Mechanisms of protein evolution

Running Title: On the evolution of proteins

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Abstract

How do proteins evolve? How do changes in sequence mediate changes in protein structure, and in turn in function? This question has multiple angles, ranging from biochemistry and biophysics to evolutionary biology. This review provides a brief integrated view of some key mechanistic aspects of protein evolution. First, we explain how protein evolution is primarily driven by randomly acquired genetic mutations and selection for function, and how these mutations can even give rise to completely new folds. Then, we also comment on how phenotypic protein variability, including promiscuity, transcriptional and translational errors, may also accelerate this process, possibly *via* 'plasticity-first' mechanisms. Finally, we highlight open questions in the field of protein evolution, with respect to the emergence of more sophisticated protein systems such as protein complexes, pathways and the emergence of pre-LUCA enzymes.

Introduction

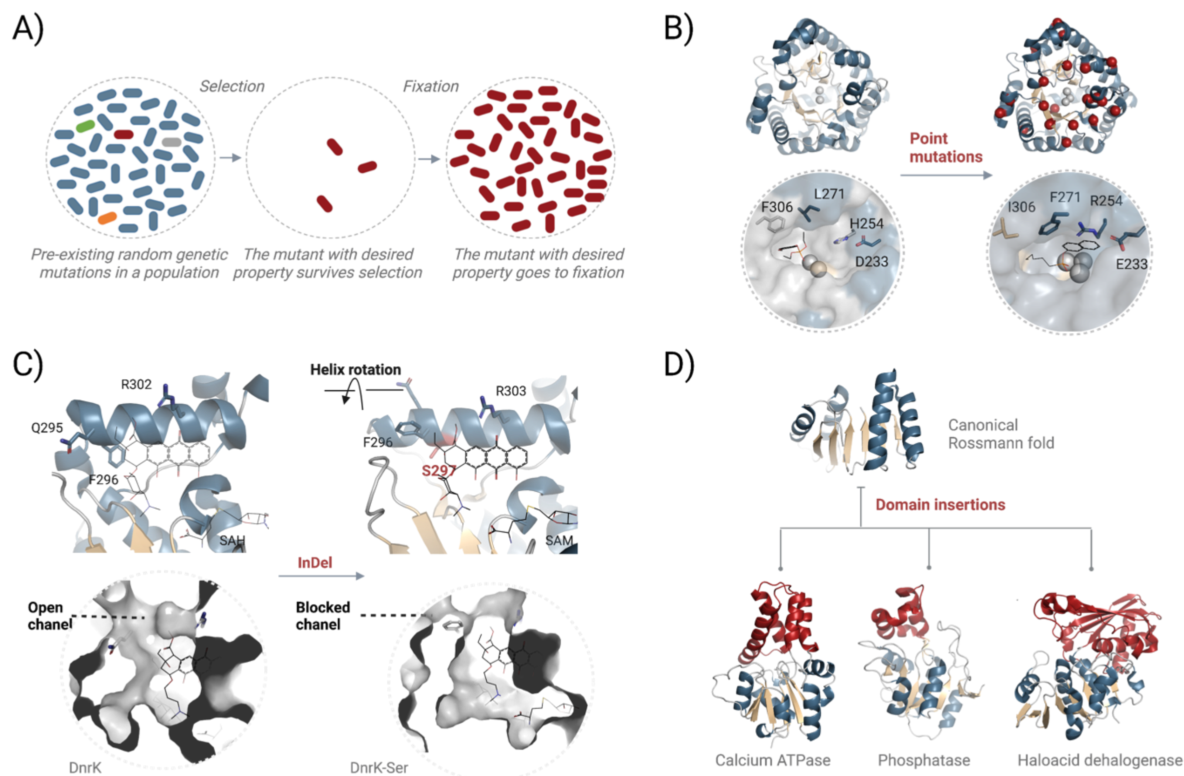
The first version of this manuscript was written by Paola Laurino, Lianet Noda-García and Prof. Dan S. Tawfik, whom the authors deeply miss.

Protein evolution encompasses a large variety of phenomena addressed by multiple disciplines including biophysics, biochemistry and evolutionary biology. The mechanistic aspects of protein evolution may be broadly phrased as: how do changes in protein sequence occur, and how do they mediate changes in protein structure, and in turn in function? Each discipline has its own angle with respect to these questions. Here we present an integrated view, through the eyes of protein scientists. We attempted to portray how multi-faceted the research of protein evolution is and discuss relatively unexplored aspects and fundamental questions that remain unanswered. However, breadth inevitably trades off with depth. Thus, we apologise if significant achievements of specific fields are not thoroughly cited.

A fundamental paradox in protein evolution is that: 'nothing evolves unless it already exists', or in other words as stated by DeVries: 'Natural selection may explain the survival of the fittest, but it cannot explain the arrival of the fittest' [1]. Mutations, insertions/deletions, and recombination mostly induce minor changes in protein structure (*micro-transitions*) that are sufficient for the rise of new functions, although in rare cases, these can generate completely new protein folds (*macro-transitions*) (**Box 1**, *1*). Our review revolves around this classic, 'Darwinian model', and covers cases where the pre-existing sequence diversity in a population give rise to new functions.

Further, we describe various mechanisms that may expedite this process. For instance, it is possible that the genomic mutations needed conferring a novel function might not be present in a population, they can however, rise by non-genetic mechanisms mediated by errors in replication, transcription and translation (phenotypic mutations)[2–4]. Thus, the upcoming new function is already present, as fortuitous, latent variation at the phenotypic level within identical genotypes (phenotypic variability) [5,6]. These changes are observed at all biological levels of organization, from single proteins to entire organisms [7,8]. Indeed, the pre-existence of protein activities as latent promiscuous functions, is by now, a well-established hypothesis understood in atomic detail [9–11]. We also highlight additional aspects of phenotypic variability that underlie the arrival of the fitter. A seemingly attractive, yet controversial hypothesis, is that phenotypic variability (and possibly also genetic changes) is directly induced by environmental challenges.

66 These so-called ‘Baldwin-effects’[12,13] may apply to protein evolution, and are presented here
67 under a general model, coined ‘plasticity-first’.
68 Much of the current work revolves around the evolution of individual biochemical
69 activities such as ligand binding (DNA, RNA, small molecules or proteins) or enzymatic
70 functions (for recent examples see [14–23]). However, beyond biochemical activity *per se*, other
71 protein features are also shaped by evolution, such as the regulation of the protein expression,
72 folding, stability and oligomerization [24–27] or avoiding undesired interactions with other
73 metabolites or proteins [28]. Further, proteins also coevolve with other proteins and biomolecules
74 with whom they interact, and with the cellular components responsible for protein synthesis,
75 maintenance, and clearance [29,30]. Here, we discuss some open questions related to these aspects.
76 As proteins have been evolving for ca. 3.7 billion years, the mechanisms underlying the
77 divergence of recently evolved enzymes [11,31] may appear largely inapplicable to the emergence
78 of the very first protein(s) [32]. There are, however, some unifying themes that we describe here
79 alongside differences and unknowns. We conclude by discussing how short and functional
80 protein fragments may have been recruited prior to the appearance of LUCA’s proteome to
81 give rise to primitive metabolic systems.
82
83 ***Box 1***
84 **Microtransitions in protein evolution**
85 Protein evolution is driven by mutations that can occur biasedly [33,34] or at random and with no
86 relation to selection [35]. Deleterious mutations are purged, whereas new challenges drive the
87 fixation of mutations that give rise to proteins with modified or new functions (**Box 1**, *2*, and
88 **Figure 1**).



89

90 **Figure 1. Darwinian evolution driven by pre-existing genetic changes, ranging from single amino acid** 91 **mutations to gene rearrangements**. **(A)** Schematic representation of Darwinian selection: selection purges most 92 of the variations in the population, leading to survival of the fittest mutant, eventually undergoing fixation. **(B)** 93 The outcome of a laboratory evolutionary trajectory of 18 consecutive point mutations (PDB codes: 1DPM, 2R1N, 94 4E3T) [36]. The original and evolved active sites are depicted with their corresponding reaction intermediates (a 95 phosphotriesterase (left) and aryl-esterase (right). The mutated positions are denoted in red. The overall structure 96 (cartoon) and the key catalytic residues remained unchanged (the catalytic metals are presented as grey spheres). 97 **(C)** A switch between two fundamentally different activities, methyltransferase (left) and monooxygenase (right) 98 may be triggered by an insertion of a single amino acid. An inserted serine at position 297 (red) induces a flip of 99 the adjacent side-chain of Phe296 (blue sticks) that reshapes the active-site (surface) and triggers the activity 100 change (PDB codes: 4WXH and 5EEG) [37]. **(D)** Domain insertions into an existing enzyme drive the divergence 101 of new functions [38,39], as exemplified here for three different enzymes that share a Rossmman-fold core domain a 102 Haloacid dehalogenase (PDB 1ZRN), a phosphatase (1N9K) and a calcium pump-driving ATPase (1SU4). The 103 canonical Rossmann fold is represented by a dehydrogenase (5KKA). For other examples of microtransitions see 104 [19–22].

105

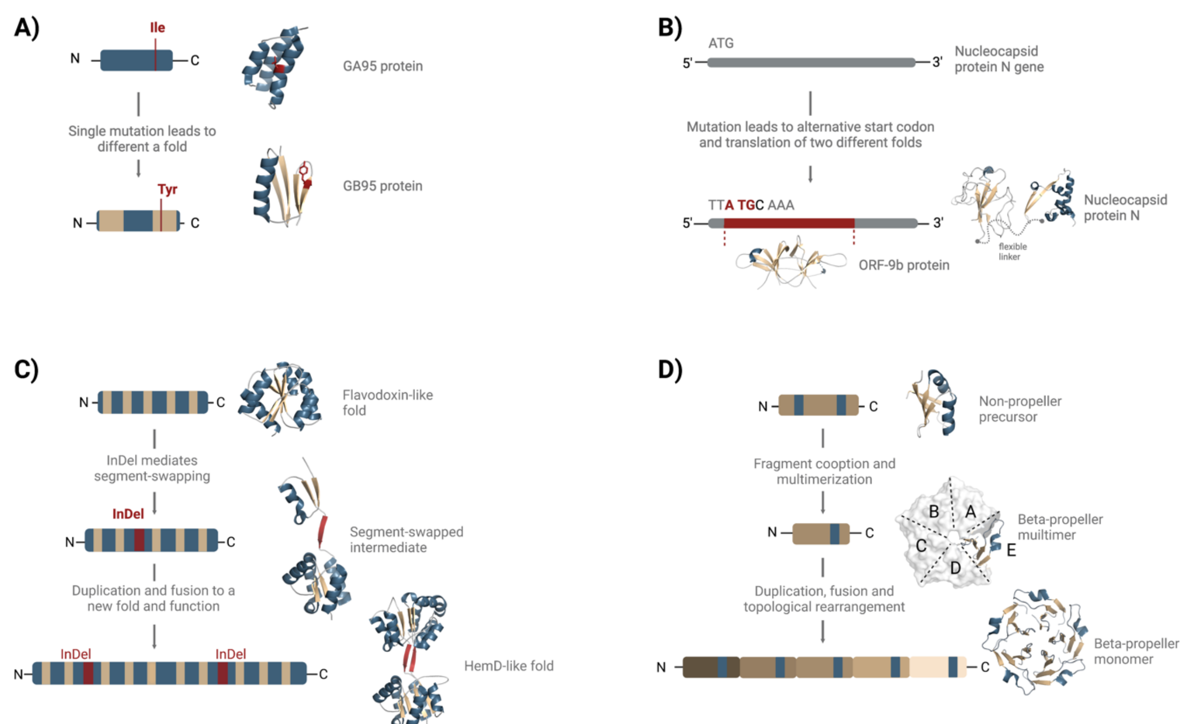
106 As exemplified in **Figure 1**, most, if not all, of the extant protein repertoire emerged by small 107 structural modifications while maintaining their basic fold. Such changes, dubbed 108 *microtransitions* (**Box 1**, *1*), have been demonstrated in the laboratory, largely *via* point 109 mutations, insertions/deletions (InDels), homologous or non-homologous recombination [23] and 110 domain fusions [40]. While the effects of point mutations have been widely explored (e.g., **Figure** 111 **1b**), we know less about how other types of genetic changes lead to new proteins. InDels, for 112 example, have high adaptive potential. For instance, a single InDel can induce functional 113 transitions [37,41] (e.g., **Figure 1c**). Additionally, domains frequently mix and match (gene fusion 114 or fission) to yield new proteins [39,42–44]. The addition of a single relatively small domain allows

Rossmann fold enzymes to catalyse different reactions e.g., calcium ATPase, phosphatase and haloacid dehalogenase (**Figure 1d**). InDels or larger genetic rearrangements are on average, even more deleterious than point mutations and therefore intensely purged [45,46]. Acceptance of mutations in general, and InDels or larger genetic rearrangements especially, typically demands compensation by other mutations (**Box 1,** *6*) [45,47].

In contrast to the divergence of functions in existing domains, the birth of new protein topology and architecture is driven primarily by duplication and fusion of short segments, as discussed in the following section.

Macrotransitions in protein evolution

Protein domains whose secondary structural elements adopt similar orientation in space are classified under the same architecture. If in addition, these elements display identical topological connections, they are further sorted under the same fold. Substantiated cases of homology between different folds are rare. Only recently, the development of sensitive homology prediction tools has allowed drawing evolutionary bridges between folds that were previously thought unrelated [48–55]. Despite these efforts, most of the evolutionary relationships between distant homologs remain a mystery. How did the first protein folds emerge? Did transitions between these architectures occur at any stage; and if so, how?



135 **Figure 2. Macrotransitions: genetic mutations induce changes in protein structure. (A)** A single amino acid
136 mutation (I45Y, red) leads to a fold change as exemplified by protein GA95 (PDB 2KDL): the all-alpha structure
137 protein acquires alpha and beta secondary elements in GB95 (PDB 2KDM). **(B)** Mutations at the DNA level can
138 lead to alternative reading frames. Such is the case for the Nucleocapsid protein N gene that gives rise to the
139 nucleocapsid N ORF-9b protein (PDB 2CME) [56]. The new protein adopts an all-beta fold, in contrast to the alpha
140 and beta elements of the original protein [57]. (**C**) An insertion (red) within the flavodoxin-like fold (PDB 1REQ),
141 results in an additional beta element that segment-swaps the original fold in two. This structural rearrangement
142 creates a protein interface that is now able to associate with another monomer, inducing the topological changes,
143 resulting in the hemD-like fold (PDB 1jr2) [53]. **(D)** Short fragments within proteins can act as building blocks to
144 create novel architectures. A fragment from a non-propeller precursor (PDB 3WHI) upon oligomerisation,
145 duplication and fusion rearrange in a monomeric propeller fold (PDB 5C2N) [58].
146

147 Studies of metamorphic proteins have provided some hints [59–62], which demonstrate that the
148 topology and architecture of protein domains can be altered, herein called *macrotransitions,* by
149 introducing a few or even one single amino acid substitution [56] (**Box 1**, *1*). Such is the case of
150 the G_A protein, a serum binding domain that is converted into G_B, an IgG-binding domain upon
151 a L45Y substitution (**Figure 2a**). This type of structural transition suggests the existence of
152 critical residues that stabilize certain tertiary interactions while abolishing others. Likewise, a
153 single protein sequence can fold into more than one structure. These sequences have more than
154 one energetically favoured minimum (scaffold plasticity) that allows the interconversion
155 between different structures upon changes in the environment such as pH; lipid or buffer
156 composition [63]. *De novo* emergence of proteins by overprinting is another example of a
157 *macrotransition*, where alternative frames of coding sequences from short segments of existing
158 proteins are translated. This phenomenon can give rise to new amino acid sequences, and
159 ultimately to new protein architectures [64–66]. For instance, by incorporating an alternative start
160 codon within the nucleocapsid protein N, an additional reading frame is created, giving rise to
161 ORF-9b protein, which adopts a new fold (**Figure 2b**) [57]. This process is not to be confused
162 with the *de novo* emergence of proteins from non-coding DNA (see open questions), where
163 arbitrary transcripts occasionally overlap with randomly attained open-reading-frames and
164 become translated[67–70]. Further architectural rearrangements can emerge through trading of
165 structurally similar regions (segment-swaps) between two or more domains, which can be
166 found in around 13% of the PDB structures [71]. This type of *macrotransition* can also be induced
167 by InDels within a protein sequence [53] as exemplified by the flavodoxin-like fold, which upon
168 insertion, duplication and fusion gave rise to a new functionality, adopting the bi-lobular
169 hemD-like architecture (**Figure 2c**). Duplication and fusion of short segments can also lead to
170 open-ended (solenoid) structures as indicated by the internal symmetry that underlines many
171 protein folds, [72,73], e.g., αα-hairpin repeats generate TRP, HEAT, Armadillo, and Ankyrin
172 structures, whereas βαβ units generate leucine rich repeats. In other instances, repeating units

173 create globular structures, such is the case for the TIM barrels [74,75] and beta-propellers (**Figure**
174 **2d**) [58,76,77]. Overall, the above-mentioned examples highlight how novel protein architectures
175 can emerge from structurally unrelated scaffolds through relatively small changes, illustrating
176 their plasticity and resilience potential.
177
178 While it is well known that mutations, gene rearrangements and InDels can cause functional
179 and structural changes in proteins, not all these mutations go to fixation. In the next section,
180 we discuss how selection and fixation occur, based on results of various directed evolution
181 experiments on individual proteins.
182
183 **Selection and fixation of mutations**
184 Following their appearance, most mutations are purged while some are fixed, by selection, but
185 also by chance (**Box 1**, *2*). This leads to the critical question: out of all possible mutations in a
186 protein, which fraction of these is neutral versus what fraction is deleterious, and to what degree.
187 Equally crucial is the frequency of potentially beneficial mutations and their effects on the
188 protein's original function and stability as this dictate whether they might be fixed or rapidly
189 purged.
190
191 The answer to these is embedded in the distribution of fitness effects of mutations
192 (DFE) – a subject of extensive research. Systematic mappings of the effects of all possible
193 single amino acid mutations in a given protein have become routine [78–81]. These mutational
194 scans yield distributions of the effects of mutations in individual proteins, and also insights
195 regarding the structural and biochemical parameters that dictate them [82,83]. The cumulative
196 knowledge of protein DFEs indicates that the vast majority of mutations, probably ≥80%, are
197 deleterious [84], with the primary reason being impaired folding and/or decreased stability [84].
198 Mutations that alter biochemical function are rare and also purged more intensely [82,83]. The
199 effects of mutations on folding and stability are complex, as they also relate to how the cellular
200 machinery deals with impaired mutants (see below). Indeed, in the short term, mildly-
201 deleterious mutations may be tolerated owing to various cellular buffering mechanisms, thus
202 facilitating protein evolution [85–87].
203
204 The evolutionary interpretation of deep mutational scans is problematic, not the least
205 because the measured 'fitness' values rarely relate to organismal fitness. Accordingly, most
206 experiments indicate higher mutational tolerance than what observed in nature amongst

207 homologous proteins, suggesting that most mutations, in laboratory conditions, do not affect
208 structure and/or function [84]. It appears that the deleterious effects of mutations are masked in
209 most laboratory experiments [83], rendering the results more relevant to the understanding of
210 short-term genetic diversity (*e.g.* population polymorphism), as opposed to long-term
211 evolutionary processes [84,88]. Similarly, when it comes to adaptation (acquiring new or modified
212 protein properties), laboratory selections may typically be too stringent, thus funnelling
213 adaptations towards one trajectory in a limited and defined environment (a single growth
214 medium, temperature, etc.). The gradual selection pressures and diverse environments that
215 underlie natural evolution may shape protein adaptation in ways that differ from what has been
216 observed in most laboratory experiments [89,90].
217
218 **Evolutionary rates of proteins**
219 When it comes to long-term evolution, the rates by which proteins evolve vary dramatically.
220 Even when comparing proteins of the same species, or orthologues only (i.e. assuming minor
221 changes in protein function), evolutionary rates (substitutions per site, per generation) typically
222 span over 2 orders of magnitude among the proteins in the same genomes. The factors that
223 dictate the rate of protein evolution is of major interest [91]. One key determinant is epistasis,
224 namely interdependency between different positions of the same gene/protein (intragenic
225 epistasis; **Box 1**, *5*). Globular proteins in general exhibit negative epistasis (deleterious effects
226 of two different mutations is greater than the sum of individual ones) [92]. As proteins evolve,
227 deleterious mutations can still be fixed. However, their acceptance depends on the pre-
228 existence of other mutations (permissive, enabling mutations) or on the subsequent
229 accumulation of compensatory mutations (**Box 1**, *6*). This context dependency of mutations
230 dictates a slower rate of evolution. Biophysical and functional constraints also affect rates of
231 protein evolution. These include high expression levels that make proteins more prone to
232 aggregation and promiscuous associations; and multi-functionality, thereby engaging a large
233 fraction of the protein's surface [91,93].The latter two constraints act primarily on the protein
234 surface -namely surface residues mutate 4-fold faster than the core residues. Interestingly, the
235 surface constraints slow down the divergence of other residues, in particular core residues,
236 resulting in an overall very slow evolutionary rate [94].
237
238 Finally, the acquisition of new functions is the strongest driving force to protein sequence
239 changes. Accordingly, mutational trajectories that lead to new protein functions have been
240 extensively studied, revealing in atomic detail the effects of mutations on protein structure and

241 function (**Box 1**, *3-9*) [95]. We note that nearly every long adaptive trajectory beyond few
242 mutations, includes multiple mutations at positions distal to the active site. Despite the
243 importance of these so-called 3rd shell mutations their contribution to the emergence of new
244 protein function remains poorly understood [96,97].
245

246 **Mutagenic hotspots**

247 Mutations that confer modified or new protein functions (adaptive mutations), may pre-exist
248 in the population when a new challenge appears, or may arise within subsequent generations –
249 for example, both pre-existing and arising mutations have been identified in insect esterases
250 that evolved towards insecticide resistance [98–100]. Mutations that are neutral or nearly neutral,
251 with respect to the protein's existing function, and are therefore not purged, may become
252 beneficial upon the emergence of a new challenge (**Box 1**, 7). Still, the occurrence of point
253 mutations is rare (10^{-9} per site, per generation, on average). Thus, the genetic diversity available
254 at any given moment is limited, especially in organisms with small population size. In cases
255 where mutation(s) with adaptive potential do not pre-exist in a population, the initial response
256 to a new challenge is critical. In this context, we review and discuss several mechanisms that
257 may hence expedite adaptation in the absence of pre-existing genetic diversity.
258

259 Cellular stresses correlate with higher mutation rates [101]. Also, the rate and type of mutations
260 vary dramatically, depending on local DNA context *e.g.* short sequence repeats [102] and in a
261 global one *e.g.* highly transcribed regions [33,103]. These so-called adaptive mutations arise due
262 to high mutability of single-stranded DNA in active transcription bubbles and from replication-
263 transcription collisions [104,105]. Similarly, highly transcribed genes may be duplicated *via* cDNA
264 intermediates (retro-genes) [106]. Duplications can vary from gene segments to whole genomes
265 and may also be considered as 'adaptive mutations', whenever they are stress induced and auto-
266 amplified [107]. Under strong selection, multiple copies of a gene mediating survival may emerge
267 within a strikingly small number of generations and disappear immediately after selection is
268 removed [108,109].
269

270 Given high replication fidelity, the above discussed mechanisms may be crucial in shortening
271 the time gap between new challenges and the arrival of mutations that promote survival [110]. It
272 is not trivial to establish direct causality between stress, the induction of genetic changes, and
273 adaptation [101]. Nonetheless, their relevance is highlighted by the existence of explicitly evolved

‘hotspots’ regions in specific genes which encode rapid heritable genetic switches, such as in surface antigen proteins of pathogenic bacteria [102,111].

Protein noise and phenotypic mutations

In most cases, mutations conferring new function are pre-existing in a population. Alternatively, the yet-to-become new function could be already present, as latent, coincidental phenotypic variation whereby a single genotype (a given gene sequence) may give rise to a range of protein sequences, structures and functions, and thereby to multiple phenotypes. If phenotypic protein variability is neutral in the environment(s) under which a protein evolved – this variability comprises ‘molecular noise’. Nonetheless, upon appearance of a new challenge phenotypic variability may provide an immediate survival advantage and increase the adaptive potential. In proteins, phenotypic variation can be displayed in multiple ways, including: (*i*) variable protein levels in a population of cells due to expression noise; (*ii*) latent, promiscuous protein conformations and activities due to drift; and (*iii*) alternative protein sequences due to transcriptional, splicing and/or translational errors.

Here, we focus on transcriptional and translational errors. For the adaptive potential of (*i*) see Refs. [112,113], regarding (*ii*), see **Box 1**, *4*. Translational and transcriptional errors are ~10^5 times more frequent than genetic mutations [114,115]. As a representative example, it was shown that in yeast ADH1 gene, transcriptional errors alone can affect almost every aspect of enzymes function including oligomerization, substrate binding, cofactor binding, metal binding and post-translational modification site (**Figure 3b**) [116]. Like genetic mutations, phenotypic mutations are not limited to single amino acid exchanges – frameshifts, alternative starts and/or stops codons, and larger rearrangements (*e.g., via* alternative splicing) are also common (**Figure 3b-d**). Overall, given the wealth of noise associated with transcription, mRNA processing and protein synthesis, protein copies that deviate from the expected translated gene sequence are abundant [114,117,118]. These so-called phenotypic mutations have a role in the evolutionary shaping of proteins [2,119], and may also provide starting points for emergence of new proteins or functions [115,120]. Short peptide segments that result from ‘illegitimate’ translation (smORFs) are also prevalent due to alternative start/stop codons, off-frame translation of coding sequences, or translation of complementary strands or even of noncoding regions [65,121–123]. Such segments might also comprise the starting material for novel proteins (**Figure 3c, d**) [124,125].

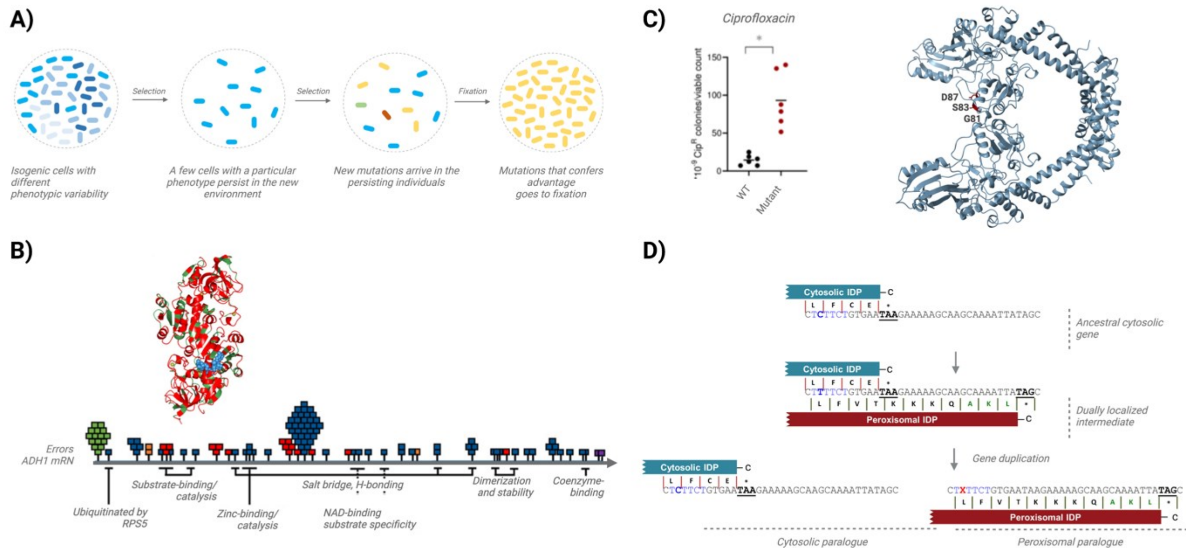


Figure 3. Plasticity-first mechanisms driving protein evolution (A) A schematic representation of selection that follows the plasticity-first mechanism. A new environmental challenge selects a subset of phenotypically variable isogenic cells. The phenotype permits the survival of cells, providing time for the occurrence of a mutation which confers an adaptive advantage. The mutant cells take over the entire population (fixation). **(B)** Transcriptional errors in yeast ADH1 mRNA mapped on to the structure. The residues with errors are highlighted in red. The scheme at the bottom shows that the mutations can affect several aspects of the enzyme: oligomerization, substrate binding, cofactor binding, metal binding and post-translational modification sites [116]. The figure panel is reprinted from the reference (116). (**C**) An *E. coli* mutant exhibiting higher mistranslation rates (phenotypic plasticity) displays higher frequency of genetic mutations that confer antibiotic resistance (adaptation). This panel is reprinted from the reference (135). The right panel shows the structure of DNA gyrase with the mutations conferring ciprofloxacin resistance highlighted in red. (**D**) Translational errors may provide the raw material to new proteins [120]. In the depicted example, a coincidental translational slippage at a TCTTTT site produces an alternative protein form with a C-terminal peroxisomal signal. In the second step, a mutation of C-to-T, that is silent with respect to the original frame, increases slippage rate, thus generating two alternative protein forms from one gene: the original cytosolic form, and a minor peroxisomal form (the AKL peroxisomal signal peptide, denoted in green). Finally, following gene duplication, a single base deletion gives rise to a new, legitimate peroxisomal paralogue, whereas the original, cytosolic gene loses the cryptic peroxisomal signal.

It is important to highlight the fact that, although phenotypic mutations are not heritable as such, the potential to make them can be [118,126–129]. For example, the frequency of transcriptional/translational errors is highly variable and sequence dependent. Codon usage strongly affects the rate of mistranslation [130]. The frequency of slippage to yield phenotypic frameshifts is directly proportional to repeat length, 8 consecutive A's being an example of programmed slippage [131–133]. Therefore, selection may favour gene sequences that increase the frequency of alternative protein variants while retaining the original wild-type protein sequence. In this manner, errors that occur largely at random can be amplified at specific sites and can also be heritable.

Although phenotypic mutations occur at higher frequency, and are shown to be important for the adaptation of organisms [134–137], the experimental evidence for their adaptive role in protein evolution is only recently emerging. Direct evidence for the evolutionary role

337 of phenotypic mutations came from the emergence of a new yeast enzyme paralogue (**Figure**
338 **3d**) [120]. The ancestral gene of isocitrate dehydrogenase (IDP) encodes two enzyme forms
339 (isozymes) – a major cytosolic form by intact translation, and a minor form that possess a C-
340 terminal peroxisomal signal peptide due to a translational frameshift. Following duplication, a
341 single nucleotide genetic deletion gave rise to a new, legitimate peroxisomal paralogue,
342 whereas the cytosolic paralogue lost the translational frameshift that leads to a peroxisomal
343 signal.
344
345 **Genetic accommodation of phenotypic mutations**
346 Phenotypic mutations may bridge the time gap between the appearance of a new challenge and
347 the emergence of a mutation that resolves it (a gap that can be much longer than intuitively
348 assumed). If a challenge persists, what initially comprises coincidental noise often becomes a
349 'legitimate' function *via* the fixation of mutations at the genomic level that refine this function.
350 For example, typically following gene duplication a weak promiscuous enzymatic activity may
351 increase in both rate and selectivity to become the primary function. This was demonstrated
352 recently in a study employing *E. coli* strains with varying levels of translation error rates. The
353 authors show that the *E. coli* mutants with higher error rates show higher frequency of
354 ciprofloxacin resistant colonies compared to WT strains (**Figure 3c**) [138]. Accordingly, lowering
355 the mistranslation rates, reduced the frequency of resistant colonies as well. It is worth noting
356 that the genotypic mutation is often different from the phenotypic mutation.
357 Promiscuous protein activities seem to have a unique evolutionary advantage –
358 mutations that increase them usually have either weak or no deleterious effects on the protein's
359 primary activity (**Box 1**, *4*, *7*, *8*). Phenotypic mutations may also have a unique advantage in
360 how they are genetically accommodated. In the yeast IDP case described above, single base
361 deletions that accommodate the new trait at the DNA level (*i.e.*, in-frame translation of the
362 peroxisomal signal to direct all protein molecules to the peroxisome) occur at the very same
363 mRNA site at which translational slippage occurs [120]. Overlaps between sites of genetic and
364 phenotypic mutations have also been observed in an *in vitro* study [131]. Thus, selection of
365 genotypes exhibiting a higher rate of a specific phenotypic mutation also gives rise to a hotspot
366 for genetic mutations that accommodate the very same trait [120,131]. Similarly, ambiguous
367 decoding (translation of a given codon to two different amino acids) was genetically
368 accommodated in certain organisms via divergence of a dedicated tRNA [117]. More recently, it
369 was also shown that phenotypic mutation can reduce the mutational load in a population by

370 efficiently purging deleterious mutation. Accordingly, phenotypic mutation exhibits negative
371 epistasis with DNA or genotypic mutation [139].
372
373 **Plasticity-first: an emerging model for protein evolution**
374 The so-called Baldwin-effect [12], or in its more modern form, the 'plasticity-first' model [13] refers
375 to the phenomena when non-hereditary molecular variability induced by an environmental
376 change enables initial survival. This buys time for the emergence and accommodation of
377 genetic mutations, ensuring long-term survival of the population in the new environment. Both
378 phenotypic plasticity and the ensuing genetic accommodation of mutations have been
379 extensively examined and debated in the context of developmental plasticity and evolutionary
380 adaptations [8]. Here, we adapted the Baldwin effect [12] and following a recent and insightful
381 review [13], present the key criteria for such a mechanism to be applied to protein evolution
382 (**Figure 3a**).
383
384 The most critical criterion for proving the 'plasticity-first' model for protein evolution is that
385 the yet-to-evolve trait becomes more variable in response to the physiological stress that
386 accompanies the new challenge. For example, the magnitude of certain promiscuous activities
387 or the frequency of translational errors may increase due to changes in metabolite
388 concentrations or pH. Similarly, if some pre-existing, cryptic genetic variation happens to
389 increase the magnitude of trait variability, this would of course promote the 'plasticity-first'
390 mechanism. This criterion is not trivial to establish, and to the best of our knowledge, has not
391 been directly examined in relation to a proven case of protein evolution.
392
393 Indeed, in many cases where the history of acquisition of new protein functions had been
394 tracked down; pre-existing promiscuous functions [140,141], or phenotypic mutations [115,120], were
395 found to have been starting points and even provide initial survival of the population [142,143].
396 However, such trajectories may be perfectly accounted by a Darwinian mutation-selection
397 model, since the pre-existence of mutants with an optimal activity in the population was not
398 examined. Therefore, a key challenge remains: to show that the latent activity was present at a
399 sufficient level to provide a selective advantage before genetic accommodation of mutations.
400
401 Increased molecular noise is inevitably associated with reduced fitness. The cost of increased
402 rate of translational errors, may be tolerable in short-term [114], but in the long-term, high error
403 rates rarely persist [101]. Overall, whilst the 'plasticity-first' model presents an elegant shortcut

404 to the ‘arrival of the fitter’, direct evidence for its role in protein functional evolution is yet to
405 be provided.

406 **Protein evolution - beyond biochemical activity**

407 Biochemical activity —be it ligand binding or catalysis— is the primary driving force of
408 evolutionary innovation. However, within their natural context, proteins are shaped by
409 additional needs and forces that are complex (see **Box 1**, *8*). Following their translation,
410 proteins fold into their native state, and must be sufficiently stable to avoid misfolding,
411 aggregation and/or proteolysis. The interactions of proteins with the cellular machineries that
412 control protein quality are therefore crucial. Chaperones and also proteases, therefore impacts
413 the type and number of mutations tolerated and thus impacts protein evolution [85,86,144].
414
415 Regulation of protein expression is another key property shaping evolutionary trajectory. As
416 indicated by their faster sequence divergence, non-coding elements are more evolvable than
417 the proteins they regulate [145]. Often, the initial steps, and even the driving force for divergence
418 may involve a new mode of transcriptional regulation. Further, the divergence of a new
419 biochemical function is often initiated by increase in expression of an existing protein with a
420 latent, promiscuous function [146]. This divergence may occur via mutations in the gene’s own
421 promoter, in genes encoding other regulatory elements, or via gene duplication (**Box 1**, *3*). By
422 the current view, most new genes, and paralogues especially, diverged in their transcriptional
423 regulation and not in their biochemical function [147,148]. A classic example is the divergence by
424 duplication of yeast Gal1/3. The ancestral, pre-duplicated gene, Gal1, encoded an enzyme, b-
425 galactosidase that also acts as transcriptional co-inducer. Upon duplication, the new paralogue,
426 Gal3, specialized as co-inducer, primarily *via* changes in the promoter that enabled faster
427 triggering of Gal1’s transcription upon appearance of lactose [146].
428
429 Changes in the regulation of protein expression can also affect the evolvability of proteins. In
430 fact, expression levels and protein concentration correlate with evolutionary rates – the higher
431 the protein amount in the cell, the slower the rate [91], although to our knowledge, direct causality
432 has not been established. In the case of Gal3 although the key adaptive step was due to the
433 changes in the promoter, [146] protein activity was also changed. Specialized as a co-inducer,
434 Gal3 lost its enzymatic activity, but gain the ability to bind to Gal80 (the transcriptional
435 repressor) with >10-fold higher affinity compared to Gal1, thus providing a distinct advantage
436 upon switching to lactose as carbon source [149]. The divergence of new genes therefore involves

437 changes in gene expression, that in turn enable changes in protein activity, and *vice versa* – in
438 other words, noncoding and coding regions coevolve [149,150]. Beyond transcription, levels of
439 translation are regulated, as are cellular protein levels (*via* changes in protein turnover rates).
440 The mechanisms and dynamics behind the coevolution of protein expression, turnover and
441 function remain to be elucidated.
442
443 Proteins seldom work as independent subunits, and often self-assemble (homomers) or
444 associated with other proteins (heteromers). About 60% of proteins are known to form
445 complexes [151]. How these multimeric assemblies emerge and if there is adaptive value for these
446 complexes is not clear. Recent experimental [152] and theoretical work [151] suggest that these
447 complexes can emerge by neutral drift just like in the case of catalytic promiscuity. Often, as
448 little as one or two mutations are enough to form new homomeric complexes [153]. Though it is
449 tempting to associate an adaptive value for these assemblies, this remains to be investigated.
450 Finally, protein evolution is also constrained by its cellular location. A new localization
451 imposes new challenges. ~30% of the yeast paralogues and ~15% of *Arabidopsis* paralogues
452 diverged in localization [154,155]. Beyond retargeting, typically by the acquisition of a signal
453 peptide[120], a change in localization enforces adaptation towards export (that may involve
454 unfolding and refolding), different pH and/or redox state, and new protein partners. Overall,
455 protein adaptation is a comprehensive process involving multiple parameters in addition to
456 biochemical activity. Foremost, it is a process of coevolution involving the protein itself, its
457 transcriptional and translational regulatory elements, the cellular protein-handling machineries
458 and other proteins and biomolecules that interact with the evolving protein.
459
460 **Open questions**
461 Beyond the series of questions mentioned above, there are, in our view, three key aspects in
462 protein evolution that remain largely unanswered.
463
464 *Multiple, interlocked protein components.* Proteins rarely confer physiological advantage on
465 their own. Typically, they are part of a system – a pathway, or whole network involving several
466 proteins, -whereby loss of any one of these proteins results in loss of function of the entire
467 system. For example, biosynthetic pathways comprise several enzymes, and loss of any of
468 which of these enzymes typically results in no product. How did these multiple, interlocked
469 protein systems (*MIPSs*) emerge in the first instance?
470

471 Many MIPSs can be unlocked – suffice to say that free-living natural bacteria with <1,400
472 genes are known, and even these genomes can probably be reduced [156]. Thus, the current state
473 of a MIPS does not reflect its initial, emergent state. Relatively simple scenarios for the
474 emergence of MIPSs have been hypothesized [32]. With respect to metabolic pathways,
475 bifunctional enzymes are commonly found, suggesting that certain pathways may have *a priori*
476 evolved to catabolize more than one nutrient, or produce more than one product, and at later
477 stages diverged and specialized (**Box 1**) [157–159]. Nonetheless, the emergence of the first MIPSs,
478 and specifically of the core biosynthetic pathways, remains enigmatic. Spontaneous occurrence
479 of reactions, alongside a few multi-functional enzymes, may have enabled the formation of key
480 metabolites, thus seeding the future pathways [160–163].
481
482 *Pre-LUCA recruitment of the first enzymes.* In the pre-LUCA world, modern enzymes did not
483 exist. Rather, ribozymes, metals, and H^+ and OH^- ions [164] may have been the principal catalysts.
484 In this scenario, it has been postulated that the first peptides could have emerged to assist these
485 early catalysts [165,166]. In fact, the exceptional abilities of peptides to chelate metals, catalyse
486 reaction by themself and concentrate in condensate to enhance their activity, make them ideal
487 seeds for the emergence of complex enzymes.[167,168] An alternative scenario includes amyloids
488 as plausible catalytic unit at the origin of life [170,171]. Not only they show an extraordinary
489 stability against UV radiation, different pH and high salt concentrations, but they also catalyse
490 diverse reactions, including their own formation and correction while being replicated. For
491 these reasons the catalytic role of prototype peptides and/or amyloids prior to the putative pre-
492 LUCA world cannot be excluded [169]. An early form of metabolism could have started *via* the
493 recruitment of small peptides with catalytic properties. These units can be seen as minimalistic
494 representations of enzymes [172–174]. Sequence and structural studies on protein domains suggest
495 that the first proteins may have emerged by repetition, fusion, recombination, and augmentation
496 of primordial peptides [175]. These peptide units can be found in modern protein domains with
497 distinct global architecture [55,176,177] and were probably catalytically active as stand-alone, even
498 if less efficient than their contemporary descendants, as well as stable enough to survive. Many
499 questions remain unanswered on how these minimal and functional structures were recruited
500 to replace pre-biotic catalysts and eventually lead to modern protein world.
501
502
503
504

505 *De novo emergence of proteins*. So far, we have addressed a large body of evidence related to
506 transitions (micro- and macro-) in proteins that have a pre-existing globular 3D-structure (and
507 function), but how does structure and function evolve in *de novo* proteins? *De novo* proteins
508 are encoded in genes that emerge from non-coding segments of the DNA sequence [178–180].
509 These new proteins are highly disordered and represent an excellent model system to study
510 how globular proteins evolved from a disordered precursor. The foldability of a *de novo* protein
511 was examined in detail, showing that it adopts a rudimentary fold, exhibits amyloid-like
512 properties and could act as a precursor for the emergence of fully folded proteins [179]. The study
513 of *de novo* proteins might provide in the future new general principles for the evolution of
514 folded proteins.
515
516 Overall, the evolution of MIPSs, the recruitment of first enzymes, and de novo emergence of
517 proteins are aspects where our knowledge is still at infancy. As our understanding of how
518 proteins evolve advances, new insights will emerge that address these and other key questions.

519 **Box 1: Concepts and mechanisms in protein evolution - a very brief guide**

520 The text focuses on a few less explored aspects of protein evolution, while more established
521 aspects are covered in this box that lists key concepts and guiding references (reviews and
522 recent papers describing specific case studies). Scientific concepts and mechanisms are
523 inevitably schematic (if not dogmatic). Alternative scenarios or mechanisms are denoted here
524 side-by-side in blue (noted as 'versus', 'alternatively', etc.). In reality, these are not mutually
525 exclusive and may be even complementary. Many concepts are also interrelated as indicated
526 in our cross-referencing.

527 (*1*) Transitions in protein evolution can be categorized to:

528 **Microtransitions** – divergence of new functions while maintaining the original architecture
529 (fold) and key active-site features (divergence within protein families and superfamilies).

530 **Macrotransitions** – transitions between different folds including the emergence of the earliest
531 protein folds.

532 (*2*) Protein sequences diverge with time (this is what evolution means). Schematically, these
533 changes may relate to drift or adaptation:

534 **Drift** – sequence changes occurring due to random sampling while preserving the protein's
535 structure and function (see *purifying selection*).

536 **Adaptation** – changes in protein properties including the acquisition of new biochemical
537 activities (see *positive selection*).

538 **Selection** may drive a reduction in the frequency of certain mutations (alleles) within a given
539 population (purging, *purifying selection*) and/or the enrichment of other mutations (*positive*
540 *selection*). Selection shapes protein traits including their biochemical activity (binding,
541 catalysis, etc.) and biophysical properties (folding, stability, etc.). Traits such as enzyme
542 selectivity relate to *positive selection*, i.e., enrichment of mutations that increase binding, or
543 catalytic efficiency with the target ligand/substrate, but also by mutations that reduce activity
544 with undesirable, non-cognate substrates [19,181] (see also *trade-offs*). The latter is often addressed
545 as '*negative selection'* (although in population genetics this term is used in relation to purifying
546 selection).

547 (*3*) ***Gene duplication*** provides the raw material for new proteins. Several different mechanisms
548 may underline the emergence of new genes via duplication [4,31,182]. Briefly, duplicated genes

may evolve towards a novel function that had not been present in the ancestral, pre-duplicated gene (*neo-functionalization*). Alternatively, a bifunctional ancestor (*generalist*) may split to two *specialist* genes (*sub-functionalization*, or *divergence before duplication*). Duplication may also provide an adaptive advantage *per se*, by increasing protein dose and thereby augmenting a weak, pre-existing promiscuous function [110].

(*4*) ***Promiscuity*** relates to the coincidental pre-existence of functions that may serve as the starting point for new functions [9–11]. If such *latent*, *promiscuous* functions come under selection, they give rise to bi-functional, *generalist* intermediates. Upon gene duplication, generalist intermediates split, giving rise to two *specialists*, each performing one function (*sub-functionalization*) [14,18,21]. Although duplication and going from *generalists to specialists* is a general trend, the opposing process of gene loss and/or *specialist to generalist* also occurs [183].

(*5*) ***Epistasis*** – the effects of mutations in different genes, but also within the same gene/protein can be non-additive, *i.e.*, *epistatic*. Epistasis has a profound impact on evolution in general, and protein evolution in particular [184–186].

(*6*) **Enabling/compensatory mutations.** The dominance of epistasis also means that many (probably most) mutations that eventually get fixed in evolving proteins are deleterious on their own (during *drift*, and certainly during *adaptation*). Their acceptance may therefore occur via two alternative mechanisms: A deleterious mutation transiently accumulates and is later followed by a *compensatory mutation* [47]. Alternatively, mutations that accumulate initially as neutral enable deleterious mutations to fix at a later stage (*enabling, permissive* mutations) [45,187,188].

Enabling and compensation (and hence epistasis) can be *local* or *specific* [184] – *i.e.*, the deleterious and enabling/compensatory mutations occur in a specific pair of residues (typically, in two contacting residues, *e.g.* within active-sites); or *global*, *nonspecific* – a given mutation may enable/compensate a range of different deleterious mutations (*e.g.* stabilizing mutations that may compensate many different destabilizing mutations).

(*7*) ***Neutrality, robustness*** relates to the ability of proteins to accumulate mutations with no change of structure, stability or function. ***Evolvability***, or ***innovability***, relate to the ability of one, or a few mutations to introduce a new structure and/or function.

While seemingly contradictory, these properties are actually complementary [189,190] - this is primarily because mutations may be neutral in one context (function, environment) yet

580 beneficial in another (*e.g.*, neutral mutations with respect to a protein's native, physiological
581 function may augment a latent, promiscuous activity; see also original-new function *tradeoff).*

582 (*8*) ***Trade-offs in protein evolution*** – mutations almost always affect more than one protein
583 trait (*pleiotropy*) and often in contradictory ways. Epistasis and trade-offs are the key elements
584 shaping the trajectories of protein evolution [36]. Several types of evolutionary trade-offs are
585 known with respect to proteins:

586 *Original vs New-function trade-off* – a mutation improving a new, evolving function is likely
587 to decrease the original one. A strong trade-off enforces *neo-functionalization*, *i.e.*, duplication
588 must occur to complete divergence and specialization (*escape from adaptive conflict*) [4]. In
589 many cases this trade-off is, initially weak, thus enabling divergence towards a bifunctional,
590 *generalist* intermediate (see *sub-functionalization* above). The magnitude of original-new
591 trade-offs tends to vary along adaptive trajectories, starting from weak trade-offs that give rise
592 to *generalist* intermediates, and shifting to strong trade-offs as selection progresses, thus
593 yielding a new *specialist* (typically after *duplication*) [36,95].

594 *Stability-activity trade-off* – most mutations decrease protein stability and thereby lead to
595 misfolding, aggregation and/or proteolysis. New-function mutations are even more so, thus
596 making their accumulation dependent on *enabling/compensatory mutations* [191,192].

597 *Folding-stability trade-off* – beyond the thermodynamic and kinetic stability of the native,
598 folded state, the folding process itself imposes severe constrains. Trade-offs between monomer
599 folding and assembly of oligomers, or between the ability of a protein to fold and the stability
600 of its final, folded state, may underline the birth of new proteins [58].

601 *Rate-accuracy trade-off* – a mutation that improves the catalytic efficiency of an enzyme may
602 reduce its selectivity. Similarly, improvement in the affinity towards the cognate ligand may
603 also increase cross-reactivity with noncognate ligands (see also *positive* versus *negative*
604 *selection*) [181].

605 (*9*) ***Diminishing returns*** – evolutionary optimizations, including protein optimizations, are
606 subject to strong diminishing returns – early mutations confer large advantages per mutation
607 but as the new, evolving trait improves, the improvement per mutations decreases.[36,95]. *Trade-*
608 *offs*, *diminishing returns* and other factors result in many proteins being suboptimal with
609 respect to individual traits such as catalytic efficiency, selectivity and stability [181,193].

610

611 (*10*) ***Phenotypic variation***
612 Variation that exists in a genetically identical population due to the noise associated with
613 various biological processes like transcription, translation, splicing etc.
614
615
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