the noncognate function. Comparison with a pre-duplication outgroup species makes clear that this is a case of quantitative subfunctionalization, illustrating the risks of assuming that because duplicate genes have redundant functions, they must have been preserved on the basis of their backup capacities.

Finally, a potentially common mode of duplicate-gene preservation in eukaryotes involves the partitioning of gene functions via the modification of transit signals for localization of mRNAs and/or proteins to particular subcellular regions (Kumar et al. 2002; Silva-Filho 2003; Krogan et al. 2006). Immediately after transcription, eukaryotic mRNAs are typically decorated with one or more RNA-binding proteins, many of which attach to specific motor proteins for delivery to a specific subcellular locality prior to translation (Besse and Ephrussi 2008; Holt and Bullock 2009; Buxbaum et al. 2015). There are numerous cases in which modifications of transit signals in post-duplication genes have lead to sub- or neolocalization. For example, following duplication of one of the subunits of cytochrome c oxidase (a terminal complex in the electron transport chain) in an ancestral vertebrate, one member came to specialize on localization to the mitochondrion, whereas the other is delivered to the golgi (Schmidt et al. 2003). Likewise, NADP-dependent isocitrate dehydrogenase has been duplicated independently in both yeast and mammals, and in both

cases the descendant copies partitioned their localizations to either the nucleus or the cytoplasm (Nekrutenko et al. 1998; Szewczyk et al. 2001).

Marques et al. (2008) suggest that about a third of duplicated genes surviving the whole-genome duplication leading to *S. cerevisiae* exhibit spatial subcellular partitioning, and similar estimates have been given for other taxa. For example, up to 25% of gene duplicates in the plant *Arabidopsis* (another descendant of a whole-genome duplication event) have experienced relocalization or sublocalization of their gene products (Byun and Singh 2013; Liu et al. 2014). There is, however, some uncertainty as to whether such partitioning is typically a cause or consequence of duplicate-gene preservation, as singleton genes in *S. cerevisiae* also appear to frequently acquire novel relocalization patterns (Qian and Zhang 2009). Although bacteria also exhibit spatial organization of translation (Montero Llopis et al. 2010; Nevo-Dinur et al. 2011), this is dictated primarily by the cellular locations of genes on the chromosome, and there appears to be less opportunity for partitioning subcellular localization following gene duplication.

The Emergence of Modular Gene Subfunctions

Taken together, these results (along with many others to appear in subsequent chapters) make clear that duplicate-gene subfunctionalization has played a major role in the evolution of structural and enzymatic features of eukaryotic cells. However, few examples have been revealed in prokaryotes. One simple reason for expecting rarity of subfunctionalization in prokaryotes is the population-size constraint associated with the mutational and energetic costs of duplicate genes (Adler et al. 2014), but another is the general absence of independently mutable regulatory elements and localization zipcodes necessary for subfunction partitioning. This raises the broader question as to how modular gene architectural features essential to subfunctionalization actually evolve.

In fact, the same types of duplication and degeneration processes that lead to the subfunctionalization of duplicate genes promote the emergence of the subfunctions themselves (Force et al. 2005). To simplify discussion, we will assume that subfunctions are defined by transcription-factor binding sites (TFBSs) or integrated regions of such sites (simply referred to here as promoters) that are separable from other such sites, both mutationally and functionally (as further elaborated on in Chapter 21). However, the same principles apply to subfunctions defined by functional motifs in coding regions, binding interfaces in multimers, or any other gene features that can be mutationally separated.

The goal is to understand how a gene that is initially ubiquitously controlled under all conditions in the same manner comes to be regulated by more specialized mechanisms while retaining the same overall expression pattern. The process envisioned here, subfunction fission, involves the progressive reconfiguration of a general-purpose enhancer via consecutive processes of partial duplication and loss of regulatory information, with each step proceeding in a nearly neutral fashion (Figure 6.6). The first phase involves the accretion of new regulatory elements, followed by the degeneration of one or more ancestral sites to yield two semi-independent promoters. The second phase involves tandem duplication of the regulatory region,

followed by the formation of two entirely independent regulatory subfunctions by complementary degenerative mutations. Other than the fact that smaller DNA elements are involved, the events during the second phase are conceptually identical to those noted above for the subfunctionalization of entire genes.

There is not necessarily a permanent allelic state under this model, as the alternative classes of shared and semi-independently regulated alleles are free to mutate back and forth (hence, the two-way arrows in the top left of Figure 6.6). Thus, it is necessary to consider the circumstances under which semi-independently regulated alleles are likely to rise to high frequency, as their presence is essential to completing the transition to an allele with two entirely independent subfunctions.

There are two reasons why gene structure is more likely to gravitate to the subfunctionalized a modular state in small populations. First, the stochastic gain of specific regulatory elements can occur either by *de novo* mutation in existing sequence to an appropriate motif or by the insertion of a pre-existing element via duplication from alternative genomic sites. The rate of *de novo* origin of an appropriate TFBS motif by mutation will depend on the mutation rate per nucleotide site and the mutational target size (amount of intergenic spacer DNA), both of which scale approximately inversely with N_e (Chapter 4; Lynch 2007). In addition, the large, more gene laden genomes of species with small N_e (e.g., eukaryotes vs. prokaryotes) have more potential sources of TFBSs for duplicative transpositions. Second, alleles with more complex regulatory regions have a higher mutational vulnerability and impose an excess energetic cost at the DNA level, both of which are small effects that will only be efficiently opposed by selection in populations with large N_e.

The salient point here is that the same population-genetic environments that favor the subdivision of gene functions following gene duplication are expected to favor the emergence of gene-structural architectures necessary to fuel subfunctionalization. Such potential for reinforcement provides further support for the contention that reductions in N_e, which naturally occurred as eukaryotes arose (and subsequently spawned the metazoan and land-plant lineages), promoted a setting for the evolution of complexity by passive mechanisms with essentially no involvement of positive selection. Consistent with such a ratchet-like march towards complexity is the observation that whereas duplicate genes gradually lose their shared expression patterns over evolutionary time, the total numbers of regulatory motifs and interacting protein partners remain roughly constant for each member of the pair, suggesting an approximate balance between gains and losses of such elements. Such patterns have been observed in yeast (Papp et al. 2003; He and Zhang 2005), mammals (Huminiecki and Wolfe 2004), and *Arabidopsis* (Arsovski et al. 2015).

Taken together, these observations raise significant questions about the necessity and sufficiency of natural selection as a determining force in the emergence of complex patterns of gene regulation and protein deployment. In sufficiently small populations, modular forms of gene structure are expected to emerge in the absence of any direct selection for such architectural features. In sufficiently large populations, such changes are opposed by selection (unless immediately accompanied by phenotypic advantages that substantially offset the mutational and energetic disadvantages).

The Passive Origin of Species via Gene Duplication

In addition to gene duplication playing a central role in the evolutionary divergence of cellular traits, the process also plays a powerful indirect role in the second major engine of evolution – the process of speciation (Lynch and Force 2000). Genetic theories of speciation have traditionally focused on two competing hypotheses (reviewed in Orr 1996; Rieseberg 2001; Coyne and Orr 2004). The Bateman-Dobzhansky-Muller model postulates the accumulation of lineage-specific gene-sequence changes that are mutually incompatible when brought together in a hybrid genome, whereas the chromosomal model invokes the accumulation of rearrangements that result in gene loss in hybrid backgrounds.

Both models are based on rather stringent assumptions. For example, the Bateman-Dobzhansky-Muller model invokes the evolution of mutually incompatible coadaptive complexes of epistatically interacting factors, few of which have yet been identified at the molecular level. Chromosomal models generally focus on major rearrangements, for which within-population fixation can be greatly inhibited by the reduction in fitness in chromosomal heterozygotes. Notably, the gene-duplication model for speciation is consistent with both the Bateman-Dobzhansky-Muller and the chromosomal models, while requiring fewer assumptions than either of them.

The passive reassignment of gene (sub)functions to novel locations following gene duplication is central to the model. To see this, consider a diploid ancestral species with an unlinked pair of duplicate autosomal genes, which then experience divergent non- or subfunctionalization in two descendent species. This results in different chromosomal locations of the active gene (Figure 6.7). Because the F_1 hybrids of such species will be "presence-absence" heterozygotes at the two independently segregating loci, 1/4 of the F_1 gametes will contain null (absentee) alleles at both loci. For a gene that is critical to gamete (haploid) function, this single divergently resolved duplication would result in an expected 25% reduction in fertility. For a zygotically (diploid) acting gene, $(1/4)^2 = 1/16$ of the F_2 offspring from the interspecific cross would lack functional alleles at both loci, and another 1/4 would carry only a single functional allele. Thus, if the gene is haploinsufficient, 5/16 of the F_2 zygotes of such a cross would be inviable (and/or sterile). With n divergently resolved duplicates, the expected fitness of hybrid progeny is $W = (1-\delta)^n$, with δ denoting the reduction in hybrid fitness per map change. For example, with $\delta = 5/16$, as in a zygotically-acting haploinsufficient viability gene, $W = 0.024$ in the F_2 generation when $n = 10$, and 5×10^{-17} when $n = 100$.

Observed rates of gene duplication indicate that this type of process is sufficiently powerful to yield nearly complete genomic incompatibility within a few million years of cessation of gene flow (Lynch and Force 2000; Shpak 2005). This is also the approximate time scale over which postzygotic isolation generally occurs in animals (Parker et al. 1985; Coyne and Orr 1997; Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002). Unfotunately, knowledge on the timescale of speciation in unicellular organisms is scant. However, genomic comparisons of the yeasts *S. cerevisiae* and *Candida albicans* imply an overall rate of microchromosomal rearrangement of ~ 2.3 / lineage / MY (Seoighe et al. 2000), likely driven in large part by divergent resolution of duplicate genes, as noted below.

The gene-duplication model for speciation is effectively a chromosomal model,

but because the rearrangements are microchromosomal, they are unlikely to cause significant pairing problems during meiosis. Such changes can then accumulate passively without any alteration in within-species fitness, only being revealed after crossing to a lineage with a deviant gene location. The gene-duplication model also masquerades as a Bateman-Dobzhansky-Muller model, in that reassignments of genes to new locations operate like epistatic interactions because the loss-of-function phenotype is determined by the total number of active alleles at the two duplicate loci in hybrid progeny.

A key feature of the gene-duplication model is that speciation can occur without any molecular evolution. All that is required is the reciprocal silencing of ancestral-gene (sub)functions in sister taxa following ancestral gene duplications. This process can also proceed via paths of neofunctionalization provided the latter occurs at the address of the ancestral gene copy in one lineage (Lynch and Force 2005), and this can lead to misinterpretations regarding the underlying genetic mechanism of postzygotic isolation. Often it is assumed that speciation is a by-product of local adaptation generating physiologically incompatible alleles. However, incompatibilities resulting from the neofunctionalization of a duplicate gene need not be a direct function of adaptive changes at the neofunctionalized locus, but simply an indirect consequence of relocation of the ancestral-gene function.

The divergent resolution of duplicate genes is by no means the only possible route to the origin of post-zygotic species isolating barriers. However, given the frequency of gene duplication, it is difficult to escape the conclusion that it is a common and pervasive mechanism for speciation. As an example, a duplicate pair of a genes involved in histidine biosynthesis was present in the ancestor of the plant *Arabidopsis thaliana,* with different copies becoming silent in different *A. thaliana* sublineages. When plants containing the reciprocally silenced genes are crossed, the hybrids (presence/absence heterozygotes at both loci) segregate out different haplotypes in the next round of gametes, with progeny lacking both copies being inviable (Bikard et al. 2009; Blevins et al. 2017). A similar scenario, involving a different gene duplication, has been found in the genus *Mimulus* (Zuellig and Sweigart 2018).

The fruit fly *Drosophila* has been one of the major workhorses for research on the genetics of speciation, and here there are also well-documented examples of the involvement of duplicate genes in reproductive isolation. In two cases, a strong phase of positive selection operating on single duplicate copies has been implicated (Ting et al. 2004; Greenberg et al. 2006), suggesting the possibility of neofunctionalization. But in some *D. melanogaster* - *D. simulans* hybrids, sterility appears to be a simple consequence of the movement of an essential gene to a new chromosomal location via an intermediate phase of gene duplication (and without a change in function) (Masly et al. 2006).

Finally, it bears emphasizing that under the gene-duplication model, certain groups of organisms are expected to be more prone to speciation than others. For lineages experiencing a doubling in genome size, the process noted above will be essentially unavoidable. Following the first map changes induced by reciprocal silencing in sister polyploid taxa, the thousands of duplicate pairs still remaining are free to become divergently resolved in subsequently isolated lineages, potentially yielding a large number of nested speciation events.

A particularly striking example of reproductive isolation by divergent resolution is provided by the *Paramecium aurelia* complex, consisting of at least 14 cryptic species that emerged after two whole-genome duplication events and are individually separated by hundreds of map changes as ancestral genes came to be represented by one, two, or three copies located on different chromosomes (McGrath et al. 2014). Although the members of the *P. aurelia* complex have evolved unique pairs of mating types, despite $> 10^8$ years of isolation, there has been no discernible morphological evolution.

Another remarkable observation that appears to be quite compatible with the gene-duplication model for the origin of isolating barriers involves the yeast *S. cerevisiae* and its close relatives, which exhibit up to hundreds of differences in gene-order changes resulting from divergently resolved pairs of gene duplicates following a whole-genome duplication (Scannell et al. 2006). Although the haploid offspring of crosses between such species are almost always sterile, engineering the chromosomes to restore large-scale colinearity increases fertility to levels of $\sim 25\%$ (Delneri et al. 2003). Because some minor gene-order differences almost certainly went undetected in these constructs, restoration to complete colinearity might have even a greater effect. Notably, Selmecki et al. (2015) demonstrated that whole-genome duplication in yeast can drive rapid adaptation by neofunctionalization by simply generating more material for molecular evolution, while also providing more opportunities for modifying gene balance by large deletions and/or chromosome loss, all of which will lead to the chromosomal repatterning essential to the gene-duplication model of speciation.

The key point here is that as in the case of phenotypic change within lineages, ample mechanisms exist for the passive origin of new species via nonadaptive processes. One potential example of such a key event, touched upon in Chapter 3, involves the base of the eukaryotic lineage – the colonization of LECA by the mitochondrion. Considering the very large number of organelle-to-nucleus gene transfers that apparently occurred soon after the establishment of the mitochondrial progenitor (Martin et al. 1998), divergent resolution of duplicated organelle genes may have provoked the passive development of isolating barriers among basal eukaryotic lineages.

Summary

- To minimize energetic costs and mutational vulnerability, all other things being equal, natural selection is expected to always favor simplicity over complexity. Yet, many aspects of cell biology are demonstrably over-designed, particularly in eukaryotes, and most notably in multicellular species.

- Constructive neutral evolution provides a vision for how organismal complexity can emerge by nonadaptive mechanisms. The key idea is that the fortuitous development of initially neutral interactions between different gene products can alter the selective environment in ways that enable the fixation of previously forbidden mutations, thereby leading to permanent mutual dependence. Although

the formalities of the theory remain to be worked out, the model provides a plausible explanation for the origin of a wide variety of cellular features, including the large number of protein subunits associated with complexes such as the electron-transport chain and the ribosome.

- Gene duplication is one of the primary mechanisms for the origin of organismal complexity, with neofunctionalization of one member of a pair providing a facile route to the origin of novel gene features. However, duplicate genes are more commonly preserved by other nonadaptive mechanisms. Most notably, subfunctionalization occurs when complementary degenerative mutations result in the partitioning of ancestral gene functions. The latter process has a higher probability of occurrence in populations with small effective sizes.

- The same processes that lead to subfunctionalization of duplicate genes promote the evolution of modular forms of gene structure upon which the process of subfunctionalization depends. Thus, by facilitating the recurrent emergence and partitioning of gene subfunctions, reduced effective population sizes can lead to the passive increase in organismal complexity without any direct selection for such changes.

- Gene duplication also provides a powerful mechanism for the passive origin of reproductively isolated species, particularly when lineages have experienced whole-genome duplications, as has happened repeatedly throughout the eukaryotic phylogeny.

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Figure 6.1. An idealized scenario by which increased complexity might arise by constructive neutral evolution – a transition from an independently functioning molecule A to an obligatory A:B interaction. Here, a fortuitous interaction with B suppresses deleterious mutational effects in A (which would otherwise be eliminated by selection), rendering A dependent on B. After the first two mutational steps, the complex has become evolutionarily established – A has acquired a conditionally silent mutation (red) that if exposed by elimination of B leads to loss of function. In the final stage, two additional mutations (white and black), potentially refining A:B function beyond its initial state, have become established. From Lukeš et al. (2011).

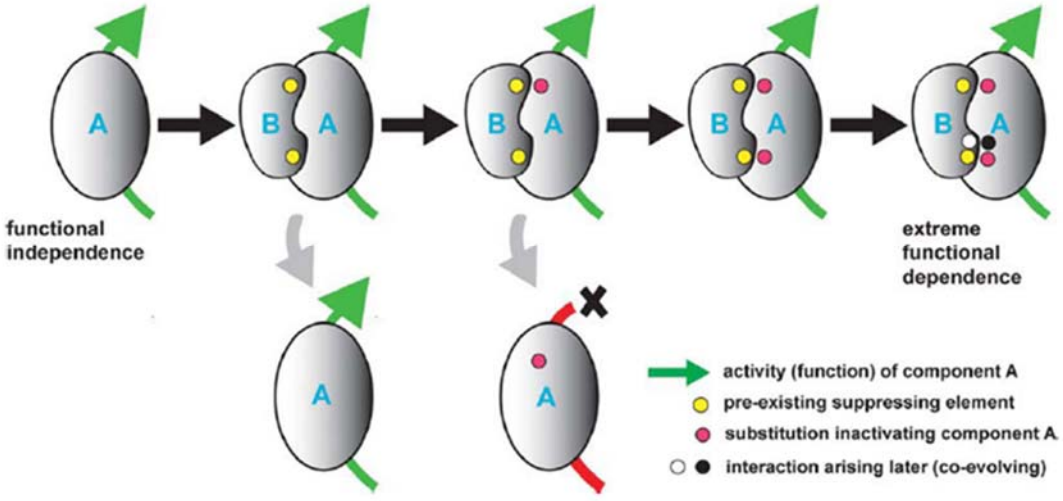
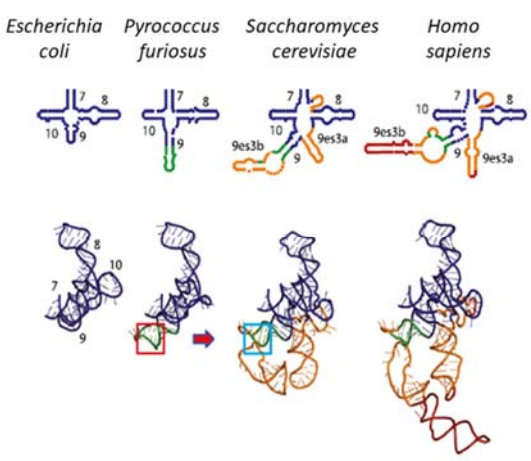


Figure 6.2. Left) Adventitious growth of one particular set of helices associated with the SSU ribosomal RNA. The nearly invariant functional core is represented by the dark blue subsections. From Petrov et al. (2015). **Right)** Sizes of the small and large rRNA subunits in various taxa. From Petrov et al. (2014).



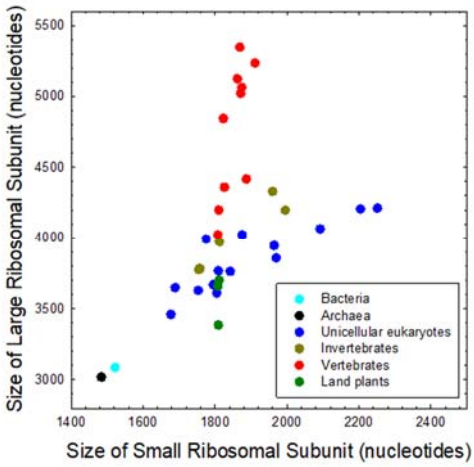


Figure 6.3. The DDC model for the alternative fates of duplicate genes. The ancestral gene is depicted as having two independently mutable subfunctions (blue and green), e.g., two regulatory regions, each driving expression in a particular tissue or environmental condition, two alternative spliced forms, or two domains with nonoverlapping functions. Solid boxes denote fully functional regulatory and coding regions, whereas open boxes denote loss of function, and a red box denotes the gain of a new beneficial function. Each pair of genes reflects the fixed haploid state of the population. Following the duplication event, the first degenerative mutation eliminates a subfunction from one of the copies. The second mutational event then dictates the final fate of the pair: subfunctionalization, with the second copy acquiring a complementary loss-of-subfunction mutation; neofunctionalization, with the second copy acquiring a novel, beneficial expression pattern at the expense of an ancestral subfunction; or nonfunctionalization, with the first copy losing all functional ability. From Force et al. (1999).

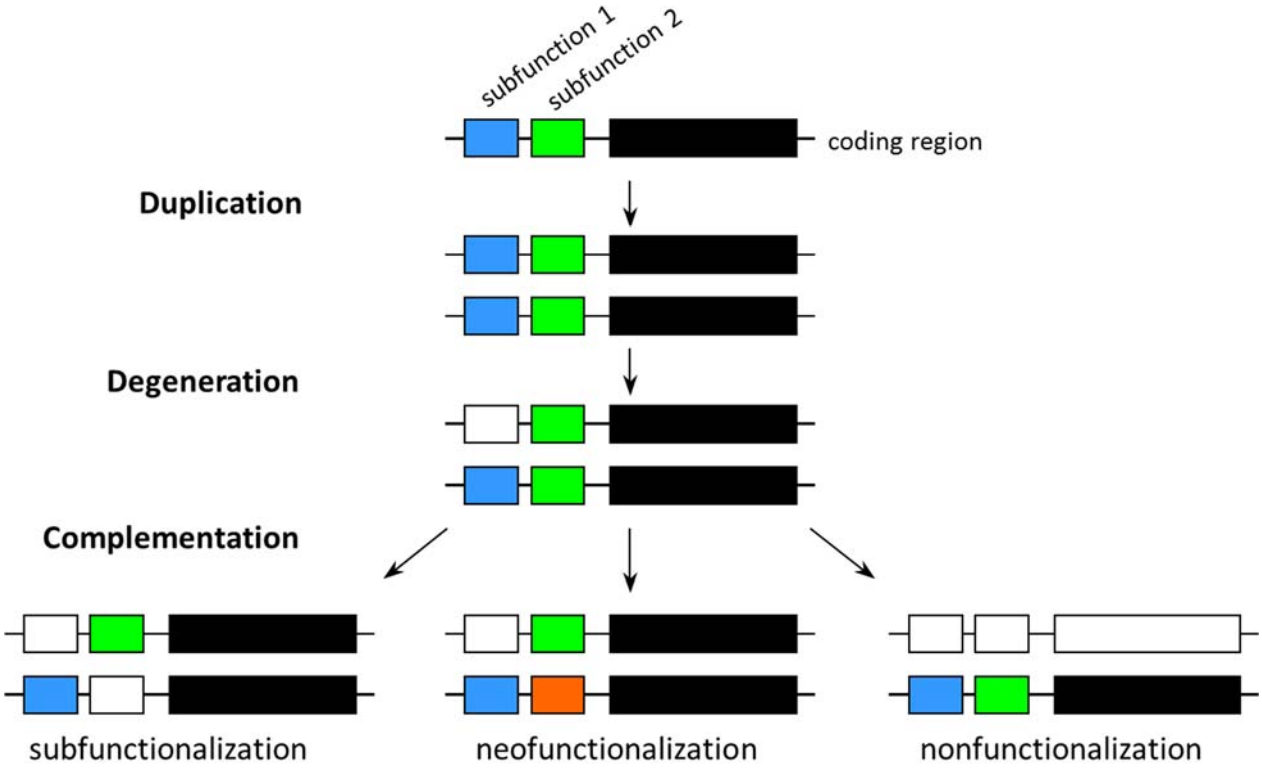


Figure 6.4. Evolution of the galactose utilization pathway following duplication subfunctionalization of the ancestral gene (Gal1/3)in the yeast *S. cerevisiae,* giving rise to the duplicates Gal1 and Gal3. Blue represents the coding regions, and orange the binding sites of the transcription factor Gal4. The loss of binding sites in Gal3 and the rearrangement of sites in Gal1 removed an adaptive conflict involving the regulatory efficiency of the single-copy gene. From Hittinger and Carroll (2007).

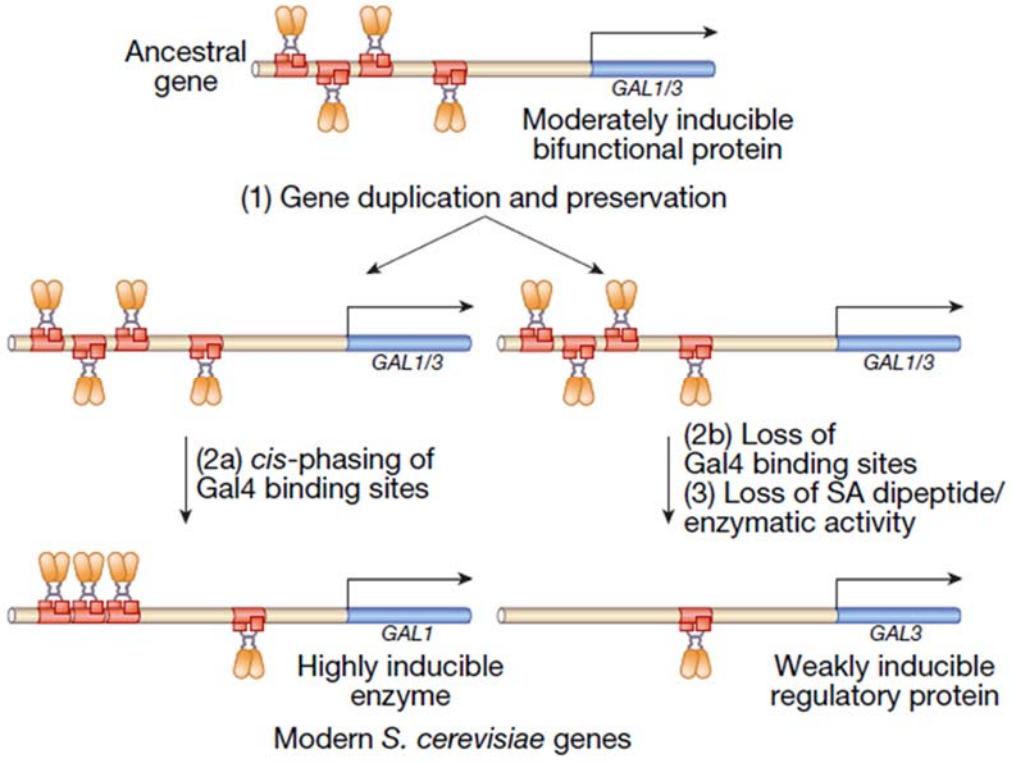


Figure 6.5. Duplication and subfunctionalization of components of the internal-membrane bound ring of vacuolar ATP synthase in the ancestral fungal genome. After their origin by gene duplication, subunits Vma3 and Vma11 acquired complementary changes in interface residues, preventing each from binding one side of Vma16, as demonstrated by experimental manipulation of protein sequences by Finnigan et al. (2011).

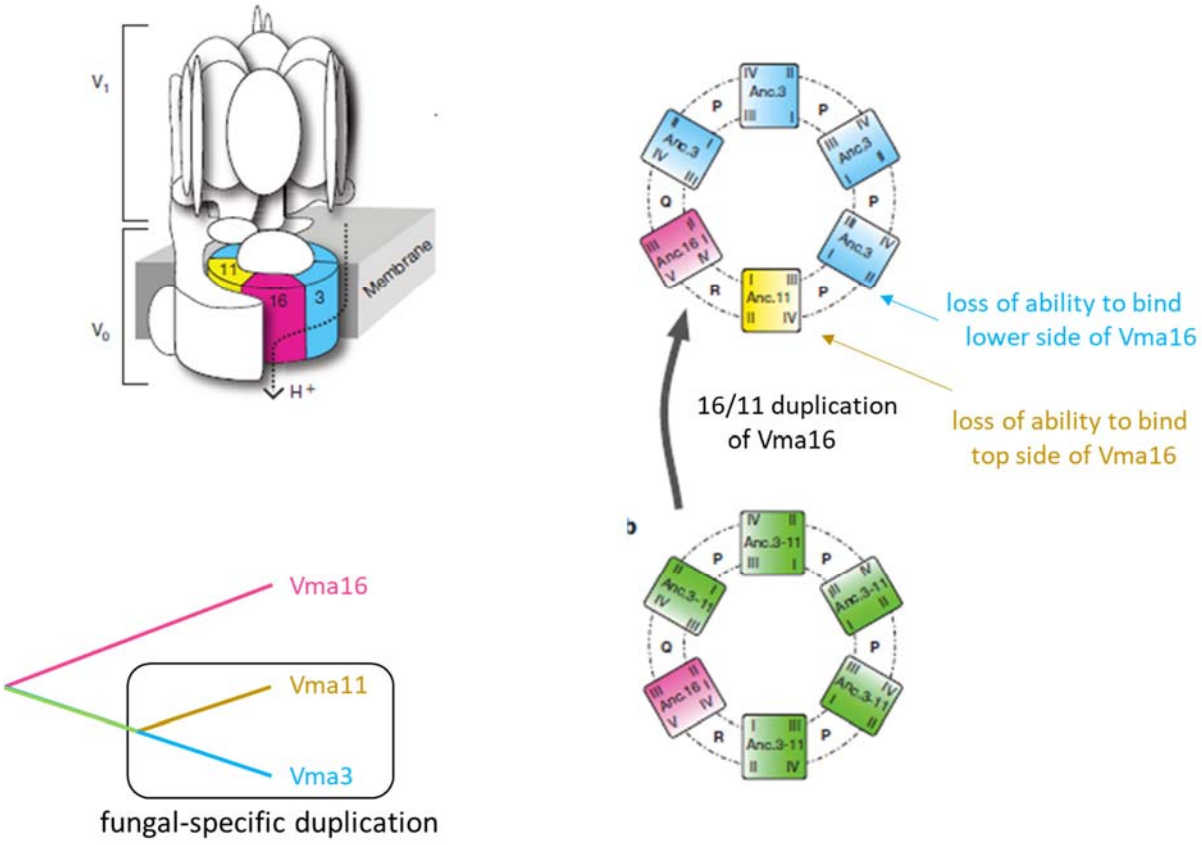


Figure 6.6. A hypothetical scenario in which a gene with two independently mutable subfunctions can arise from an ancestral state with a single generalized expression mechanism. Regulatory regions are depicted on the left, with each regulatory element color-coded according to the transcription factor that binds to it. On the right, the patterns of allele-specific utilization of transcription factors are color coded. Transcription factors denoted by black and white are ubiquitously expressed, whereas those denoted by green and red are expressed in single, non-overlapping conditions. The original gene has a promoter that requires occupancy of three transcription-factor binding sites (one white and two black) for expression. In the first phase of gene evolution, the regulatory region undergoes the sequential accretion of green and red elements, which together are redundant with respect to the white element, which is then lost in a neutral fashion by degenerative mutation. At this point, the evolved allele has a semi-independent mode of expression, as the two black elements are still essential for expression in both tissues. In the second phase, the entire enhancer region is tandemly duplicated, with each component then losing a complementary (red/green) element. The resultant gene now has two independent subfunctions denoted by the green and red open boxes, as a mutation in either region has effects that are confined to a single condition. Note that throughout all of these evolutionary steps, there has been no change in the pattern of expression of the gene.

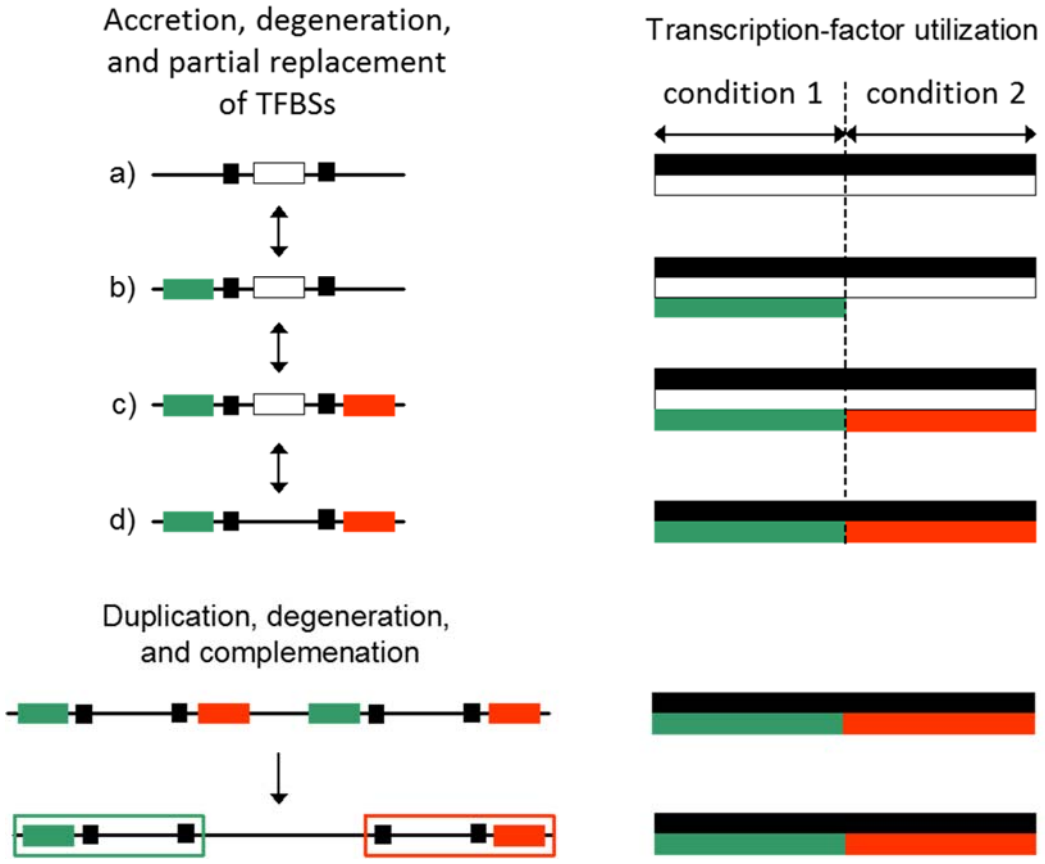


Figure 6.7. Divergent silencing of an ancestral duplicate gene in two geographically isolated lineages. One gene copy is denoted by a white box, the other by a green box, with × denoting an inactivated gene. Gene pairs represent alleles at diploid loci. Progeny other than those indicated might have compromised fitness, e.g., individuals with just a single active gene.