

Tales from the crypt(ic): neutral mutations can breathe life into adaptation

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Adaptation via natural selection requires inherited changes in an organism's *phenotype*. So how might neutral mutations (changes in genotype that do not affect phenotype) possibly affect the pace or diversity of outcomes of adaptation? The answer lies in the fact that how a genotype translates to a phenotype is inherently dependent upon context. The phenotypic consequence of a mutation may change due to interactions either with other mutations in the genome (known as epistasis), or with the physical environment (known as a genotype-by-environment, $G \times E$, interaction). Given the fact that it is impossible to test a mutation's phenotypic consequence in all possible genomic and environmental contexts, the best we can really say is that a mutation is neutral until proven otherwise. Such neutral mutations constitute so-called "cryptic" genetic variation. Until recently, there have been relatively few experimental tests of whether cryptic variation plays any role in the adaptation of populations, or how. On page XX of this issue, Zheng *et al.* (1) demonstrate that the accumulation of neutral mutations in a protein allowed for faster adaptation in an environment selecting for a novel function, and that this effect required the combined impact of epistasis and $G \times E$ interactions.

The impact of neutral mutations upon adaptation has often been framed from the point of view of the phenotype, rather than the genotype: an ancestral phenotype that remains unchanged in the face of genomic mutations is considered mutationally robust. However, whether mutational robustness spurs adaptation in the longer term or suppresses it has been debated. On one hand, by the very definition of mutational robustness, fewer mutations affect a robust phenotype, and thus cannot lead to adaptation. On the other hand, a broader cloud of genotypes can be tolerated in a mutationally robust phenotype, even under purifying selection in an initial environment; these mutations form a "neutral network" that may serve as a genetic resource should the genotype be confronted with a new environment to adapt to. Importantly, there may be mutations within that network that in the new environment are deleterious on their own but beneficial in combination (due to epistasis); being allowed to accumulate cryptically, shielded from $G \times E$ interactions in the original environment, can make those mutations accessible (Fig. 1). Theoretical analysis has revealed that mutational robustness may either speed or slow adaptation, depending on whether high-fitness mutations (or those generating novelty) are relatively rare across the neutral network, or are relatively common, respectively (2). A recent study with ribozymes suggests there can be extensive intersections of genotype networks that can promote such innovation (3).

The work by Zheng *et al.* provides a critical empirical test of these questions: they asked whether generating a broad pool of cryptic genetic variation in a protein can accelerate and diversify adaptation when that protein is asked to take on a new activity. This builds upon analogous work from the same laboratory using *in vitro* evolution of RNA molecules (4), but now examines protein function in live cells,

taking advantage of fluorescence-activated cell sorting (FACS) to select upon the *in vivo* activity of yellow fluorescent protein (YFP). To generate cryptic genetic variation, they mutagenized *yfp*, introduced the resulting pool into *Escherichia coli* cells, and then imposed purifying selection by sorting for the 20% of cells in each of four replicate populations with yellow fluorescence levels centered most closely upon the ancestral phenotype. After four rounds, they created a new selective environment by switching the FACS to select for a novel activity, green fluorescence (ancestral YFP is weakly fluorescent at that wavelength), carrying forward the top 0.1% of YFP variants with the highest green fluorescence for four rounds.

The first key finding was that the generation of cryptic variation in YFP prior to selection for green fluorescence increased the rate of adaptation compared to control populations initiated without prior generation of diversity. However, it is worth noting that the benefit of cryptic variation was most prominent in the first round after the transition to selection for green fluorescence. Over the next three rounds, the adaptation rate of each pool was roughly similar, although the overall difference remained after four rounds. This stands in contrast to results from the *in vitro* evolution of a ribozyme to use a novel substrate (4), in which the initial boost in adaptation continued to grow for five rounds in a row, and only then did the gap begin to close. While it will take many more examples before generalizations may be made, it is certainly clear that the quantitative effect of cryptic genetic variation is likely to be different across systems and selective pressures.

The second key finding was that the cryptic genetic diversity generated in the first environment permitted evolutionary trajectories that would not otherwise have been accessible. Zheng and colleagues made it possible to observe this by reconstructing all possible mutational intermediates building to the high green fluorescence *yfp* genes observed in the final populations. When green fluorescence was directly selected from the ancestral *yfp* without cryptic variation, the network of mutational intermediates almost exclusively featured steps that, in any order, would have been beneficial. This, and the fact that these populations all ended up with very similar genotypes, demonstrated a constraint on selection. In contrast, nearly all trajectories observed from the pool with cryptic variation featured steps that would have been deleterious in the environment selecting for green and therefore would not have survived without the initial generation of diversity. Furthermore, these four populations began with their own unique initial mutations that resulted in a wider variety of genotypes after four rounds of selection.

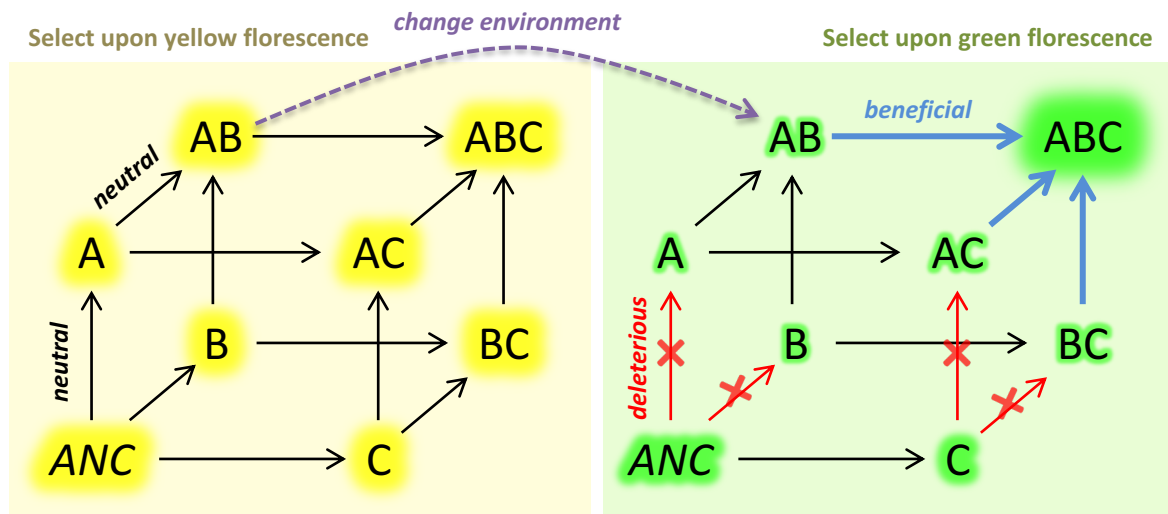
Cryptic variation matters most when the fitness landscape is very rugged, containing peaks consisting of unique combinations of mutations that work well together, but not with other beneficial mutations. While this "sign" epistasis (in which a mutation may be either beneficial or deleterious depending on the genetic background)(5) is observed commonly within single molecules, such as proteins (6), epistatic interactions between beneficial mutations in different genes have tended to only modify the magnitude of benefit, and generally show weaker interactions

(i.e., smoother fitness landscapes)(7-9). This suggests that the effect of initial cryptic variation may be more subtle at the level of cellular physiology.

Fluorescent proteins and ribozymes in laboratory conditions represent excellent model systems, but is there evidence that cryptic genetic variation has actually played a role in the evolution of novel traits during Earth's history? Remarkably, the answer appears to be yes. Through resurrection and biochemical analysis of reconstructed evolutionary intermediates for a family of hormone receptors, it has been possible to identify mutations that occurred in these proteins that did not change activity on their own, but were essential for the evolution of novel hormone binding properties >400 million years ago (10). A clever genetic screen even allowed researchers to attempt to "replay the tape" to reveal how many mutations could have occurred that would have set the stage for novelty to arise without disrupting the current function: they found only the one amino acid change that occurred historically (11). The rarity of useful cryptic variation in this case was likely due to the fact that this genetic screen was constrained to find mutations that interact well with the one particular set of "future" mutations known to have occurred historically, rather than simply opening the door to any set of other mutations to provide the desired novel phenotype.

By demonstrating the role of epistasis and the avoidance of G×E interactions through the change of selective conditions, the work of Zheng *et al.* significantly advances our understanding of how cryptic variation – phenotypes that are mutationally robust – can actually aid in adaptation. The authors suggest that future efforts to utilize directed evolution for practical purpose take these principles into effect, as is already done when considering folding stability and directed evolution of proteins (12). From a fundamental perspective, perhaps the biggest question is whether the observations from evolving single RNA or protein molecules will also apply at the level of the whole cell; if so, we can hope to move toward a predictive understanding of these phenomena.

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Under selection for yellow fluorescence, many mutations that do not change phenotype (indicated by glow) can accumulate as cryptic genetic variation. Upon selection changing to green fluorescence, every path from ANC (ancestral) to the brightest ABC combination, such as A→B→C would be blocked by deleterious mutations (indicated in red). If these mutations had already accumulated neutrally, such as AB, they could enable rapid evolution of green fluorescence (beneficial mutations indicated in blue).