

Gene duplications, robustness and evolutionary innovations

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Summary

Mutational robustness facilitates evolutionary innovations. Gene duplications are unique kinds of mutations, in that they generally increase such robustness. The frequent association of gene duplications in regulatory networks with evolutionary innovation is thus a special case of a general mechanism linking innovation to robustness. The potential power of this mechanism to promote evolutionary innovations on large time scales is illustrated here with several examples. These include the role of gene duplications in the vertebrate radiation, flowering plant evolution and heart development, which encompass some of the most striking innovations in the evolution of life. *BioEssays* 30:367–373, 2008. © 2008 Wiley Periodicals, Inc.

Mutational robustness

Mutational robustness is a biological system's ability to withstand mutations. It has two fundamental mechanistic causes, redundancy of parts and the distributed architecture of biological systems.⁽¹⁾ The robustness caused by these mechanisms exists on multiple levels of biological organization. Cases in point include random mutagenesis experiments of various proteins. They suggest that only a small fraction of mutations affect protein function adversely. For instance, a study of the bacteriophage T4 lysozyme generated more than 2000 random amino acid changes in the protein. Only 16% of them affected lysozyme function.⁽²⁾ Other examples come from regulatory gene networks, such as the molecular network specifying fruit fly segments. Such networks may tolerate considerable quantitative variation in interactions among network genes.^(3–6) Examples at the highest level of organization include macroscopic traits. Even substantial genetic variation—ultimately caused by mutations—may leave such

traits unchanged. Take the vulva of the nematode worms *Caenorhabditis elegans* and *Pristionchus pacificus*.⁽⁷⁾ These organisms shared a common ancestor 200–300 million years ago. Their vulvae are very similar, yet the genetic and cellular networks producing them have diverged greatly. For example, whereas in *C. elegans* one specific cell—the anchor cell—induces vulva development, multiple gonadal cells are responsible for this induction in *P. pacificus*.⁽⁸⁾ Similarly, the same key signaling molecules, such as Wnt, may play a positive role in the network for vulval induction in *C. elegans*, but a negative role in *P. pacificus*.^(9,10) In sum, mutational robustness is everywhere, from proteins to organisms.

Gene duplications cause robustness

Many different kinds of mutations can affect a genome. They include point mutations, insertions, deletions and chromosome rearrangements. Among them, duplications of genes and genomes are unique: only they, as a rule, increase mutational robustness.

Two principal lines of evidence are germane. The first comes from efforts to eliminate (“knock out”) a gene's expression to help determine gene function. To the dismay of many a graduate student, such gene knock-outs often do not show a phenotypic effect, rendering them of limited use in some functional studies.⁽¹¹⁾ Gene duplications are often responsible for such absent effects.^(1,12,13) Genome-scale efforts to eliminate each of thousands of genes in a genome lead to similar results,^(14,15) namely that only a fraction of genes have a phenotypic effect in the laboratory.

A second line of evidence comes from molecular evolution studies. Duplicate genes experience relaxed selection shortly after their duplication. They can tolerate more nucleotide changes than their single-copy counterparts. The phenomenon is evident most clearly on a whole-genome scale,^(16,17) where recent gene duplicates in various eukaryotes tolerate 10-fold more amino acid changes than old duplicates.⁽¹⁶⁾ The accumulating changes may cause duplicates to diversify their function and sometimes quite rapidly.^(1,18,19)

Robustness facilitates evolutionary innovations

Narrowly defined, a biological system is *evolvable* if it can produce non-lethal, heritable phenotypic variation through mutations. More broadly defined, evolvability is the ability to produce phenotypic diversity, novel solutions to the problems

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organisms face and evolutionary innovations. Evolvability in the narrow sense is a prerequisite for evolvability in the broader sense.^(20,21)

The requirement that phenotypic variation is non-lethal hints at an intimate link between evolvability and mutational robustness. This link is increasingly evident through a combination of computational approaches and laboratory evolution studies.^(22–29) For instance, recent experiments assessed the evolvability of the enzyme cytochrome P450. In response to a given number of random mutations induced through error-prone PCR, the thermodynamically stable and mutationally robust variant of this enzyme evolved the ability to hydroxylate several new substrates more readily.⁽²²⁾ Another example involves two ribozymes, the class III self-ligating ribozyme and the Hepatitis D virus antigenomic ribozyme.⁽²⁴⁾ These ribozymes are very different in sequence, structure and their biochemical activity (ligation versus cleavage). One of them is a product of laboratory design, the other is of biotic origin. Despite these differences, a laboratory experiment succeeded at transforming one into the other by following a mutational path that involved some 40 point mutations.⁽²⁴⁾ Importantly, along about half of this mutational path, the catalytic function of the evolving molecule did not change dramatically relative to the starting sequence, indicating that this function is robust to some extent. Halfway on this mutational path, however, a series of only four nucleotide changes allowed the molecule to adopt the activity of the target enzyme. While traversing this narrow region, one enzyme's catalytic activity was transformed into that of the other.

While molecular traits are perhaps easiest to explore in evolution experiments, the robustness of more complex, morphological traits has been subject to similar analyses. Robustness of morphological and developmental traits is called canalization.⁽³⁰⁾ Historically, such traits were the first whose robustness received serious scrutiny, preceding the current literature about robustness in molecules and networks by decades. The most-important line of pertinent work, beginning in the late 1940s with experiments by Schmalhausen and Waddington, focuses on morphological traits that normally show very little variation in wild populations.^(23,31–34) Through specific mutations, drug treatment or environmental stress, such variation can be induced in a population. This variation is heritable, as can be shown in artificial selection experiments: over multiple generations in which organisms with a variant trait reproduce preferentially, phenotypic change accumulates in the population. The variation exposed in such traits thus reflects genetic variation, caused by past mutations, but variation to which the organism is normally robust. Only in the special conditions of the experiment does that variation become expressed and available to natural selection. Several researchers, most notably Wilkins⁽¹³⁾ suggested that gene duplications may be one of several causes of canalization.^(11,13,35–37)

In sum, multiple lines of experimental evidence suggest that robustness facilitates a system's ability to produce new variation and evolutionary innovation. The principle at work is simple.⁽³⁸⁾ It can be understood in terms of the genotype space that evolving populations explore. Think of a population as a cloud of points in a vast space of genotypes. Mutations drive the spreading of the cloud through this space, whereas selection confines the cloud to regions with favored phenotypes. Populations of robust individuals explore this space especially rapidly. The reason is that fewer mutations have deleterious effects with a disfavored phenotype if robustness is high. Ultimately, this leads to a greater diversity of genotypes in populations of robust individuals. This greater diversity, although itself not phenotypically visible, may facilitate the emergence of new phenotypes. For example, mutations in a genotypically more diverse population produce more diverse phenotypes.^(38,39) Also, as the literature on canalization shows, in the right environment such genotypic diversity may become phenotypically visible and subject to selection.

Gene duplications facilitate evolutionary innovation

Gene duplications cause robustness. Robustness, in turn, facilitates evolvability. Syllogistic necessity would dictate, then, that gene duplications facilitate evolvability. This last assertion is not new. Some 40 years ago, Ohno⁽⁴⁰⁾ already made it for gene and genome duplications. According to him, duplications are key to evolutionary innovation. What is new, however, is that now can we understand this role of gene duplications as a special case of a more general principle, namely that robustness facilitates evolvability. This principle manifests itself in the laboratory experiments discussed above, experiments that successfully evolved phenotypes ranging from new enzyme functions to morphological characters.

Laboratory experiments can demonstrate that gene duplications cause robustness, for example through systematic gene deletions. Laboratory evolution experiments can also demonstrate that robustness facilitates evolutionary innovations. However, laboratory experiments have one key weakness. They can only study evolutionary innovations that arise on modest laboratory time scales, such as modifications of existing enzymatic functions. To what extent does robustness promote evolutionary innovations on larger, geological time scales? Comparative studies, although they do not provide the conclusive proof of the laboratory, can address this question. They can examine spectacular evolutionary innovations that arose on time scales of hundred millions of years. I will now briefly review several such innovations and their association with gene duplications. They help us extrapolate from the laboratory to larger time scales. They give us a glimpse of how powerful robustness as an enabler of evolvability might be, when acting over hundreds of millions of years.

Flowering plant evolution

Flowering plants (angiosperms) are the most-diverse and evolutionary successful group of land plants. Their approximately 250,000 species outnumber those of all other plant taxa. Since their great radiation some 100 million years ago, flowering plants have come to dominate terrestrial ecosystems. Many of their key evolutionary innovations relate to reproductive functions. Among them are closed carpels that shield the female germ cells and prevent self-fertilization, the endosperm, a triploid tissue that nourishes a seedling, and, most visibly, flowers themselves. The prototypical angiosperm flower consists of four different floral organs—sepals, petals, stamens and carpels—that arise sequentially from a floral meristem. A myriad variations exist on the number, arrangement and synorganization of these four organs. Together, they account for the most visible aspects of angiosperm diversity.⁽⁴¹⁾

The key to understanding angiosperm diversity lies in understanding angiosperm development, in particular the development of the flower. The identity of floral organs is specified combinatorially by a network of transcription factors that are expressed in the developing flower. The earliest and simplest incarnation of this insight is the so-called ABC model of flower development, established first in *Arabidopsis thaliana* and *Antirrhinum majus*.⁽⁴²⁾ According to this model, the combined action of three classes of transcription factors called A, B and C are necessary to specify floral organ identity. A class A transcription factor expressed by itself specifies sepals; A and B are jointly necessary to specify petals; B and C jointly specify stamens; C alone specifies carpels. Accumulating evidence required some model modifications,⁽⁴³⁾ but the model's central notion, combinatorial specification of organ identity, is well corroborated.

Most of the well-studied transcription factors involved in flower organ specification are MADS box proteins. MADS box proteins are ubiquitous in eukaryotes. Flowering plants have experienced a wave of duplication in these genes^(44–48) (Fig. 1). Yeast, nematode and fruit fly genomes contain only between one and four MADS box genes;^(48,49) the most-recent common ancestor of gymnosperms and angiosperms may have had as few as 7 MADS box genes.^(44,50) In contrast, the two completely sequenced genomes of the angiosperms *Arabidopsis thaliana* and rice each contain more than 70 MADS box genes.^(46,47)

Some duplicate MADS box genes have preserved identical functions since their duplication, which underscores the notion that duplication causes robustness. Examples include the *SEPALLATA* genes, of which *Arabidopsis* contains several duplicates (*SEP1–4*). These genes are jointly responsible for converting leaf-like structures into petals, stamens and carpels.^(51–53) Loss-of-functions of individual members of this family, however, yield no strong phenotypic effects, indicating their redundancy and robustness to such mutations.^(51,53)

Another example is the *CAULIFLOWER* (*CAL*) gene. A loss of function in this gene has no phenotype. However, in combination with mutations in its closely related duplicate *APETALA1* (*AP1*) gene, mutations in *CAL* give a characteristic cauliflower-like phenotype.⁽⁵⁴⁾

In addition to such redundancy, many duplicate MADS box gene functions have also diversified within and among species.^(55,56) This is expected if robustness caused by gene duplications provides the substrate for morphological evolution. Examples involve again the *SEP* genes. While redundant in *Arabidopsis*, *SEP* homologs have adopted different functions in other plants. A case in point is a tomato *SEP* homolog that is involved in fruit ripening but not in floral organ identity.⁽⁵⁷⁾ The *SEP* gene family has experienced further expansion in the monocotyledons. Based on divergent expression patterns in different grasses, it has been suggested that *SEP*-like genes may have influenced the morphological diversification of grass inflorescences.⁽⁵⁸⁾

Another example involves the *AGAMOUS* (*AG*) gene family, whose name derives from *AGAMOUS*, a class C gene involved in carpel and stamen formation. This gene has experienced a duplication in the lineage leading to the eudicotyledons, creating two duplicate gene lineages.^(56,59) In *Antirrhinum*, ectopic expression of the *AG* family member *PLENA* transforms sepals into carpels, but ectopic expression

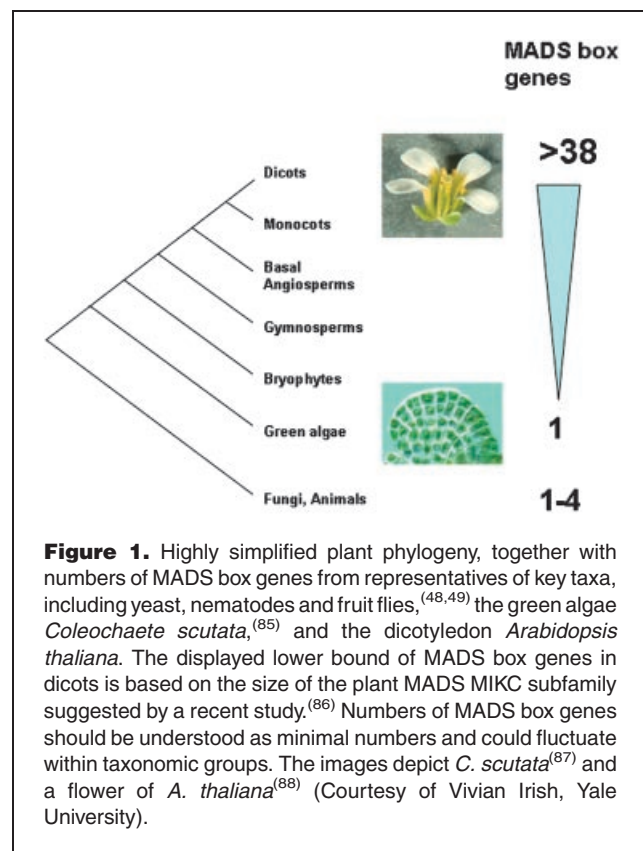


Figure 1. Highly simplified plant phylogeny, together with numbers of MADS box genes from representatives of key taxa, including yeast, nematodes and fruit flies,^(48,49) the green alga *Coleochaete scutata*,⁽⁸⁵⁾ and the dicotyledon *Arabidopsis thaliana*. The displayed lower bound of MADS box genes in dicots is based on the size of the plant MADS MIKC subfamily suggested by a recent study.⁽⁸⁶⁾ Numbers of MADS box genes should be understood as minimal numbers and could fluctuate within taxonomic groups. The images depict *C. scutata*⁽⁸⁷⁾ and a flower of *A. thaliana*⁽⁸⁸⁾ (Courtesy of Vivian Irish, Yale University).

of its paralog *FARINELLI* (*FAR*) does not.⁽⁶⁰⁾ The different loss-of-function phenotypes in the two genes show that they have adopted different functions.⁽⁶⁰⁾ Conversely, *Arabidopsis* contains two paralogs of *AG*, the *SHATTERPROOF* genes *SHP1* and *SHP2*, which have adopted new functions in fruit ripening.⁽⁶¹⁾

Taken together, examples like these suggest that the robustness originally caused by a duplication has facilitated evolutionary diversification on the molecular level. Such diversification is a prerequisite for morphological evolution.

Vertebrate diversification

No evolutionary account of gene duplications would be complete without Hox genes. Hox genes show a tightly linked (clustered) organization in many organisms. Their spatiotemporal expression pattern along the head–tail axis is colinear with their chromosomal order in a cluster. Hox genes are involved in the patterning of many structures along the head–tail body axis, including the hindbrain, the vertebral column and the limbs.⁽⁶²⁾ Many invertebrates have a single tightly linked cluster of Hox genes that underwent at least two duplications during vertebrate evolution. This means that many vertebrates have four Hox gene clusters labeled a–d. The cluster of the most recent common vertebrate ancestor likely had 14 Hox genes, of which 13 are left in vertebrates. The genes in the four vertebrate clusters are thus subdivided into 13 paralogous groups labeled 1 through 13.⁽⁶³⁾

Vertebrates are characterized by numerous innovations relative to their chordate ancestors.⁽⁶⁴⁾ These include a more-elaborate brain with three specialized regions (forebrain, midbrain and hindbrain), cartilage and mineralized structures—bone and teeth—that serve many roles from support to feeding. The evolution of bone in turn gave rise to the most obvious and striking vertebrate innovations. These include a differentiated vertebral column, hinged jaws and paired appendages. The latter permit many different forms of locomotion, including walking, swimming and flying, which made many ecological niches accessible when they first arose. Various duplicate Hox genes are critical for the proper embryonic development of these traits, suggesting important roles for Hox genes in morphological evolution.

Again, despite their duplication hundreds of million years ago, many Hox gene duplicates have retained partially redundant functions, remnants of the robustness that gene duplications cause. For example, zebrafish *Hoxa2* and *Hoxb2* function redundantly in embryonic patterning of the second pharyngeal arch,⁽⁶⁵⁾ and the mouse *Hox8* genes have redundant roles in positioning of the hindlimbs.⁽⁶⁶⁾ While some aspects of Hox gene function are conserved, others have diverged. Here, a recurring theme is functional divergence through diverging gene expression rather than diverging biochemical function.^(67–70) A case in point are the duplicate Hox genes *Hoxa3* and *Hoxd3*,⁽⁶⁷⁾ The developmental defects

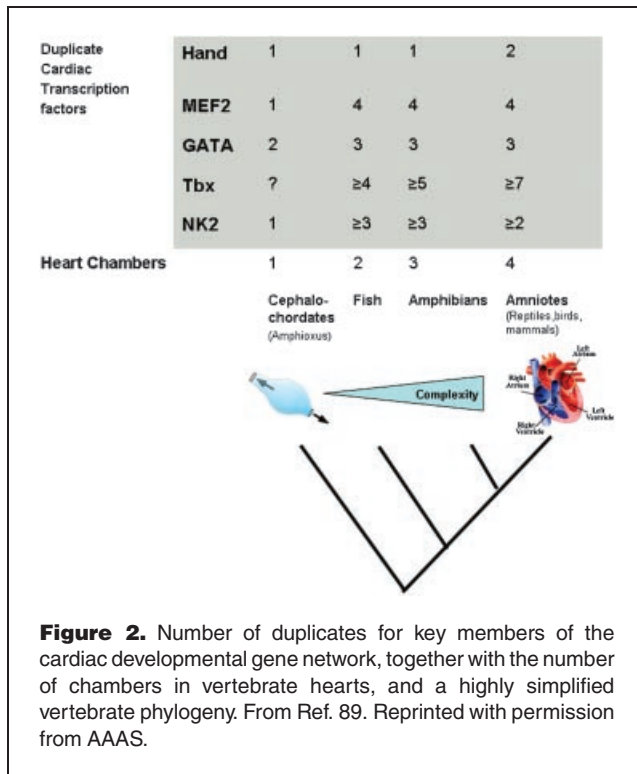
found in loss-of-function mutations of either gene are very different. *Hoxa3* mutants are defective in pharyngeal tissues, whereas *Hoxd3* mutants show malformed cervical vertebrae.^(71,72) Gene swapping experiments suggest that the two genes can carry out the same biological functions. Quantitative expression changes may thus be responsible for their observed differences.⁽⁶⁷⁾ Similarly, for the duplicate mouse *Hoxa1* and *Hoxb1* genes, the gene product of either gene, when expressed in the right way, can drive the developmental program mediated by the other paralog.⁽⁷⁰⁾ The genes have adopted different functions in hindbrain development, mediated partly by spatiotemporal expression differences.

In sum, Hox genes have been duplicated early in the vertebrate radiation. The remnants of the resulting mutational robustness are still visible. The vertebrate radiation has produced myriad innovations and great morphological diversity. Because Hox genes play critical roles in the development of the very traits involved in this radiation, it would be surprising if their diversification had played no role in the vertebrate radiation.

Heart evolution

Gene duplications have been associated not only with spectacular evolutionary radiations, but also with evolutionary innovations in individual traits. One of them is the heart. In organisms too large for diffusion to distribute nutrients and oxygen, a pump driving fluid circulation through the body becomes necessary. The prototypical invertebrate heart and that of ancestral chordates is a simple contractile tube with bidirectional blood flow. In contrast, the amniote (reptile, bird and mammalian) heart is a sophisticated four-chambered pump with two atria and two ventricles that separate oxygen-poor from oxygen-rich blood. During the evolution of vertebrates, the heart grew increasingly complex. Fish hearts have a single atrium and a single ventricle, whereas amphibian hearts have two atria and one ventricle. Additional vertebrate innovations include septae to separate the heart's chambers, valves to enforce unidirectional flow, as well as a conduction system for synchronized and powerful pumping.⁽⁷³⁾

Heart development in vertebrates and invertebrates is controlled by a core network of transcription factor genes, including *NK2*, *MEF2*, *GATA*, *Tbx* and *Hand* (reviewed in Refs. 74,75). Like many other genes, these genes have more duplicates in vertebrates than in their chordate ancestor⁽⁷⁴⁾ (Fig. 2). One of these genes, *MEF2* (myocyte enhancer factor 2), is involved in the expression of contractile muscle proteins. The fruit fly *Drosophila* has only one *MEF2* gene. Loss of its expression eliminates expression of contractile proteins in muscle cells.^(76,77) In vertebrates, there are four *MEF2* duplicates showing partially redundancy,⁽⁷⁸⁾ a remnant of the robustness caused by their ancient duplication. Loss of function of *MEF2c*, one of these duplicates, eliminates a subset of contractile proteins in the heart, and also abolishes



formation of the right ventricle.⁽⁷⁹⁾ The population of cells from which the right ventricle is formed is specific to amniotes. The MEF2c function in it is thus probably a new acquisition. This example illustrates again the theme that single developmental regulators have broad functions, but their paralogs after duplication may adopt more specialized, restricted and yet novel functions. Perhaps the most-striking example of this principle is the *Hand* (heart and neural crest derivatives expressed transcript) gene. Zebrafish and amphibians, both of which have only one ventricle, express a single copy of this gene. The zebrafish *Hand* gene is necessary for ventricle formation.⁽⁸⁰⁾ Mice express two duplicates of *Hand*. Among other defects, loss-of-function mutants in *Hand1* are defective in left ventricle formation, whereas loss-of-function mutants in *Hand2* fail to form the right ventricle.^(81–84) The functions of the two duplicates have become partitioned such that each is associated with formation of a morphological partition of an organ.

Conclusions

The above examples suggest that gene duplications lead to robustness and that this robustness allows subsequent molecular and morphological diversification. However, like almost every generalization in biology, this one comes with caveats. First, it simplifies, and perhaps excessively so. Can there be too much robustness, an excess of robustness that impedes innovation, for example caused by excessive gene duplication? What causes of robustness other than gene

duplications have been important for evolutionary innovations in the history of life? Are the driving forces of gene duplication and preservation linked to or independent from the evolutionary innovations they might cause? We do not have answers to these questions, but they provide fertile ground for further investigation.

Second, any observation based on comparative studies (as opposed to laboratory experiments) cannot easily distinguish between correlation and causation. This is the price to pay if we want to see how observations from laboratory evolution experiments extrapolate onto geological time scales. In fact, gene duplications are probably not *causal* to the radiations I discussed: the robustness they cause may be necessary, but it is certainly not sufficient. To see why, consider that complex evolutionary change may require both natural selection and variation. Variation without natural selection leads nowhere. The nematode vulva discussed earlier may be a case in point. We know that the vulva development network is robust to genetic change, because it has changed substantially in the last 200 million years. However, in this vast amount of time, the vulva itself has changed little. This is an example where robustness exists – although not necessarily caused by gene duplications—where robustness allows genetic variation to occur and a network to change, but where the key impetus for morphological evolution—natural selection—may be missing. A similar case could be made for large-scale gene duplication events that are not associated with dramatic evolutionary radiations. Examples include the teleost-specific genome duplications.

In sum, variation without selection does not lead to innovation, but the same holds for selection without the right kind of variation. One could view gene duplications as just one of many sources of genetic variation. However, gene duplications are unique and different from the many point mutations, deletions and rearrangements that genomes are bombarded with. Only they increase robustness, which facilitates the production of evolutionary innovations. The flowering plant radiation, vertebrate evolution and complexification of hearts indicate how powerful the principle of robustness as a facilitator of evolvability might be.

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