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Experimental evolution and proximate mechanisms in biology

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ABSTRACT

Biological functions – studied by molecular, systems and behavioral biology – are referred to as proximate mechanisms. Why and how they have emerged from the course of evolution are referred to as ultimate mechanisms. Despite the conceptual and technical schism between the disciplines that focus on each, studies from one side can benefit the other. Experimental evolution is an emerging field at the crossroads of functional and evolutionary biology. Herein microorganisms and mammalian cell lines evolve in well-controlled laboratory environments over multiple generations. Phenotypic changes arising from the process are then characterized in genetics and function to understand the evolutionary process. While providing empirical tests to evolutionary questions, such studies also offer opportunities of new insights into proximate mechanisms. Experimental evolution optimizes biological systems by means of adaptation; the adapted systems with their mutations present unique perturbed states of the systems that generate new and often unexpected output/performance. Hence, learning about these states not only adds to but also might deepen knowledge on the proximate processes. To demonstrate this point, five examples in experimental evolution are introduced, and their relevance to functional biology explicated. In some examples, from evolution experiments, updates were made to known proximate processes – gene regulation and cell polarization. In some examples, new contexts were found for known proximate processes – cell division and drug resistance of cancer. In one example, a new cellular mechanism was discovered. These cases identify ways the approach of experimental evolution can be used to ask questions in functional biology.

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1. Introduction

As pointed out by ethologist Nikolaas Tinbergen and evolutionary biologist Ernst Mayr, there exist two distinct mechanisms of

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life: proximate and ultimate [1,2]. Proximate mechanisms refer to what organisms are, studied by molecular, systems and behavioral biology; and ultimate mechanisms refer to why organisms have come to be as they are, the subject in evolutionary biology. Fields on the two sides have been developing in parallel with little crosstalk. The question is why should functional biologists care about evolution?

The answer is simply because all biological phenomena with their underlying proximate mechanisms are products of evolution. These mechanisms, one way or another, are representations of the environments organisms have experienced in the long past. Knowing why and how the mechanisms have evolved in history might explain their characteristics presently observed.

Consider ribulose-1,5-bisphosphate carboxylase (RubisCo), the enzyme responsible for fixing atmospheric carbon dioxide into biomass [3]. It is notoriously inefficient in catalysis [4] and constitutes a limiting step in biomass increase on the Earth. This inefficiency is because oxygen competes with carbon dioxide in the active center of Rubisco for a side reaction, inhibiting carbon fixation. However, there exist other carboxylases in nature that fix carbon dioxide while insensitive to oxygen [5]. These differential sensitivities to oxygen are rooted in evolutionary history. RubisCo happened to originate in ancestors to modern photosynthetic organisms – cyanobacteria, algae and most plants – at a time when the atmospheric oxygen was extremely low. Hence, selective pressure against the interference of oxygen to carbon fixation was lacking. On the other hand, the oxygen-insensitive carboxylases, found primarily in bacteria, evolved independently from RubisCo and somehow did not make to the metabolic pathways of photosynthesis [6]. In other words, the oxygen limitation of biomass increase seen in modern photosynthetic organisms might not be due to laws of physics and chemistry but simply an unfortunate accident in history [7].

This example of ancient adaptation requires information about geological record of the Earth and about phylogeny and biochemical properties of RubisCo. The demanding scope of knowledge and the reliance on evolutionary record in this approach [8,9] hinder its application in broad contexts. Indeed, an easier bridge between proximate and ultimate mechanisms is provided by an emerging field, namely experimental evolution. It takes advantage of short generation time of microbes (dozens of minutes) or mammalian cell lines (hours). Organisms are subject to reproduction through hundreds to thousands of generations – the age of *Homo sapiens* thus far is ten thousand generations [10] – under well-defined selective pressure in laboratory environment. Evolved populations and individual organisms are then characterized at both genotypic and phenotypic level. In a nutshell, experimental evolution optimizes a biological system by means of adaptation. The evolutionary dynamics can be precisely resurrected from characterizing organisms archived/frozen across all stages of evolution. This analysis reveals how the system is perturbed by a sequence of mutations to assume functional changes that increase fitness.

Experimental evolution has been extensively reviewed elsewhere [11–17]. In this review, five examples will be discussed in-depth to demonstrate how experimental evolution can be utilized to do unique service to functional biology. In two examples, experimental evolution of model systems for gene regulation and cell polarization uncovered unexpected properties of the systems. In another two examples, the classic topics in cell biology – cell division and drug resistance of cancer – found their connection to multicellularity, an unsolved problem in evolution. In the last, evolving organisms in a cyclic environment in laboratory revealed that an existing gene network can be reprogrammed by a single amino acid substitution to generate a new behavior.

2. New insights into known proximate mechanisms

2.1. Reversing mode of regulation in gene expression

A classic model of regulation in gene expression, the *lac* operon is arguably the most understood molecular system with several decades of research [18]. To express the structural genes for the metabolism of lactose, lactose binds to a transcription factor lacI that has been sitting on the promotor region of the operon DNA to repress transcription of the structural genes. This binding changes conformation of the transcription factor, which in turn falls off the promoter DNA, and expression of the operon ensues. This paradigm of inducer-repressor received a surprising update recently. Poelwijk et al. discovered that as few as three amino acid substitutions at lacI were sufficient to convert the inducer molecule (lactose) into co-repressor [19]. That is, the binding of lactose to the mutant lacI facilitates repression of the structural genes, the opposite of what it does to wildtype lacI.

This discovery was made through experimental evolution. A synthetic operon was made where lacI was used to regulate expression of two proteins, one conferring resistance to the antibiotic chloramphenicol; and the other, sensitivity to sucrose. Hence, expression of the operon was beneficial to the host bacterial cell in the presence of chloramphenicol but deleterious in the presence of sucrose. A library of lacI mutants were introduced using error-prone PCR. A population of cells each carrying a different lacI mutant were competed in a cyclic environment. In this environment chloramphenicol and sucrose alternated their presence, and inducer was added with either chloramphenicol or sucrose (Fig. 1a). When inducer was added with chloramphenicol, competition confirmed that wildtype took on optimal fitness. When inducer was added with sucrose, however, wildtype lacI was selected against, and the mutants that converted inducer into co-repressor swept the population after multiple cycles of environmental shift.

This work exposes functional flexibility at the level of a single macromolecule: It takes only a few mutations at a regulatory protein to turn an inducible system into a repressible one. A few distinct genotypes were identified with the reversed mode of regulation but shared a common mutation that substituted serine 97 at the dimer interface with proline (red residue in Fig. 1b), which is known to devastate the allosteric transition, i.e., the inducer-caused conformational change needed for the function of wildtype lacI [20]. Then the allostery required of the reversed regulation has to be mediated through alternative sets of amino acid residues (Fig. 1b), a phenomenon not obvious from existing knowledge. In the future, crystal structures of these unique mutant proteins combined with their molecular dynamics simulations will bear great hope in elucidating the mechanistic basis of allostery, a fundamental problem in biochemistry [21].

2.2. Recovery of damaged cell polarization

Cell polarization defines a spatial axis of the cell that is essential for division, migration, differentiation, etc. [22,23] Its molecular mechanism has been elucidated, and the underlying gene network empirically mapped out in budding yeast [22]. At the center is GTPase Cdc42, a master regulator that concentrates at a specific spot in the cell membrane where a new bud starts to form. Its functioning is regulated dynamically by an ensemble of mechanisms to ensure bud formation with precise space and time (Fig. 2a). This process is an example of self-organization at the molecular level. The question is how a single site of concentrated Cdc42 is specified on the 2D surface of cell membrane in the face of diffusion (green arrows, Fig. 2a). Many mathematical models have been built to account for this localization event as an emergent

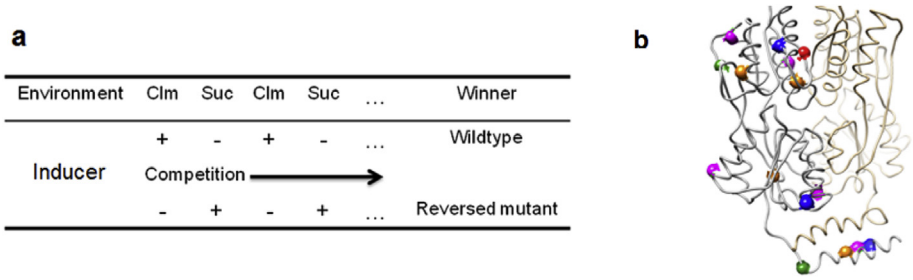

Fig. 1. Reversion in the mode of regulation in gene expression through experimental evolution. **a**. Different experimental regimes select for different phenotypes. Clm, chloramphenicol; Suc, sucrose. **b**. Structure of lacI dimer is shown. The mutation at the critical residue (red) works together with alternative sets of additional mutations (residues differentially colored; one color indicating one set) to reverse the mode of regulation.

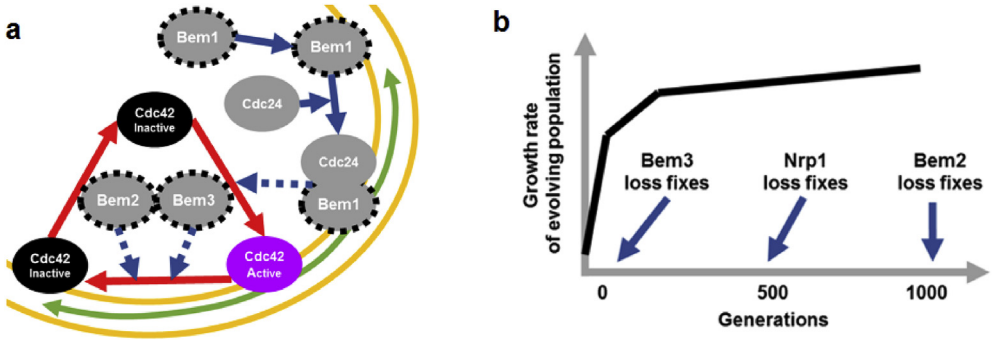

Fig. 2. The gene network of cell polarization is evolutionarily robust to dramatic genetic perturbation. **a**. A core pathway for cellular polarization with dynamic regulation of active Cdc42 (magenta oval). Cell membrane is depicted as yellow curves. Ovals with dashed outline are those whose loss restores normal polarization. **b**. Evolutionary dynamics of restoring growth rate from Bem1 deletion. The arrows mark time points when various adaptive mutations became fixed in the population.

property of underlying biochemical interactions [24–27]. While details are still debated [22,28,29], a core mechanism has reached consensus — a positive feedback where Cdc42 is continuously recycled between inactive and active forms and between being cytoplasmic and membrane-bound (red arrows). Shown in Fig. 2a, two steps in the feedback are under heavy regulation. A scaffold protein Bem1 is first embedded into cell membrane and holds Cdc24 and inactive Cdc42 (black oval) together so that the former activates the latter by replacing its associated guanosine diphosphate with guanosine triphosphate. Meanwhile, Bem2 and Bem3 both promote hydrolysis of this guanosine triphosphate, inactivating Cdc42. Modelled in the framework of Turing's diffusion-reaction equations, this feedback quantitatively recapitulates Cdc42 localization [29].

This positive feedback with its sophisticated regulation is considered essential for the molecular dynamics required of proper bud formation [30]. However, a new result from experimental evolution overthrows this belief [31]. Laan et al. deleted the gene for Bem1, which destroyed normal bud formation and severely reduced growth grate, but not killing the cell. Using this sick genotype as ancestor, they passaged populations of cells for one thousand generations. Shown in Fig. 2b, the retarded growth rate picked up rapidly within the first twenty generations and kept increasing later though with slowed pace. Sequencing whole genome of the populations across all evolutionary stages revealed that the initial pickup of growth rate was caused by mutations that knocked out Bem3 and that the further optimization was caused by functional loss of Bem2. The loss of Nrp1 — a protein with no obvious connection to cell polarization based on current knowledge — also helped; and, puzzlingly, the benefit of losing Bem2 relied on losing Nrp1first. Characterization of the mutants showed that as growth rate recovered with addition of the mutations, bud formation went back to normal.

This result begs rethinking of mechanisms behind cell polarization. It first provides a test of robustness for the current models. While those that require Bem1 automatically fail the test (e.g. [29]), others (e.g. [30]) can be further examined quantitatively to see if zeroing Bem1 and Bem3 restore polarization partially as seen in the experiment. Indeed, most models take Bem1 as a necessary component. This raises the possibility that cell polarization studied in the chassis organism, budding yeast, is only an evolutionary anecdote. If yes, can we still build a general model to understand the fundamentals of cell polarization? Laan et al. provide an approach to address this question: Systematic genetic perturbations followed by evolutionary recovery will eliminate non-essential details and narrow down to the basic elements, with which a general model of cell polarization can be built.

Note that these elements are not necessarily genetic or molecular entities and can be relationships. For instance, in Fig. 2a, both Bem1 and Bem3 are regulators of the positive feedback that recycles Cdc42 between active and inactive forms. From a dynamical system's point, it is not surprising that removing both regulators generates a pattern of Cdc42 localization similar to that of the original system with both regulators intact as long as the feedback still exists. Therefore, the basic element here is not the regulator proteins but the feedback loop.

3. Finding new biological contexts for known proximate mechanisms

3.1. Cell division and multicellularity

Origin of multicellularity is a major problem unsolved in biology due to its ancient occurrence that has left few traces for

investigation. Study on this topic has been constrained to inference of the evolutionary history until a recent work that captured in laboratory an event of de novo evolution of a primitive form of multicellular organisms from unicells [32]. Ratcliff et al. reasoned that an environment that favors increase in organismic size might select for multicellularity and implemented this idea simply by utilizing gravity. Culture of budding yeasts was let sit still for some time so that bigger and heavier cell clumps would quickly fall to the bottom, and then the cells at bottom of the tube were sampled to inoculate a new culture while the rest discarded. When the new culture grew to high density, the same procedure was executed again. After repeating this cycle 60 times, the population was fixed with multicellular yeasts. During cell division, mother and daughter cells did not separate; so that one individual clump was formed with multiple cells sticking to each other, appearing snowflake-shaped. The real surprise came when the authors found that, after several rounds of mitosis, an old cell at center of the snowflake underwent apoptosis where the clump fractured into multiple smaller clumps, effectively reproducing (Fig. 3a).

Genome sequencing revealed that the clumping was caused by a mutation that knocked out the function of a transcription factor, Ace2, which normally activates the machinery for septum destruction after cytokinesis [33]. This initial adaptation of multicellularity set stage for later mutations that increased apoptosis in aged cells to generate smaller clumps for faster growth [34].

One important message of this work is the demonstration that a single mutation with simple molecular mechanisms can reorganize organisms to shift the level of individuality from unicell to multicell. Once this initial transition occurred, a set of adaptive changes surfaced – e.g., larger and more hydrodynamic clumps – with which the clumps became the unit that responded to selection. Ace2 is a well-studied molecule in cell biology. Here, experimental evolution uncovers the paramount importance of this master regulator of cytokinesis to the evolution of multicellularity.

Another interesting observation is that when the same selection experiment was repeated with another yeast species, multicellular individuals did evolve but, instead of taking over the population, coexisted stably with unicells [35]. Echoing this contingency at the species level, independent selection experiments with the original species repeatedly discovered the loss of Ace2 function as a gateway to multicellularity, only after which were mutations at diverse gene targets selected to further optimize multicellular traits [34,36]. In the future, one exciting place to seek explanation for this evolutionary divergence and convergence lies in the architecture of protein-protein interaction network (Fig. 3b).

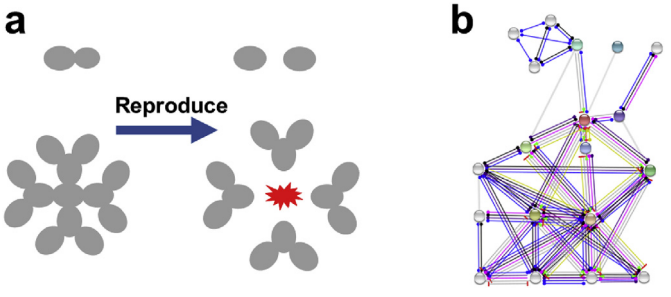

Fig. 3. Evolution of multicellularity and the architecture of the protein-protein interaction network for a critical gene. **a**. Modes of reproduction for unicellular (upper) and multicellular (lower) yeasts. The red symbol indicates the apoptotic cell. **b**. The protein-protein interaction network of Ace2. Nodes represent proteins and edges interactions. Ace2 is shown red. The network was retrieved from STRING-DB.org [37].

3.2. Drug resistance of cancer and de-evolution of multicellularity

Experimental evolution has been used to study cancer for decades. This field saw a rejuvenation with recent advances in sequencing and microfluidic technologies. Wu et al. built a microfluidic device to see how Multiple Myeloma cancer cells evolve resistance to the drug doxorubicin [38]. They found that resistance did not occur in environments with uniform concentration of the drug; but increased 16-fold within five generations in environments with a drug gradient. This observation adds evidence to the importance of spatial structure in environments to drug resistance. They sequenced the transcriptome from the population of resistant cells. Mutations occurred in many functional groups – cell cycle, apoptosis, protein folding, etc. – confirming the established mechanisms of drug resistance in cancer.

Novel insight arrived when the authors put the mutations in an evolutionary context. They examined the age for the genes with changed amino acids and those with dramatic shifts in expression level. It turned out the adaptive mutations in the cancer cells were biased towards old genes that had prokaryotic origins, compared to the average genes in human genome. This discovery lends a rare empirical support to an intriguing but highly controversial hypothesis that cancer is the relapse of multicellular life to the ancestral unicellular form, where group-level cooperation succumbs to cheating, and fitness of individual cells is foremost [39,40]. It will be of great interest to see if the same pattern comes out evolving other types of cancer towards a variety of drugs.

4. Discovering new proximate mechanisms

4.1. A new behavior as an adaptation to cyclic environments

Loss of function at the molecular level has been a major mode of gaining organismic fitness in experimental evolution [41,42], as also seen in two examples discussed so far [31,32]. This observation indicates the relative difficulty in evolving novel molecular cellular functions. Yi and Dean [43] provided an example of the opposite. To study adaptation to complex environments where fitness has more components than just growth rate, the authors designed a cyclic selective regime with two phases. Initially, bacterial cells were grown in batch culture until food was depleted and growth rate reduced; then a glass capillary carrying fresh food was lowered into the culture to attract the starving cells, which would respond by chemotaxis (the ability of cells swim up a gradient of attractant chemicals). The cells that moved into the capillary were then used to inoculate the next fresh batch culture. This environment selects for fast growth and strong chemotaxis. However, both functions are known to be costly. Given finite output of energy, there might be a trade-off between the two. In the first five hundred generations, growth rate slowed while chemotaxis improved, confirming the anticipated trade-off. The evolving population then stayed stagnant close to the predicted fitness optimum for two hundred generations. Until mutant cells with fast growth and strong chemotaxis, seemingly breaking the trade-off previously established, swept the population.

Functional characterization of the ancestral and evolved strains uncovered two distinct adaptive strategies (Fig. 4a). In the early stage, cells developed hypermotility throughout the selective phases, conferring advantage for capillary competition but also cost in the growth phase when motility was not needed. Hence, a trade-off between growth and chemotaxis was seen. Later, the “breakthrough” came with a new behavior: In comparison to the ancestor, the cells suppressed motility in the growth phase and cranked up later when the typical timing of capillary competition was approaching. This way, the cells did the right things at the right

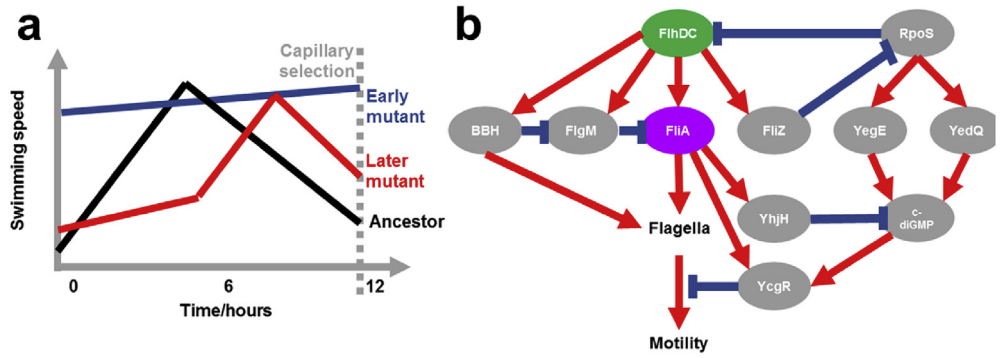

Fig. 4. Evolution of the chemotaxis/motility system. **a**. A cyclic environment selects for two adaptive strategies (blue and red). Cells were grown in batch culture in the first 11.5 h until capillary was used to select for chemotaxis (dashed line). **b**. The gene network of motility. Red and blue edges indicate activation and inhibition, respectively. Magenta oval denotes the node mutated to adapt to the cyclic environment as in **a**; green, to the temporally uniform environment as in Ref. [46].

times, not wasting energy as did their predecessors. Surprisingly, this new behavior was sufficiently enabled by a single amino acid substitution at, yet again, a transcription factor, responsible for chemotaxis and motility. This time, however, the mutation was subtler than functional knockout. It weakened DNA binding of the transcription factor and led to less expression of the flagellar proteins in the growth phase, reducing motility. It also increased the fraction of motile cells (there was always a subpopulation that was nonmotile), boosting overall motility after the growth phase. How exactly the latter was achieved at the cellular level remains unknown.

This work elucidates how a new behavior can emerge from reprogramming an existing gene network by tweaking a hub node with mutations. While studies of gene network have focused on its robustness [44,45], this work shows an empirical example of evolvability — the flexibility to assume changed dynamic outputs by small genetic modifications. In a similar work [46], growth and chemotaxis were also both selected — albeit simultaneously without temporal separation. Adaptation arose in the regulatory hierarchy at a level higher than that of the gene mutated here (Fig. 4b). Together, these studies present unique perturbed states of the system on which further study might generate intuition on how the network works.

5. Conclusion

Five examples are discussed in this review to illustrate how ultimate processes implemented in laboratory (selections for given functions) can inform proximate mechanisms in ways otherwise impossible. In each case, selection experiments successfully discovered mutant organisms that adapted to some unfamiliar environment; and follow-up genetic/genomic and functional characterizations uncovered the underlying proximate mechanisms. These exposed mechanisms would *elude* conventional approaches in functional biology. Take the cell polarization case for example, yeast geneticists have long known the deleterious effect of inactivating Bem1 *or* Bem3 based on classic single-gene knockout experiments. However, they never expect the double knockout to recover cell polarization because 1) this recovery is not obvious at all from molecular biology and 2) systematic characterization of double knockouts is impractical due to the enormous number of gene combinations. In experimental evolution, however, the double knockout mutant rapidly surfaced from the fierce competition among dozens of millions of individuals. These individuals constantly explore the combinatorial space of mutations through the natural process of reproduction. In consequence, interesting mutants, even complex ones with multiple mutations, are easily discovered as long as they excel at the criteria of selection. These unique mutants then provide promise of new insights to the proximate mechanisms, highlighting the power of experimental evolution.

There has been a realization in functional biology in replacing the gene-centric view with the emphasis on modules in order to deepen our understanding of biological functions [47]. A module is a group of components (gene products and their interactions) that work together to fulfill certain function. The gene networks for cell polarization (Fig. 2a) and chemotaxis (Fig. 4b) are examples of modules. As life itself, modules are products of adaptation and genetic drift in complex and variable environments over the long past. They not only need to fulfill the proximate functions at this moment; but also, are to keep the possibility of accommodating changes required of potential environmental shifts. Hence, both proximate and ultimate capabilities are intrinsic to the concept of module. In the past, the concept of module is discussed primarily in the context of proximate mechanisms [47]. The proximate-ultimate duality of modules identified here calls for refining existing modules. The works on cell polarization [31] and chemotaxis [43] are along this line. With evolutionary tinkering of the gene networks, non-essential details (e.g., Bem1 and Bem3 regulators of cell polarization) are discarded and critical nodes (e.g., FliA transcription factor) and relationships (e.g., the Cdc42 positive feedback in cell polarization) pinpointed in search for the basic elements of proximate-ultimate functionality. Such refining will have the merit of simplifying modules that are redundant and complex to reveal the functional backbone and/or identifying the source of robustness and evolvability. It is hard to imagine how such endeavor would become possible without the contribution of experimental evolution.

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