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The role of robustness in phenotypic adaptation and innovation

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Abstract: Phenotypes that vary in response to DNA mutations are essential for evolutionary adaptation and innovation. Therefore, it seems that robustness, a lack of phenotypic variability, must hinder adaptation. The main purpose of this review is to show why this is not necessarily correct. There are two reasons. The first is that robustness causes the existence of genotype networks—large connected sets of genotypes with the same phenotype. I discuss why genotype networks facilitate phenotypic variability. The second reason emerges from the evolutionary dynamics of evolving populations on genotype networks. I discuss how these dynamics can render highly robust phenotypes more variable, using examples from protein and RNA macromolecules. In addition, robustness can help avoid an important evolutionary conflict between the interests of individuals and populations—a conflict that can impede evolutionary adaptation.

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1 **The role of robustness in phenotypic adaptation and innovation**

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32 **Abstract.** Phenotypes that vary in response to DNA mutations are essential for
33 evolutionary adaptation and innovation. Therefore it seems that robustness, a lack of
34 phenotypic variability, must hinder adaptation. The main purpose of this review is to
35 show why this is not necessarily correct. There are two reasons. The first is that
36 robustness causes the existence of genotype networks, large connected sets of genotypes
37 with the same phenotype. I discuss why genotype networks facilitate phenotypic
38 variability. The second reason emerges from the evolutionary dynamics of evolving
39 populations on genotype networks. I discuss how these dynamics can render highly
40 robust phenotypes more variable, using examples from protein and RNA macromolecules.
41 What is more, robustness can help avoid an important evolutionary conflict between the
42 interests of individuals and populations, a conflict that can impede evolutionary
43 adaptation.

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47 **Introduction.** A feature or *phenotype* of an organism is robust if it persists when
48 perturbed. Phenotypes encompass a broad range of traits, from macroscopic, visible traits,
49 to molecular traits, such as the expression level of a gene, or the three-dimensional
50 conformation of a protein.

51 The perturbations that can affect a phenotype fall into two broad categories. The
52 first comprises environmental perturbations. These include changes in an organism's
53 exterior environment, such as changes in temperature, in available nutrients, or in the
54 abundance of other organisms, such as potential prey. They also include changes in an
55 organism's internal environment, such as temporal fluctuations in gene expression levels,
56 which are caused by ubiquitous intracellular noise. The second category of changes are
57 mutations, changes in an organism's DNA, its *genotype*. Mutations affect an organism
58 more permanently than environmental change, because the changes they cause are readily
59 inherited from generation to generation. For this reason, they are especially important
60 study objects for students of evolution. I will here focus on robustness to genetic
61 mutations – mutational robustness. A huge body of literature shows that living systems
62 on all levels of organization – from macromolecules to whole organisms – are to some
63 extent robust to mutations (Kitano 2004; Stelling et al. 2004; Wagner 2005a; Wagner
64 2005b). I note that mutationally robust systems are often also robust to environmental
65 change (Ancel & Fontana 2000; Lehner 2010; Masel & Siegal 2009; Meiklejohn & Hartl
66 2002; Szollosi & Derenyi 2009; Wagner 2005b), even though exceptions may exist
67 (Cooper et al. 2006; Milton et al. 2003). Thus, most observations I make here about
68 mutational robustness apply to environmentally robust systems as well.

69 During a population's evolutionary adaptation to a new environment, which takes
70 place over multiple generations, its members undergo mutations. Most of these mutations
71 are detrimental, and only few change an existing phenotype into a new, better-adapted
72 phenotype (Eyre-Walker & Keightley 2007; Sawyer et al. 2007; Soskine & Tawfik 2010;
73 Wloch et al. 2001). Very occasionally, one or more mutations may also bring forth
74 evolutionary innovations – new phenotypes that are qualitatively different and superior to
75 existing phenotypes. The ability of mutations to bring forth new phenotypes is important
76 to Darwinian evolution. I will refer to it as *phenotypic variability*.

77 Phenotypic variability and robustness may seem opposite properties, because in a
78 robust system, mutations do not easily change a phenotype. The main purpose of this
79 review is to show why this view is not necessarily correct, and why robustness can
80 instead be a prerequisite for phenotypic variability.

81 To make this point beyond individual case studies – anecdotes of natural history –
82 one needs to study the relationship between genotypic change and the resulting
83 phenotypic change *systematically*, either experimentally, through comparative data, or
84 through computational modeling. I will focus here on protein and RNA macromolecules,
85 for which this has become possible in recent years, but similar principles also hold for
86 very different classes of systems (ESM and (Wagner 2011c)).

87 Other recent reviews have focused on phenotypic variability and its relationship
88 to recombination (Masel & Trotter 2010), enzyme promiscuity (Khersonsky & Tawfik
89 2010), commonalities among different system classes (Wagner 2011b), and phenotypic
90 constraints (Wagner 2011a). In contrast, this review’s focus is the role of robustness in
91 phenotypic variability. In part one I introduce some concepts and discuss the most
92 difficult problem organisms face in evolutionary adaptation and innovation. Parts two and
93 three focus on the two main respects in which robustness affects phenotypic variability.
94 Specifically, part two shows that robustness influences how genotypes with the same
95 phenotype are organized in a vast space of genotypes. This *static* or *structural* influence
96 of robustness contrasts with its influence on the evolutionary *dynamics* of populations
97 that is the focus of part three. A brief part four discusses the evolutionary conflict
98 between the interests of an individual and that of a population in producing phenotypic
99 variation. It points out that robustness as a variability mechanism can avoid this conflict. I
100 will not discuss the important but controversial claim that mechanisms endowing living
101 systems with robustness originated in evolution *because* they favor variability, for lack of
102 sufficient evidence (Pigliucci 2008).

103 **Exploring the new while conserving the old.** Proteins and RNA catalyze all
104 chemical reactions, provide structural support, help cells and organisms move, guide cell
105 communication, and carry out many other functions. The *genotype* of each such molecule,
106 is a sequence of amino acids or RNA nucleotides. *Genotype space* comprises all
107 sequences of a given length L . It is astronomically large, and comprises 20^L protein

108 genotypes and 4^L RNA genotypes. A molecule's *phenotype* refers to the fold or
109 conformation that it forms in space, and to the biochemical function that this fold makes
110 possible. New phenotypes in molecules arise through genotypic changes that cause
111 phenotypic change. Macromolecules are well-studied, with many known adaptations and
112 innovation (Cheng 2006; Khersonsky & Tawfik 2010; Li 1997; Wagner 2011c).

113 Evolutionary change takes place in populations of organisms. Each member of a
114 population has some genotype. For the purpose of this review, it is useful to think of a
115 population as a collection of genotypes in genotype space. The members of this
116 population change their location in this space through mutations. An especially important
117 class of mutations are point mutations, which transform a genotype into one of its
118 neighbors – a genotype that differs in one amino acid or nucleotide. The individuals in an
119 evolving population also have some phenotype. Natural selection preserves individuals
120 with well-adapted phenotypes, and eliminates mutants with poorly adapted phenotypes.

121 Somewhere in genotype space a superior genotype may exist whose phenotype is
122 better adapted to the current environment. The central problem in evolutionary adaptation
123 is how a population can find such a superior genotype. The problem is difficult, because
124 this genotype may exist far away from the population's current genotype, and because the
125 vast majority of mutant phenotypes a population explores are inferior, not superior to
126 existing phenotypes (Eyre-Walker & Keightley 2007; Sawyer et al. 2007; Soskine &
127 Tawfik 2010). What is more, during a population's "search" for this genotype, an
128 existing well-adapted phenotype must be preserved. Perhaps the most compact way to
129 express this problem is with an analogy from politics: evolving populations need to be
130 both "conservative" and "progressive" at the same time. A tall order indeed.

131 Two generic features of genotype space make it possible to reconcile these
132 conflicting demands. The first is the existence of connected genotype networks, vast sets
133 of genotypes whose members all have the same phenotype (Figure 1). These sets extend
134 far through genotype space, and can be traversed in many small steps of individual
135 mutations with little or no phenotypic change. Their existence has first been suggested by
136 computational models of phenotype formation (Lipman & Wilbur 1991; Schuster et al.
137 1994), but they also exist in real macromolecules. A paradigmatic example is the family
138 of oxygen-binding globins. They comprise hundreds of known members that are

139 connected through single amino acid changes to a common ancestor. They share a
140 common fold and biochemical function – oxygen binding – but have diverged in more
141 than 95 percent of their amino acid residues (Goodman et al. 1988; Hardison 1996).
142 Macromolecules like this are the rule rather than the exception (Bastolla et al. 2003; Rost
143 2002; Thornton et al. 1999; Todd et al. 1999).

144 Genotype networks are sometimes called neutral networks (Schuster et al. 1994).
145 However, evolutionary change on such networks is usually all but neutral. That is, such
146 change may affect fitness. For example, weakly deleterious mutations are more abundant
147 than neutral mutations in most macromolecules, but they are often followed by
148 compensatory changes that allow a preservation of phenotype. Similarly, in large
149 populations the simultaneous occurrence of multiple mutations can help a population
150 “tunnel” through a region of low fitness in genotype space, and thus help preserve a
151 phenotype (Eyre-Walker et al. 2002; Hartl & Clark 2007; Kern & Kondrashov 2004;
152 Kulathinal et al. 2004; Sawyer et al. 2007; Weinreich & Chao 2005). Because phenotype
153 preservation does not require neutrality of individual mutations, I refrain from using the
154 word neutrality in this context.

155 The second central feature of genotype space regards the collection of those
156 genotypes that can be reached from any one genotype through one or few mutations. This
157 collection is also called a genotype’s *neighborhood*. Neighborhoods are important,
158 because the set of different phenotypes in a neighborhood are easily accessible by
159 mutation. The size of this set is thus a simple measure of how phenotypically variable a
160 genotype is in response to mutations (Wagner 2008). The second feature of genotype
161 space is that neighborhoods of different genotypes typically contain different novel
162 phenotypes (Figure 1, see also the ESM).

163 The first feature, genotype networks, allows individuals in a population to
164 preserve their phenotype, while changing their genotype in many small steps that,
165 cumulatively, can add up to substantial divergence. Because of the second feature, the
166 different genotypes on a genotype network can explore different phenotypes, precisely
167 because their neighborhoods contain different novel phenotypes.

168 Thus far, I implicitly assumed that one genotype has one phenotype, a
169 simplification that helps illustrate key concepts in simple terms. However, it is important

170 to be aware that many molecules can form multiple folds and exert multiple functions
171 (O'Brien & Herschlag 1999; Tokuriki & Tawfik 2009b). Such multifunctionality can play
172 an important role in the origin of novel phenotypes (Khersonsky & Tawfik 2010), and
173 can further enhance the variability caused by genotype networks (Wagner 2011c, Ch. 13).

174 **In bringing forth genotype networks, robustness facilitates phenotypic**
175 **variability.** To appreciate the role of robustness in phenotypic variability, it is useful to
176 define robustness in the genotype space framework. Specifically, a genotype is to some
177 extent robust to mutations if it has some neighbors with the same phenotype P as itself.
178 One can show that robustness thus defined is both necessary and sufficient for the
179 existence of genotype networks (see ESM).

180 Now compare, as a thought experiment, two kinds of genotypes. The first is a
181 minimally robust genotype, that is, a genotype that has no neighbors with phenotype P .
182 Figure 2a shows such a hypothetical, minimally robust genotype G (black circle) with
183 eight neighbors (dashed black lines). The second genotype is a genotype with some
184 robustness, as exemplified by the left-most hypothetical genotype G (black circle) in
185 Figure 2b. Half of the eight neighbors of this genotype have the same phenotype P as
186 itself (solid lines), whereas the other half (dashed dark blue lines) have new phenotypes
187 (not shown in the figure), all of which might be different from each other.

188 Which of these two genotypes is phenotypically more variable, in the sense that it
189 can access a greater number of novel phenotypes through mutation? The answer is
190 genotype G in Figure 2a. Because it is minimally robust, all of its eight neighbors have a
191 phenotype different from P . In contrast, the left-most genotype in Figure 2b is more
192 robust but less phenotypically variable, because only some of its neighbors have a
193 phenotype different from P . This is the core argument why robustness reduces
194 phenotypic variability, cast in the abstract but precise language of genotype space.

195 Figure 2b also illustrates why this argument is flawed. The robust left-most
196 genotype G has neighbors with the same phenotype P as itself, one of which, G' , is
197 shown as the middle circle in Figure 2b. This neighbor G' itself has a neighborhood,
198 which contains five genotypes with new phenotypes (dashed medium blue lines) that may
199 not occur in the neighborhood of G itself. G' has three further neighbors (solid lines) that
200 have the same phenotype P , one of which is shown as G'' . The neighborhood of G''

201 contains four genotypes with novel phenotypes (dashed light blue lines). In comparison
202 with the non-robust genotype, from which up to 8 different novel phenotypes are
203 accessible, the robust genotype can – merely through the neighbors shown in the figure –
204 access up to $(4+5+4=13)$ new phenotypes, more than if it was not robust. This argument
205 takes only into account the neighborhoods of G' and G'' , not the neighborhoods of
206 several other neighbors of G with the same phenotype P . Thus, the actual number of
207 different accessible phenotypes may be even higher for the robust genotype.

208 Three concrete examples show how much higher. These examples are based on
209 three different natural RNA molecules, a guide RNA, a ribozyme, and a telomerase
210 (Figure 2c, same color coding as Figures 2a and 2b). The phenotype in question is RNA
211 secondary structure, a planar fold that occurs when an RNA molecule folds onto itself
212 through internal base pairing. Secondary structure is essential for the function of many
213 RNA molecules, and thus in itself a phenotype worthy of study. It can be predicted
214 computationally using known biophysical RNA folding principles (Hofacker et al. 1994).

215 Each of the three panels of Figure 2c shows, in a black bar, the maximally
216 possible number of novel secondary structure phenotypes accessible to an RNA genotype
217 if its phenotype were minimally robust (as in Figure 2a). This number equals the total
218 number of neighbors of an RNA molecule, which equals three times the molecule's total
219 length L in nucleotides, because every one of the molecule's nucleotides can mutate into
220 three other nucleotides. For example, for the guide RNA with length $L=40$, these would
221 be $3 \times 40 = 120$ neighbors and novel phenotypes.

222 The dark blue bars indicate the *actual* number of accessible new phenotypes in
223 the neighborhood of a genotype. This number was obtained by computing the minimum
224 free energy secondary structure phenotype of each neighbor of a genotype with an RNA
225 folding algorithm (Hofacker et al. 1994). For the guide RNA, this number is 40, many
226 fewer than the maximally 120 new phenotypes without robustness, thus confirming the
227 principle illustrated Figures 2a and 2b.

228 The medium and light blue bars indicate the total number of different phenotypes
229 that are accessible up to two and three mutations away from the starting genotype. This
230 number – obtained again by computing the structures for all genotypes in these
231 neighborhoods – is much larger than the 120 maximally attainable phenotypes in the

232 absence of robustness. Specifically, for the guide RNA discussed here 746 (medium blue
233 bars) and 1174 (light blue bars) distinct new phenotypes become accessible two and three
234 mutations away. Thus, robustness allows access to many novel RNA phenotypes.

235 I note that the genotypes considered in this computation (up to two mutations
236 away from G and with the same phenotype P) are a tiny fraction of the genotypes that
237 form a typical genotype network. For example, a simple calculation shows that there are
238 only 7.2×10^3 total genotypes that differ from the guide RNA with $L=40$ in no more than
239 two mutations. However, the size of this guide RNA's genotype network – which can be
240 computed – equals approximately $9.1 \times 10^{17} (\pm 3.3 \times 10^{16})$ genotypes, and is thus more than
241 14 orders of magnitude larger (Jörg et al. 2008). (Astronomically large genotype
242 networks are typical for natural RNA molecules.) It is currently not feasible to compute
243 the number of distinct phenotypes near a genotype network this large, but this number
244 would surely also be astronomical.

245 Taken together, these observations mean that the mere existence of robustness
246 makes a dramatic difference in phenotypic variability. The difference is that between the
247 few novel phenotypes accessible in the immediate neighborhood of a non-robust
248 genotype, and the extremely large number of new phenotype accessible from the
249 neighbors of a genotype network. By bringing genotype networks into existence,
250 robustness makes vastly more new phenotypes accessible.

251 I will next discuss two lines of experimental evidence that indicate how important
252 this principle is for the discovery of new molecular phenotypes.

253 The first experiment revolves around one natural and one synthetic ribozyme
254 (Schultes & Bartel 2000). The natural ribozyme which catalyzes its own cleavage is
255 encoded by the human hepatitis delta virus. The synthetic RNA is the so-called class III
256 self-ligating ribozyme, which joins an oligonucleotide substrate to its own 5' end. The
257 two ribozymes are unrelated in sequence and fold. (Schultes & Bartel 2000). Schultes and
258 Bartel (Schultes & Bartel 2000) were able to design a mutational path through RNA
259 genotype space that starts from either one of the ribozymes and leaves its native activity
260 largely intact, until it reaches a hybrid ribozyme that is more than 40 mutational steps
261 from each starting point. This hybrid can act both as a self-cleaving ribozyme and as a
262 ligase, albeit with lower catalytic activity than the starting enzymes. By constructing a

263 hybrid ribozyme and constructing a path through sequence space back to its ancestors,
264 this work makes two key points. First, many consecutive changes in a genotype are
265 possible that do not affect an RNA's (catalytic) phenotype. Without robustness, this
266 would not be the case. Second, these changes can be very important intermediate steps to
267 create a new catalytic function. Similar principles have been suggested for other
268 ribozymes (Beckert et al. 2008; Huang & Szostak 2003).

269 This experiment demonstrated the role of robustness in the origin of new
270 phenotypes using an engineered path through genotype space. Biological evolution does
271 not use such pre-meditated paths, but random changes in evolving populations. The next
272 experiment shows that robustness is also highly relevant in such populations (Hayden et
273 al. 2011). The experiment revolves around the concept of cryptic variation. Cryptic
274 variation is genotypic variation in a population that is not visible on the level of
275 phenotype (Gibson & Reed 2008). An example is variation in genotypes on the same
276 genotype network. Cryptic variation cannot exist without mutational robustness. The
277 experiment asks whether cryptic variation can help a population find a new and superior
278 genotype during an evolutionary search.

279 The study system was again an RNA ribozyme, the so-called *Azoarcus* ribozyme
280 (Tanner & Cech 1996), which is a naturally occurring ribozyme that can ligate a short
281 RNA fragment to its own end. One can subject populations of ribozymes like this to
282 repeated cycles of mutagenesis, and to selection to maintain or to modify this catalytic
283 activity (Beaudry & Joyce 1992).

284 The experiment in question compared two different kinds of populations, one that
285 consisted mostly of identical or similar genotypes, all of them copies of a single ribozyme
286 sequence, and another that consisted of many diverse genotypes (Figure 3). Ribozymes in
287 the two kinds of populations had similar catalytic activities on a specific RNA substrate,
288 such that the average activities of the populations were indistinguishable. In other words,
289 the first kind of populations contained little or no cryptic variation, whereas the second
290 kind contained lots of it. The experiment then changed the chemical environments in
291 which these populations existed. That is, it exposed both kinds of populations to a new,
292 chemically modified RNA substrate, on which the starting ribozyme has low catalytic

293 activity. Both kinds of populations then experienced repeated rounds of mutagenesis and
294 selection that favored high activity on the new substrate (Hayden et al. 2011).

295 Populations with much cryptic variation adapted up to six times faster to the new
296 chemical environment (Figure 3b) (Hayden et al. 2011). They did so through genetic
297 changes that improved the ribozyme's catalytic activity on the new substrate. DNA
298 sequencing of thousands of genotypes from evolving populations subsequently showed
299 why: There exists a superior genotype, and populations with much cryptic variation
300 discover this genotype faster, because they are genotypically more diverse, and contain
301 genotypes that are already close in genotype space to the superior genotype. In sum, this
302 experiment shows that cryptic variation – a consequence of robustness – can accelerate
303 evolutionary adaptation to a new chemical environment. Similar phenomena are likely to
304 exist in proteins (Amitai et al. 2007; Bloom et al. 2007), even though we do not yet have
305 proof that cryptic variation can accelerate the rate of adaptation for them.

306 **Robustness can facilitate phenotypic variability by affecting evolutionary**
307 **dynamics on large genotype networks.** Thus far, I argued that robustness can facilitate
308 variability through its *static, structural* role in organizing genotypes with the same
309 phenotype into genotype networks. I will next turn to a second role of robustness, which
310 builds on the first role: Robustness can increase variability through its influence on the
311 evolutionary *dynamics* of populations on genotype networks. To this end, I will first
312 introduce some further terminology from the genotype space framework. After that, I will
313 explain how robustness can affect the evolutionary dynamics of populations, and then
314 discuss a mix of pertinent experimental, computational, and comparative data.

315 Some phenotypes have very large associated genotype networks and are formed
316 by many different genotypes. Others have much smaller genotype networks and are
317 formed by fewer genotypes (Ciliberti et al. 2007a; Ciliberti et al. 2007b; Jörg et al. 2008;
318 Li et al. 1996; Samal et al. 2010; Schuster et al. 1994). This difference in genotype
319 network size is accompanied by a difference in the average robustness of genotypes
320 encoding these phenotypes. Specifically, the larger a phenotype P 's genotype network is,
321 the greater is also the average fraction of each genotype's neighbors with this phenotype
322 P . In other words, genotypes on a large genotype network are more robust to mutations
323 than genotypes on a small genotype network (Reidys et al. 1997; Wagner 2008). This

324 observation allows one to extend the definition of robustness I used thus far – the number
325 of a *genotype*'s neighbors with the same phenotype. Specifically, one can define the
326 robustness of a *phenotype* as the average robustness of all genotypes encoding it.
327 Phenotypes with large genotype networks are more robust.

328 Now consider a population of initially identical genotypes with the same
329 phenotype *P*. Subject the population to repeated cycles (“generations”) of mutations and
330 selection that confines the population to the genotype network of the phenotype *P*. After
331 a given number of generations, examine the neighborhood of each individual in the
332 population, and enumerate the number of *different* or *unique* phenotypes that occur in
333 these neighborhoods. That is, if the same phenotype is formed by two or more genotypes
334 in these neighborhoods, count it only once. This number is a measure of the phenotypic
335 variability of an entire population, not just of a single individual. It encompasses all
336 phenotypes that a population can access through a single nucleotide change in some
337 individual.

338 To understand how phenotypic variability is affected by phenotypic robustness, it
339 is necessary to examine how populations evolve on genotype networks that vary in size.
340 Recent work on populations of evolving RNA molecules has done that for
341 computationally predicted secondary structure phenotypes (Wagner 2008). It found that
342 populations whose phenotypes have greater robustness also show greater phenotypic
343 variability. This observation is based on thousands of randomly sampled phenotypes, and
344 is thus independent of any one particular phenotype. It is a generic feature of RNA
345 genotype space.

346 To understand this observation, one needs to understand two different phenomena
347 with opposite effects on phenotypic variability. The first of these is the number of
348 different phenotypes in the neighborhood of any one genotype. This number will be
349 lower for highly robust phenotypes, because their genotypes have, on average, more
350 neighbors with unchanged phenotype.

351 The second phenomenon is the rate at which a population spreads through a
352 genotype network. This rate is determined by the likelihood that a mutation is deleterious,
353 that is, that it does not preserve the phenotype *P*. Individuals suffering deleterious
354 mutations are eliminated from the population, which slows the population's

355 diversification. The greater the incidence of such mutations, the slower a population
356 spreads through genotype space. A lack of robustness thus acts like a brake on the
357 genotypic diversification process of a population. This diversification process is
358 important, because the fraction of unique phenotypes in the neighborhoods of two
359 genotypes increases with the distance between them (Figure 1). This means, as I
360 discussed earlier, that populations with greater (cryptic) genotypic diversity can access
361 more novel phenotypes through mutations. They have greater phenotypic variability.

362 In sum, considering only the first phenomenon, high phenotypic robustness entails
363 low variability. In contrast, considering the second phenomenon, high robustness entails
364 high variability. In evolving populations, these two phenomena have opposite effects on
365 variability. For RNA secondary structure phenotypes, the second phenomenon – greater
366 population diversity – exerts the dominant influence on phenotypic variability. This is
367 why more robust phenotypes have higher phenotypic variability overall (Wagner 2008)).

368 Observations from computational analyses like these can help us appreciate that
369 we must study the dynamics of evolving populations – not just individual genotypes – to
370 understand the quantitative link between robustness and phenotypic variability. A
371 combination of experimental evolution work and comparative analyses further indicate
372 that robustness also matters for real molecules.

373 One class of experiments worth highlighting regards chaperones, proteins that
374 assist other proteins in folding, and help maintain their fold and function. Chaperones can
375 reduce the effects of environmental stress, such as high temperature, and they can
376 eliminate the deleterious effects of some mutations that reduce protein stability and
377 abolish a protein's activity (Fares et al. 2002; Hartl & Hayer-Hartl 2002). In the language
378 of genotype space, one could say that a chaperone increases the size of the genotype
379 network of a particular phenotype, because it can render some mutations neutral that
380 would otherwise be deleterious or lethal. In other words, a chaperone can make a
381 phenotype more robust. Recent laboratory evolution experiments on four different
382 enzymes expressed in *E. coli* support this notion. Specifically, populations of these
383 enzymes tolerated twice as many amino acid changes and evolved greater genotypic
384 diversity when large amounts of a chaperone were present. One of these enzymes, a
385 phosphotriesterase that can hydrolyze the pesticide paraoxon, was also subjected to

386 laboratory evolution for activity on a new catalytic substrate, 2-naphtylhexanoate.
387 Populations of this enzyme attained higher activity and specificity on the new substrate
388 when the chaperone was overexpressed. In sum, high robustness – in this case induced by
389 a chaperone – is associated with superior evolutionary adaptation (Tokuriki & Tawfik
390 2009a).

391 Laboratory evolution experiments of enzymes also provide relevant evidence
392 independent from that of chaperones (Amitai et al. 2007; Bloom et al. 2006; Bloom et al.
393 2005). A case in point is cytochrome P450, which belongs to an enzyme superfamily
394 whose members hydroxylate many different substrate molecules. The relevant
395 experiments mutagenized different variants of this enzyme that differed in their
396 thermodynamic stability, and in their robustness to mutations. The stable and more robust
397 variants of cytochrome P450 more readily evolved the ability to hydrolyze new substrates,
398 such as the anti-inflammatory compound naproxen (Bloom et al. 2006; Bloom et al.
399 2005).

400 Experiments like these can show how robustness can facilitate evolutionary
401 adaptation on short, laboratory timescales. They are silent about how this relationship
402 translates onto the enormous timescales on which proteins diversified in life's history.
403 Only a comparative analysis of the phenotypic diversity of proteins – a record of past
404 evolutionary innovation – can answer this question. That is, it can answer whether highly
405 robust protein phenotypes have adopted many different functions in their evolutionary
406 history.

407 Such an analysis has become possible with the ability to estimate the robustness
408 of protein folds (not just genotypes) to point mutations (England & Shakhnovich 2003),
409 and to estimate the functional diversity of proteins, for example through well-catalogued
410 enzyme functions. A recent study of 112 ancient protein folds showed that highly robust
411 folds have evolved greater functional diversity, using different and complementary
412 measures of functional diversity (Ferrada & Wagner 2008).

413 In sum, evidence that ranges from computational to comparative and
414 experimental suggests that more phenotypic robustness can increase the ability of RNA
415 and protein molecules to adapt and diversify in evolution. The computational work I
416 discussed earlier in this section helps explain why: Phenotypic robustness accelerates the

417 spreading of populations through a genotype network, makes a broader spectrum of
418 phenotypes accessible through mutation, and thus increases the odds of encountering a
419 beneficial phenotype.

420 **Robustness can help avoid conflicts between individuals and populations**
421 **in bringing forth phenotypic variation.** It is sometimes stated that biological systems
422 bring forth novel features because this ability has been “selected for”. This assertion is
423 naïve and problematic. To see why, consider mutator alleles, variants of genes that can
424 increase an organism’s mutation rate (and phenotypic variability) dramatically
425 (Sniegowski et al. 2000; Taddei et al. 1997). Mutators can be quite abundant in bacterial
426 populations (Taddei et al. 1997). A facile explanation for their abundance resorts to the
427 advantage they confer to a *population*: they help create many new phenotypes. Even
428 though most of these new phenotypes may be deleterious, the few beneficial phenotypes
429 may help the population survive in a challenging environment. However, this advantage
430 is overshadowed by a great disadvantage to the *individual* – typically just one in a large
431 population – who first acquires a mutator: Because most mutations are deleterious,
432 carrying the mutator genotype is detrimental to this individual, and will thus often lead to
433 its extinction (Sniegowski et al. 2000). A conflict thus exists between the interests of a
434 population and that of an individual. How this conflict is resolved may depend on details
435 of a population’s life history and environment. Sometimes the conflict may be resolved in
436 favor of the population, at other times in favor of the individual. In the latter case,
437 variability would be reduced. Thus, the emergence of phenotypic variability in evolution
438 is not a foregone conclusion. Similar conflicts exist for other mechanisms that facilitate
439 phenotypic variability (Kirschner & Gerhart 1998).

440 Robustness as a variability principle, however, has a remarkable property: it
441 can avoid this conflict. In RNA and proteins, where more robustness promotes greater
442 variability, the interests of the individual and the lineage can be perfectly aligned. This is
443 a simple consequence of how robustness influences the evolutionary dynamics of
444 populations. Consider a population where stabilizing selection maintains a well-adapted
445 phenotype. If this phenotype is highly robust, it is not easily perturbed through mutation
446 or environmental changes, because the two kinds of robustness are usually correlated
447 (Ancel & Fontana 2000; Lehner 2010; Masel & Siegal 2009; Meiklejohn & Hartl 2002;

448 Szollosi & Derenyi 2009; Wagner 2005b). Such robustness is advantageous for an
449 individual that has this phenotype, because this individual experiences fewer
450 perturbations with deleterious effects. At the same time, it is also advantageous for
451 populations of such individuals. The reason lies in the evolutionary dynamics I discussed
452 in the preceding section: Robust phenotypes in both RNA and protein molecules show
453 greater phenotypic variability, and can become phenotypically more diverse on
454 evolutionary time scales (Ferrada & Wagner 2008; Wagner 2008). Robustness can thus
455 benefit both an individual and its lineage. Evolutionary conflicts are among the most
456 serious impediments to adaptation, which makes their avoidance here even more
457 significant (Futuyma 2009). Their general role in the evolution of phenotypic variability
458 needs further study.

459 **Summary and open questions.** In sum, I have distinguished between two
460 roles of robustness in evolutionary adaptation and innovation, a structural and a dynamic
461 role. First, robustness causes the existence of genotype networks, complex web-like
462 structures formed by genotypes with the same phenotype, which facilitate phenotypic
463 variability. Second, a robust phenotype can help the evolutionary exploration of new
464 phenotypes in macromolecules by accelerating the dynamics of change in an evolving
465 population.

466 Many open questions persist in this young research field. They fall into two
467 broad classes. The first regards quantitative aspects of evolutionary dynamics. How do
468 the sizes of evolving populations and their mutation rates interact with robustness to
469 influence phenotypic variability? Do the principles I discuss here also apply in
470 environments that change rapidly and continually, where populations always track a
471 moving optimal phenotype? Do these principles apply to systems with extremely high or
472 low robustness? Does robustness also accelerate the evolutionary exploration of new
473 phenotypes in systems other than macromolecules, such as evolving regulatory circuits or
474 metabolic networks? Population genetic models and computational analyses of genotype-
475 phenotype relationships are beginning to tackle these questions (Ancel & Fontana 2000;
476 Draghi et al. 2010; Draghi & Wagner 2008; Espinosa-Soto et al. 2010; Rodrigues &
477 Wagner 2011). However, we still lack a sufficient body of concordant evidence from
478 different approaches to draw general conclusions.

479 A second class of questions regards evolutionary changes in robustness itself.
480 Experimental and comparative work suggests that the robustness of macromolecules can
481 change on evolutionary time scales (Montville et al. 2005; Sanjuan et al. 2006; Wagner &
482 Stadler 1999). If robustness benefits both individuals and populations, then natural
483 selection may favor robust phenotypes. If so, the robustness of phenotypes might increase
484 over time. Only tentative evidence exists that naturally occurring phenotypes may be
485 unusually robust (Cowperthwaite et al. 2008; Jörg et al. 2008). We do not yet know the
486 causes of this robustness, we do not yet have relevant evidence from other system classes,
487 and we are ignorant about the population genetic conditions under which such an increase
488 would occur. Only with such evidence will we be able to answer a last and most
489 fundamental question: Does robustness evolve in a way that facilitates evolutionary
490 adaptation and innovation?

491

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499 **Figure Captions**

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501 **Figure 1: Connected genotype networks facilitate accessibility of diverse phenotypes.**

502 The figure schematically represents a hypothetical set of genotypes (small open black
503 circles) in genotype space (rectangle) that share the same phenotype and form a genotype
504 network; neighboring genotypes are connected by black lines. Colored circles indicate
505 genotypes with different phenotypes. The two large dashed circles denote the
506 neighborhood of two different genotypes on the genotype network. The upper left
507 neighborhood contains two novel phenotypes (blue and orange), whereas the lower right
508 neighborhood contains two different novel phenotypes (beige and green). The figure
509 illustrates that many different novel phenotypes can be accessed from a connected
510 genotype network that spreads far through genotype space. Note that a two-dimensional
511 figure like this cannot capture many features of high dimensional genotype spaces. These
512 include that individual genotypes can have thousands of neighbors, not just the few
513 shown here, and that the phenotypes shown in color also have vast genotype networks
514 that are not shown. Adapted from (Wagner 2011c). **Permission from OUP requested.**
515

516 **Figure 2: Robustness makes many phenotypic variants accessible to mutations.**

517 Circles in a) and b) represent genotypes with some hypothetical phenotype P , straight
518 lines connect a genotype to its neighbors (not all neighbors of a genotype are shown as
519 circles), solid lines connect a genotype to a neighbor with the same phenotype P , dashed
520 lines connect a genotype to a neighbor with a new phenotype. **a)** The hypothetical
521 genotype G shown here has no robustness, that is, no neighbors with the same phenotype.
522 All eight of its neighbors have new phenotypes. It thus shows maximal phenotypic
523 variability. **b)** All three genotypes shown are to some extent robust, that is, they have
524 neighbors with the same phenotype P . Dark, medium, and light blue dashed lines point to
525 genotypes with new phenotypes that are one, two, and three mutations away from the
526 left-most genotype in b). Robustness makes more new genotypes accessible. See text for
527 details. **c)** illustrates this principle through the actual number of new accessible
528 phenotypes for three different natural RNA molecules (horizontal axis), and for
529 computationally predicted (Hofacker et al. 1994) minimum free secondary structure

530 phenotypes in their neighborhood. Each of these molecules has some phenotype P (not
531 shown). The black bar in each of the three panels indicates the maximally possible
532 number of different phenotypes one mutation away from an RNA genotype G . This
533 number is equal to $3L$, where L is the number of nucleotides in a molecule. It would be
534 attained only in the absence of robustness, as in panel a). Dark, medium, and light blue
535 bars indicate, just as in panel b), the number of distinct new phenotypes that are
536 accessible in the neighborhood of the molecule G (“1 mutation away”), in the
537 neighborhoods of all its neighbors G' with phenotype P (“2 mutations away”), and in the
538 neighborhood of the neighbors G'' of G' with phenotype P (“3 mutations away”). Data in
539 c) are averages (error bars: one standard error of the mean) from ten inversely folded
540 (Hofacker et al. 1994) RNA genotypes per RNA secondary structure phenotype. The
541 individual RNA molecules have been obtained from the functional RNA database
542 (<http://www.ncrna.org/frnadb>) (Kin T et al. 2007). They include a guide RNA
543 (*Trypanosoma brucei*, fRNAdb accession number L25590, $L=40$ nt), a hammerhead
544 ribozyme (*Schistosoma mansoni*, acc. no: AF036740, $L=43$), and a telomerase
545 (*Moneuplotes crassa*, acc. no: AF061109; $L=33$ nt). See (Jörg et al. 2008, Table S1) for
546 predicted secondary structure phenotypes P of these RNA molecules.

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549 **Figure 3: Cryptic variation can facilitate evolutionary adaptation. a)** The large
550 rectangles in both panels represent a genotype space into which a hypothetical genotype
551 network is inscribed (gray open circles connected by gray lines). The colored circles
552 symbolize individuals in a population on this genotype network. The left population (blue
553 circles) is less genotypically diverse, and thus contains less cryptic variation than the
554 population on the right (yellow circles). **b)** A laboratory evolution experiment showing
555 how fast two populations of ribozymes with indistinguishable phenotype (catalytic
556 activity on an RNA substrate) but different amounts of cryptic genotypic variation adapt
557 evolutionarily to a new RNA substrate. As in panel a), blue and yellow correspond to
558 populations with little and much cryptic variation. The horizontal axis shows time in
559 generations, the vertical axis shows a measure of the biochemical activity of each

560 population on the new RN substrate. The population with more cryptic variation adapts
561 faster (Hayden et al. 2011).

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