Anton Crombach *Editor*

Evolutionary Systems Biology

Advances, Questions, and Opportunities

Second Edition

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ISBN 978-3-030-71736-0 ISBN 978-3-030-71737-7 (eBook)
https://doi.org/10.1007/978-3-030-71737-7

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

In 2012, a first book on *Evolutionary Systems Biology* was published (Adv. Exp. Med. Biol. 751), addressing the question of what this (back then emerging) field precisely is. The book put on display a rich variety of topics and approaches that touched upon a combination of the three terms, *evolution*, *systems*, and *biology*. Now, almost a decade later, this new book provides a moment to take a pause and reflect on the current status and future aspirations of Evolutionary Systems Biology.

The core goal of *Evolutionary Systems Biology* is to study the genotype-phenotype (GP) mapping—in the broad sense of the term. The GP map is an abstraction to capture that high-level phenomena are generated by lower-level mechanisms. This multilevel reasoning can be applied at various scales, e.g., RNA and other macromolecules that fold into 2- or 3-dimensional structures, networks of metabolites that determine a cell's physiological response, or gene regulatory networks that form patterns across tissues in multicellular organisms. Moreover, *Evolutionary Systems Biology* addresses not only the question of how the GP map works, but especially how evolution shapes the mapping, and vice versa, how the mapping shapes evolutionary opportunities. Soyer and O'Malley (BioEssays 35:696–705, 2013) concluded that this is not a definition of a well-demarcated field, nor a discipline, but that it hints at Evolutionary Systems Biology becoming a general mode of research: it prescribes how one looks at biology and how one asks questions about biological systems. The exact methodology and precise topic are only of secondary importance. Given my experience in the field and my interactions with colleagues, I think this assessment remains spot on and provides a viable way forward for thinking about Evolutionary Systems Biology in the years to come.

This book should thus be viewed as a snapshot of the current state of affairs in Evolutionary Systems Biology. My goal has been to capture some of the key developments during this last decade. Perhaps the most important one is a reduction in fear for "big" systems approaches, which has to happen as we increasingly make use of genome-wide data sets and large parameter scans—be they experimental (mutants, genetic screens), computational simulations, or complex mathematical models. In addition, each of the chapters is from a researcher that I know to be a cross-disciplinary scientist, either because they themselves have done both modeling

and experiments or because they work extensively with colleagues who are on opposite sides of the wet-dry spectrum. As a result, the book brings a combination of topics and viewpoints.

The first chapters (Chaps. 1–3) address perennially difficult topics of complexity and predictability from a theoretical point of view. Beslon et al. (Chap. 1) probe the origins of complexity and ask why evolutionary systems come up with "complex" and "complicated" solutions, while a simple solution could be easily constructed by a human. Hogeweg (Chap. 2) exploits *in silico* evolution to mitigate the parameter curse and at the same time shows that models with additional degrees of freedom can be more informative than overly minimalist ones. In turn, Kaneko and Furusawa (Chap. 3) show that even if biological systems are high-dimensional, complex entities, their evolutionary paths may collapse to a much lower-dimensional phenotypic space. This provides a step towards a mechanistic explanation for the results of Chaps. 1 and 2, and an opportunity to develop a general theory for predicting evolution.

The next two chapters (Chaps. 4 and 5) can be considered critical reviews of success stories. Crombach and Jaeger (Chap. 4) provide a progress report on their use of reverse engineering and *in silico* evolution. They review their studies on the evolution of a regulatory network used by fly embryos to lay down their body plan. In a similar vein, Onimaru and Marcon (Chap. 5) document their work on data-driven modeling of the fin-to-limb transition in vertebrates. Both chapters address what a systems-level approach, innovative experiments, and (evolutionary) models may bring. Importantly the chapters also address the practical features that enabled the studies. One common basis for these successes is the availability of a wealth of knowledge on the model systems studied—this know-how was obviously generated in an era and through disciplines that did not stress the "system" component, e.g., molecular biology and genetics. My hope is that new technologies and novel theoretical insights enable us to generate similarly rich knowledge bases in a "leapfrog" manner for many other (non-model) systems of interest.

Complementing Chaps. 1–4 that use *in silico* evolution, Chaps. 6 and 7 elaborate on experimental evolution in the wet-lab. Helsen and Jelier (Chap. 6) thoroughly review how studying "evolution in the lab" has helped our understanding of phenomena like clonal interference and evolvability. Moreover, they highlight the novel approaches that are shaping the emerging field of evolutionary systems genetics. Next, Baier and Schaerli (Chap. 7) capture the fast-moving field of evolutionary synthetic biology. They elaborate on the advantages and challenges of using synthetic systems to study evolutionary dynamics, from simple engineered regulatory circuits to entire artificial genomes. From gene regulation, Johnson et al. (Chap. 8) then move to the state of the art in our understanding of the metabolic system. They argue that taking an explicit evolutionary viewpoint, especially if combined with (novel) omics approaches, will enable progress on various fronts including eco-evolutionary systems where single cell dynamics are interlocked with population-level behavior.

In the three closing chapters, we move back from data to analysis and modeling. Two notions permeating all of Evolutionary Systems Biology are robustness and

evolvability. Aguilar-Rodríguez and Payne (Chap. 9) discuss these concepts from various angles within the frame of transcriptional regulation. As such, they provide a crucial foundation to many of the topics discussed in the rest of the book, and beyond. Chapters 10 and 11 make convincing cases for improving the computational and mathematical toolkit that we use to investigate evolving biological systems. Salazar-Ciudad et al. (Chap. 10) argue for the inclusion of epigenetic factors (f.i. cellular behavior, a tissue context), which are commonly ignored in purely genetic models, to improve the explanatory power of models (cf. Chap. 2). And Jaeger and Monk (Chap. 11) advocate the study of dynamical modularity through a decomposition of a system's behavior, contrasting more traditional methods that focus solely on (gene) interaction structure. Both chapters remind us that the set of glasses we look through has a strong influence on the results we observe and the conclusions we draw.

Finally, I would like to thank Orkun Soyer for the opportunity to embark on the adventure of this second book on *Evolutionary Systems Biology*, and the editorial team at Springer Nature led by Larissa Albright, Noreen Henson, and Sofia Valsendur for making it happen. I thank the authors and external reviewers for their hard work, wonderful contributions, and abundance of patience. I hope this book helps guide the field to transition into a valuable, general approach of studying biology.

Villeurbanne, France Anton Crombach

Contents

Of Evolution, Systems and Complexity

Guillaume Beslon, Vincent Liard, David P. Parsons, and Jonathan Rouzaud-Cornabas

Abstract The question of complexity in biological systems is recurrent in evolutionary biology and is central in complex systems science for obvious reasons. But this question is surprisingly overlooked by evolutionary systems biology. This comes unexpected given the roots of systems biology in complex systems science but also given that a proper understanding of the origin and evolution of complexity would provide clues for a better understanding of extant biological systems. In this chapter, we will explore the links between evolutionary systems biology and biological systems complexity, in terms of concepts, tools and results. In particular, we will show how complex models can be used to explore this question and show that complexity can spontaneously accumulate even in simple conditions owing to a "complexity ratchet" fuelled by sign epistasis.

1 Introduction

The link between evolution and complexity is as old as the evolutionary theory itself.[1] However, it is still largely controversial. There is a kind of a general agreement that complexity has globally increased along evolutionary history (McShea, 1996)—although it may have decreased in some lineages, typically endosymbionts and marine cyanobacteria (Batut et al., 2014)—and that all extant organisms can

[1] "[. . .] if we know of a long series of gradations in complexity, each good for its possessor, then, under changing conditions of life, there is no logical impossibility in the acquirement of any conceivable degree of perfection through natural selection" (Darwin, 1859, page 204, Chap. VI).

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_1

be considered "complex", but the question becomes highly controversial when it comes to the origin of complexity, i.e. to its ultimate causes (Mayr, 1961).

The question of the evolutionary origin and dynamics of biological complexity should logically be a central matter of interest in evolutionary systems biology (ESB). Yet, although not completely absent (Soyer & Bonhoeffer, 2006), it is surprisingly overlooked compared to questions about modularity or robustness, for instance. This is surprising as a proper understanding of the evolutionary origin of biological complexity could provide valuable clues to analyse extant biological systems and help decipher the structure-function relationship in biology.

In this chapter, we will first discuss the specific aspects of ESB questions with a focus on the question of the evolution of complexity (Sect. 2). We will then discuss why and how "complex models" are required to tackle this question in particular and ESB questions in general (Sect. 3). Finally, in Sect. 4, we will present a series of experiments based on the Aevol model that shed a new light on the old question of the evolution of complexity.

2 Of Evolution, Systems and Complexity

The definition of evolutionary systems biology is a recurrent matter of discussions within the community (Loewe, 2016; Soyer & O'Malley, 2013). But although defining precisely ESB and its relations to evolutionary biology on one side and systems biology on the other side could be an important matter for science policy, it is of low practical importance in performing science itself. Indeed, a field can afford to be fuzzily defined. What is important is to identify a coherent corpus of concepts, questions, tools and methods that are shared within a given scientific community. Ultimately, the latter determines what is considered a valid result within the boundaries of a given science and what is not.

Linked with the attempts to define ESB, one could typically discuss whether ESB embraces evolutionary biology and systems biology, whether ESB addresses those questions that are shared by both fields or whether ESB comes with its own set of questions. However, if, as suggested above, we consider this problem at the level of concepts, questions and tools, it immediately appears that, in terms of concepts, ESB unifies both fields, while, in terms of questions, ESB appears more as an intersection—if not as a disjunction—of evolutionary biology and systems biology.

Logically, ESB concepts include many concepts that originate, on the one side, from evolutionary biology (typically *Selection*, *Fitness*, *Population*, *Mutations*, *Drift*, *Epistasis*, *Fitness Landscape*, *Genotype-to-Phenotype (G2P) Map*, etc.) and, on the other side, from systems biology (*Regulation Networks*, *Metabolic Networks*, *Pathways*, *Motifs*, *Architecture*, etc.). Given the strong roots of systems biology itself in complex systems science, it is not surprising to find also concepts initially formulated in this domain, typically *Multiscale Systems*, *Complex Networks*, *Self-Organisation*, *Modularity*, *Feedback*, etc. Some concepts, initially formulated in one field, have strongly benefited from the interaction with the other and have fructified

therein. Typically, the concepts of *Robustness* and *Evolvability* that initially emerged in the context of complex systems science (Simon, 1962) are now fully integrated in the corpus of evolutionary biology (De Visser et al., 2003; Pigliucci, 2008; Wilke et al., 2001; Woods et al., 2011). Finally, two concepts have acquired a specific status within ESB: *Fitness Landscapes* and *Genotype-to-Phenotype Maps*. Initially formulated within the context of evolutionary biology and genetics (Alberch, 1991; Wright, 1932), they quickly became central in ESB, probably because they directly match systems biology and complex systems science concepts (respectively, *Energy Landscapes* and *Multiscale Systems*), hence enabling direct exchanges between both fields.

When it comes to questions, the integrative trend that we observe for concepts seems to be reversed, and ESB questions seem more to intersect than to unify evolutionary and systems biology. In fact, one could easily understand that ESB, that has emerged as a scientific field after both its "parent fields", cannot address the same questions they do. To be recognised as an independent scientific field, ESB must identify its own, independent, corpus of questions: questions that cannot be answered (or even addressed) within the fields of evolutionary biology or systems biology alone but that, on the opposite, require to link both fields. Given this, it appears that the questions addressed by ESB belong to two families:

(*i*) *What kind of system is likely to result from a given evolutionary process?*
(*ii*) *Knowing its properties, how is a given system likely to evolve?*

Of course, both kinds of questions are very general and lead to more specific ones (e.g. How may a change of the mutation rate/mutational pattern/population size/etc. impact a given characteristic of the system?), but all ESB questions ultimately belong to one of these two families. Importantly, both families of questions cannot be addressed by evolutionary biology or systems biology alone as they require manipulating concepts originating from both fields. Typical examples are the influence of a system's robustness on its evolution (Wilke et al., 2001) and the influence of evolutionary conditions on a system's modularity (Kashtan & Alon, 2005) or evolvability (Crombach & Hogeweg, 2008).

Surprisingly, among the properties of biological systems, *complexity* has received relatively little attention within the field of ESB. This is surprising because the question of the origin of biological complexity is a recurrent source of debate in evolutionary biology (Dawkins, 1997; Gould, 1996; McShea, 1996); because it is at the heart of several unsolved questions, among which the well-known C-value enigma (Elliott and Gregory, 2015; Thomas Jr., 1971); and because it clearly requires integrating concepts from evolutionary biology, complex systems sciences and systems biology.

It is difficult to identify the reasons for this lack of interest, but it is worth noting that this question is also absent from the systems biology corpus. This may have two explanations. First, systems biology focuses on extant organisms which are all considered complex. Considering that biological epistemology is rooted in classification and comparison, complexity can easily be ignored as a question. Second, and more importantly, from the outside of evolutionary biology,

complex biological systems are generally implicitly believed to be produced by selection (Lukeš et al., 2011). Complexity therefore seems a non-question, and systems biology focuses mainly on the *function* of pathways, networks or elements, following the naive idea that they are there because they have been selected for while they could very well flourish by the sake of random drift rather than by selective necessity (Lynch, 2007).

Finally, and directly related to the questions, is the matter of tools and methods. Methods change very quickly in science, and evolutionary biology, systems biology and evolutionary systems biology have all followed the "big data bioinformatics" trend (Greene et al., 2014), accumulating deep sequencing data on an ever-increasing number of organisms, systems and conditions. Now, accumulating data is of low interest if this data is not to be integrated into a coherent explanation framework—an unfortunate trend in the current "big data-machine learning" era (Pigliucci, 2009). On that matter, both evolutionary biology and systems biology share the same tradition of explanatory modelling to seek for unifying principles. Typical explanatory models comprise fitness landscapes and population genetics (in evolutionary biology) or complex networks and dynamical systems (in systems biology). But these tools only integrate concepts from their origin field. Addressing ESB questions requires tools that integrate concepts originating from both fields (e.g. tools integrating concepts such as fitness or drift *and* concepts such as modularity or complexity). In other words, we need tools to observe how systems evolve when systems biology provides us with tools to understand systems (independently from their evolution) and evolutionary biology provides us with tools to understand the evolution of independent items (e.g. genes or traits) but lacks tools to understand the evolution of systems integrating a large number of interacting elements. What ESB requires is a set of tools enabling to observe and analyse the evolution of systemic properties within the framework of Darwinian evolution.

3 Of Complex Evolution Models

Darwinian evolution has the advantage of being relatively simple to reproduce artificially. Indeed, since the emergence of Evolutionary Algorithms (EAs) in the 1950s and 1960s, it has been shown that virtually any data structure (variables, sets, vectors, matrices, programmes, networks, trees, etc.) can evolve in silico as long as it is subjected to a selection process and to a replication-with-variation process. While EAs use this capacity for optimisation purposes, it can also be used to design models of evolution. Now, since there is no limit to the complexity of the data structures that can evolve within such a framework, one can design data structures with specific systemic properties and experimentally—but computationally—study how these properties evolve under various evolutionary constraints (Fig. 1). This approach emerged within the field of artificial life (O'Neill, 2003; Ray, 1991) and differs from other modelling approaches in evolutionary biology by the use of "complex models", i.e. models in which the "digital organisms" (Adami, 2006) are

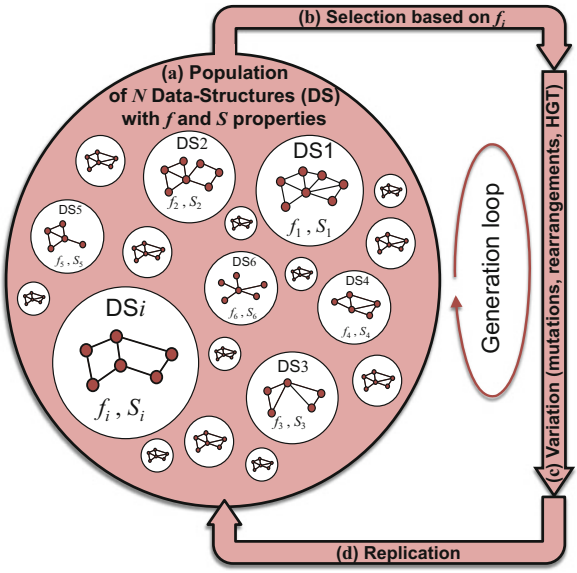

Fig. 1 Sketch of in silico experimental evolution. The model uses a population (**a**) of N data structures (DSs) (DS1, DS2,...DSi,...DSN), each associated with a computable fitness f_i and quantifiable systemic properties S_i. DSs are submitted to a generational loop composed of a selection process (**b**) based on f_i (but generally independent of S_i), a mutation process (**c**) that can modify the DSs (hence modifying f_i and S_i) and a replication process (**d**) that populates the forthcoming generation. The population is let to evolve under monitoring, usually for thousands of generations, before the trajectories $f_i(t)$ and $S_i(t)$ are analysed

purposefully complex, redundant and degenerated. This introduces many degrees of freedom in the organisms' genotype-to-phenotype map, hence allowing to study the evolution of organisms' structures in parallel with the evolution of their phenotypes and fitnesses. We will hereby call this approach "in silico experimental evolution" (ISEE) to emphasise its methodological similarities with in vivo experimental evolution (Batut et al., 2014; Hindré et al., 2012).

ISEE methodology is in some way closer to in vivo experimental evolutionary assays than to classical population genetic models. Indeed, after the initial design of the data structure (see Fig. 1) and of the experimental conditions, ISEE practitioners let populations evolve in controlled conditions. Then, exactly as experimentalists would, they analyse a posteriori the winning lineages (i.e. the final data structure's systemic properties) and the underlying evolutionary dynamics (including the sequence of fixed mutational events) to relate the digital organism's characteristics with the evolutionary conditions. Although ISEE is of course limited by its use of digital rather than biological organisms, it offers numerous advantages compared to in vivo assays. One is of course time. ISEE offers the possibility to simulate the evolution of hundreds of populations for hundreds of thousands of generations. But more than that, it allows for perfect fossil records of the simulations (Adami, 2006;

Hindré et al., 2012), hence making it possible to decipher contingent events from deterministic ones, a classical issue in evolution. Last but not the least, it allows for "impossible experiments" (O'Neill, 2003), i.e. experiments that would be infeasible in vivo, either because the experimental conditions would be impossible to set up or because too many confounding factors would interact within biological organisms.

Compared to other modelling approaches in systems biology, the interest of ISEE is straightforward: it allows for the simulation of the evolution of any systems biology model provided that one can define mutation and selection operators on that model (the former is generally relatively straightforward, but the latter may be tricky as it requires to be able to estimate the fitness corresponding to any parameter set). Compared to other modelling approaches in evolutionary biology (typically population genetics and quantitative genetics), ISEE is particularly suited to study the evolution of systems. Indeed, there is virtually no limit to the complexity of the data structures that could evolve within a computer, as long as variation and selection operators can be defined for these data structures. In the context of ESB, this allows to use data structures in which systemic properties (S in Fig. 1) can vary by mutations and to observe how these properties evolve under different conditions. As long as the underlying data structure is redundant (formally if the application DS $\rightarrow$ f is non-injective in Fig. 1), the systemic properties S can evolve independently from the fitness of organisms f. It is then possible to study the indirect interplay between evolution and these systemic properties. Indeed, in the last 20 years, many different data structures, including programmes (Adami, 2006; Wilke et al., 2001), networks (Espinosa-Soto & Wagner, 2010; Kashtan & Alon, 2005), differential equation systems (Soyer & Bonhoeffer, 2006), etc., have been used to study properties such as robustness (Wilke et al., 2001), evolvability (Crombach & Hogeweg, 2008) or modularity (Clune et al., 2013; Espinosa-Soto & Wagner, 2010; Kashtan & Alon, 2005). However, surprisingly, only few ISEE assays have tackled the question of complexity (Adami et al., 2000; Lenski et al., 2003; Soyer & Bonhoeffer, 2006). Apart from the aforementioned reasons, another issue limits the capacity to study complexity with digital models. Indeed, most ISEE data structures have a fixed complexity. For instance, in most network models, the strength of the connections can evolve, while the size of the network cannot (number of nodes, number of connections). In other words, one could state that most complex models are not complex enough to tackle the question of complexity.

4 Of Evolution of Complexity

4.1 Introduction

The evolutionary origin of complexity is a historical source of controversy. Basically, there are two main theoretical bodies, each emphasising one of the two main engines of evolution: variation and selection. A naive (but common) interpretation

of Darwinian theory states that selection is the driving force at the origin of extant structures. In this view, extant complexity is "naturally" due to selection, which can act through several mechanisms (Lukeš et al., 2011): complexity could be selected simply because complex organisms intrinsically have a higher fitness or because complex environments select for complex organisms, directly (Albantakis et al., 2014) or through interactions with other species or mates (Zaman et al., 2014); because complex organisms are more robust or more evolvable than simple ones (Soyer & Bonhoeffer, 2006); because multi-part systems require complex regulation mechanisms (Maslov et al., 2009); or because new genes are recruited to compensate for the negative pleiotropic effects previous mutations brought about (Pavlicev & Wagner, 2012). In contrast, according to neutralist theories, complexity increases by the action of random variations that spontaneously accumulate complexity, at least in some lineages. For instance, in S. J. Gould's "drunkard's walk" model, variation disperses lineages in the space of complexity levels, hence resulting in the emergence of lineages with ever-increasing complexities (Gould, 1996). In the "Zero-Force Evolutionary Law" (McShea & Brandon, 2010), variation disperses redundant components of the evolving system, hence increasing its complexity. Finally, in "Constructive Neutral Evolution" (Lukeš et al., 2011), complexity arises because mutations create dependencies in multi-component systems. So, while the precise mechanism by which variation may increase complexity depends on the authors, all neutral models agree on the idea that the main driving force is random drift.

There are many reasons why studying the evolution of complexity is difficult. First, definitions and measures of complexity are not firmly established, especially for biological systems (Adami, 2002). Second, biological systems are multiscale systems, and complexity can simultaneously rise and fall on different scales, as exemplified by the C-value enigma (Elliott & Gregory, 2015), by the strong streamlining of obligate bacterial symbionts (Moran, 2007) and by "major transitions" (Maynard Smith & Szathmary, 1997). Finally, another difficulty is the lack of experimental tools. Indeed, most of the above-mentioned hypotheses are based on thought experiments and/or on extant organisms without any possibility to observe the transition between the emergent life, supposedly "simple", and extant complex life forms.

By using complex models, ISEE can overcome these difficulties. Complexity is easier to define and measure on models than it is on real organisms, and, in a simulation, complexity can be monitored all along the evolutionary process on perfect fossil records. Moreover complex models can integrate different scales, and complexity can be quantified simultaneously at these different scales. Last but not the least, ISEE makes it possible to perform "impossible experiments" (O'Neill, 2003) in which multiscale organisms evolve in environments which are more or less demanding in terms of complexity. Indeed, one can define environments in which simple organisms can easily thrive (at least as easily as complex organisms) and environments in which complex organisms are likely to have a better fitness than simple ones. Then, by comparing the evolutionary outcomes in these two conditions,

it is possible to decipher the relative effect of selective and neutralist forces on the dynamics of complexity.

We used the Aevol in silico experimental evolution platform to implement this research programme. We let populations of initially simple individuals evolve in two different environments: a simple one and a complex one. The analysis of the winning lineages in the two contexts revealed that complexity evolves in both contexts and that complex organisms are not more complex in demanding environments. Moreover, in both environments, complexity increases are driven by selection, although in simple environments, simple organisms are far fitter than complex ones. These seemingly antagonistic results together show that complexity is driven by a ratchet mechanism, powered by selection but clicking in a direction opposite to the long-range selection gradient.

4.2 The Aevol Model

Aevol (https://www.aevol.fr) is an in silico experimental evolution platform developed by the Inria Beagle team. This chapter is not intended to promote Aevol, and since the platform has been described in numerous publications (Batut et al., 2014; Beslon et al., 2010; Knibbe et al., 2007; Liard et al., 2020; Rutten et al., 2019), we will confine ourselves to its core principles and focus on the structure of the information coding scheme as it is of utmost significance in our experiments.

The rationale of Aevol is that the structure of the fitness landscape of an organism is likely to be strongly determined by the structure of the biological information coding of this organism (i.e. by the structure of its genotype-to-phenotype (G2P) map) and by the variety of mutational operators that act on its genome. That is why Aevol uses a data structure that mimics precisely the biological genomic structure and that is decoded through a bio-like G2P map (Fig. 2a–d). "Organisms" are then embedded in an evolutionary loop that includes classical selection operators and a large variety of mutations operators including structural ones (Fig. 2f–h).

The core principles of Aevol make it ideally suited to study the evolution of biological complexity. Aevol is a multiscale model. As such, complexity can be monitored at different levels (genomic, proteomic and phenotypic), and the set of mutational operators includes large-scale chromosomal rearrangements (including duplications and deletions). Hence, genomic complexity can vary by gene duplication-divergence, possibly driving complexity to higher levels. Finally, similar to "real" biological G2P mappings, Aevol's mapping is redundant meaning that complexity can evolve partly independently at the different levels.

4.2.1 Information Coding in Aevol

In Aevol, each individual owns a genome containing its heritable information (Fig. 2a). The genome is a binary double-stranded sequence that is decoded in

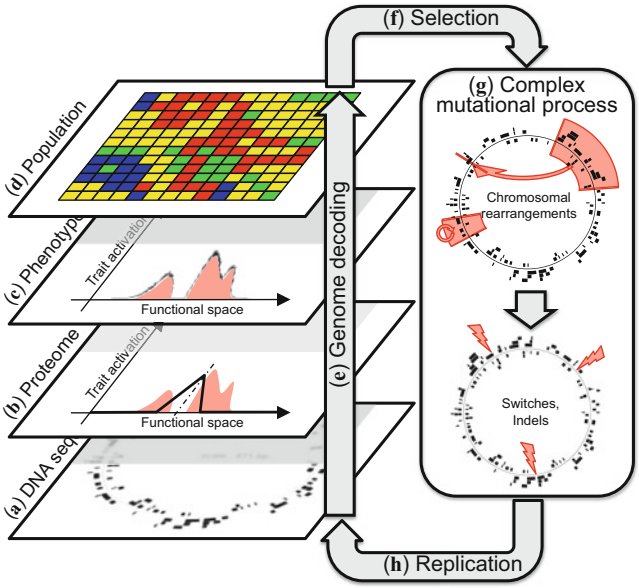

Fig. 2 The Aevol model. Genomes (**a**) contain genes decoded into proteins. Functional levels (proteome and phenotypes) use a mathematical abstraction where proteins are represented by triangular functions (**b**) and phenotypes are computed as the sum of all the proteins' functions (**c**). Fitness is computed through a curve-fitting task; the closer the phenotype from a "phenotypic target" (in red on parts **b** and **c**), the fitter the organism. Aevol is based on a generational loop with selection (**f**), a complex mutational process including rearrangements and mutations (**g**) and replication (**h**)

two steps: *transcription* and *translation*. Transcription relies on consensus signals (promoters) and hairpin-like structures (terminators) for transcription initiation and termination, respectively. Translation involves consensus ribosome binding sites and an artificial genetic code based on triplet codons (including START and STOP) which is used to compute the protein sequences. Importantly, these processes introduce redundancy and degeneracy: complex genomes can encode for simple proteomes (e.g. if all genes have the same sequence), and complex proteomes can be encoded on compact sequences (e.g. if genes share sequences by overlapping).

Given the sequence of a protein, Aevol computes its functional contribution. Now, although mimicking biological processes at the sequence level is feasible, it is impossible to compute the function of a protein from its primary structure in a realistic way. That is why Aevol uses an abstract mathematical formalism to describe the functional levels (proteins and phenotype). In Aevol, all functions are expressed in a one-dimensional continuous "functional space" (more precisely on the $[0, 1]$ interval) by an activation value in the $[-1, 1]$ interval (upper and lower bounds corresponding to maximum activation and maximum inhibition, respectively). In this space, proteins are described as triangle-shaped functions (Fig. 2b), themselves described by three parameters (mean m, height h and half-width w) computed from

three interlaced variable-length binary codes in the primary structure of the protein. Once all kernels have been computed from the protein set, they are summed to compute the phenotype (Fig. 2c).

Finally, in Aevol, the fitness is computed as the exponential of the difference between the phenotypic function and a "phenotypic target" indirectly representing the abiotic conditions the organisms evolve in. Classically, in Aevol, the target function is defined by a sum of Gaussians, hence requiring a virtually infinite number of protein-triangles to be perfectly fitted.

4.3 Designing an Impossible Experiment

The abstract mathematical formalism used to model functional levels in Aevol makes it possible to design experiments allowing to quantify the relative contribution of neutral and selective forces in the evolution of complexity. Indeed, it is difficult to quantify these relative contributions in a complex environment where they are both expected to increase complexity. Now, if one can let initially simple organisms evolve in a simple environment, then selective forces are supposedly inactive, and the evolutionary outcome shall only reflect neutral force contribution.

Using Aevol, we can easily design such an "impossible experiment": given Aevol's G2P map, we can design two kinds of environments. As stated above, in Aevol, genes are decoded into triangular kernel functions, and the sum of these kernels gives the organism's phenotype. As a result, because triangular phenotypic targets have the same shape as protein kernel functions, they don't require a complex proteome structure to be fitted (but they *can* be fitted by a complex proteome). On the opposite, Gaussian-shaped functions are impossible to fit with a finite number of protein-triangles. We used this property to design two phenotypic target functions, one simple and the other complex (Fig. 3). Then, we sampled random genomes to find "trivial" organisms with only one gene and let these initially trivial organisms evolve in both environments to quantify the final levels of complexity. Finally, since the mutation rate is also likely to influence complexity (Knibbe et al., 2007), we tested three different mutation rates: $\mu = 10^{-4}$, 10^{-5} and 10^{-6} mut.bp^{-1}.gen^{-1}. We simulated 100 independent evolution threads per condition with a constant population size (1024 individuals). Simulations were seeded with clonal populations of simple individuals with a single gene generated by random sampling of 5000 bp genomes. All simulations lasted 270,000 generations.

In silico experimental evolution makes it possible to store perfect fossil records. Hence, once the evolutionary runs are finished, one can take any organism at any generation and retrieve its lineage, including fixed mutations. Given an organism, one can also measure its characteristics, including its complexity, robustness and

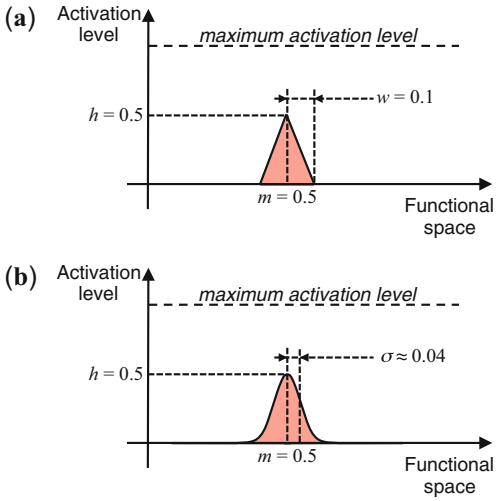

Fig. 3 The two phenotypic targets used in our experiments. (**a**): The simple target is an isosceles triangle of mean $m = 0.5$, half-width $w = 0.1$ and height $h = 0.5$. This shape can be perfectly fitted by a single protein-triangle (see Sect. 4.2). (**b**): The complex target is a Gaussian-shaped curve of mean $m = 0.5$, standard deviation $\sigma \approx 0.03989$ and a maximum value $h = 0.5$. Given the protein model in Aevol, this target cannot be perfectly fitted with a finite number of proteins

evolvability.[2]Here we measured fitness, complexity, robustness and evolvability of the ancestors of the best final organism (at generation 270,000) from generation 0–250,000 (the last 20,000 generations being ignored as ancestors cannot be considered to be fixed when they are too close to the final generation).

Following Adami (2002), we considered complexity as the quantity of information an evolving system integrates from its environment. Here we quantified complexity at two levels: the sequence level and the functional level. At the sequence level, *genomic complexity* (C_G) is directly measured by the number of "essential" base pairs on the genome (i.e. base pairs which, if mutated, would change the phenotype of the organism). Note that it may be very different from the genome size since the genome can accumulate non-coding sequences. It can also be shorter than the functional information stored on the genome since genes can share sequences by means of overlapping (see Fig. 4b). Measuring *functional complexity* C_P is not as straightforward. At the phenotypic level, complexity is directly driven by selection that imposes that the phenotype fits the phenotypic target. Now, the phenotypic function can result from the sum of a variable number of protein kernel functions. However, simply counting the proteins would overestimate complexity as

[2]In Aevol, robustness and evolvability are estimated by Monte Carlo sampling. 10,000,000 offspring of a given individual are generated. Robustness is estimated by the fraction of neutral offspring, and evolvability is estimated by the mathematical expectation of fitness improvement on the forthcoming generation.

two proteins can have different sequences but the same function. Hence, we used a finer description of the proteomic information to measure the functional complexity C_P: C_P is defined as the number of parameter values used to encode the protein set (i.e. the number of different m, different w and different h values in the kernel set).

To study the long-term fate of simple vs. complex organisms, we also defined a qualitative classification procedure. A trivial option would have been to define a threshold on the quantitative measures, but this would be arbitrary. Hence, we classified organisms according to their functional structure: in Aevol, if all the proteins of an organism have the same m and w (i.e. all the proteins have the same function, possibly with different levels of activity h), then their functions produce a triangular phenotype with the same characteristics. We used this property to define two classes. Organisms are called "simple" if all their proteins have the same function (in mathematical terms, if the sum of all kernel functions is a kernel). Importantly simple organisms may contain many different proteins differing in their efficiency h. Hence, simple organisms can have variable levels of genomic (C_G) and functional (C_P) complexities. Figure 4 shows examples of simple and complex organisms.

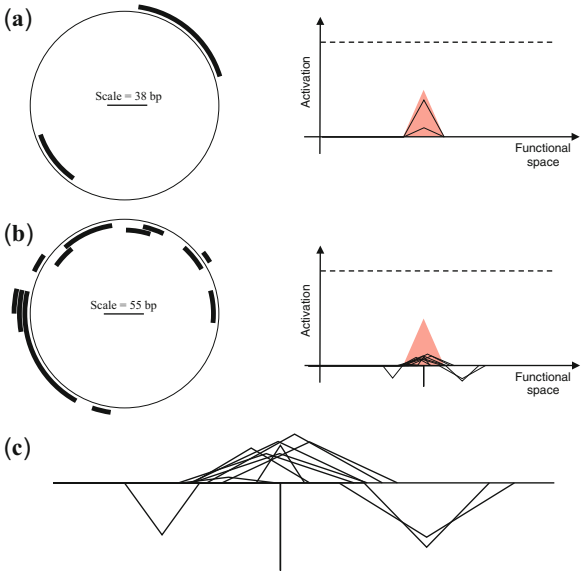

Fig. 4 A simple (**a**) and a complex (**b**) organism. Both evolved for 250,000 generations under a mild mutation rate (10^{-5} mut.bp^{-1}.gen^{-1}) in simple conditions (triangular target). Left: Circular genomes and genes (black arcs). Notice the non-coding sequences on both genomes and also the genes overlap on the genome of the complex organism (**b**). Right: Proteins (black triangles) and phenotypic targets (red-filled triangle). Panel (**c**) zooms on the protein structure of the complex organism. The simple organism has two genes, two proteins and a functional complexity $C_P = 4$. The complex organism has 12 genes, 11 functional proteins and $C_P = 24$

4.4 Results: The Complexity Ratchet

Among the 300 simulations that evolved in a complex environment, 298 were classified as "complex" at generation 250,000. However, strikingly, only 71 of the 300 simulations that evolved in a simple environment are "simple" at generation 250,000, the remaining 229 being "complex". Even more strikingly, a comparison of the mean fitness of the simple and complex organisms (in a simple environment) shows that simple organisms are far fitter with a mean fitness $\overline{f_{\text{simple}}} = 0.99 \pm 0.008$ (the maximum fitness in Aevol being $f_{\max} = 1$) than the complex ones ($\overline{f_{\text{complex}}} = 0.42 \pm 0.32$). This shows that in a simple environment, complex organisms have no direct selective advantage over simple ones. We also verified that they have no robustness and evolvability advantage (simple organisms being actually much more robust than complex ones).

A natural interpretation of these results is that complexity is a transitory state and that complex organisms will become simpler as their fitnesses improve. However, this is firmly contradicted by the dynamics of simple vs. complex individuals. First, simples' fitnesses grow very quickly at the beginning of the simulations to reach their maximum in a few thousands of generations (often less than 1000). Second, among the 236 individuals that were complex at generation 10,000, 227 were still complex at generation 250,000. This shows that complexity (or simplicity) belongs to organisms' identities and that, once fixed at the very beginning of the simulations, hardly can it change thereafter.

When comparing the final complexity levels in the simple and complex environments, we found no significant difference. Together with the previous observation that, in a simple environment, complexity accumulates despite the fitness advantage of simple organisms, this suggests that complexity is driven by a strong neutral process, strong enough to overcome the large difference of fitness between simple and complex organisms in the simple environment (see above). However, in both environments, results show that a high mutation rate strongly limits complexity at both the genomic and functional levels, which is not consistent with a neutral process as diffusion is likely to occur at a faster pace with a high mutation rate. Moreover, when relaxing the selection pressure (i.e. letting the populations evolve further without selection), we observed that complexity quickly drops to zero, hence invalidating neutralist hypotheses.

Altogether, these results are puzzling: on the one hand, they show that complexity accumulates despite selection (simple organisms being fitter than complex ones), and on the other hand, they suggest that complexity is not driven by a neutral process either. To disentangle the relationship between drift and selection, we analysed the evolution of complexity and fitness during the 250,000 generations of the experiments (Fig. 5).

Figures 5a, b illustrate that the dynamics are different for the two complexity measures but similar for the two environmental conditions. Indeed, in both complex and simple environments, C_G quickly plateaus, while C_P increases all along evolution (less obviously so for the highest mutation rate). Moreover, Fig. 5c shows

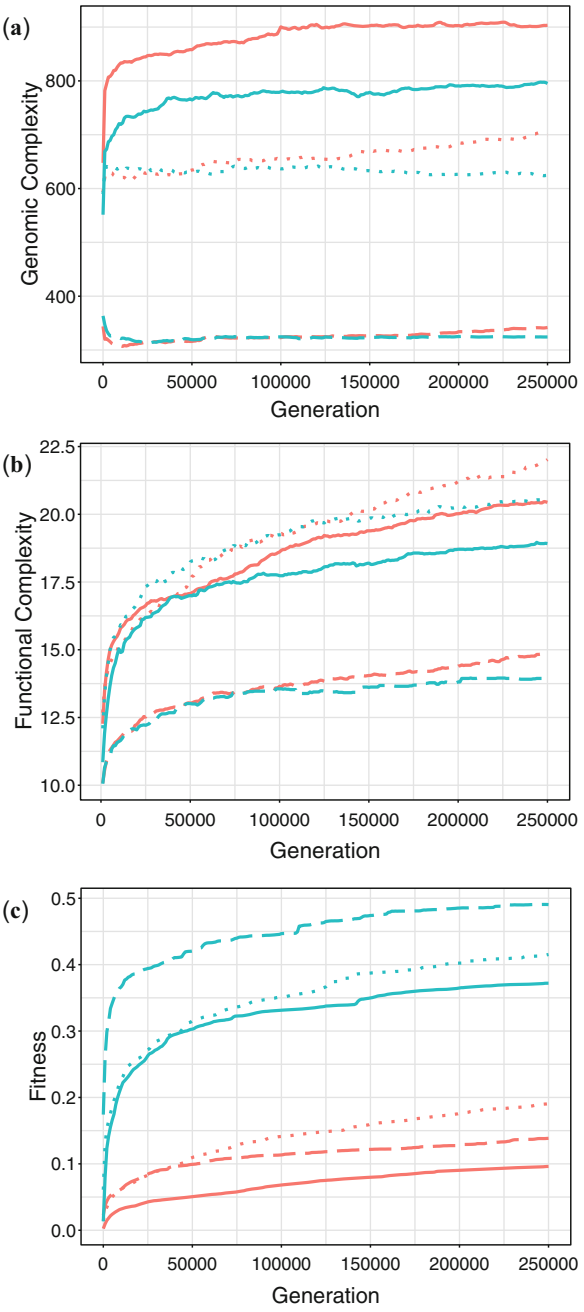

Fig. 5 (**a**) Mean genomic complexity C_G along generations for organisms that evolved in the simple environment (blue lines) and in the complex environment (red lines) and for the three different mutation rates. None of the differences are statistically significant at generation 250,000 except the effect of a harsh mutation rate (p-value $<10^{-4}$). (**b**) Mean functional complexity C_P along generations for organisms that evolved in the simple environment (blue lines) and in the complex environment (red lines) and for the three different mutation rates. None of the differences are statistically significant at generation 250,000 except the effect of a harsh mutation rate (p-value $<10^{-3}$). (**c**) Mean fitness f along generations for organisms that evolved in the simple environment (blue lines) and in the complex environment (red lines) and for the three different mutation rates. The effects of mutation rates on fitness are all statistically significant at generation 250,000 in complex conditions and for the 2 extreme mutation rates in simple conditions. Plain line, $\mu = 10^{-6}$. Dotted line, $\mu = 10^{-5}$. Dashed line, $\mu = 10^{-4}$

that, even though simple organisms are fitter than complex ones (see above), paradoxically, the latter improve their fitnesses while increasing in complexity. This shows that, in our experiments, complexity is driven by a strong sign epistasis mechanism that, in the fitness landscape of Aevol, sets simple functional structures and complex ones apart. This sign epistasis initiates a "complexity ratchet" (Liard et al., 2018, 2020) that pushes organisms towards greater complexity, meanwhile improving their fitness but staying far below the fitness of simple organisms.

This means that the negative complexity-fitness correlation observed in the simple environment is due to some initial contingent events ("frozen accidents"). Depending on their very first evolutionary steps, organisms initiate different evolutionary trajectories: some initiate a "gene optimisation" trajectory (typically through substitutions), while others initiate a "gene duplication-divergence" trajectory. In a complex environment, both strategies ultimately converge as complexity is required to adapt to the Gaussian target. But in a simple environment, the gene optimisation strategy and the gene duplication strategy are antagonistic because of sign epistasis. Hence, once organisms have set themselves on either trajectory, they can hardly switch to the other, and the longer the evolutionary history, the harder the switch becomes. We verified this theory by evolving populations without any chromosomal rearrangement mechanism. We observed that, in these new conditions, 98% of the simulations lead to simple organisms (to be compared to the 23.7% of simple organisms in presence of both point mutations and rearrangements), confirming that the complexity ratchet is indeed fuelled by duplications. This also shows that a minimum diversity of mutational operators is required for the ratchet to be effective and suggests that including such a variety of operators is mandatory to observe the whole complexity of the evolutionary process.

By evolving initially simple organisms in a simple environment, we were able to observe the effect of a "complexity ratchet". Now, by comparing the complexity levels in simple and complex environments, we can estimate the power of this ratchet. Indeed, we first showed that complexity is not higher in complex environments, showing that the ratchet is at least as powerful as direct selection for complexity. Second, we have observed an effect of the mutation rate on the complexity levels and on fitness levels (Fig. 5): high mutation rates strongly limit the maximum level of genomic complexity (Fig. 5a). This suggests that evolution of complexity must be analysed in a multiscale framework: the complexity ratchet drives complexity at the functional level, but the functional level has to be encoded within the genomic sequence. Hence, mutational robustness, by bounding the amount of information a genome can store (Knibbe et al., 2007; Wilke et al., 2001), loosely bounds the functional complexity. Yet, at the functional level, complexity continuously increases (Fig. 5b), driven by the complexity ratchet that slowly increases fitness (Fig. 5c).

5 Conclusion

In this chapter, we have explored the relationship ESB maintains with complexity. We successively discussed the apparently low interest of ESB for the question of complexity. We then showed how complex models can be used to explore how evolving systems accumulate complexity. Finally, we presented an experiment that revealed the existence of a "complexity ratchet" fuelled by sign epistasis (Liard et al., 2018, 2020). Within the field of ESB, this result deserves a specific discussion. Indeed, it shows that, when analysing a biological system, there may be no relationship between the complexity of the structure and that of the function. Because of the complexity ratchet, a simple function can very well be carried out by very complicated systems, even when simple solutions exist. Interestingly, sign epistasis has recently been identified in signalling cascades (Nghe et al., 2018), a system that is well known for its unnecessary complexity (Soyer & Bonhoeffer, 2006).

On the methodological side, complex models raise a difficult question: given the complexity of these models, how can one ensure that the results (and, here, the complexity ratchet) are valid, i.e. transferable to "real" living systems but also to evolutionary biology and to systems biology that don't make use of this kind of models (and hence don't trust them). On that question, the answer has been given more than 20 years ago by Volker Grimm in an enlightening article (Grimm, 1999) "The decisive thing with modelling is not the model per se, but what the model and working with the model does to our mind. [...] If the whole process of modelling has succeeded, something will have happened in our head, namely that an understanding of relationships has emerged. We should then be in a position to communicate our insights to others without referring to the model". It has no meaning to discuss whether Aevol is "true" or "false"; but if we are able to explain the complexity ratchet in biological terms independently from the model that revealed it, then the question of the model's rightness no longer matters.

References

Adami, C. (2002). What is complexity? *BioEssays, 24*(12), 1085–1094.

Adami, C. (2006). Digital genetics: Unravelling the genetic basis of evolution. *Nature Reviews Genetics, 7*(2), 109.

Adami, C., Ofria, C., & Collier, T. C. (2000). Evolution of biological complexity. *PNAS, 97*(9), 4463–4468.

Albantakis, L., Hintze, A., Koch, C., Adami, C., & Tononi, G. (2014). Evolution of integrated causal structures in animats exposed to environments of increasing complexity. *PLoS Computational Biology, 10*(12), e1003966.

Alberch, P. (1991). From genes to phenotype: Dynamical systems and evolvability. *Genetica, 84*(1), 5–11.

Batut, B., Knibbe, C., Marais, G., & Daubin, V. (2014). Reductive genome evolution at both ends of the bacterial population size spectrum. *Nature Reviews Microbiology, 12*(12), 841.

Beslon, G., Parsons, D. P., Sanchez-Dehesa, Y., Pena, J.-M., & Knibbe, C. (2010). Scaling laws in bacterial genomes: A side-effect of selection of mutational robustness? *Biosystems, 102*(1), 32–40.

Clune, J., Mouret, J.-B., & Lipson, H. (2013). The evolutionary origins of modularity. *Proceedings of the Royal Society B, 280*(1755), 20122863.

Crombach, A., & Hogeweg, P. (2008). Evolution of evolvability in gene regulatory networks. *PLoS Computational Biology, 4*(7), e1000112.

Darwin, C. (1859). *On the origin of species by means of natural selection*. J. Murray.

Dawkins, R. (1997). Human chauvinism. *Evolution, 51*(3), 1015–1020.

De Visser, J. A. G., Hermisson, J., Wagner, G. P., Meyers, L. A., Bagheri-Chaichian, H., Blanchard, J. L., et al. (2003). Perspective: Evolution and detection of genetic robustness. *Evolution, 57*(9), 1959–1972.

Elliott, T. A., & Gregory, T. R. (2015). What's in a genome? the c-value enigma and the evolution of eukaryotic genome content. *Philosophical Transactions of the Royal Society B, 370*(1678), 20140331.

Espinosa-Soto, C., & Wagner, A. (2010). Specialization can drive the evolution of modularity. *PLoS Computational Biology, 6*(3), e1000719.

Gould, S. J. (1996). *Full house: The spread of joy from plato to Darwin*. Harmony Books.

Greene, C. S., Tan, J., Ung, M., Moore, J. H., & Cheng, C. (2014). Big data bioinformatics. *Journal of Cellular Physiology, 229*(12), 1896–1900.

Grimm, V. (1999). Ten years of individual-based modelling in ecology: What have we learned and what could we learn in the future? *Ecological Modelling, 115*(2–3), 129–148.

Hindré, T., Knibbe, C., Beslon, G., & Schneider, D. (2012). New insights into bacterial adaptation through in vivo and in silico experimental evolution. *Nature Reviews Microbiology, 10*(5), 352.

Kashtan, N., & Alon, U. (2005). Spontaneous evolution of modularity and network motifs. *PNAS, 102*(39), 13773–13778.

Knibbe, C., Coulon, A., Mazet, O., Fayard, J.-M., & Beslon, G. (2007). A long-term evolutionary pressure on the amount of noncoding DNA. *Molecular Biology and Evolution, 24*(10), 2344–2353.

Lenski, R. E., Ofria, C., Pennock, R. T., & Adami, C. (2003). The evolutionary origin of complex features. *Nature, 423*(6936), 139–144.

Liard, V., Parsons, D., Rouzaud-Cornabas, J., & Beslon, G. (2018). The complexity ratchet: Stronger than selection, weaker than robustness. In T. Ikegami, N. Virgo, O. Witkowski, M. Oka, R. Suzuki & H. Iizuka (Eds.), *Artificial life conference proceedings* (pp. 250–257). MIT Press.

Liard, V., Parsons, D. P., Rouzaud-Cornabas, J., & Beslon, G. (2020). The complexity ratchet: Stronger than selection, stronger than evolvability, weaker than robustness. *Artificial Life, 26*, 38–57.

Loewe, L. (2016). Systems in evolutionary systems biology. *Encyclopedia of Evolutionary Biology, 4*, 297–318.

Lukeš, J., Archibald, J. M., Keeling, P. J., Doolittle, W. F., & Gray, M. W. (2011). How a neutral evolutionary ratchet can build cellular complexity. *IUBMB Life, 63*(7), 528–537.

Lynch, M. (2007). The frailty of adaptive hypotheses for the origins of organismal complexity. *PNAS, 104*(suppl 1), 8597–8604.

Maslov, S., Krishna, S., Pang, T. Y., & Sneppen, K. (2009). Toolbox model of evolution of prokaryotic metabolic networks and their regulation. *PNAS, 106*(24), 9743–9748.

Maynard Smith, J., & Szathmary, E. (1997). *The major transitions in evolution*. Oxford University Press.

Mayr, E. (1961). Cause and effect in biology. *Science, 134*(3489), 1501–1506.

McShea, D. W. (1996). Metazoan complexity and evolution: Is there a trend? *Evolution, 50*(2), 477–492.

McShea, D. W., & Brandon, R. N. (2010). *Biology's first law: The tendency for diversity and complexity to increase in evolutionary systems*. University of Chicago Press.

Moran, N. A. (2007). Symbiosis as an adaptive process and source of phenotypic complexity. *PNAS, 104*(suppl 1), 8627–8633.

Nghe, P., Kogenaru, M., & Tans, S. J. (2018). Sign epistasis caused by hierarchy within signalling cascades. *Nature Communications, 9*(1), 1–9.

O'Neill, B. (2003). Digital evolution. *PLoS Biology, 1*(1), e18.

Pavlicev, M., & Wagner, G. P. (2012). A model of developmental evolution: Selection, pleiotropy and compensation. *TREE, 27*(6), 316–322.

Pigliucci, M. (2008). Is evolvability evolvable? *Nature Reviews Genetics, 9*(1), 75.

Pigliucci, M. (2009). The end of theory in science? *EMBO Reports, 10*(6), 534–534.

Ray, T. S. (1991). An approach to the synthesis of life. *Artificial Life II* 371–408.

Rutten, J. P., Hogeweg, P., & Beslon, G. (2019). Adapting the engine to the fuel: Mutator populations can reduce the mutational load by reorganizing their genome structure. *BMC Evolutionary Biology, 19*(1), 191.

Simon, H. A. (1962). The architecture of complexity. *Proceedings of the American Philosophical Society, 106*(6), 467–482.

Soyer, O. S., & Bonhoeffer, S. (2006). Evolution of complexity in signaling pathways. *PNAS, 103*(44), 16337–16342.

Soyer, O. S., & O'Malley, M. A. (2013). Evolutionary systems biology: What it is and why it matters. *BioEssays, 35*(8), 696–705.

Thomas Jr., C. A. (1971). The genetic organization of chromosomes. *Annual Review of Genetics, 5*(1), 237–256.

Wilke, C. O., Wang, J. L., Ofria, C., Lenski, R. E., & Adami, C. (2001). Evolution of digital organisms at high mutation rates leads to survival of the flattest. *Nature, 412*(6844), 331.

Woods, R. J., Barrick, J. E., Cooper, T. F., Shrestha, U., Kauth, M. R., & Lenski, R. E. (2011). Second-order selection for evolvability in a large *Escherichia coli* population. *Science, 331*(6023), 1433–1436.

Wright, S. (1932). The roles of mutation, inbreeding, crossbreeding and selection in evolution. In *Proceedings of the Sixth International Congress of Genetics* (pp. 356–366, Vol. 1).

Zaman, L., Meyer, J. R., Devangam, S., Bryson, D. M., Lenski, R. E., & Ofria, C. (2014). Coevolution drives the emergence of complex traits and promotes evolvability. *PLoS Biology, 12*(12), e1002023.

Modeling Complex Biological Systems: Tackling the Parameter Curse Through Evolution

Paulien Hogeweg

Abstract As we all know, "Nothing in biology makes sense except in the light of evolution" Dobzhansky (Am Biol Teach 35(3):125–129, 1973). Among the challenges of modeling complex biological systems is to determine the relevant parameters. The common practice is to extract parameters from the literature, or to determine them from ongoing experiments, or by collectively fitting the parameters to the experimental results the model tries to explain. Doing so ignores, or at least does not exploit, Dobzhansky's wisdom. In this perspective paper, we argue and demonstrate the importance of using evolutionary methods to derive relevant parameters. We show that by doing so, we can debug experimental and modeling artifacts.

1 Introduction

The holy grail of systems biology is to match experimental and modeling results. In pursuing this lofty goal, one should keep in mind that wet experiments and dry in silico modeling face different opportunities and limitations to the challenge of unraveling complex biological systems. A common heuristic for experiments is to keep conditions as constant as possible, and limiting the variability of the biological material, e.g., by working with clonal populations, or preferring males over females in medical research because of less hormonal variation. This way a simplest "input-output" system is approached, without accounting for the (variable) state, i.e., considering an $< I, O, \Omega >$ dynamical system (defined in terms of a set of inputs (I), a set of outputs (O), and a function linking input and output (Ω)) instead of a full $< I, O, S, \Omega, \Sigma >$ dynamical system, in which in addition the internal state (S) and internal state changes (Σ) are considered. In contrast, modeling approaches do focus on state changes of the system and use either the full system specification

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_2

$< I, O, S, \Omega, \Sigma >$ or simplify it to $< S, \Sigma >$ considering fixed external conditions (no input beyond initial condition) and observing state changes as outputs.

In silico modeling requires full specification of processes and parameters. An often used heuristic is to compose a very simple model, in which it is possible to survey the full parameter space. This way the result takes the form "the observed *in vivo* or in vitro behavior is possible within the proposed model". In such simple models, the parameters are often composites of potentially measurable quantities and often not validated beyond the fact that they produce the observed behavior. On the other hand, large-scale models try to integrate measurements of many different experiments, often having to add "reasonable" values for unknown parameters, and determine whether these parameters and inferred interactions incorporated in the model indeed produce particular experimental results.

In both cases, one should keep in mind the warning signal put up by James Watson (as quoted by Francis Crick 1988, pp. 59–60 Crick, 1988) "no good model ever accounted for all the facts, since some data was bound to be misleading if not plain wrong. A theory that did fit all the data would have been 'carpentered' to do so and would thus be open to suspicion."

The relevance of this warning signal is preeminently exposed in the history of the study of the lactose operon (*lac* operon). Both models and experiments agreed for a long time that the *lac* operon coded for a bistable switch, (e.g., Griffith, 1968; Novick & Weiner, 1957; Ozbudak et al., 2004), although this notion was challenged early on on theoretical grounds by Savageau (1999). This conclusion is now on theoretical and experimental grounds falsified, (e.g., Afroz et al., 2014; Ozbudak et al., 2004; Rao & Koirala, 2014; Savageau, 2011; Van Hoek & Hogeweg, 2006, 2007; Zander et al., 2017). Here we will relate how evolutionary systems theory contributed to this reversed conclusion.

2 Case Study: The *lac* Operon and Bistability

We will use the *lac* operon to illustrate the power of evolutionary modeling to understand the "how and why" of a particular well-studied regulatory circuit. To this end, we will review an earlier published model and results (Van Hoek & Hogeweg, 2006, 2007), emphasizing the methodology, from a conceptual as well as from a "hands-on" point of view. For details of the model, quantitative results, and mathematical analysis, the reader is referred to the original publications (Van Hoek & Hogeweg, 2006, 2007).

2.1 Background: "State of the Art"

The *lac* operon has been seen for many years as the prototype example of a bistable switch. Indeed the very concept of gene regulation was discovered by Jacob

and Monod (1961) by the observation of population heterogeneity and hysteresis when *E. coli* was grown at different concentrations of an inducer. An artificial, not metabolized, inducer was used, in order to be able to create constant conditions for the experiment. These and subsequent experimental results were soon supported by a simple theoretical model, showing that the positive feedback loop of the import of an inducer on the internal inducer concentration was sufficient to explain the bistability (Novick & Weiner, 1957). This model is taught in many "introduction to biological modeling" courses. Such a so-called mini-model shows that for some parameters, the model can account for the observed behavior and therewith that such a positive feedback is potentially sufficient to explain the observations. For such a compact mini-model, there are only a few parameters; these parameters can be fitted to match the experimental results but cannot be measured in a model-independent way.

Subsequent experimental results elucidated many details of the structure of the *lac* operon and its regulation by a combination of the inducer (lactose or an artificial substitute (IPTG or TMG)) and the preferred resource glucose (see scheme in Fig. 1). A shorthand description is the *lac* operon is an AND gate: it is *ON* when there is lactose and no glucose and *OFF* otherwise.

Subsequent large-scale modeling (e.g., Wong et al., 1997; Yildirim & Mackey, 2003) incorporated this accumulated experimental knowledge. Consequently these models contain many parameters, which were taken from the literature or estimated as "reasonable". Also these models concluded that yes indeed the *lac* operon of *E. coli* encodes a bistable switch. However, close scrutiny of the parameters used revealed large differences between those used in different models. Moreover some parameters were adjusted in order to ensure bistability.

Finally, the notion that the *lac* operon encoded a bistable switch was also reinforced by an evolutionary mini-model which showed bistability to be advantageous (e.g., Thattai & Van Oudenaarden, 2004).

Although the agreement between models, experiments, and optimization consideration may seem conclusive, the parameter uncertainties and their adjustments to match experimental outcomes suggest that we should heed Watson's warning quoted above.

Our research was triggered by a then recent paper of (Setty et al., 2003) which reported direct measurements of the transcription rate of the operon (by coupling a GFP reporter to the operon) for many combinations of the artificial inducer IPTG and cAMP (high cAMP concentrations correspond to low glucose concentration; see scheme of the lactose operon in Fig. 1). The resulting promoter function is not a simple AND gate (*ON* for high inducer and high cAMP (i.e., low glucose) and *OFF* otherwise) but shows distinct (non-zero) expression levels for, respectively, low inducer high cAMP, high inducer low cAMP, and low inducer low cAMP (see Fig. 3b).

They fitted the data to a phenomenological promoter function (see Fig. 1) and obtained a good fit. However, they also showed that this function is quite sensitive to its parameters. They concluded that "the promoter is selected to perform an elaborate computation in setting the transcription rate" (Setty et al., 2003).

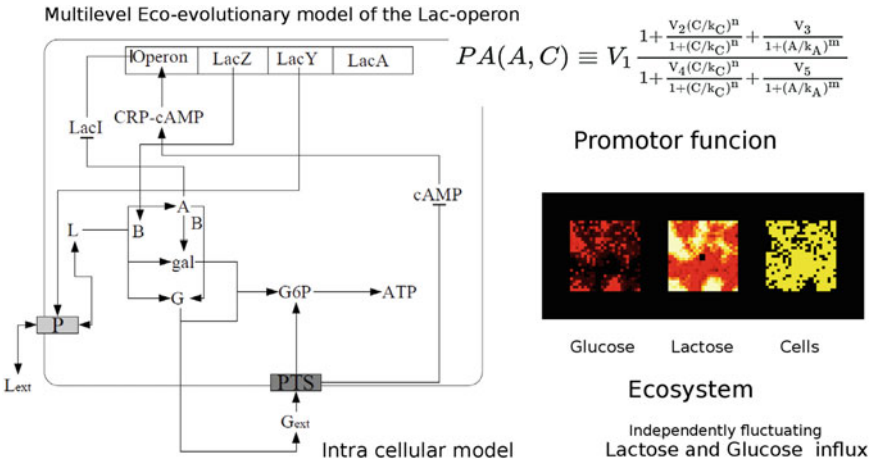

Fig. 1 Overview of the model, which includes the intracellular as well as the intercellular interactions. On the left, the intracellular metabolism and gene regulation related to lactose and glucose utilization are shown as implemented in the model. Expression of the *lac* operon is regulated by the concentration of allolactose (A) and cAMP (C). Allolactose is derived from lactose, and cAMP is inhibited by the influx of glucose in the cell. The expression of the operon is needed for the production of allolactose. It codes for the pump to get lactose into the cell, as well as the enzyme β-galactosidase which transforms lactose to allolactose. Because allolactose induces the *lac* operon, by inhibiting LacI (which inhibits the operon), and needs the expression of the operon to be produced, there is a positive feedback loop which might lead to bistability. On the upper right, the form of the promoter function, dependent on allolactose (A) and cAMP (C), is given as fitted to experimental data by Setty et al. (2003). The V parameters are functions of the following physiological parameters: RNA polymerase and its dissociation constant for binding to the free promoter site and to the site when occupied by CRP (the cAMP-associated transcription factor), as well as its transcription rate dependent on the site occupancy (α and β); the "leakage" of the promoter (γ), i.e., its expression when not induced; the concentration of LacI and its dissociation constant; and the CRP concentration and its dissociation constant. It are these (more physiological) parameters which are subject to mutation and selection in the model; see main text. Finally, in the lower right, the "ecosystem" is depicted, showing the local variation of the external concentration of glucose and lactose and the presence of the cells at arbitrary point in time. For details, see Van Hoek and Hogeweg (2006)

Heeding Dobzhansky's dictum (Dobzhansky, 1973) we wondered if we could "make sense" of the form of the promoter function from an evolutionary point of view, i.e.:

1. Should we expect such a promoter function to evolve given the known and/or hypothesized details of the metabolic pathways involved?
2. What is the functionality that is in fact being selected?

To answer these questions, we used an evolutionary systems biology approach.

3 Eco-evolutionary Model of the *lac* Operon

We constructed a multilevel agent-based eco-evolutionary model. The model includes the within-cell physiological dynamics related to the *lac* operon, cell growth, and reproduction and competition between cells in a spatially explicit environment, which is modified by the cell metabolism (see Fig. 1).

The model for cell-level physiological dynamics is adapted from the model of (Wong et al., 1997), using their parameter values. However, the promoter function of (Setty et al., 2003) was incorporated, and its parameters were subjected to evolution. The rationale for using fixed parameters for all processes except those of the promoter function was that we wanted to study how the promoter function evolved, given the constraints set by the rest of the system. Important, for example, are the relatively slow protein dynamics.

Thus the model includes the following components (Fig. 1):

- A promoter function. We used the same (phenomenological) function that (Setty et al., 2003) used to (successfully) fit their experimental data (see Fig. 1). The parameters of the function were subject to evolution, i.e., subjected to mutation and selection. Importantly, after initial trials in which "nothing happened" (i.e., no evolutionary adaptation was observed), we realized we should not use the dimension (parameter) reduction used to simplify the model fitting, but the underlying binding reactions instead. This increases the number of parameters from the 7 shown in Fig. 1 to 11 more physiological parameters (see the legend of Fig. 1). Thus we create a redundant genotype-to-phenotype (GP) mapping. Such a redundant GP mapping has been shown to strongly improve evolutionary search. (For a recent extensive review on the role of GP maps in evolution, see Manrubia et al., 2020.)
- Intracellular molecular interactions, including protein expression and degradation, transport into the cell of lactose and glucose, and ATP production, as modeled by (Wong et al., 1997). In addition, the cells grow as a function of ATP production, causing dilution of the protein concentrations. When a cell reaches a certain predefined size, it can divide.
- Ecology: the cells are embedded on a spatial grid. Resources, i.e., lactose and glucose, flux into the medium and are taken up by the cells. The cells compete for the resources as well as empty grid cells. Cells divide after reaching a certain size and die with a probability which depends on the global cell density.

The aim of this evolutionary model is to alleviate the "parameter curse," inherent in detailed models. Paradoxically, but unavoidably, extra (semi-arbitrary) parameters have to be set in the evolutionary model, in this case, for example, the cost (in terms of ATP) of protein expression and the definition of the environment in which the evolution takes place. The latter involves relative changes in external and internal resource concentration when resources are consumed, as well as the temporal changes of the influx of the resources into the environment. Fortunately, for the environmental parameters, we could use an "adequacy" criterion, i.e., in order

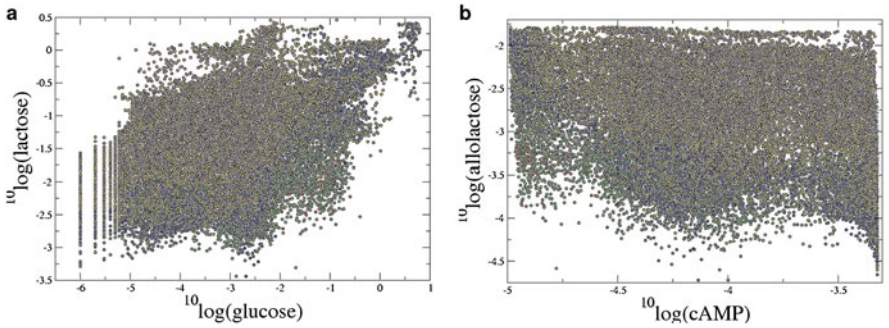

Fig. 2 Coverage of the state space, i.e., the conditions the cells experience over time. On the left, the external conditions, i.e., the concentrations of glucose and lactose, encountered. On the right, the realized internal conditions which directly impact on the expression of the *lac* operon, i.e., the concentrations of cAMP and allocatose. Because of the dynamics of the model, all these concentrations cannot be directly manipulated as input but are the result of the model dynamics. Glucose and lactose were influxed in independently Poisson-distributed blocks of certain duration and concentration. We tuned frequency and amount of influx in such a way that all circumstances were encountered regularly, as shown in the pictures

to select for the full operon function, all combinations of concentrations of glucose and lactose as well as the resulting internal concentrations of allolactose and cAMP should be regularly encountered by the cells. As these concentrations are not directly imposed, but result from the consumption and metabolism, we tuned the timing and amount of influx of glucose and lactose in such a way that this requirement is met (see Fig. 2 for the resulting coverage of the state space).

3.1 Analysis of the Eco-evolutionary Dynamics of the Model

Darwin distinguished "natural selection" from "artificial selection", where the latter referred to selection by breeders for certain properties preferred by them. The above described eco-evolutionary model of the *lac* operon (artificial as it is) incorporates in this sense "natural selection": no a priori fitness criterion is defined. Instead the environmental conditions are constantly shifting, not only due to fluctuating external influx of glucose and lactose but importantly also through the current population of cells and the variation of the promoter functions of neighboring cells which defines their uptake of the resources, and therefore the local resource conditions. (Indeed recent experiments have shown the importance of micro-scale gradients in the functioning and evolution of bacterial colonies Dal Co et al., 2019; Van Vliet et al., 2017). These local conditions determine the immediate fitness. Long-term integration of immediate fitness will determine, in the long run, what evolves. This is indeed what evolution is about. However, it makes life harder for the modeler, because there is not one obvious observable (fitness) to evaluate whether or not the

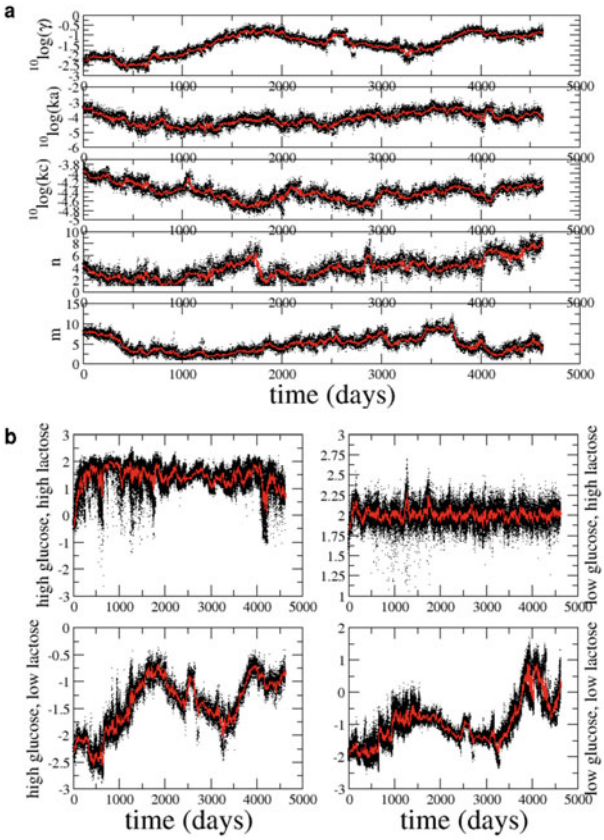

Fig. 3 First observations on the evolutionary dynamics of the model. (**a**) The evolution through time of individual parameters does not show any clear trend. (**b**) Depicts the evolution over time of some phenotypic features, namely, the promoter activity the evolving promoter function would have when encountering the four extremes of high and low cAMP and allolactose concentration (although the circumstances in which it finds itself are different). The phenotypic features show a somewhat clearer evolutionary trend, although they also do not convincingly show that anything other than drift occurs

model is actually evolving something. As shown in Fig. 3a, b, looking at changes in the parameter values over time is hardly informative, although looking at the change over time of some selected phenotypic features indicates something beyond neutral drift might be happening. However, further analysis and experiments with the model are needed to establish this, as discussed below.

There is at all times plenty of variation in the population. To get a more detailed understanding of what is evolving, we extracted the last common ancestor of the population at the end of the simulation. This cell obviously was most successful in producing surviving offspring, thus, in hindsight, being per definition the fittest. This works quite well (as shown below), but one should keep in mind that the success of

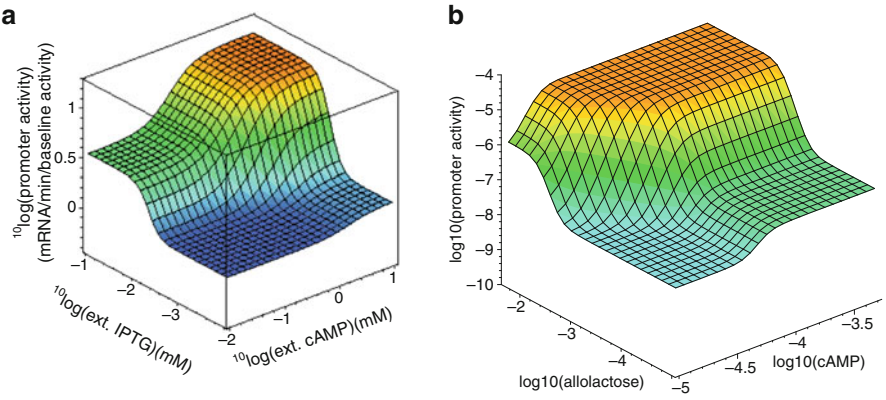

Fig. 4 Similarity of the shape of the promoter function of the best evolved common ancestor (**b**) with the promoter function of Setty et al. (2003); see panel (**a**). Shown is the activity in the state space as experienced by the cells (compare Fig. 3), which is not the same in both cases

the last common ancestor could be caused simply by chance or importantly by later occurring mutations. Several independent evolutionary runs were performed. To select the "best" evolved promoter function, we pairwise competed the last common ancestors of the various runs and selected the one which won most often. This best promoter function is depicted in Fig. 4 alongside the promoter function of (Setty et al., 2003) as fitted to their measured data. The similarity is striking, especially realizing that *no* fitting was involved in setting up the model.

So far so good: apparently the shape of the promoter function as determined in the experiment, with the "fine-tuned parameter values" noted by Setty et al. (2003), is explained by the "natural selection" in our eco-evolutionary model, given the background metabolic processes as modeled previously (Wong et al., 1997). But why? We can now study its behavior in different external resource concentrations. The results are given in Fig. 5a for various concentrations of external lactose, either with high or low glucose concentrations. In contrast to the common expectation that the natural promoter function of the *lac* operon of *E. coli* codes for a bistable switch, the promoter function evolved in our eco-evolutionary model does not, despite its similarity to the measured promoter function. What is wrong?

3.2 Internal Validation of the Model

It turns out that nothing is wrong with the model and that, indeed, the *lac* operon of *E. coli* does not encode a functional bistable switch. This insight is first of all obtained from the model itself. Realizing that experiments were almost always done with artificial inducers (IPTG or TMG), which are not metabolized, we tested our promoter function by stimulating it with IPTG, adjusting the model accordingly. As

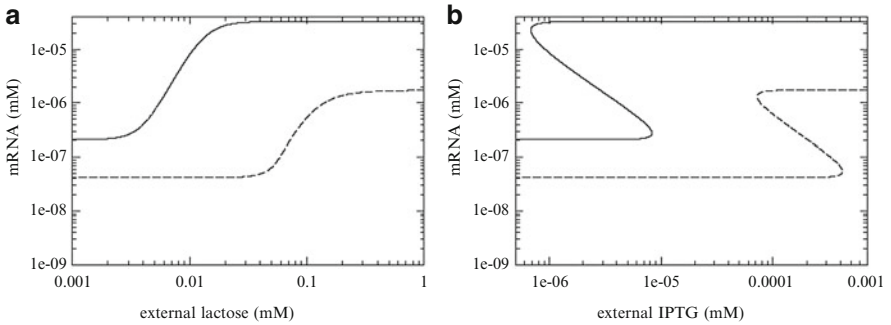

Fig. 5 The best evolved promoter function does not code for a bistable switch in its natural environment and the model, i.e., when it is induced by lactose (**a**). However, when studied with an artificial inducer (IPTG) as commonly done in the lab, it does code for a bistable switch (**b**). Solid line, low glucose concentration; dotted line, high glucose concentration (Reproduced from Van Hoek and Hogeweg, 2006)

seen in Fig. 5b, the model with the evolved promoter function in that case recovers a strong bistable switch, both for low and for high concentrations of external glucose. The difference in behavior is due to the fact that artificial inducers are not metabolized, whereas lactose is. This is in fact the advantage of using these artificial inducers in experiments, as it allows to control the conditions. However, because they are not metabolized, the positive feedback loop is strengthened, causing bistability under a much wider set of circumstances than is the case for lactose, which is metabolized (Díaz-Hernández & Santillán, 2010). In other words, we conclude that the common notion that the *lac* operon is coding a bistable switch is an experimental artifact, derived from the preference to do experiments in controlled conditions. As mentioned above, in our eco-evolutionary model, as in nature, conditions are extremely non-controlled.

3.3 Experimental Validation of the Model Results

Currently, the consensus opinion has shifted away from considering the *lac* operon as a bistable switch. Although the model results described above, in my opinion, support this conclusion strongly, the *communis opinio* is based on more recent, more conventional systems biology experiments as well as wet evolutionary experiments. Strikingly, the paper entitled "Multistability in the lactose utilization network of *Escherichia coli*" mentions in passing in the supplementary material "During induction with lactose, as opposed to IPTG, TMG. . . . . .the steady state distribution after 4 hours of growth is always uni-modal, and we never observe hysteresis" (Ozbudak et al., 2004). Strikingly, despite its title, this paper is frequently cited as evidence for the gradual response instead of bistability.

A combined experimental and modeling paper (Zander et al., 2017) confirmed in their carefully parameterized model that the *lac* operon is not bistable. However, they showed in the model and in experiments that overexpression of LacI (the repressor of the operon which is repressed by the inducer (allolactose or artificial inducer)) does induce bistability. In fact, their results show that the wild-type promoter function is only just not bistable. Similarly, in our model, we see that over evolutionary time some individuals of the variable population do show bistability for lactose. Moreover, we see that when, as we did, evolution starts off with a bistable promoter, it evolves away from bistability by increasing the default expression of the operon without induction (the γ parameter (see Fig. 3a top panel)). Note that decreasing LacI expression implies less repression of the operon and therewith an increase of leakage (i.e., γ). Indeed we showed analytically that the occurrence of bistability (i.e., a Hopf bifurcation) depends primarily on a low enough value of γ (Van Hoek & Hogeweg, 2006). These results suggest that long-term evolution avoids bistability but minimizes the (costly) expression of the operon when not induced as much as possible without becoming bistable.

Interestingly, LacI is in fact itself also regulated by the *lac* operon (Semsey et al., 2013). This autoregulation leads to a further smoothing of the response. This is another indication that avoidance of bistability is an evolved feature.

Also interesting are the results of the evolutionary experiments of (Quan et al., 2012). They evolved *E. coli* cells on four different media, only glucose, only lactose, glucose and lactose, and alternating glucose and lactose, and studied the bistability of the evolved lac operons, using artificial inducer (TMG). Even with artificial inducer, they only observed bistability consistently in the glucose-only medium and in a subset of cases in the lactose-only medium. These results, counterintuitive as they may seem at first sight, can be understood in terms of the above discussion. On glucose-only medium, the operon should never be expressed. Therefore, the "leakage" expression without inducer should be low. When this is low enough, bistability is even seen with lactose as inducer (see also Fig. 6b).

Conversely, if they evolved on lactose only, the operon should be active all the time. Whether or not it is bistable under other circumstances is irrelevant. Hence, in some replicates, it remains bistable for artificial inducer as it was the initial wild type, and in other cases, bistability is lost by neutral evolution. In the other two cases, a graded response is observed even for TMG. In contrast to our eco-evolutionary model, where we tuned the parameters so that all environmental conditions were experienced regularly, these evolutionary experiments severely limited the environmental conditions experienced by the cells. Therefore, the cells adapted quickly to the subset of conditions encountered. Likewise when we varied the environmental conditions or internal parameters (e.g., cost of gene expression) and only a subset of conditions occurred, different promoter functions evolved (Van Hoek & Hogeweg, 2006).

Finally I mention the study of (Afroz et al., 2014), who studied bistability for a number of carbon sources. They report no bistability for the *lac* operon, but do find bistability for others, e.g., L-arabinose. It would be interesting to see whether the modeling methodology we used here would for L-arabinose indeed predict bistability.

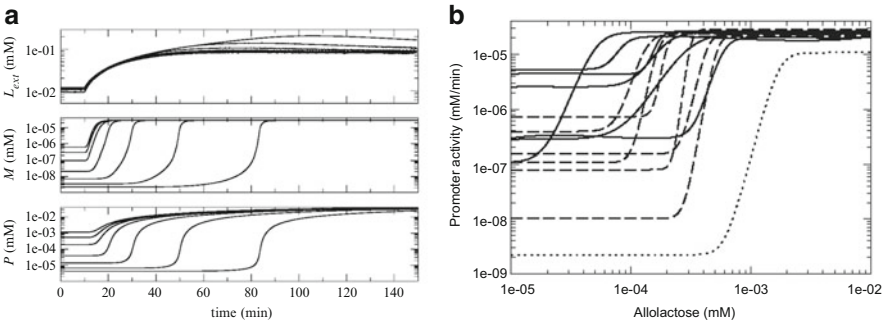

Fig. 6 (**a**) Delay in activation of the lac operon when external lactose becomes available, dependent on un-induced activity level: delays longer than average division time of E. coli for un-induced values compatible with bistability. (**b**) Evolved promoter activity dependent on internal allolactose in various replicates. Dotted line, initial bistable promoter. Dashed and solid line, evolved promoters in, respectively, the deterministic and the stochastic model. The stochastic model evolves even farther from bistability by increased un-induced expression (Reproduced from Van Hoek and Hogeweg, 2006, 2007)

3.4 Why Avoid Bistability?

The theoretical and experimental results discussed so far show that bistability is evolutionary avoided. But why? When some phenotypic feature evolves, this does not automatically imply that it has an adaptive benefit, as it may be produced by neutral drift. Indeed neutral drift can generically lead to well-defined, apparently nonrandom phenomena which are attractors of the stochastic dynamical system defined by the mutational operators employed, a striking example of which is shown in (Cordero & Hogeweg, 2006). The fact that an evolutionary model and empirical data converge to the same outcome, as is the case in both (Cordero & Hogeweg, 2006) and the current model, also does not preclude a neutral explanation. For example, the avoidance of bistability simply could be due to the fact that a larger part of parameter space generates a graded response, rather than bistability, which is indeed so for the natural system with lactose as inducer, whereas the parameter space leading to bistability is much larger in the case of artificial inducer (as shown here and argued in Savageau, 1999, 2011). Apart from neutral drift, another non-adaptive explanation of an evolved phenotypic feature might be that it is a side effect of the positive selection acting on an apparently unrelated feature, when the same mutations affect both. The above mentioned bistability of the *lac* operon when evolved on glucose medium is a nice example of this. Important for the discussion here is that whether or not the studied feature is generated by adaptive or neutral evolution, or as a side effect, does not affect the main conclusion of this paper, i.e., that an evolutionary perspective is very helpful to debug matching theoretical and experimental results.

In the present case, we see a clearly adaptive signature in the outcome. First of all, competition experiments mentioned above clearly show the fitness advantage of the evolved promoter function, in the type of environment in which it evolved (but note that in competition experiments, the environment unavoidably differs from the native environment because of the presence of the competitor). Another indication of the adaptive relevance of the evolved promoter function is that under different environmental circumstances, clearly different promoter functions evolve, as discussed above in this model as well as in experiments (Quan et al., 2012).

Moreover we can pinpoint why a sufficiently high value of γ and therewith the avoidance of bistability give an evolutionary advantage. In Fig. 6a, we show the onset of mRNA and protein production when external lactose becomes available. For promoters with very low activity when no external lactose is available, the delays are very long, in fact longer than the average division time of *E. coli*. Obviously such long delays are detrimental. Cells which avoid such delays consume the external lactose earlier, leaving less resource for cells with longer delays. Slow protein dynamics plays an important role in these long delays. This underscores the importance of using the large-scale parameterized model for the cell metabolism in our eco-evolutionary model. Note that bistability, and therewith hysteresis, would even further aggravate the delays. We conclude that it is the transient, non-equilibrium situation which determines the long-term evolutionary outcome.

In contrast, an earlier evolutionary model "explained" the advantages of bistability (Thattai & Van Oudenaarden, 2004). In their mini-model, instantaneous switching was assumed. In addition, their model was stochastic. Bistability ensured heterogeneity in the population, so that some cells were pre-adapted to a changing environment. This raises the question whether it is the lack of stochasticity in gene expression in our model which prevents evolution to exploit the advantages of bistability. We modified the model to incorporate stochastic gene expression (Van Hoek & Hogeweg, 2007) and conducted a similar set of experiments in the stochastic model. Figure 6b shows that the stochastic model evolved even farther away from bistability by increasing the expression in the absence of lactose (i.e., γ). Indeed, again the explanation is in terms of delays, which are even more severe in the stochastic model (Van Hoek & Hogeweg, 2007). Moreover, the stochasticity only marginally increases the heterogeneity of the population, relative to the genetic and environmental heterogeneity prevalent in the eco-evolutionary model (Van Hoek & Hogeweg, 2007). Likewise through metagenomic analysis, extreme heterogeneity is commonly observed in natural bacterial populations at a micro-scale (e.g., Preheim et al., 2011; Vetsigian et al., 2011).

4 Discussion

The evolutionary systems biology approach discussed in this paper proved to be surprisingly powerful. We showed that the measured promoter function (Setty et al., 2003) was evolutionary favored, which was our original aim. The modeling

was moreover rich in "results++". i.e., in unexpected novel insights. Foremost the insight emerged that the assumption of bistability of the *lac* operon, which was supported by experiments, mini-models, large-scale models, and evolutionary optimization models, is in fact incorrect. How could this false notion be sustained for so many years? The need for well-defined conditions in experiments, and therewith the use of artificial inducer, clearly was the primary cause, combined with the construction of models and the setting of parameters such that the model results match the experimental results. In contrast, we used an unsupervised modeling approach and observed a striking match to some experimental results, namely, the shape of the promoter function together with the totally unexpected evolutionary trend away from bistability. Moreover, we could determine that bistability was avoided in order to avoid delays in activation and therewith gain a competitive edge. Our results also indicate that examining monomorphic, clonal populations in experiments or models may lead to artifacts, in the sense that it does not reflect what happens in natural populations.

Having argued that the *lac* operon does not encode a bistable switch, we should reflect on what we mean with such a statement. Bacteria, including *E. coli*, adapt to a prevailing environment very quickly, as shown in evolutionary experiments, e.g., those of (Quan et al., 2012) discussed above, and stressed by (e.g., Dekel and Alon, 2005). This we also see in our model: if the environment switches too fast, regulation is largely lost, as only an average environment is experienced by the cells. As another example, when cost of protein expression is set very high, bistability may evolve but occurs at very high glucose concentrations, which were very seldom if ever encountered. In such a case, like in the glucose-only environment of (Quan et al., 2012), bistability occurs as a side effect which does not harm the system. Thus, indeed as stated by Setty et. al., the promoter function can be fine-tuned easily. It is therefore even more remarkable that, given that a full set of environmental conditions is encountered (which is not the case in the abovementioned examples), evolution of wild-type *E. coli* and the model converge to an unequivocal solution.

In the eco-evolutionary model discussed here, we only evolved some of the large number of parameter values which needed to be specified, and for which the experimental evidence is not unequivocal. However, because of the evolution of the parameters determining the phenomena of interest, their precise value might not matter too much and certainly was not tuned/fitted for the results obtained. In that sense, the parameter curse which encumbers large-scale models was somewhat alleviated. This was enough to debug the results obtained from models in which parameters were fitted or tuned to match the experimental results.

Finally I like to note that the general approach advocated here, i.e., non-supervised, multilevel eco-evolutionary modeling, can be generalized beyond evolving parameters in a fixed model structure as done here. Giving the models many degrees of freedoms to adjust model structure, we have repeatedly seen surprising convergence to biological systems, leading to novel insights in their functioning as well as novel insights in evolution itself (e.g., Cuypers & Hogeweg, 2012, 2014; van Dijk et al., 2019; Van Hoek & Hogeweg, 2009).

References

Afroz, T., Biliouris, K., Kaznessis, Y., & Beisel, C. L. (2014). Bacterial sugar utilization gives rise to distinct single-cell behaviours. *Molecular Microbiology, 93*(6), 1093–1103.

Cordero, O. X., & Hogeweg, P. (2006). Feed-forward loop circuits as a side effect of genome evolution. *Molecular Biology and Evolution, 23*(10), 1931–1936.

Crick, F. (1988). What mad pursuit: a personal view of scientific discovery. *New York: Basic Books, 13*(182), 8.

Cuypers, T. D., & Hogeweg, P. (2012). Virtual genomes in flux: an interplay of neutrality and adaptability explains genome expansion and streamlining. *Genome Biology and Evolution, 4*(3), 212–229.

Cuypers, T. D., & Hogeweg, P. (2014). A synergism between adaptive effects and evolvability drives whole genome duplication to fixation. *PLoS Computational Biology, 10*(4), e1003547.

Dal Co, A., Van Vliet, S., & Ackermann, M. (2019). Emergent microscale gradients give rise to metabolic cross-feeding and antibiotic tolerance in clonal bacterial populations. *Philosophical Transactions of the Royal Society B, 374*(1786), 20190080.

Dekel, E., & Alon, U. (2005). Optimality and evolutionary tuning of the expression level of a protein. *Nature, 436*(7050), 588–592.

Díaz-Hernández, O., & Santillán, M. (2010). Bistable behavior of the lac operon in *E. coli* when induced with a mixture of lactose and TMG. *Frontiers in Physiology, 1*, 158.

Dobzhansky, T. (1973). Nothing in biology makes sense except in the light of evolution. *The American Biology Teacher, 35*(3), 125–129.

Griffith, J. (1968). Mathematics of cellular control processes I. Negative feedback to one gene. *Journal of Theoretical Biology, 20*(2), 202–208.

Jacob, F., & Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology, 3*(3), 318–356.

Manrubia, S., Cuesta, J. A., Aguirre, J., Ahnert, S. E., Altenberg, L., Cano, A. V., Catalán, P., Diaz-Uriarte, R., Elena, S. F., García-Martín, J. A., et al. (2020). From genotypes to organisms: State-of-the-art and perspectives of a cornerstone in evolutionary dynamics. arXiv preprint arXiv:2002.00363.

Novick, A., & Weiner, M. (1957). Enzyme induction as an all-or-none phenomenon. *Proceedings of the National Academy of Sciences of the United States of America, 43*(7), 553.

Ozbudak, E. M., Thattai, M., Lim, H. N., Shraiman, B. I., & Van Oudenaarden, A. (2004). Multistability in the lactose utilization network of *Escherichia coli*. *Nature, 427*(6976), 737.

Preheim, S. P., Timberlake, S., & Polz, M. F. (2011). Merging taxonomy with ecological population prediction in a case study of vibrionaceae. *Applied and Environmental Microbiology, 77*(20), 7195–7206.

Quan, S., Ray, J. C. J., Kwota, Z., Duong, T., Balázsi, G., Cooper, T. F., & Monds, R. D. (2012). Adaptive evolution of the lactose utilization network in experimentally evolved populations of *Escherichia coli*. *PLoS Genetics, 8*(1), e1002444.

Rao, C. V., & Koirala, S. (2014). Black and white with some shades of grey: the diverse responses of inducible metabolic pathways in *Escherichia coli*. *Molecular Microbiology, 93*(6), 1079–1083.

Savageau, M. (1999). Design of gene circuitry by natural selection: analysis of the lactose catabolic system in *Escherichia coli*. *Biochemical Society Transactions, 27*(2), 264–270.

Savageau, M. A. (2011). Design of the lac gene circuit revisited. *Mathematical Biosciences, 231*(1), 19–38.

Semsey, S., Jauffred, L., Csiszovszki, Z., Erdőssy, J., Stéger, V., Hansen, S., & Krishna, S. (2013). The effect of laci autoregulation on the performance of the lactose utilization system in *Escherichia coli*. *Nucleic Acids Research, 41*(13), 6381–6390.

Setty, Y., Mayo, A. E., Surette, M. G., & Alon, U. (2003). Detailed map of a CIS-regulatory input function. *Proceedings of the National Academy of Sciences, 100*(13), 7702–7707.

Thattai, M., & Van Oudenaarden, A. (2004). Stochastic gene expression in fluctuating environments. *Genetics, 167*(1), 523–530.

van Dijk, B., Meijer, J., Cuypers, T. D., & Hogeweg, P. (2019). Trusting the hand that feeds: Microbes evolve to anticipate a serial transfer protocol as individuals or collectives. *BMC Evolutionary Biology, 19*(1), 201.

Van Hoek, M., & Hogeweg, P. (2006). In silico evolved lac operons exhibit bistability for artificial inducers, but not for lactose. *Biophysical Journal, 91*(8), 2833–2843.

Van Hoek, M., & Hogeweg, P. (2007). The effect of stochasticity on the lac operon: An evolutionary perspective. *PLoS Computational Biology, 3*(6), e111.

Van Hoek, M. J., & Hogeweg, P. (2009). Metabolic adaptation after whole genome duplication. *Molecular Biology and Evolution, 26*(11), 2441–2453.

Van Vliet, S., Winkler, A. R., Spriewald, S., Stecher, B., Ackermann, M., et al. (2017). Local interactions lead to spatially correlated gene expression levels in bacterial groups. *BioRxiv* 109991.

Vetsigian, K., Jajoo, R., & Kishony, R. (2011). Structure and evolution of *Streptomyces* interaction networks in soil and in silico. *PLoS Biology*, 9(10), e1001184.

Wong, P., Gladney, S., & Keasling, J. D. (1997). Mathematical model of the *lac* operon: Inducer exclusion, catabolite repression, and diauxic growth on glucose and lactose. *Biotechnology Progress, 13*(2), 132–143.

Yildirim, N., & Mackey, M. C. (2003). Feedback regulation in the lactose operon: a mathematical modeling study and comparison with experimental data. *Biophysical Journal, 84*(5), 2841–2851.

Zander, D., Samaga, D., Straube, R., & Bettenbrock, K. (2017). Bistability and nonmonotonic induction of the *lac* operon in the natural lactose uptake system. *Biophysical Journal, 112*(9), 1984–1996.

Direction and Constraint in Phenotypic Evolution: Dimension Reduction and Global Proportionality in Phenotype Fluctuation and Responses

Kunihiko Kaneko and Chikara Furusawa

Abstract A macroscopic theory for describing cellular states during steady growth is presented, based on the consistency between cellular growth and molecular replication, as well as the robustness of phenotypes against perturbations. Adaptive changes in high-dimensional phenotypes were shown to be restricted within a low-dimensional slow manifold, from which a macroscopic law for cellular states was derived, which was confirmed by adaptation experiments on bacteria under stress. Next, the theory was extended to phenotypic evolution, leading to proportionality between phenotypic responses against genetic evolution and environmental adaptation. The link between robustness to noise and mutation, as a result of robustness in developmental dynamics to perturbations, showed proportionality between phenotypic plasticity by genetic changes and by environmental noise. Accordingly, directionality and constraint in phenotypic evolution were formulated in terms of phenotypic fluctuation and the response against environmental change. The evolutionary relevance of slow modes in controlling high-dimensional phenotypes is discussed.

1 Introduction

In a chapter in the previous volume of Evolutionary Systems Biology (Kaneko, 2012a; Soyer, 2012), we discussed the evolutionary fluctuation-response relationship, which states that if phenotypic variance due to noise is high, then evolution rapidly occurs. This suggests a correlation between short-term phenotypic dynamics

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_3

and long-term evolutionary responses. This, in some sense, is a quantitative expression of Waddington's genetic assimilation (Waddington, 2014).

Can we push this viewpoint forward to determine the direction of phenotypic evolution in a high-dimensional phenotypic space (i.e., with a large degree of freedom components)? Can one predict which traits are likely to evolve among many components before evolution progresses?

To answer the question, we first investigate the characteristics of responses of phenotypes with a large degree of freedom. We review the general relation in phenotypic responses of cells over many components, demonstrating that global proportionality exists among all logarithmic changes in concentrations against adaptation to different environmental conditions. We first discuss this proportionality as a general consequence of steady exponential growth cells, following (Kaneko et al., 2015). We show that when a cell grows and divides while maintaining its composition, the abundances of each component increase at the same rate; this constraint supports the global proportional relationship.

However, the growth rate constraint is not enough to explain the experimental observations. Global proportional changes across all components are confirmed even across many different environmental conditions. This result cannot be explained by the constraint of steady growth alone. We will see that another important constraint, imposed by the robustness in a phenotypic state shaped throughout evolution, is essential. From this evolutionary robustness, phenotypes can change mostly along only one or in a few dimensions, although the original phenotypic space is high-dimensional as a consequence of the huge diversity in the components of cells. Based on (Furusawa & Kaneko, 2018; Kaneko, 2012a), we demonstrate this evolutionary dimension reduction both through theory and simulations, whereas its consequence is consistent with experimental observations.

This constraint in phenotypic changes is extended to changes that occur in evolution. We demonstrate that long-term phenotypic changes via evolution and short-term changes via adaptation are highly correlated. Global proportionality in the phenotypic changes by environmentally induced adaptation and those by genetically induced evolution is confirmed across all components, both in simulations and laboratory evolution experiments (Furusawa & Kaneko, 2015).

In contrast, response and fluctuation are *two sides of the same coin*, as has been demonstrated by statistical mechanics (see also the first volume of Evolutionary Systems Biology (Kaneko, 2012a and Kaneko & Furusawa, 2018)). Hence, a similar correlation in concentration fluctuations is expected across all components. Indeed, we demonstrate a proportional relationship between fluctuations by gene mutation and those by noise over the concentration of all components. Recall that the variances in each trait (phenotype) due to genetic variation are proportional to the evolution rate of the trait according to the fundamental theorem of natural selection by Fisher (1930). Hence, the evolution rate of each trait is correlated with its variance by noise, which is predetermined before mutation and selection. This means that the evolutionary potential of each trait is determined in advance by the phenotypic changeability which is affected by environmental variation or noise before genetic changes occur. This enables the prediction of phenotypic evolution.

Among the high-dimensional phenotypic space, evolution progresses along the direction in which the variation by the noise or environmental response is larger, which is predetermined before mutation. Although genetic variation itself is random and undirected, phenotypic evolution tends to show directionality.

2 Constraint in a Steady-Growth System: Global Proportionality Law

To describe changes in the cellular state in response to environmental changes, we introduce a simple theory by assuming that cells undergo steady growth. When a cell grows and reproduces in this steady state, all components, e.g., expressed proteins, must be approximately doubled (Furusawa & Kaneko, 2003; Kaneko, 2006).

Consider a cell consisting of M chemical components. In the cellular state under steady-growth conditions, the cell number increases exponentially over time, as does the cell volume V, as given by $dV/dt = \mu V$. In a steady-growth cell, the abundance of all components increases at the same rate, preserving the concentration of each component during the cell cycle.

To formulate the constraint for steady growth, let us denote the concentration $x_i (> 0)$ for each component $i = 1, \cdots, M$. The cellular state is represented as a point in an M-dimensional state space. Here, each component i is synthesized or decomposed relative to other components at a rate $f_i(\{x_j\})$, for instance, by the rate equation in chemical kinetics. Additionally, all concentrations are diluted by the rate $(1/V)(dV/dt) = \mu$, so that the time change of a concentration is given by

$$dx_i/dt = f_i(\{x_j\}) - \mu x_i. \tag{1}$$

For convenience, let us denote $X_i = \log x_i$ and $f_i = x_i F_i$. Then, Eq. (1) can be written as $dX_i/dt = F_i(\{X_j\}) - \mu$, which assumes that $x_i \neq 0$, i.e., all components exist. Then, the stationary state is given by the fixed-point solution $F_i(\{X_j^*\}) = \mu$ for all i.

In response to environmental changes, the term $F_i(\{X_j\})$ and growth rate μ change, as does each concentration x_i^*; however, the $M - 1$ conditions $F_1 = F_2 = \cdots = F_M$ must be satisfied. Thus, a cell must follow a one-dimensional curve in the M-dimensional space (see Fig. 1) under a given change in the environmental conditions (e.g., against changes in stress strength). Now, consider intracellular changes in response to environmental changes. Here each environmental change given by a type of stress a is parametrized by a single continuous parameter E^a (such as the temperature, degree of nutrient limitation, etc.). Using this parameterization E^a, the steady-growth condition leads to $F_i(\{X_j^*(E^a)\}, E^a) = \mu(E^a)$.

We consider the parameter change from E_0 to E, where each X_j^* changes from X_j^* at E_0 to $X_j^* + \delta X_j$, which is accompanied by a change from μ to $\mu + \delta\mu$. Assuming a gradual change in the dynamics x_j, we introduce a partial derivative

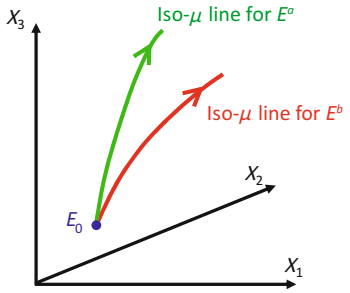

Fig. 1 Schematic representation of our theoretical analysis: changes in gene expression in a high-dimensional state space under different perturbations E^a and E^b are presented. Upon a given environmental change, the phenotypic changes in component concentrations follow a curve satisfying the constraint showing that the growth rates of all components are identical, i.e., an iso-μ line $F_1 = F_2 = \cdots = F_M$, in an M-dimensional state space. For a different environmental vector, the locus in the state space follows a different iso-μ line

of $F_i(\{X_j^*(E)\})$ by X_j at $E = E_0$, which gives the Jacobian matrix J_{ij}. Assuming that the environmental change is small and that phenotypic changes are sufficiently small and follow only the linear term in δX_j, we obtain

$$\sum_j J_{ij}\delta X_j(E) + \gamma_i \delta E = \delta\mu(E) \tag{2}$$

with $\gamma_i \equiv \frac{\partial F_i}{\partial E}$. Under our linear conditions, $\delta\mu \propto \delta E$, so that $\delta\mu = \alpha\delta E$ holds for a constant α. Accordingly, we obtain $\sum_j J_{ij}\delta X_j(E) = \delta\mu(E)(1 - \gamma_i/\alpha)$. Hence, $\frac{\sum_j J_{ij}\delta X_j(E)}{\delta\mu(E)} = \frac{\sum_j J_{ij}\delta X_j(E')}{\delta\mu(E')}$ so that

$$\frac{\delta X_j(E)}{\delta X_j(E')} = \frac{\delta\mu(E)}{\delta\mu(E')} \tag{3}$$

is obtained over all j. The formula can be compared with experimental observations. Note that it can be applied to any component. For example, one can use the concentration of either mRNA or protein, depending on the available experimental data.

3 Experimental Confirmation

To explore the relationship between changes in global gene expression and growth rate, we analyzed transcriptome data of *Escherichia coli* obtained under three environmental conditions, osmotic stress, starvation, and heat stress, as presented in Matsumoto et al. (2013). In the experiments, the cells were initially cultured

under minimal medium at 37 °C. After the transient response to the introduction of a given stress, the cells were harvested when the growth rate reached a constant value. The data were taken only from an exponentially growing steady state. For each stress condition, three levels of stresses (s = high, medium, low) were used, so that the absolute expression levels, represented by x_j for jth gene, were measured over 3×3 conditions.

Through transcriptome analysis under different environmental conditions, we calculated the change in gene expression levels between the original state and for a system experiencing environmental stress. We investigated the difference in gene expression using a log scale ($X_j = \log x_j$), that is, $\delta X_j(E) = X_j(E) - X_j^O$ (i.e., $\log(x_j(E)/x_j^O)$), for genes j, where E represents a given environmental condition and X_j^O represents the log-transformed gene expression level under the original condition (Kaneko et al., 2015).

To examine the validity of the theory for global changes in expression induced by the environmental stresses, we plotted the relationship between the differences in expression $(\delta X_j(E_{s_1}^a), \delta X_j(E_{s_2}^a))$ in Fig. 2a for s_1 = low and s_2 = medium, where a is heat stress. A common proportionality was observed in concentration changes across most mRNA species, which is consistent with the theory (Kaneko et al., 2015).

According to our theory, the proportion coefficient in the expression level should agree with the growth rate. Here, for each condition, the change in the growth rate $\delta\mu(E_s^a)$ was also measured (a is either osmotic, heat, or starvation stress). The slope fitted from the data agrees well with the common ratio $\delta X_j(E_{s_1}^a)/\delta X_j(E_{s_2}^a)$.

In this respect, the theory based on the steady-growth state and linearization of changes in stress works well for analyzing transcriptome changes in bacteria. Indeed, the relevance of growth rate to global trend in transcriptome changes was noted in several experiments (Brauer et al., 2008; Keren et al., 2013; O'Duibhir et al., 2014; Regenberg et al., 2006), and the formulation in the last section can provide a step to understand such global trend (see also Klumpp et al., 2009; Scott et al., 2010). Here, however, the steady-growth theory is not sufficient. First, the global proportionality is satisfied even under a stress condition that reduces the growth rate to below 20% or as compared to the standard. Such expansion of the linear regime is beyond the simple theory. The other important point missed by this steady-growth theory will be discussed in the next section.

4 Global Proportional Changes in Gene Expression Beyond the Simple Theory

Until now, we have compared the responses against a given type of environmental condition with different strengths. In general, possible environmental changes are described by a vector as $\mathbf{E}$. In the study, the changes are given by $\mathbf{E} = \lambda^a \mathbf{e}^a$ with different strengths λ^a by fixing $\mathbf{e}^a$. However, one can also compare expression

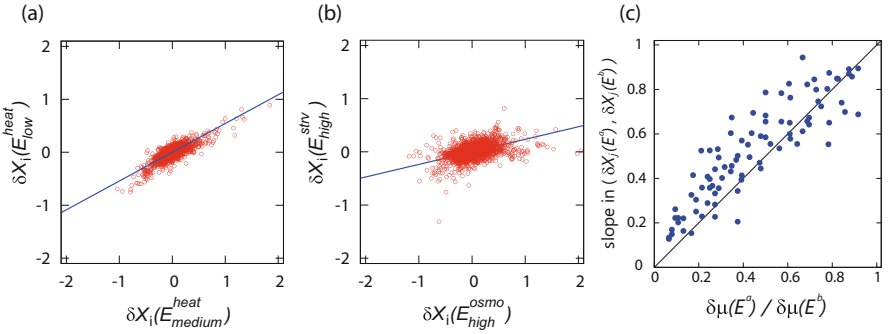

Fig. 2 Examples of common proportionality in *E. coli* gene expression changes $\delta X_j(E^a_{s_1})$ and $\delta X_j(E^b_{s_2})$ for genes in *E. coli*. δX_j represents the difference in the logarithmic expression level of a gene j between non-stressed and stressed conditions, where a, b represents the stress type and s_1 and s_2 represent stress strengths, e.g., low, medium, and high. Reproduced from Kaneko et al. (2015). (**a**) The relation between $\delta X_j(E^a_{s_1})$ and $\delta X_j(E^a_{s_2})$ for a = heat stress and s_1 =medium and s_2 =low. The fitted line indeed agrees well with that expected from the growth-rate change in Eq. (3). (**b**) The relation between $\delta X_j(\mathbf{E}^a_s)$ and $\delta X_j(\mathbf{E}^b_s)$, where a and b are osmotic stress and starvation, respectively, and s =high. (**c**) Relation between the slope of the change in protein expression and the change in the growth rate. The abscissa represents $\delta\mu(\mathbf{E}^a)/\delta\mu(\mathbf{E}^b)$, whereas the ordinate is the slope in $\delta X_j(\mathbf{E}^a)/\delta X_j(\mathbf{E}^b)$. The slope was obtained by fitting the protein expression data. Reproduced from Furusawa and Kaneko (2018)

changes across different types of stress conditions. In this case, the environmental change $\mathbf{E}$ is no longer represented by a scalar variable, and the responses against the environmental changes given by different vectors $\mathbf{e}^a$ and $\mathbf{e}^b$ must be compared.

However, the above theory cannot predict the common proportionality. This is because each one-dimensional curve upon a given type of environmental change is generally located along a different direction in the state space (see Fig. 1). This can also be understood using Eqs. (1)–(3) in Sect. 2. To compare the responses against different types of environmental changes using Eqs. (2) and (3), one needs $\gamma_i^a \equiv \frac{\partial F_i}{\partial \lambda^a}$, which depends on the type of environmental (stress) condition a. Hence, rather than Eq. (3), we obtain

$$\frac{\delta X_j(\mathbf{E}^a)}{\delta X_j(\mathbf{E}^b)} = \frac{\delta\mu(\mathbf{E}^a)}{\delta\mu(\mathbf{E}^b)} \cdot \frac{\sum_i L_{ji}(1-\gamma_i^a/\alpha^a)}{\sum_i L_{ji}(1-\gamma_i^b/\alpha^b)} \tag{4}$$

Then, because $\gamma_i^a \neq \gamma_i^b$, in general, the proportionality cannot be determined as in Eq. (3). Although the theory cannot predict the simple proportional relationship, one can plot the experimental data as in Fig. 2a, even across different conditions for osmotic pressure, heat, and starvation. An example of such plot is given in Fig. 2b (see also Kaneko et al., 2015). Further support of the proportionality was obtained from the changes in concentrations of thousands of proteins (rather than mRNA) under different conditions by using proteome analysis (Schmidt et al., 2016). Interestingly, in both cases, a strong correlation between $\delta X_j(\mathbf{E}^a)$ and $\delta X_j(\mathbf{E}^b)$ was

still observed for all components j, even under different environmental conditions. Although more genes deviated from the common proportionality, lowering the correlation coefficients as compared with those for the same type of stress, the global proportionality still held for most genes (note that Fig. 2b includes more than 1000 points, so that a single line fits most of these points). Thus, the global proportionality is still valid. Further, as shown in Fig. 2c, the slope approximately agrees with the rate of growth change, as in Eq. (3). Additionally, other data suggested such a correlation in mRNA abundance under different environmental conditions (Keren et al., 2013; Stern et al., 2007).

Because gene expression dynamics are very high-dimensional, this correlation suggests that a strong constraint exists in adaptive changes in expression dynamics that cannot be explained by the simple theory assuming only steady growth. The global proportionality is beyond the scope of the simple theory presented in Eqs. (2) and (3). Thus, this proportionality is not generic in any dynamical systems satisfying only steady growth.

In summary, two questions remain: how to evaluate a broad range of linearity regime and evaluate proportionality under different environmental conditions. To answer these questions, some factor other than steady growth must be evaluated.

Of course, cells are not only constrained by steady growth but also are a product of evolution. Through evolution, cells can efficiently and robustly reproduce themselves under external conditions. Therefore, the above two features may result from evolution. In the next section, we examine the validity of the hypothesis that evolutionary robustness constrains intracellular dynamics to exhibit global proportionality in the adaptive changes of many components.

5 Emergence of Global Proportionality Through Evolution: Formation of a Dominant Mode

5.1 Catalytic Reaction Network Model for Numerical Evolution

The above hypothesis regarding the consequence of evolutionary robustness is difficult to evaluate experimentally, as the experimentally available data are only from organisms that currently exist as a result of evolution: one cannot compare them with the data before evolution. Hence, we used numerical evolution for some models.

To this end, we utilize simple cell models consisting of a large number of components and numerically evolve them under a given fitness condition to determine how the phenotypes of many components evolve. Two models are adopted in which phenotypes are generated by dynamical process for intracellular components. One is a catalytic reaction network model (Furusawa & Kaneko, 2003, 2012) in which catalysts are synthesized with the aid of other catalysts so that the concentrations of

a set of catalytic molecules constitute the phenotypic state space. The other model adopts a gene regulation network (Ciliberti et al., 2007; Glass & Kauffman, 1973; Kaneko, 2007; Mjolsness et al., 1991) in which proteins are expressed as a result of mutual activation or inhibition from other proteins. Both dynamics involve a large number of components, i.e., chemical concentrations or protein expression levels, which determine the phenotypes. The growth rate or fitness is determined by these phenotypes.

In these models, genes govern the network structure and parameters for the reactions that establish the rules for such dynamical systems. The phenotype of each organism, as well as the growth rate (fitness) of a cell, is determined by such reaction dynamics, whereas the evolutionary process consists of selection according to the associated fitness and genetic change in the reaction network (i.e., rewiring of the pathway). The global proportionality in concentration changes across components is confirmed in both the models after evolution.

Here, we explain the results of analysis using the catalytic network. Despite its simplicity, this model captures the basic characteristic of cells such as the power-law abundance and log-normal fluctuations of cellular components and adaptation with fold-change detection, among other factors (Furusawa & Kaneko, 2003; Furusawa et al., 2005; Furusawa & Kaneko, 2012; Kaneko & Furusawa, 2006).

In the model, the cellular state is represented by the numbers of k chemical species, i.e., $(N_1, N_2, \cdots, N_k)$, whereas their concentrations are given by $x_i = N_i/V$ with the volume of the cell V (Furusawa & Kaneko, 2003, 2012). There are $m(< k)$ resource chemicals $S_1, S_2, \cdots, S_m$ whose concentrations in the environment and within a cell are given by $s_1, \cdots, s_m$ and $x_1, \cdots, x_m$, respectively. Each reaction leading from one chemical i to another chemical j was assumed to be catalyzed by a third chemical ℓ, i.e., $i + \ell \rightarrow j + \ell$. The resource chemicals are transported into the cell with the aid of other chemical components named as "transporters." We assumed that the uptake flux of nutrient i from the environment is proportional to $Ds_i x_{t_i}$, where chemical t_i acts as the transporter of nutrient i and D is a transport constant. For each nutrient, there is one corresponding transporter, represented by $t_i = m + i$. The other $k - 2m$ chemical species are catalysts synthesized from other components via the catalytic reactions mentioned above. The catalytic reactions result in nutrient transformation into cell-component chemicals. With the uptake of nutrient chemicals from the environment, the total number of chemicals $N = \sum_i N_i$ in a cell increases. A cell, then, is divided into two cells when the total number of molecules exceeds a given threshold.

Here, to achieve a higher growth rate, the synthesis of the cell components must progress concurrently with nutrient uptake. Hence, the cellular growth rate depends on the catalytic network, which is determined by genes. With evolution, this growth rate, i.e., the fitness, can be increased.

Because of this fitness, the evolutionary procedure is carried out as follows: First, we prepared n parent cells with slightly different reaction networks, randomly generated with a given connection rate. We applied stochastic reaction simulation of the above model and selected n/L cells with high growth rates. From each of the n/L parent cells, L mutant cells were generated by replacing a certain fraction of

reaction paths, whose rate is determined by the mutation rate. Next, we obtained n cells of the next generation, which contained slightly different reaction paths. We repeated the same procedure to obtain the next generation population and so on.

The simulation of evolutionary dynamics was performed under a constant environmental condition $\mathbf{o} = \{s_1^o, \cdots, s_m^o\}$. Under the original condition, the concentrations were set at $s_1^o = s_2^o = \cdots = s_m^o = 1/m$, at which evolution progresses so that the cell growth rate, i.e., inverse of the average division time, is increased.

5.2 *Emergent Global Proportionality Through Evolution*

Using the model described in the last subsection, we analyzed the response of the component concentrations to the environmental change from the original condition. Here, the environmental condition is given by the external concentration $\{s_1, \cdots, s_m\}$. We then changed the condition to $s_j^{(\varepsilon,\mathbf{E})} = (1-\varepsilon)s_j^o + \varepsilon s_j^{\mathbf{E}}$, where ε is the intensity of the stress and $\mathbf{E} = \{s_1^{\mathbf{E}}, \cdots, s_m^{\mathbf{E}}\}$ denotes the vector of the new, stressed environment, in which the values of component $s_1^{\mathbf{E}}, \cdots, s_m^{\mathbf{E}}$ were determined randomly to satisfy $\sum_j s_j^{\mathbf{E}} = 1$. For each environment, we computed the reaction dynamics of the cell to obtain the concentration $x_j^{(\varepsilon,\mathbf{E})}$ in the steady-growth state, from which the logarithmic change in the concentration $\delta X_j^{(\varepsilon,\mathbf{E})} = \log(x_j^{(\varepsilon,\mathbf{E})}/x_j^{\mathbf{o}})$ was obtained; the change in growth rate μ, designated as $\delta\mu^{(\varepsilon,\mathbf{E})}$, was also computed.

We next examined whether changes in $\delta X_j^{(\varepsilon,\mathbf{E})}$ satisfy the common proportionality across all components under a variety of environmental changes for the ranges $0 \leq \varepsilon \leq 1$. We examined the degree of proportionality both for the random networks before evolution and those after evolution under the given environmental conditions. We also tested whether the proportion coefficient is consistent with $\delta\mu^{(\varepsilon,\mathbf{E})}$.

We computed the response of expression to the same type of stress, i.e., the same vector $\mathbf{E}$ with different intensities ε. We first examined the correlation between changes in component concentrations $\delta X_j^{(\varepsilon_1,\mathbf{E})}$ and $\delta X_j^{(\varepsilon_2,\mathbf{E})}$ caused by different magnitudes of environmental change ($\varepsilon_2 = \varepsilon_1 + \varepsilon$, at $\varepsilon > 0$). For a small environmental change ($\varepsilon = 0.02$), the correlation was strong both for the random and evolved networks, whereas for a larger environmental change ($\varepsilon = 0.08$), the correlation coefficients were significantly smaller for the random networks (Furusawa & Kaneko, 2018). We then computed the relationship between the ratio of the growth rate changes $\delta\mu^{(\varepsilon_1,\mathbf{E})}/\delta\mu^{(\varepsilon_2,\mathbf{E})}$ and fitted slope in $(\delta X_j^{(\varepsilon_1,\mathbf{E})}, \delta X_j^{(\varepsilon_2,\mathbf{E})})$ across all components. Figure 3a shows the ratio of the slope to $\delta\mu^{(\varepsilon_1,\mathbf{E})}/\delta\mu^{(\varepsilon_2,\mathbf{E})}$ (which turns to be unity when Eq. (3) is satisfied) as a function of the magnitude of environmental change ε_1. These results demonstrated that for the evolved network, Eq. (3) is maintained under large environmental changes, whereas it holds only

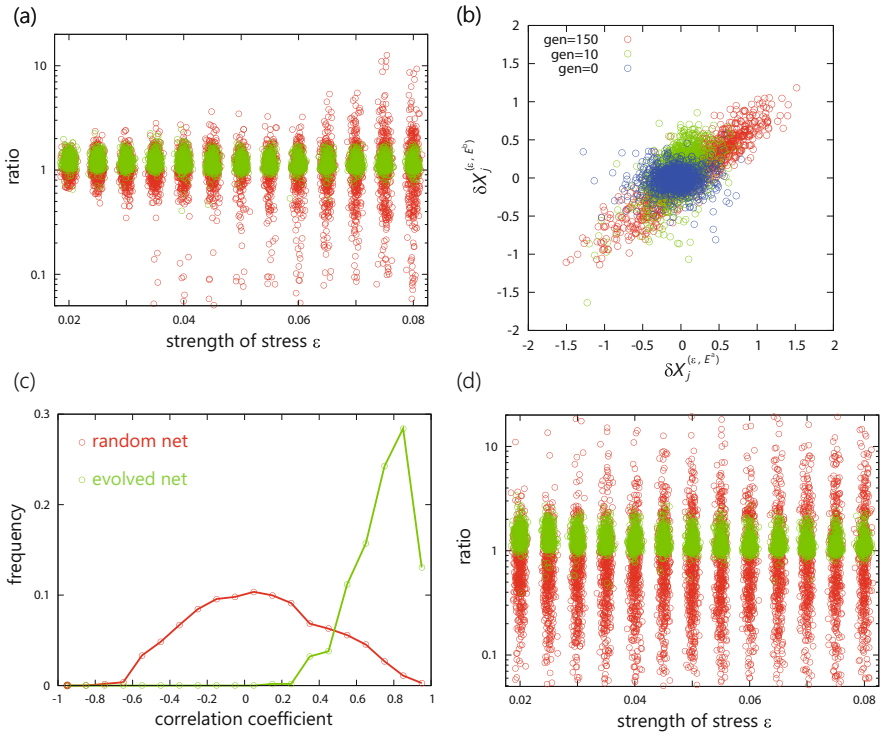

Fig. 3 Common proportionality in concentration changes in response to the stresses in the catalytic reaction network model. Reproduced from Furusawa and Kaneko (2018). (**a**) Ratio of the slope in the concentration changes by the same type of stress to the growth rate change as a function of the intensity of stress ε. For the random (red) and evolved networks (green), against 100 randomly chosen environmental vectors $\mathbf{E}$. The ratio in the ordinate becomes unity when Eq. (3) is satisfied. (**b–d**) Common proportionality in concentration changes in response to different types of stress in the catalytic reaction network model. (**b**) Concentration changes across different types of environmental stressors. For three different networks from different generations, $(\delta X_j(\mathbf{E}^a), \delta X_j(\mathbf{E}^b))$ are plotted by means of different randomly chosen vectors $\mathbf{E}^a$ and $\mathbf{E}^b$. (**c**) Distributions of coefficients of correlation between the changes in component concentrations $\delta X_j^{(\varepsilon,\mathbf{E}^a)}$ and $\delta X_j^{(\varepsilon,\mathbf{E}^b)}$. Red and green curves represent the distributions of a random network and evolved network (150th generation) obtained from 1000 pairs of $\mathbf{E}^a$ and $\mathbf{E}^b$, respectively. The magnitude of environmental change ε is fixed at 0.8. (**d**) Ratio of the slope in the relation between concentration changes to the growth rate change, plotted as a function of the intensity of stress ε in the case of different types of stress

against small changes for random networks. The expansion of the linear regime by evolution was confirmed.

Next, we examined the correlation of concentration changes under different types of environmental stressors. Figure 3b shows examples of $(\delta X_j^{(\varepsilon,\mathbf{E}^a)}, \delta X_j^{(\varepsilon,\mathbf{E}^b)})$ obtained by three networks from different generations. For the initial random networks, there was no correlation, whereas a modest correlation emerged in the

tenth generation. Later, over evolution, common proportionality was observed; for instance, in the 150th generation, the proportionality reached more than 2 digits. To demonstrate the generality of the proportionality over a variety of environmental variations, we computed the coefficients of correlation between $\delta X_j^{(\varepsilon,\mathbf{E}^a)}$ and $\delta X_j^{(\varepsilon,\mathbf{E}^b)}$ for a random choice of different vectors $\mathbf{E}^a$ and $\mathbf{E}^b$. Figure 3c shows the distributions of the correlation coefficients obtained by the random network and evolved network (150th generation). Remarkably, global proportionality was observed even under different environmental conditions that had not been experienced through the course of evolution.

We first computed the relationship between $\delta\mu^{(\varepsilon_1,\mathbf{E}^a)}/\delta\mu^{(\varepsilon_2,\mathbf{E}^b)}$ and the slope in $(\delta X_j^{(\varepsilon_1,\mathbf{E}^a)}, \delta X_j^{(\varepsilon_2,\mathbf{E}^b)})$, from which the ratio of the slope in $(\delta X_j^{(\varepsilon_1,\mathbf{E}^a)}, \delta X_j^{(\varepsilon_2,\mathbf{E}^b)})$ to $\delta\mu^{(\varepsilon_1,\mathbf{E}^a)}/\delta\mu^{(\varepsilon_2,\mathbf{E}^b)}$ is shown in Fig. 3d as a function of ε_2. The slope of δX agrees rather well with the growth rate change as given by Eq. (3) for the evolved networks as compared to the random networks.

The global proportionality over all components across various environmental conditions suggests that changes $\delta X_j^{(\varepsilon,\mathbf{E})}$ across different environmental conditions are constrained mainly along a one-dimensional manifold after evolution has progressed (even) under a single environmental condition. To verify the existence of such constraints, we carried out the principal component (PC) analysis of the data of $\delta X_j^{(\varepsilon,\mathbf{E})}$ across different environmental changes $\mathbf{E}$ and ε. As shown in Fig. 4a, we plotted the data in the space with the first three PC axes. In the evolved network, high-dimensional data from $X_j^{(\varepsilon,\mathbf{E})}$ were located along a one-dimensional curve. Note that the contribution of the first PC reached 74% in the data. In contrast, the data from the random network were scattered, and no clear structure was visible, as shown in Fig. 4b. Furthermore, for the evolved network, the value of the first PC agrees rather well with the growth rate (Furusawa & Kaneko, 2018).

We then examined the evolutionary course of the phenotype projected on the same principal component space, as depicted in Fig. 4a. As shown in Fig. 4c, the points from $\{X_j\}$ generated by random mutations in the reaction network are again located along the same one-dimensional curve. Thus, the phenotypic changes are highly restricted, both genetically and non-genetically, within an identical one-dimensional curve. As shown in Fig. 4a, c, variation in the concentration due to perturbations was much larger along the first PC than along the other components. This suggests that relaxation is much slower in the direction of the first component than in the other directions.

In summary, we observed emergent global proportionality which is far beyond the trivial linearity in response to tiny perturbations. After evolution, the linearity region expanded to a level with an order-of-magnitude change in the growth rate. Additionally, the proportionality over different components across different environmental conditions was enhanced through evolution. In this global proportionality, evolutionary dimension reduction in phenotypic dynamics underlies changes in the high-dimensional phenotypic space across a variety of environmental conditions, genetic variations, and noise, which are confined to a common one-

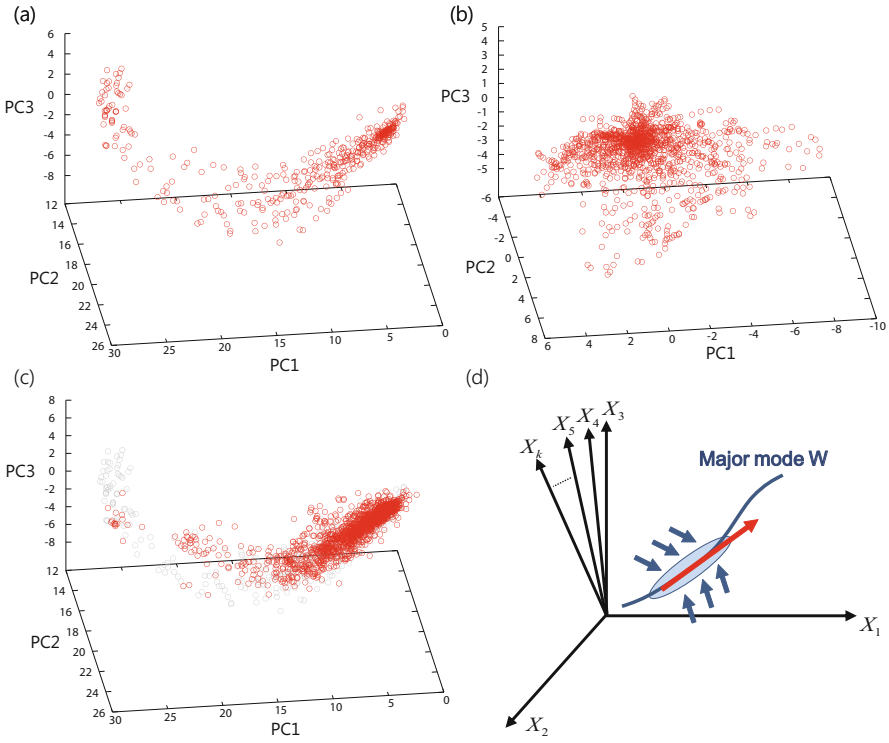

Fig. 4 (**a**–**c**) The change in $X_j^{(\varepsilon,\mathbf{E})}$ with environmental changes in principal component space. Reproduced from Furusawa and Kaneko (2018). Component concentrations $X_j^{(\varepsilon,\mathbf{E})}$ at randomly chosen various **E** and ε values are presented for (**a**) evolved and (**b**) random networks. In (**a**), the contributions of the first, second, and third components were 74%, 8%, and 5%, respectively. In (**c**), change in component concentration X_j because of mutations is plotted, where mutations were added to the evolved reaction network by randomly replacing 0.5% of the reaction paths. The red dots show the concentrations of components after mutations, which are projected onto the same principal component space, as depicted in (**a**). The gray dots represent the concentration changes caused by environmental changes for the reference, which are identical to those shown in (**a**). (**d**) Schematic representation of the dimension reduction hypothesis. In the state space of **X**, dominant changes are constrained along the mode **W** following the major axis and its connected manifold, whereas the attraction to this manifold is much faster

dimensional manifold. The first principal component mode corresponding to the one-dimensional manifold is highly correlated with the growth rate.

Notably, this dimension reduction by evolution has also been observed in some other models. First, even when the fitness for selection does not affect the growth rate but rather some other quantity (such as the concentration of a component), the phenotypic change is mainly constrained along a one-dimensional manifold. Second, even when the environmental condition (e.g., concentrations of external nutrients) is not fixed but rather fluctuates over generations, restriction within the one-dimensional manifold is observed (Sato & Kaneko, 2020). Third, there are

preliminary reports that the evolution of gene regulation networks and spin-glass models (Sakata et al., 2009) also show dimension reduction of phenotypic changes, as observed in our results.

6 Evolutionary Dimension Reduction Hypothesis

Following the observation of global proportionality and dimension reduction from the high-dimensional phenotypic space in the last section, we proposed the following hypothesis:

Phenotypic dynamics involve a large number of variables, and their state space is generally high-dimensional. However, phenotypic changes induced by environmental perturbations are constrained mainly along a low-dimensional (often one-dimensional) manifold (major axis). Along this manifold, the phenotypic dynamics are much slower than those across the manifold. Further, phenotypic changes induced by evolutionary changes (i.e., due to genetic changes governing phenotypic dynamics) also progress mainly along this manifold. The fitness gradually changes along the manifold, whereas it rapidly decreases across the manifold.

Indeed, the results described in the last section support the hypothesis, where the dominance of the first principal mode in a phenotypic change emerges after evolution, and the phenotypic changes as a result of mutation are constrained along the principal mode axis (see Fig. 4c). Moreover, expression data from bacterial evolution studies support this hypothesis, as described later.

This hypothesis is plausible considering the evolutionary robustness of phenotypes: first, in most cases, phenotypes (e.g., concentrations of chemicals) are shaped as a result of complex dynamics involving a large degree of freedom. For examples, these dynamics can be determined by catalytic reaction or gene regulatory networks, which are determined by genes. In general, networks with higher fitness are rare, and thus a mutation selection process is needed to achieve higher fitness. Because of the complexity of the dynamics, stochastic perturbations may influence the final phenotype in general, unless the networks are evolved to reduce the influence of perturbations (Kaneko, 2012a).

As evolution progresses, robustness of the state against perturbations will be acquired. Otherwise, because of inevitable noise during the dynamics, a rare fitter state is not sustained. Increased robustness to perturbations is expected to result from evolution (see also Ciliberti et al., 2007; Kaneko, 2007). Accordingly, in the state space, the dynamics provide flow to the selected (fitter) phenotype against perturbations for most directions, as shown schematically in Fig. 4d. Strong contraction to the attracted state is shaped by evolution. However, there is (at least) one exceptional direction that does not possess such a strong contraction. This is the direction along which evolution to increase fitness progresses. Along this direction, phenotypic states can be changed rather easily by perturbations. Otherwise, it is difficult for evolution to progress. Hence, as schematically shown in Fig. 4d, only

along the direction in which evolutionary changes proceed, the relaxation is slow, whereas for other orthogonal directions, the change is much faster.

Now, let us consider the relaxation dynamics to the original state (attractor) at a given generation, as represented by Eq. (2). The relaxation dynamics are represented by a combination of eigenmodes with negative eigenvalues. The magnitude of (negative) eigenvalues will be large, except for one (or a few) eigenvector, whereas that along the direction of evolution will be much closer to zero, that is, relaxation along the direction of evolution is slower. Hence, variance along the largest principal component will be dominant, as demonstrated numerically in Fig. 4a, c.

Indeed, in a recent study Sato and Kaneko (2020), the eigenvalues of the Jacobian matrix in Eq. (2) were numerically computed by using the catalytic reaction dynamics adopted in the last subsection. The results confirmed that one eigenvalue was close to zero and all other (negative) eigenvalues had much larger magnitudes. This separation of one eigenvalue occurred because of evolution.

This hypothesis indicates that only one mode dominates in Eq. (2). Although the original dynamics are high-dimensional, most changes occur along the one-dimensional manifold **W**, corresponding to the eigenvector for the smallest eigenvalue (or its nonlinear extension). Let us denote this direction as $\mathbf{w_0}$. From this dominance of the single dominant mode W, the global proportionality in Eq. (3) is naturally derived across different conditions **E**. Because changes in all components X_j, δX_js are constrained along the dominant mode W, they are given by the projection of the change in W onto each X_j axis, which is in turn given by $\cos\theta_j$, where θ_j is the angle between W and each axis X_j. This angle is determined by the given phenotypic state only and is independent of the type of environmental perturbation. Thus, the proportionality observed over different strengths of an identical environmental condition is valid across different types of environmental perturbations.

Note that the change along **W** is parametrized by the growth rate $\delta\mu$ because it shows tight one-to-one correspondence with the principal coordinate. Then δW is represented as a function of $\delta\mu$. Given that $\delta W \propto \delta\mu(\mathbf{E})$ for small $\delta\mu$, then Eq. (3) can be extended to different environmental vectors as follows:

$$\frac{\delta X_j(\mathbf{E})}{\delta X_j(\mathbf{E}')} = \frac{\delta\mu(\mathbf{E})}{\delta\mu(\mathbf{E}')}. \tag{5}$$

Indeed, the above argument can be formulated explicitly by using linear algebra for the relaxation dynamics to the original state (attractor) at a given generation, as represented by Eq. (2). By using $\mathbf{L} = \mathbf{J}^{-1}$, it follows that $\boldsymbol{\delta X} = \mathbf{L}(\delta\mu\mathbf{I} - \boldsymbol{\gamma}\delta E)$, where **I** is a unit vector $(1, 1, 1, \ldots 1)^T$. The matrix **L** is represented by $\sum_k \lambda_k \mathbf{w}_k \mathbf{v}_k^T$, with their eigenvalues λ_k and the corresponding right (left) eigenvectors $\mathbf{w}_k$ ($\mathbf{v}_k$), respectively. Note that the hypothesis in the present section postulates that the magnitude of the smallest eigenvalue of **J** (denoted as $k = 0$) is much smaller than that of the others. In other words, the absolute eigenvalue of λ_0 for $\mathbf{L} = \mathbf{J}^{-1}$ is much greater than others. Then, the major response to environmental changes is given only by the projection to this mode for λ_0. In other words, the major

axis for the change $\boldsymbol{\delta X}$ is given by $\mathbf{w_0}$. Using this reduction to this mode $\mathbf{w_0}$, and through straightforward calculation (see Furusawa & Kaneko, 2018 for details), we obtain $\boldsymbol{\delta X} = \lambda^0 \mathbf{w_0}(\delta\mu(\mathbf{v_0} \cdot \mathbf{I}))$. Accordingly, the global proportionality relationship in Eq. (5) is reproduced.

In summary, we can explain two basic features observed in experiments and simulations using the above theoretical formulation:

1. **Overall proportionality in expression level changes across most components and across various environmental conditions:** This is because high-dimensional changes are constrained to changes along the major axis, i.e., eigenvector $\mathbf{w_0}$.
2. **Extended region of global proportionality:** Because the range in variation along $\mathbf{w_0}$ is large, the change in the phenotype is constrained to points near this eigenvector, causing the proportionality range of phenotypic change to extend via evolution. Furthermore, as long as the changes are nearly confined to the manifold along the major axis, global proportionality reaches the regime nonlinear to $\delta\epsilon$.

7 Global Proportionality Between Responses by Environmental and Evolutionary Adaptations

According to the hypothesis in the last section, the change due to genetic variation would also be constrained along with this major axis $\mathbf{w}_0$, as the most changeable direction $\mathbf{w}_0$ is the direction in which evolution has progressed and will progress. Indeed, in the simulation described in Sect. 5, the phenotypic changes caused by the mutational change are constrained along the manifold spanned by the first principal mode in the environmentally induced phenotypic changes (see Fig. 4). Accordingly, we expect to observe global proportionality between the concentration changes induced by a given environmental condition (stress) ($\delta X_j(Env)$) and those derived from evolution with genetic changes ($\delta X_j(Gen)$), as given by

$$\frac{\delta X_j(Gen)}{\delta X_j(Env)} = \frac{\delta\mu(Gen)}{\delta\mu(Env)}. \tag{6}$$

For example, when cells are subjected to a stressed condition Env, the growth rate is reduced so that $\delta\mu(Env) < 0$, whereas the expression levels change $\delta X_j(Env)$ accordingly. Next, the cells evolve under this given stressed condition over several generations along with genetic changes. After n generations under genetic evolution, the growth rate recovers to some degree so that the growth rate shows a difference of $\delta\mu(Gen)$ from the original (non-stressed) state, satisfying $0 \geq \delta\mu(Gen) \geq \delta\mu(Env)$. The accompanied expression change, denoted by $\delta X_j(Gen)$, is then expected to satisfy

$$\frac{\delta X_j(Gen)}{\delta X_j(Env)} = \frac{\delta \mu(Gen)}{\delta \mu(Env)} \leq 1 \tag{7}$$

across most components j in a similar manner as in Eq. (5). Because $|\delta\mu(Gen)|$ is reduced with the progression of evolution, changes in the components introduced by the environmental change are reduced. Thus, there is an evolutionary tendency that the original expression pattern is recovered. This is reminiscent of the Le Chatelier principle in thermodynamics.

We next examined if the above relationship would hold in numerical simulation and bacterial evolution experiments.

7.1 Verification by the Reaction Network Model

We employed the catalytic reaction network model in Sect. 5. After evolving the cell as described in the section under the given environmental condition, we switched the nutrient condition at a given generation. This caused the growth rate to decrease, which was later recovered through genetic evolution over generations. We computed the phenotypic changes induced by the environmental and evolutionary changes to examine the validity of the above relationship.

After altering the nutrient conditions, the abundances of all the components were changed. The average change of these abundances was denoted by $\delta X_j^{Env} \equiv \langle X_j(1)\rangle - \langle X_j(0)\rangle = \log \frac{\langle N_j(1)\rangle}{\langle N_j(0)\rangle}$, where generation 1 refers to the time point immediately after the environmental change and generation 0 denotes the generation right before this nutrient change. Similarly, we defined the response by genetic evolution after m generations by $\delta X_j^{Gen}(m) = \langle X_j(m)\rangle - \langle X_j(0)\rangle$. Figure 5a, b shows the plot of δX_j^{Env} versus $\delta X_j^{Gen}(m)$ for $m = 5$ and 50. The proportionality was observed between the environmental and genetic responses over all components.

Let us now define this proportion coefficient $r(m)$ for $\frac{\delta X_j^{Gen}(m)}{\delta X_j^{Env}}$ across components j. According to Eq. (6), this agrees with the growth rate change given by the ratio of $\delta\mu^{Gen}(m) = \mu(m) - \mu(0)(\leq 0)$ to $\delta\mu^{Env} = \mu(1) - \mu(0)(< 0)$ at each generation m. In Fig. 5c, the proportion coefficient $r(m)$ was plotted against this growth rate recovery $\delta\mu^{Gen}(m)/\delta\mu^{Env}$. The agreement between the two is clearly discernible. This proportion coefficient $r(m)$ is initially close to 1 (i.e., $m \sim 1$); with increasing generation m, the value decreased toward zero, in conjunction with recovery of the growth rate $\delta\mu^{Gen}(m)/\delta\mu^{Env}$. Thus, as stated in Le Chatelier principle mentioned in the previous section, evolution shows a common tendency to reduce changes in components introduced by environmental change.

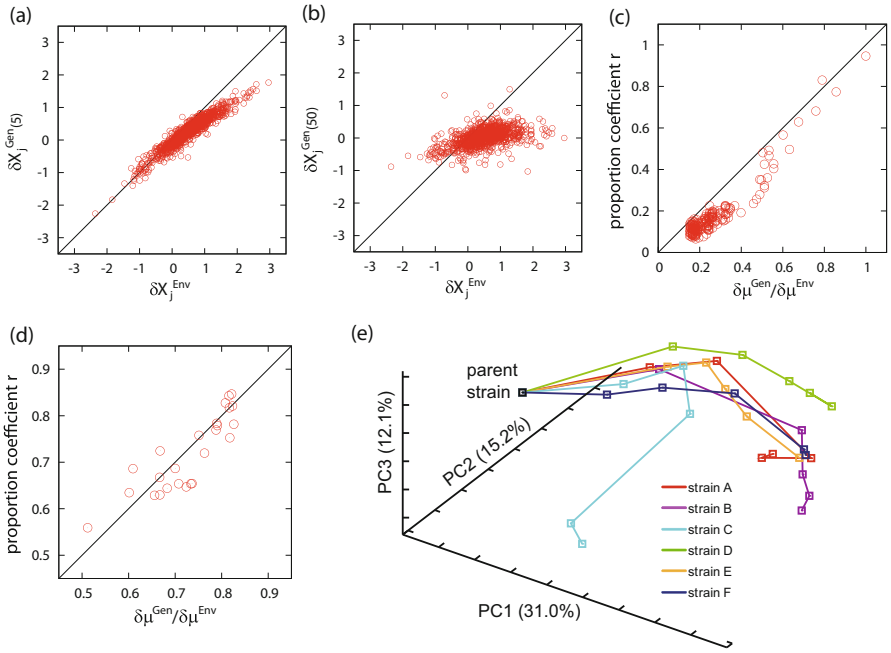

Fig. 5 (**a**–**c**) Response to environmental change versus response by evolution in the reaction network model. Reproduced from Furusawa and Kaneko (2015). Relationship between the environmental response δX_j^{Env} and genetic response $\delta X_j^{Gen}(m)$. (**a**) and (**b**) show the plots for $m = 5$ and 50, respectively. The black solid lines are $y = x$ for reference. (**c**) Relationship between growth recovery rate $\delta\mu^{Gen}(m)/\delta\mu^{Env}$ and the proportion coefficient $r(m)$. The proportion coefficient $r(m)$ was obtained by using the least squares method for the relationship of δX_j^{Env} and $\delta X_j^{Gen}(m)$ for $m = 1 \sim 200$. The black solid line is $y = x$ for reference. (**d**, **e**)Response to environmental change versus response by evolution in *E. coli* adaption to ethanol stress. (**d**) Relationship between growth recovery rate $\delta\mu^{Gen}(n)/\delta\mu^{Env}$ and the proportion coefficient $r(n)$. The proportion coefficient $r(n)$ was obtained by using the least squares method for the relationship of δX_j^{Env} and $\delta X_j^{Gen}(n)$ for $n = 384$, 744, 1224, 1824, and 2496 h. The growth recovery rate $\delta\mu^{Gen}(n)/\delta\mu^{Env}$ was calculated based on the experimental measurements. Among the six independent culture lines (see Horinouchi et al., 2015 for details), the results of five culture lines without genome duplication are plotted. The black line is $y = x$ for reference. (**e**) Changes in PCA scores during adaptive evolution. Starting from the parent strain, changes in the expression profiles during adaptive evolution are plotted as orbits in the three-dimensional PCA plane

7.2 *Experimental Confirmation by Laboratory Evolution*

To verify the relationship given by Eq. (7), we analyzed time-series transcriptome data obtained in an experimental evolution study of *E. coli* under conditions of ethanol stress (Horinouchi et al., 2010, 2015). In this experiment, after cultivation of approximately 1000 generations (2500 h) under 5% ethanol stress, 6 independent ethanol-tolerant strains were obtained, which exhibited an approximately twofold

increase in specific growth rates compared with the ancestor. For all independent culture series, mRNA samples were extracted from approximately 10^8 cells at six different time points, and the absolute expression levels were quantified by microarray analysis. All mRNA samples were obtained from cells in an exponential growth phase (see Horinouchi et al., 2015 for details).

Using the expression data taken at several generations through adaptive evolution, we analyzed the common proportionality in expression changes. The environmental response of the jth gene δX_j^{Env} is defined by the log-transformed ratio of the expression level. Similarly, the evolutionary response at n hours after the exposure to stress $\delta X_j^{Gen}(n)$ is defined by the log-transformed ratio of the expression level at n hours to that of the non-stressed condition. We found a common trend between the environmental and genetic responses over all genes (Furusawa & Kaneko, 2015).

Furthermore, as shown in Fig. 5d, the agreement between $r(n)$ and the growth recovery ratio $\delta\mu^{Gen}(n)/\delta\mu^{Env}$, as predicted by Eq. (7), was discernible, where $\delta\mu^{Gen}(n)$ and $\delta\mu^{Env}$ are the growth rate differences at n h and 24 h after the exposure to stress, respectively. These results demonstrate that the evolutionary dynamics with growth recovery were accompanied by gene expression changes, which were reduced from those introduced by the new environment.

How does $\{X_j\}$ change in the state space of a few thousand dimensions? As it is hard to see a high-dimensional state space, we used the first, second, and third principal components determined from the data for each generation of *E. coli* gene expression. (Approximately 31% of the change in each data set can be explained by the first component, whereas 15% is explained by the second component). When the data points were plotted in the space of each principal component P^i axis, they were distributed mainly along the P^1 axis direction, whereas the spread in the P^2 and P^3 axes was limited. Furthermore, the value of this first component was approximately proportional to the growth rate. This is consistent with the finding that the growth rate is a major factor in determining the change in expression of each gene. Now, Fig. 5e shows the cell state changes by projecting the expression state X_j at each generation onto these three component axes. Here, six independent data are superimposed, which were obtained by repeating the same experiment. Mutations occurred at different sites by each experiment; each of the six strains has a different genetic sequence. Nevertheless, all experimental samples changed with the same curve. The phenotypic changes to increase fitness are rather deterministic as compared to random changes in genetic sequences. Shaping the relevant phenotypic change is a priority in evolution, whereas several possibilities exist to achieve such changes genetically.

8 Evolutionary Fluctuation-Response Relationship

In the previous section, we discussed the relationship between environmental and evolutionary responses. According to statistical physics, response and fluctuation are proportional (Einstein, 1926; Kubo et al., 1976). In evolution, analogously, we

previously proposed an evolutionary fluctuation-response relationship: the phenotypic response throughout the evolutionary course is proportional to the phenotypic variance induced by noise (Kaneko, 2012a; Sato et al., 2003). Consider a system characterized by a gene parameter a and phenotypic trait X. We can then evaluate the change in X against that in the gene parameter value from a to $a + \Delta a$. Then, the proposed fluctuation-response relationship is given by

$$\frac{\langle X\rangle_{a+\Delta a} - \langle X\rangle_a}{\Delta a} \propto \langle(\delta X)^2\rangle, \tag{8}$$

where $\langle X\rangle_a$ and $\langle(\delta X)^2\rangle = \langle(X - \langle X\rangle)^2\rangle_a$ are the average and variance of the phenotypic trait X for a given system parameterized by a, respectively.

If a is assigned as a parameter specifying the genotype (e.g., number of substitutions in the DNA sequence), this relationship implies that the evolutionary rate, i.e., change in average phenotype per generation, is proportional to the variance of the isogenic (clonal) phenotypic distribution, denoted by $V_{ip} = \langle(\delta X)^2\rangle$. In fact, several model simulations and some experiments support this type of evolutionary fluctuation-response relationship (Kaneko & Furusawa, 2006; Sato et al., 2003).

This evolutionary fluctuation-response relationship is associated with the phenotypic variance V_{ip} of isogenic individuals, which is caused by noise (for its measurement, see e.g., Bar-Even et al., 2006; Elowitz et al., 2002; Furusawa et al., 2005). In standard population genetics, in contrast, the phenotypic variance due to genetic variation, named as genetic variance V_g, is considered. In fact, the so-called fundamental theorem of natural selection proposed by Fisher (1930) states that the evolutionary rate is proportional to V_g. Thus, for both the evolutionary fluctuation-response relationship and Fisher's theorem to be valid, V_{ip} and V_g must remain proportional throughout the evolutionary course. Indeed, such a relationship between these two variances was confirmed through evolutionary simulations of a catalytic reaction network model and gene regulation network (Kaneko, 2007; Kaneko & Furusawa, 2006).

The origin of such a relationship can be explained as follows: In general, developmental dynamics to shape the phenotype are quite complex, and the final state may be diverted by perturbations in the initial condition or by those that occur during the dynamics. Even if the fit phenotype is shaped by developmental dynamics, the perturbations due to noise during the dynamics may result in different non-fit states. Thus, the phenotype may be rather sensitive to noise. After evolution progresses, the robustness of the fitted state to noise is increased. In this case, the global attraction to the target phenotype is shaped. This agrees with the hypothesis in Sect. 6.

Genetic changes, in contrast, can also cause perturbations to such dynamics. As the robustness to noise is shaped, the robustness against genetic changes is also expected to increase. Through evolution, as the dynamics become increasingly robust to noise, they also become more robust to genetic changes, resulting in a correlation between the two types of robustness. As the robustness to noise is increased, the phenotypic variance V_{ip} will decrease; similarly, an increase in the robustness

to genetic mutation leads to a decrease in V_g. Hence, throughout evolution, both V_{ip} and V_g decrease in correlation (or in proportion). Thus, proportionality between V_{ip} and V_g is expected, as observed in the evolution simulations (Kaneko, 2007; Kaneko & Furusawa, 2006). Furthermore, this proportionality of the two variances is extended to that between the phenotypic variances of X_i for each component i by noise (written as $V_{ip(i)}$) and that by genetic variation (denoted by $V_g(i)$) (Furusawa & Kaneko, 2015; Kaneko, 2012a,b) (see also Stearns et al. (1995) for possible experimental support). This, indeed, is expected by assuming that the relaxation of phenotypic changes is much slower along the dominant mode **W**, and phenotypic fluctuations due to noise are nearly confined to this axis.

9 Discussion

In summary, we demonstrated the 2-by-2 global proportionality of phenotypic changes occurring between responses and fluctuations and between the perturbations due to environmental (noise) and genetic changes. This proportionality is explained by evolutionary dimension reduction, which states that phenotypic changes due to environmental changes and genetic variation are constrained along a unique low-dimensional manifold, as observed in bacterial and numerical evolution experiments. Furthermore, expression changes induced by environmental stress, for most genes, are reduced through evolution to recover the growth, which is analogous to the Le Chatelier principle in thermodynamics (Callen, 1985; Kubo et al., 1976).

We demonstrated in numerical evolution that high-dimensional phenotypic change is mainly constrained along with the mode $\mathbf{w^0}$, the eigenvector corresponding to the eigenvalue of the relaxation dynamics closest to zero. The change in the phenotypic state is larger along the direction of $\mathbf{w^0}$, and the variable W along this direction slowly returns to the steady-state value. The timescale of this mode is distinctively slower than others, as confirmed directly by evolution simulation of catalytic reaction and gene regulation networks (Sato & Kaneko, 2020). This separation of the timescale of the slowest mode from others is theoretically expected in order to make the robustness of the fitted state and plasticity along the evolutionary course compatible.

Formation of one (or few) slow mode as given by W separated from other modes is significant in evolutionary biology. It may be possible that this type of mode is straightforwardly given by the expression of some specific genes that changes more slowly than others. However, it may be more natural that this W is expressed as a collective change in the expressions of several genes rather than as a single protein. Because the slow mode is expressed by the first principal component, determination of genes whose expression levels contribute more to the first principal component will improve the understanding of how plasticity and robustness are compatible.

The slow, dominant mode W emerges from evolution but accelerates evolution. When faster and slower variables coexist and interact, the slower mode generally functions as a control variable for the faster variables. Accordingly, if the slower

mode is modified by a genetic change, most faster variables will be influenced simultaneously. Furthermore, because the mode W can influence the fitness μ, the phenotypic evolution will be feasible simply by the change in this slow mode W.

In contrast, if many variables change in a similar timescale, the genetic changes introduced to each will influence each other and make directional phenotypic changes difficult to progress. This situation is reminiscent of the proverb "Too many cooks spoil the broth." The emergence of slow modes governing the others has also been observed in the evolution of pattern formation (Kohsokabe & Kaneko, 2016).

The correlation between evolutionary and environmental responses raises a question regarding how the two processes with quite different timescales are correlated. The presence of the slow mode W suggests a possible answer to this question. Adaptive dynamics, which originally show a much faster timescale than the evolutionary change, will be slowed along the mode W, whereas the evolutionary change, which originally has a much slower timescale, is fastest, along the direction of the mode W. Thus, along the dominant mode W, the timescales of phenotypic adaptation and evolution can approach each other.

Of course, further studies are needed to establish a phenomenological theory for phenotypic evolution. The generality of the evolutionary dimension reduction and resultant constraint in phenotypic evolution must be explored. The condition required for the emergence of dimensional reduction should also be determined. The models we studied satisfy the following conditions: (1) phenotypes with higher fitness are shaped by complex high-dimensional dynamical systems and (2) the fraction of such fit states is rare in the state space and in the genetic rule space. These two features are also consistent with our theoretical argument. The evolution of a statistical physics model on interacting spins that preliminarily supports the dimension reduction also satisfies these conditions (Sakata et al., 2009; Sakata & Kaneko, 2020). Studies of statistical physics and high-dimensional dynamical systems are needed to reveal the condition for evolutionary dimension reduction. Further experimental confirmation of the dimension reduction, as well as the directionality and constraint in phenotypic evolution, is needed, including in multicellular organisms.

Note that dimension reduction, or separation of a slow eigenmode from other faster modes, has been discussed in several other topics. They include protein dynamics (Tlusty et al., 2017; Togashi & Mikhailov, 2007), laboratory ecological evolution (Frentz et al., 2015), learning in brain (Sadtler et al., 2014), and neural networks (Hopfield, 2015; Schreier et al., 2017), among others. As a possible relationship, the *sloppy parameter hypothesis* by Daniels et al. (2008) is proposed, which suggests that many parameters employed in biological models are irrelevant. Further studies are necessary to explain the universality of such evolutionary dimension reduction.

There are some limitations to dimension reduction. In the studies described here, we assumed steady-growth state, i.e., exponential phase. Under nutrient-limited conditions, however, there occurs a transition from such exponential growth to the stationary phase with suppressed growth, as has been investigated both experimentally (Gefen et al., 2014; Novick, 1955) and theoretically (Himeoka &

Kaneko, 2017; Maitra & Dill, 2015). As such phase with suppressed growth may not be selected as a robust fitted state, whether the dimension reduction is valid to it requires further analysis.

Acknowledgments The authors would like to thank Takuya U. Sato and Tetsuhiro Hatakeyama for stimulating discussions. This research was partially supported by a Grant-in-Aid for Scientific Research (S) (15H05746) from the Japanese Society for the Promotion of Science (JSPS) and Grant-in-Aid for Scientific Research on Innovative Areas (17H06386 and 17H06389) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

References

Bar-Even, et al. (2006). Noise in protein expression scales with natural protein abundance. *Nature Genetics, 38*, 636–643.

Brauer, M. J., et al. (2008). Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast. *Molecular Biology of the Cell, 19*(1), 352–367.

Callen, H. B. (1985). *Thermodynamics and an introduction to thermostatistics* (2nd ed.). New York, NY: Wiley.

Ciliberti, S., Martin, O. C., & Wagner, A. (2007). Robustness can evolve gradually in complex regulatory gene networks with varying topology. *PLoS Comparative Biology, 3*, e15.

Clausius, R. (1865). Uber verschiedene für die Anwendung bequeme Formen der Hauptgleichungen der mechanischen Wärmetheorie. *Annalen der Physik, 201*(7), 353–400.

Daniels, B. C., Chen, Y. J., Sethna, J. P., Gutenkunst, R. N., & Myers, C. R. (2008). Sloppiness, robustness, and evolvability in systems biology. *Current Opinion in Biotechnology, 19*(4), 389–395.

Einstein, A. (1926). *Investigation on the theory of Brownian movement* (Collection of papers ed. by R. Furth). New York, NY: Dover (reprinted, 1956).

Elowitz, M. B., Levine, A. J., Siggia, E. D., & Swain, P. S. (2002). Stochastic gene expression in a single cell. *Science, 297*(5584), 1183.

Fisher, R. A. (1930). *The genetical theory of natural selection*. Oxford: Clarendon Press.

Frentz, Z., Kuehn, S., & Leibler, S. (2015). Strongly deterministic population dynamics in closed microbial communities. *Physical Review X, 5*(4), 041014.

Furusawa, C., & Kaneko, K. (2003). Zipf's law in gene expression. *Physical Review Letters, 90*, 088102.

Furusawa, C., et al. (2005). Ubiquity of log-normal distributions in intracellular reaction dynamics. *Biophysics, 1*, 25–31.

Furusawa, C., & Kaneko, K. (2012). Adaptation to optimal cell growth through self-organized criticality. *Physical Review Letters, 108*(20), 208103.

Furusawa, C., & Kaneko, K. (2015). Global relationships in fluctuation and response in adaptive evolution. *Journal of the Royal Society, Interface, 12*, 20150482.

Furusawa, C. & Kaneko, K. (2018). Formation of dominant mode by evolution in biological systems. *Physical Review E, 97*(4), 042410.

Glass, L., & Kauffman, S. A. (1973). The logical analysis of continuous, non-linear biochemical control networks. *Journal of Theoretical Biology, 39*, 103–129.

Gefen, O., Fridman, O., Ronin, I., & Balaban, N. Q. (2014). Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity. *Proceedings of the National Academy of Sciences, 111*(1), 556–561.

Himeoka, Y., & Kaneko, K. (2017) Theory for transitions between log and stationary phases: Universal laws for lag time. *Physical Review X, 7*, 021049.

Hopfield, J. J. (2015). Understanding emergent dynamics: Using a collective activity coordinate of a neural network to recognize time-varying patterns. *Neural Computing, 27*(10), 2011–2038.
Horinouchi, T., et al. (2010). Transcriptome analysis of parallel-evolved *Escherichia coli* strains under ethanol stress. *BMC Genomics, 11*, 579.
Horinouchi, T., et al. (2015). Phenotypic convergence in bacterial adaptive evolution to ethanol stress. *BMC Evolutionary Biology, 16*, 802.
Kaneko, K. (2006). *Life: An introduction to complex systems biology* Heidelberg and New York: Springer.
Kaneko, K. (2007). Evolution of robustness to noise and mutation in gene expression dynamics. *PLoS One, 2*(5), e434.
Kaneko, K. (2012a). Phenotypic plasticity and robustness: Evolutionary stability theory, gene expression dynamics model, and laboratory experiments. In *Evolutionary systems biology* (pp. 249–278). Springer.
Kaneko, K. (2012b). Evolution of robustness and plasticity under environmental fluctuation: Formulation in terms of phenotypic variances. *Journal of Statistical Physics, 148*(4), 687–705.
Kaneko, K., & Furusawa, C. (2006). An evolutionary relationship between genetic variation and phenotypic fluctuation. *Journal of Theoretical Biology, 240*(1), 78–86.
Kaneko, K., Furusawa, C., & Yomo, T. (2015). Universal relationship in gene expression changes for cells in steady-growth state.*Physical Review X, 5*, 011014.
Kaneko, K., & Furusawa, C. (2018). Macroscopic theory for evolving biological systems akin to thermodynamics. *Annual Review of Biophysics, 47*, 273–290.
Keren, L, et al. (2013). Promoters maintain their relative activity levels under different growth conditions. *Molecular Systems Biology, 9*, 701.
Klumpp, S., Zhang, Z., & Hwa, T. (2009). Growth rate-dependent global effects on gene expression in bacteria. *Cell, 139*(7), 1366–1375.
Kohsokabe, T., & Kaneko, K. (2016). Evolution-development congruence in pattern formation dynamics: Bifurcations in gene expression and regulation of networks structures. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution, 326*(1), 61–84.
Kubo, R., et al. (1976). *Thermodynamics: An advanced course with problems and solutions*. North-Holland.
Maitra, A., & Dill, K. A. (2015). Bacterial growth laws reflect the evolutionary importance of energy efficiency. *Proceedings of the National Academy of Sciences, 112*(2), 406–411.
Matsumoto, Y., Murakami, Y., Tsuru, S., Ying, B. Y., & Yomo, T. (2013). Growth rate-coordinated transcriptome reorganization in bacteria. *BMC Genomics, 14*(1), 808.
Mjolsness, E., Sharp, D. H., & Reinitz, J. (1991). A connectionist model of development. *Journal of Theoretical Biology, 152*, 429–453.
Novick, A. (1955). Growth of bacteria. *Annual Review of Microbiology, 9*(1), 97–110.
O'Duibhir, E. et al. (2014). Cell cycle population effects in perturbation studies. *Molecular Systems Biology, 10*(6), 732.
Regenberg, B., et al. (2006). Growth-rate regulated genes have profound impact on interpretation of transcriptome profiling in *Saccharomyces cerevisiae*. *Genome Biology, 7*(11), R107.
Sakata, A., Hukushima, K., & Kaneko, K. (2009). Funnel landscape and mutational robustness as a result of evolution under thermal noise. *Physical Review Letters, 102*, 148101.
Sakata, A., & Kaneko, K. (2020). Dimensional reduction in evolving spin-glass model: Correlation of phenotypic responses to environmental and mutational changes. arXiv:2001.03714.
Sato, K., Ito, Y., Tomo, T., & Kaneko, K. (2003). On the relation between fluctuation and response in biological systems. *Proceedings of the National Academy of Sciences of the United States of America, 100*(24), 14086–14090.
Sato, T. U. & Kaneko, K. (2020). Evolutionary dimension reduction in phenotypic space. *Physical Review Research, 2*(1), 013197.
Sadtler, P. T., Quick, K. M., Golub, M. D., Chase, S. M., Ryu, S. I., Tyler-Kabara, E. C., Byron, M. Y., & Batista, A. P. (2014). Neural constraints on learning. *Nature, 512*(7515), 423–426.
Schmidt, A., et al. (2016). The quantitative and condition-dependent *Escherichia coli* proteome. *Nature Biotechnology, 34*(1), 104.

Schreier, H. I., Soen, Y., & Brenner, N. (2017). Exploratory adaptation in large random networks. *Nature Communications, 8*, 14826.

Scott, M., Gunderson, C. W., Mateescu, E. M., Zhang, Z., & Hwa, T. (2010). Interdependence of cell growth and gene expression: Origins and consequences. *Science, 330*, 1099–1102.

Soyer, O. S. (2012). *Evolutionary systems biology*. Springer.

Stearns, S. C., Kaiser, M., & Kawecki, T. J. (1995). The differential genetic and environmental canalization of fitness components in *Drosophila melanogaster*. *Journal of Evolutionary Biology, 8*, 539.

Stern, S., Dror, T., Stolovicki, E., Brenner, N., & Braun, E. (2007). Genome-wide transcriptional plasticity underlies cellular adaptation to novel challenge. *Molecular Systems Biology, 3*(1), 106.

Tlusty, T., Libchaber, A., & Eckmann, J. P. (2017). Physical model of the genotype-to-phenotype map of proteins. *Physical Review X, 7*(2), 021037.

Togashi, Y., & Mikhailov, A. S. (2007). Nonlinear relaxation dynamics in elastic networks and design principles of molecular machines. *Proceedings of the National Academy of Sciences, 104*(21), 8697–8702.

Waddington, C. H. (2014). *The strategy of the genes*. Allen and Unwin.

Life's Attractors Continued: Progress in Understanding Developmental Systems Through Reverse Engineering and In Silico Evolution

Anton Crombach and Johannes Jaeger

Abstract We present a progress report on our efforts to establish a new research program for evolutionary systems biology, based on reverse engineering and *in silico* evolution. The aim is a mechanistic understanding of the genotype-phenotype map and its evolution. Our review focuses on the case study of the gap gene network in dipteran insects (flies and midges). This network is the top regulatory tier of the segmentation gene hierarchy, generating a pattern of overlapping expression domains that subdivide the embryo during early embryogenesis. It is one of the best-understood developmental regulatory networks today. We have studied this system in a comparative way, across three species: the vinegar fly, *Drosophila melanogaster*; the scuttle fly, *Megaselia abdita*; and the moth midge, *Clogmia albipunctata*. In this context, we discuss methodological challenges concerning data processing and model fitting, consider different functional decompositions of the gap gene network, and highlight novel insights into network evolution by compensatory developmental system drift. Finally, we discuss the prospect of simulating the phylogenesis of the gap gene network using in silico evolution. We conclude by arguing that our case study is a first step toward a more systematic empirical investigation into the principles of network evolution.

1 Introduction

A few years ago, we proposed a comparative research program into the function and evolution of developmental systems based on reverse engineering and in

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_4

silico evolution (Jaeger & Crombach, 2012).[1] The program's main objective is to move beyond static descriptions of regulatory network *structure*, toward a more processual perspective, based on the analysis and comparison of network *dynamics* arising from the interactions between genetic and non-genetic factors (DiFrisco & Jaeger, 2019, 2020). This objective stems from our desire to shed light upon a fundamental question in biology: how phenotypic variability is generated and shaped by developmental processes. The variability of phenotypic traits among individuals in a population provides the raw material for evolution by natural selection. In the famous words of Hugo De Vries, we are interested not in the survival but the *arrival* of the fittest (Wagner, 2011).

Developmental processes not only constrain or bias but, more importantly, *generate* phenotypic variability and determine the variational properties of morphological traits (Salazar-Ciudad, 2006; Wagner, 2014; Wagner & Altenberg, 1996). In abstract terms, development can be represented as a mapping from genotype to phenotype (the G–P map), which relates variation at the genotypic level to the variability of phenotypes (Alberch, 1991; Burns, 1970; Pigliucci, 2010; Soyer and O'Malley, 2013). Mathematically, G–P maps may either be interpreted as purely correlational maps (e.g., in quantitative genetics) or as characterizing causal-mechanistic processes (e.g., in evolutionary developmental and systems biology) (DiFrisco & Jaeger, 2019). In the latter sense, G–P maps are thought to be best captured by models of developmental gene regulatory networks (GRNs) (Davidson & Erwin, 2006; Wagner, 2014). If required, such networks can be expanded to include non-genetic regulatory factors.

Continuing on this path, we need empirical studies to establish the structure and dynamics of developmental regulatory networks, if we are to understand the origins of phenotypic variability (Jaeger & Crombach, 2012). This entails the decomposition of a system into building blocks using genetic and molecular methods and to *recompose* it to show how the building blocks and their interactions generate the orchestrated overall behavior of the process (Bechtel, 2011, 2012; Bechtel & Abrahamsen, 2005, 2010; Brigandt, 2013, 2015; DiFrisco & Jaeger, 2019, 2020). Considering the complexity of development in even the simplest multicellular organisms, this recomposition poses a formidable challenge.

To manage the regulatory complexity involved in characterizing developmental systems, we need dynamic mathematical models. These models integrate the components of a system by defining how these parts interact, which in turn leads to the characteristic dynamics of the system. We previously presented four distinct approaches that yield such models of developmental regulatory networks and their evolution (Jaeger & Crombach, 2012). These consist of (1) *ensemble approaches*, (2) *forward modeling*, (3) *reverse engineering*, and (4) in silico *evolution*. Ensemble modeling involves the simulation of entire categories of dynamic networks to

[1] While our argument focuses on developmental processes in multicellular organisms, it is directly extendable to other ontogenetic processes (metabolic, physiological, etc.) that constitute the life cycle of uni- and multicellular organisms.

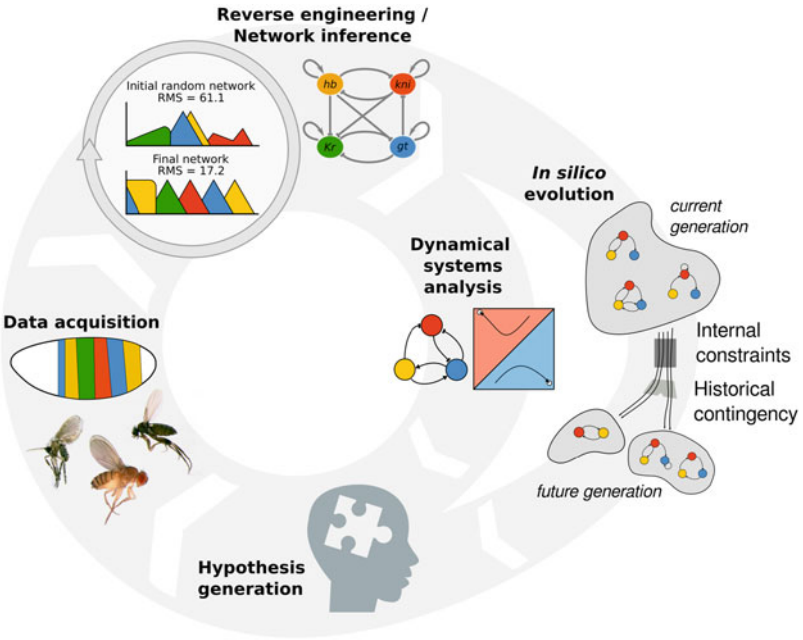

Fig. 1 The systems biology cycle for reverse engineering evolving developmental processes (modified after Kitano, 2002). Researchers *generate hypotheses* about regulatory mechanisms underlying observable developmental dynamics (e.g., gene expression patterns). *Data are acquired* that capture all relevant features of those developmental dynamics, shown as expression domains on a schematic fly embryo. For our comparative study, we used data from three species of dipteran insects: the moth midge *Clogmia albipunctata*, the vinegar fly *Drosophila melanogaster*, and the scuttle fly *Megaselia abdita* (left to right). Dynamical models (gene circuits) are then fitted to the data in order to *infer the regulatory structure of the underlying network*. Root mean square (RMS) deviation indicates quality of the fit (lower is better). The best circuits and the topology of their phase spaces (trajectories, attractors, and their basins, shown schematically for a bistable switch network) are then analyzed using the tools of *dynamical systems theory*. Comparative analyses distinguish between essential and accidental features of the system. In addition, we can use fitted circuits as start and end points of *in silico evolutionary simulations*, which reveal possible evolutionary pathways between mechanisms and how much they depend on internal constraints or historical contingency. Gap genes: *hunchback (hb)*, yellow; *Krüppel (Kr)*, green; *knirps (kni)*, red; *giant (gt)*, blue

reveal their shared general properties. Forward modeling is the classical bottom-up approach to biophysical modeling, where models are formulated in physicochemical terms and parameters are determined by measurement. In contrast, our proposal for a research program in evolutionary systems biology is based on reverse engineering and *in silico* evolution, which are employed together in a complementary fashion (Fig. 1) (Jaeger & Crombach, 2012).

Over the last decade, much progress has been made in deciphering the evolution and functioning of developmental systems. Examples range from exploratory, computational studies (Hagolani et al., 2019; Jiménez et al., 2015, 2017; Vroomans

et al., 2015, 2016, 2018) to approaches combining theory and experiment (Bailleul et al., 2019; Raspopovic et al., 2014; Sadier et al., 2019; Zagorski et al., 2017) (see also the chapter by Onimaru and Marcon). Here, we primarily focus on describing our own recent work on the gap gene system of dipteran insects (flies, midges, and mosquitoes), which we already used as a case study in (Jaeger & Crombach, 2012) (see Fig. 1). A concise overview on this work can be found in (Jaeger, 2018). It shows how reverse engineering can be used to analyze the dynamics and evolution of a complex regulatory network involving multiple regulatory genes—illustrating the high-dimensional and nonlinear nature of the G–P map and its influence on the evolutionary process. In particular, our work reveals how we can explain the phenomenon of developmental system drift in a mechanistic manner. Moreover, it demonstrates the multifaceted nature of a complex regulatory process and how alternative ways of subdividing it help in understanding its overall behavior. All of these aspects together yield a much deeper level of insight into the dynamics of development and evolution than could be achieved by genetic and molecular experimental processes alone.

In the rest of this chapter, we will reflect on a number of methodological and conceptual issues in reverse engineering and *in silico* evolution, which should be of general interest to practitioners in evolutionary systems biology. In particular, we document the major challenges we have faced, and the progress we have made to overcome them, during our efforts to reverse-engineer the gap gene system across three different evolutionary lineages of dipteran insects. The chapter is composed of four sections. In the first, we focus on methodological bottlenecks imposed by the reverse-engineering approach. In the second, we discuss a bottom-up strategy to decompose and recompose a complex dynamical system such as the gap gene network in order to generate mechanistic explanations of the underlying regulatory process. We also briefly touch upon a complementary top-down strategy (Verd et al., 2019). Due to space constraints, we refer to the chapter by Jaeger and Monk for an in-depth account of this approach. In the third part, we connect these explanations to network evolution. Finally, we conclude with an outlook on future work using *in silico* evolution that will shed light on the evolutionary transitions that have occurred in insect body segmentation.

2 Reverse Engineering with Gene Circuits

Let us first provide a brief outline of our reverse-engineering approach. It requires (1) a suitable model of the regulatory system and (2) an efficient and reliable method for inferring the parameters of the model by fitting it to (3) data that suitably represent the observable dynamics of the system, and (4) finally, we need a conceptual framework and analytical tools to extract biological insights from the resulting set of model fits. We will discuss each of these steps in turn.

We use gene circuits as models of the gap gene network (Mjolsness et al., 1991; Reinitz & Sharp, 1995). Gene circuits are dynamical models, historically derived

from recurrent Hopfield neural networks (Hopfield, 1984). In mathematical terms, a gene circuit is defined as a set of coupled ordinary differential equations (ODEs). Each equation describes the change in concentration g over time t of the product (mRNA or protein) of gene a in nucleus i:

$$\frac{dg_i^a}{dt} = R^a \Phi(u^a) - \lambda^a g_i^a + D^a(n)(g_{i-1}^a + g_{i+1}^a - 2g_i^a) \tag{1}$$

with R, λ, and $D(n)$ representing rates of production, decay, and diffusion, respectively. Diffusion rates depend on nuclear density, i.e., the number of nuclear divisions n that the system has undergone at time t.

Gene regulation is modeled by a sigmoid regulation-expression function Φ. This function captures the coarse-grained dynamics of eukaryotic transcriptional regulation, that is, a switch-like activation with a saturating response:

$$\Phi(u^a) = \frac{1}{2}\left(\frac{u^a}{\sqrt{(u^a)^2 + 1}} + 1\right), \tag{2}$$

$$u^a = \sum_{b \in G} w^{ba} g_i^b + \sum_{m \in M} e^{ma} g_i^m + h^a \tag{3}$$

Regulatory interactions are represented by an interconnectivity matrix of regulatory weights $w \in W$ and external input weights $e \in E$ for regulators that are not themselves regulated by gap genes. In our case study, internal regulators include the trunk gap genes $G = \{hb, Kr, gt, kni\}$, and external inputs are provided by maternal gradients and the products of terminal gap genes $M = \{\text{Bcd, Cad, Tll, Hkb}\}$. Each weight w and e determines whether a specific regulatory interaction exists (non-zero weights) and, if so, whether it is activating (positive) or repressing (negative). These matrices abstract from the complex (and largely unknown) biochemical details of eukaryotic transcriptional regulation. h is a threshold parameter that captures the transcriptional state of a gene in the absence of any spatially specific regulators.

Reverse engineering means fitting gene circuits to spatiotemporal gene expression data. This is framed as an optimization problem where values need to be estimated for the parameters W, E, h, R, D, and λ, such that the simulated model reproduces the expression data as closely as possible. The two weight matrices W and E are central to this challenge. They represent the regulatory structure of the network, which cannot be measured directly, but must be inferred from data. In other words, gene circuits are used as an analytical tool to understand the genetic regulatory mechanisms driving the observed dynamics of gene expression.

To determine the difference between model output and gene expression data, we can use different residuals or cost functions. Originally, we used the ordinary least squares (OLS) approach (Jaeger et al., 2004a,b; Manu et al., 2009a,b), which was later replaced by weighted least squares (WLS), which takes the variability of the data into account (see Sect. 3.2). Thus, our cost function is defined as:

$$\text{cost} = \sum_{a \in G} \sum_{t \in T} \sum_{i \in N(n)} v_i^a(t) \left(g_i^a(t) - \text{data}_i^a(t) \right)^2 \tag{4}$$

The value of this function depends on the number of data points used for fitting. To make the score of a fit comparable between different simulation settings, we use the root mean square deviation (RMS; see Fig. 1):

$$\text{RMS} = \sum_{a \in G} \sum_{t \in T} \sum_{i \in N(n)} \sqrt{\frac{1}{N_{data}} \left(g_i^a(t) - \text{data}_i^a(t) \right)^2} \tag{5}$$

Since the RMS still depends on the scale of the data, we scale mRNA data to match protein data (Crombach et al., 2012). The result is an RMS which is a normalized measure for the differences between model output and observed expression levels.

We find optimal parameter sets by minimizing the cost function (Eq. 4). This poses a challenging nonlinear global optimization problem. Several algorithms have been used to solve this challenge. Initial studies using gene circuits used a global optimization approach called parallel Lam simulated annealing (pLSA) (Chu et al., 1999; Lou & Reinitz, 2016). pLSA is a robust optimization method that is computationally costly. If high-performance computing facilities are available, it remains our preferred fitting method. Several alternatives, mostly based on evolutionary computation and scatter search, have been proposed to reduce computational cost (Abdol et al., 2017; Fomekong-Nanfack et al., 2007; Jostins & Jaeger, 2010; Perkins et al., 2006). Until recently, however, none of these alternatives were able to match the robustness and reliability of pLSA on our particular problem. This appears to have changed with a new method called FIGR, which converts the problem of fitting nonlinear gene circuit models (based on sigmoid regulation-expression functions) to a classification problem on models with purely on–off regulatory dynamics (Heaviside step function), which can be solved using standard machine learning approaches (Fehr et al., 2019). This method leads to significant speedup without a loss of robustness, which effectively removes the computational bottleneck of fitting models to data (see Sect. 3).

After optimization is performed, model fits with an RMS below a certain threshold are carefully inspected for visible expression defects and selected for analysis. We also assess the determinability of the inferred parameter values using statistical methods (Ashyraliyev et al., 2009a,b, 2008; Crombach et al., 2012). In the best case, this yields a consensus network structure, where the qualitative nature of each interaction (activation or repression) is shared between a large majority of the selected fits. Alternatively, we end up with a set of different network variants that can all explain the observed expression dynamics. In both cases, solutions must be compared to and validated with experimental evidence. From the set of validated best-fitting solutions, we choose representative examples for further analysis of regulatory mechanisms and phase space topology (Manu et al., 2009a; Verd et al., 2018, 2014, 2017). These analyses result in mechanistic and structural explanations for the dynamical behavior of the system (Fig. 1) (DiFrisco & Jaeger, 2019).

3 Challenges in Reverse-Engineering Gap Gene Networks

The gap gene network is active in early embryogenesis, during the blastoderm stage, before the onset of gastrulation (Jaeger, 2011). Pioneering genetic work in *D. melanogaster* established that gap genes form the top-most zygotic layer of the hierarchical gene regulatory network governing segment determination. They encode short-lived transcription factor proteins that diffuse through the syncytial blastoderm embryo. Here, we consider the four trunk gap genes *hunchback* (*hb*), *Krüppel* (*Kr*), *giant* (*gt*), and *knirps* (*kni*). They generate a set of broad, overlapping expression domains subdividing the embryo along its main (or anteroposterior, A–P) body axis (Fig. 2). No tissue growth is involved in this process. Gap genes are activated by maternal gradients of Bicoid (Bcd), Hb, and Caudal (Cad) proteins and cross-regulate each other. Together with the maternal factors, they activate the pair-rule genes, whose periodic two-segment patterns are exemplified by the seven stripes of *even-skipped* (*eve*) expression. Pair-rule genes, in turn, regulate the molecular pre-pattern of the segment polarity genes, expressed in 14 single-cell-wide stripes, which demarcate the boundaries of the embryonic parasegments that form later in development.

The molecular pre-pattern of the segment polarity genes is widely conserved across insects and other arthropods, but the peculiar way by which *D. melanogaster* arrives at it is not (Chipman, 2020; Clark et al., 2019; Davis & Patel, 2002; Rosenberg et al., 2009). The simultaneous subdivision of the pre-gastrulation embryo observed in *D. melanogaster* is called long-germband segment determination. Outside the dipteran lineage, it also occurs in some groups of beetles and the Hymenoptera (ants, bees, and wasps; see Fig. 2). In contrast, other insect lineages determine most of their segments later in development, after gastrulation has occurred, through sequential addition and tissue growth. This sequential mode is called short-germband segment determination.

To better understand how the derived long-germband mode of segment determination in *D. melanogaster* originated and evolved, we set out to perform a comparative analysis of the mechanisms underlying gap gene expression across several distantly related species of dipteran insects. To achieve this, we reconstructed the gap gene system *in silico* in two non-model species. The first is the phorid scuttle fly *Megaselia abdita*, which belongs to a basally branching cyclorrhaphan lineage (Wiegmann et al., 2011). *M. abdita* possesses a Bcd gradient, but no maternal Cad expression (Fig. 2) (Lemke et al., 2008; Stauber et al., 1999, 2008, 2000). Gap gene expression in this species is very conserved compared to *D. melanogaster*, which allows us to study how compensatory evolution maintains patterning output in the presence of changing maternal inputs (Wotton et al., 2015a,b). In addition, we attempted to reverse-engineer the gap gene system of the psychodid moth midge *Clogmia albipunctata* (Fig. 2). In this basally branching dipteran lineage (Jiménez-Guri et al., 2013), there is no *bcd* gene, and posterior gap gene expression differs markedly from that of both other species (García-Solache et al., 2010; Janssens et al., 2014; Rohr et al., 1999).

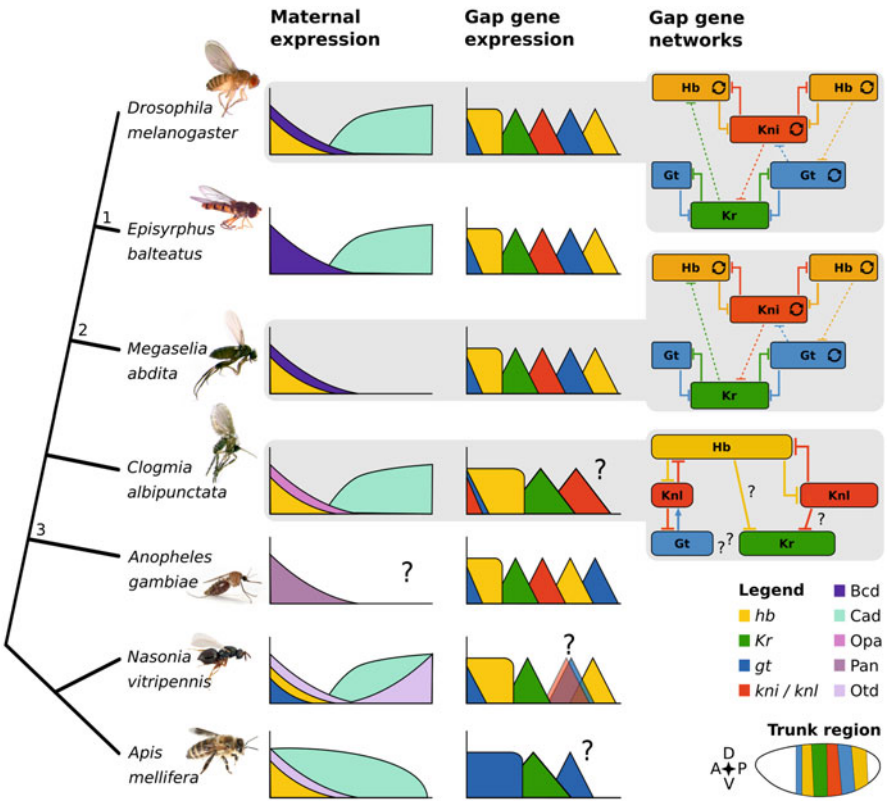

Fig. 2 Evolution of the gap gene system in holometabolan insects. A simplified phylogenetic tree is shown, featuring selected holometabolan insect lineages with (partially) known gap gene expression and regulation. The top branch of the tree represents the dipteran lineage (flies, midges, and mosquitoes) and the bottom branch the hymenopteran lineage (bees and wasps). The vinegar fly *Drosophila melanogaster*, hoverfly *Episyrphus balteatus*, and scuttle fly *Megaselia abdita* represent cyclorrhaphan lineages; the moth midge *Clogmia albipunctata* and the malaria mosquito *Anopheles gambiae* depict nematoceran lineages within the Diptera. *Apis mellifera* is the honeybee and *Nasonia vitripennis* the jewel wasp. Key evolutionary events are numbered on the tree: 1 and 3, loss of maternal hunchback (Hb); 2, loss of maternal Caudal (Cad) expression. The second column shows maternal morphogen gradients (Yoon et al., 2019), while column three depicts the relative position of gap gene expression domains along the A–P axis (Jaeger, 2011; Lemke et al., 2010; Lynch et al., 2006; Olesnicky et al., 2006; Pultz et al., 2005; Wilson & Dearden, 2011; Wilson et al., 2010). Column four shows the structure of the three reverse-engineered gap gene networks described in the text, with inhibitory interactions indicated by T-bars and self-activation by circular arrows (activation by maternal gradients is omitted for simplicity). Question marks indicate incomplete or missing information: *C. albipunctata* may have an additional posterior gap gene, and several of its gap gene interactions remain undetermined (Crombach et al., 2014; García-Solache et al., 2010). The posterior morphogen of *A. gambiae* is unknown. Data is ambiguous regarding the ordering of posterior *gt* and *kni* in *N. vitripennis* (Olesnicky et al., 2006). The schematic embryo in the bottom-right corner shows the location of gap gene expression patterns in the trunk region of cyclorrhaphan flies (anterior is to the left; dorsal is up). Gene/protein names in the legend: *hunchback* (*hb*), *Krüppel* (*Kr*), *giant* (*gt*), *knirps* (*kni*), *knirps-like* (*knl*), Bicoid (Bcd), Caudal (Cad), Odd-paired (Opa), Pangolin (Pan), Orthodenticle (Otd). Image sources: all dipterans by Wotton et al. (2015a), except *A. gambiae* by Muhammad Mahdi Karim (Wikimedia Commons), *A. mellifera (carnica)* from Makro Freak (Wikimedia Commons), and *N. vitripennis* from the New Zealand Arthropod Collection (flickr.com)

3.1 Bottleneck No. 1: Quantitative Data

Reverse-engineering complex regulatory systems poses significant methodological challenges, particularly if attempted in non-model organisms, where robust and efficient experimental protocols are often lacking. The first major challenge consists in the generation of suitable data for model fitting and model validation. This is the principal bottleneck, especially if we are aiming to understand processes of pattern formation or morphogenesis that require spatially resolved time series of gene expression data.

The quality of a reverse-engineered model crucially depends on the quality of the data used for fitting. However, the amount of work and the technical complications involved in generating high-quality spatial gene expression data can be daunting. Progress in high-throughput methodology for image bioinformatics is being made but is slower than in other areas, such as sequencing. For instance, it took over 5 years to generate the gap protein expression data used to fit the initial set of gene circuit models for *D. melanogaster* (Ashyraliyev et al., 2009b; Jaeger et al., 2004a,b; Manu et al., 2009a,b). These data were based on immunofluorescence staining protocols and confocal microscopy, combined with a quantitative data processing pipeline to measure the concentration of maternal gradients and gap gene proteins at high spatiotemporal and cellular resolution (Surkova et al., 2008a,b).

Clearly, for our comparative project, we had to find a good compromise between data quality and effort. As we struggled to raise suitable antibodies for immunofluorescence in non-model dipteran species (for a partial success story in *C. albipunctata*, see Janssens et al., 2014), we chose instead to demonstrate that post-transcriptional regulation of gap genes is not essential for the establishment and dynamics of their expression domains in *D. melanogaster* (Becker et al., 2013). This implied that we could use gap gene mRNA expression data for model fitting. Such data are much easier to acquire in non-model systems than protein expression data.

We proceeded to develop a fast and robust approach to generate semiquantitative mRNA expression data based on colorimetric *in situ* hybridization protocols, imaging by widefield microscopy, and a standardized, interactive pipeline for "medium-throughput" data processing and consistent staging of embryos into homologous developmental time classes (Crombach et al., 2012). Our measurements are semiquantitative in the sense that they only capture the timing and location of gene expression precisely, while accurate levels of expression cannot be assayed due to the nonlinear nature of the staining protocol. Expression data are available through a database called SuperFly, which contains expression data from over 1500 embryos from all 3 species (http://superfly.crg.eu) (Cicin-Sain et al., 2015). This unique data set of spatiotemporal gene expression patterns provided the basis for our comparative study of gap gene regulation dynamics across dipteran species. Once pipeline and database were in place, it only took a few months for three researchers to generate and process all the data, which means that the approach is scalable to much larger systems.

A related challenge, that tends to receive little attention, is appropriate embryo staging and the establishment of homology between developmental stages in different species. The staging scheme used in *D. melanogaster* resolves expression patterns down to intervals of about 7 min in the late blastoderm (the whole blastoderm stage lasts for about 90 min) (Surkova et al., 2008b). This scheme is based on morphological features, such as the progression of membrane invagination during cellularization, that are not necessarily conserved in other species. For this reason, we had to establish equivalent staging schemes for *M. abdita* and *C. albipunctata*. This required the identification of suitable morphogenetic landmarks: the occurrence of specific nuclear divisions and nuclear movements, the appearance of the head furrow, pole cell formation, and the onset and progression of cellularization during the blastoderm stage. The task was complicated by the possibility of heterochronic shifts in developmental timing between landmarks and by the presence of extraembryonic membranes (amnion and serosa) in *M. abdita* and *C. albipunctata*, which are heavily reduced in *D. melanogaster* (Schmidt-Ott et al., 2010). Despite considerable differences between species, our efforts resulted in a consistent sequence of homologizeable developmental stages, which put us in the exceptional position to draw detailed comparisons between the dynamical properties of gap gene expression between all three species (Jiménez-Guri et al., 2014; Wotton et al., 2014).

Next, we ensured that our semiquantitative data from *D. melanogaster* would yield the same kind of fitted gene circuit models as the (more accurate and fully quantitative) original set of protein expression data (Crombach et al., 2012). Not surprisingly, these efforts yield model solutions with more dispersed parameter values compared to the original protein fits (Ashyraliyev et al., 2009b).[2] Moreover, parameter determinability analysis indicates that the level of statistical confidence generally decreases when we use semiquantitative mRNA data. These shortcomings can be overcome, however, using experimental approaches such as RNA interference (RNAi) to empirically validate the nature and strength of a given regulatory interaction. More importantly, in *D. melanogaster* at least, we can still reconstruct a consensus network structure that includes all the relevant developmental mechanisms identified in gap gene circuits fit to protein expression data (Ashyraliyev et al., 2009b; Crombach et al., 2012; Jaeger et al., 2004b).

All of this is encouraging news for attempts at fitting gene regulatory network models to spatiotemporal gene expression data in general. We expect many developmental systems to be more sensitive to alterations in the timing and location of gene expression than to the precise level of expression for specific factors. This needs to be confirmed case by case, of course. But if it is a general trend, then fast semiquantitative approaches will be sufficient to yield robust and consistent results for reverse engineering in many developmental systems.

[2] We also pruned data and time points from our data set to test the minimal requirements for success. This led to the insight that the data should have the right spatiotemporal resolution to capture the expression features one wants to explain, such as transitions between different types of dynamics or the appearance of expression boundaries and domains (Crombach et al., 2012).

3.2 *Bottleneck No. 2: Model Fitting*

Another bottleneck for reverse engineering lies in the global optimization procedure required for fitting nonlinear dynamical models to expression data. We have already mentioned above that computational efficiency is no longer the problem today than it was a few decades ago. This is mainly due to the incessant increase in available computational power, but also because of more effective optimization algorithms (e.g., scatter search and FIGR) becoming available (see Sect. 2). However, a number of other problems remain, which have to do with the determinability of parameter values. As discussed in the previous section, fits to mRNA expression data result in higher dispersion and lower determinability. Another factor that affects determinability is the presence of correlations between parameter values (Ashyraliyev et al., 2009a, 2008). Such correlations are known to exist between rates of production, diffusion, and degradation in kinetic systems. Moreover, correlations can arise from redundancies in the regulatory network. For instance, auto-activation can compensate for ubiquitous activation of gene expression, and vice versa, or higher rates of maternal activation can be compensated by stronger rates of cross-repression between gap genes.

Other parameter correlations are not so obvious to detect. They require parameter determinability analysis to be uncovered. One of these cases affects repression of trunk gap genes by terminal gap genes *tll* and *hkb*. It turns out that all but one of these regulatory interactions—the repression of *hb* by Hkb—are functionally redundant (Ashyraliyev et al., 2009b). This not only corroborates experimental observations (Jaeger, 2011) but also allows us to eliminate these parameters from optimization, since their inferred values would not carry useful regulatory information. In turn, this leads to the practical benefits of speeding up the fitting procedure and reducing the dispersion of other parameter values. Model fitting, therefore, is most efficient if informed by functional considerations, without unnecessarily constraining the range of possible parameter values for the interactions for which we can extract information from the expression data. Naturally, acquiring these insights is heavily problem- and context-dependent and must be done case by case in an empirical manner.

Two other aspects of model fitting are worth noting. First, fitting quality and biological accuracy of inferred parameter values improved greatly when we introduced a weighted least squares (WLS) cost function, which takes both average expression patterns and their standard deviations into account (Ashyraliyev et al., 2009b, 2008) (see Sect. 3). As a general rule, protein expression shows variances that are roughly proportional to expression levels (Surkova et al., 2008a). For this reason, we introduced artificial variances to levels of mRNA expression in our semiquantitative data, which mimicked the pattern observed for protein data (Crombach et al., 2012). This greatly improved the determinability of parameter estimates, by putting a high penalty on the expression of genes in areas of the embryo where they should not be expressed. As a second step for quality control, two independent experts inspected each circuit for remaining small domains of ectopic expression. In our experience, these circuits tend to lead to artifacts in

the inferred parameter values and need to be removed as outliers that confuse the analysis of network structure.

In conclusion, we recommend an iterative procedure for reverse engineering where the values of inferred parameters are increasingly constrained through empirical considerations and determinability analysis. More generally, good model fits require data that capture the timing and location of relevant expression features and take variability of gene expression into account. Getting the exact levels of gene expression right is not crucial, at least for the gap gene system (see also Uzkudun et al., 2015). Our semiquantitative approach with mRNA instead of protein data is robust across model and non-model species and requires much less time and effort than methods based on immunofluorescence. A trained technician or postdoc is able to gather a data set for model fitting in about 3–4 months.

Taken together, this means we have overcome the two main bottlenecks involved in reverse-engineering systems of spatial pattern formation: the acquisition of microscopy data and the fitting of a consistent set of network models. This establishes the reverse-engineering approach as a method that is widely applicable in evolutionary systems biology.

4 The Art and Science of Network Decomposition

Once we have obtained a set of validated gap gene circuits with a consensus network structure (or a small set of network variants), we must find a way to analyze these models to extract biological insight from them. What we need to do to understand the G–P map as a causal regulatory process is to reconstruct how parts of the system govern specific features of the developmental dynamics that propagate the process from its initial to its final state. Importantly, this can be done in different ways that are all equally valid. Here we discuss a bottom-up approach that enables a first recomposition of parts of the network. This approach is complemented by a top-down analysis that we briefly elaborate on here and that is described in more detail in the chapter by Jaeger and Monk.

Our starting point is the consensus network structure of the gap gene system in *D. melanogaster* (Figs. 2 and 3). Simply by looking at this representation of the network, we can infer certain regulatory principles. For instance, the two double-negative (hence positive) feedback loops between pairs of gap genes with complementary expression patterns are easy to notice: *hb* and *kni*, as well as *Kr* and *gt* (Fig. 3b). The bistable switching behavior typically driven by such positive feedback loops explains that only one of the genes in each pair can be present in an embryonic nucleus at a given time.

In other cases, however, visual inspection and mental simulation are not sufficient. For example, what do the weak repressive interactions between overlapping gap domains do (Fig. 3c)? Upon their discovery, these interactions elicited some controversy (Jaeger, 2011), and no specific regulatory role for them could be determined based on qualitative evidence from genetics. This only changed with quantitative, high-resolution data and gene circuit models, which revealed that these

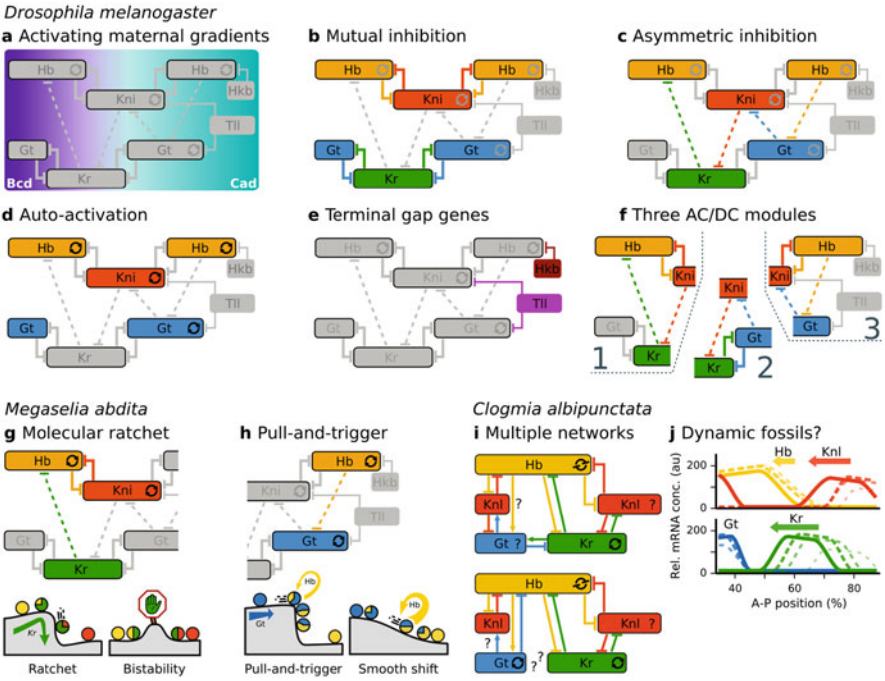

Fig. 3 Comparative analysis of dipteran gap gene networks. (**a**–**e**) The bottom-up decomposition of the *D. melanogaster* gap gene network reveals the five basic regulatory mechanisms shown here. (**f**) A complementary, top-down decomposition reveals three dynamical modules. Each module has the structure of an AC/DC circuit (Verd et al., 2019) (see the chapter by Jaeger and Monk). (**g**) A molecular ratchet involving *hb*, *Kr*, and *kni* can explain the shift of the anterior *hb* domain boundary in *M. abdita* and the lack of such a shift in *D. melanogaster*, where this boundary is set through bistability instead. (**h**) In *M. abdita*, posterior Gt activates *hb*, which triggers *hb* auto-activation once Hb concentration has sufficiently built up ("pull-and-trigger" mechanism), while *D. melanogaster* exhibits a smooth shift based on *hb* auto-activation only. (**i**) Reverse-engineering the *C. albipunctata* gap gene system resulted in a set of four different network structures, two of which are shown here. (**j**) Gap domain shifts are much more pronounced in *C. albipunctata* than in *D. melanogaster* or *M. abdita*, suggesting that these shifts may be a dynamic vestige (or "fossil"), a remnant inherited from the ancestral short-germband segmentation process. Inhibitory interactions are indicated by T-bars, self-activation by circular arrows, and self-inhibition by circular T-bars. For gene names, see Fig. 2. See main text for details

interactions are involved in dynamic positional shifts of posterior gap domains toward the anterior (Jaeger et al., 2004b; Surkova et al., 2008a). These domain shifts are an important expression feature that can be explained neither by genetic experiments (since the shifts are an emergent property of the system and do not depend on a specific mutation) nor mental simulation of the system (since the interactions that generate them are too numerous and interconnected to simply "think them through"). This is why we need dynamical models for network analysis and mechanistic explanations (DiFrisco & Jaeger, 2019, 2020).

4.1 Bottom-up Decomposition into Regulatory Mechanisms

Our validated gene circuit models allow us to directly track the influence of each specific regulatory interaction in the gap gene network, in each nucleus separately, and at any time point during segmental patterning. To achieve this, effective regulatory contributions are calculated as regulator concentration g_t^a multiplied by regulatory weights from matrices W and E (see Eq. (3)) and plotted against each other for visual inspection (see Fig. 6C in Crombach et al., 2012). Regulatory inputs can be integrated over an embryonic region and time interval of interest. This enables us to precisely characterize the relative importance of individual contributions to every observable expression feature that is correctly reproduced by the model.

Using this kind of graphical analysis, we were able to distinguish five basic developmental mechanisms involved in gap gene expression in *D. melanogaster* (Fig. 3a–e) (Crombach et al., 2012; Jaeger et al., 2004a,b):

1. Gap genes are predominantly activated by maternal factors Bcd (in the anterior) and Cad (in the posterior region of the embryo), especially during early stages when gap protein levels are still low (Fig. 3a) (Hoermann et al., 2016; Jaeger et al., 2007). Bcd acts in a graded spatially distributed manner, and Cad mainly contributes to activation in the posterior region of the embryo, where its early concentration levels are uniformly high. This activating mechanism does not correspond to classical morphogen-based patterning, since maternal inputs are not specific enough on their own to precisely position gap domain boundaries (Jaeger et al., 2008; Jaeger & Reinitz, 2006; Jaeger & Verd, 2020). As Bcd and Cad concentrations reduce over time, maternal activation diminishes, yet its role remains important as it contributes to the dynamics of gap domain shifts (Verd et al., 2017).
2. Strong mutual repression between complementary gap domains of *hb* and *kni* as well as *Kr* and *gt* establishes the basic staggered arrangement of gap gene expression domains (Fig. 3b) (see above). This double-negative (hence positive) feedback mechanism lies at the core of gap gene regulation and is conserved among dipteran species (Crombach et al., 2014, 2016) (Fig. 2). In addition, it is required for the sharpening of gap domain boundaries over time (Jaeger, 2011).
3. Gap genes with overlapping expression domains weakly repress each other. These interactions show a posterior bias such that Hb represses *gt*, Gt represses *kni*, Kni represses *Kr*, and Kr represses *hb*, but not the other way around (Fig. 3c). As mentioned above, this asymmetric cascade of repressive interactions causes the temporal shift of posterior gap domains toward the embryo's anterior.
4. Auto-activation boosts expression levels and contributes to the sharpening of expression domain boundaries of *hb*, *Kr*, and *gt* at later stages, when gap protein levels are sufficiently high (Fig. 3d). However, auto-activation does not influence the determination of positional information and is dispensable for gap gene patterning in principle (Perkins et al., 2006). Our models indicate that auto-activation is present in all three species (Crombach et al. 2012, 2014, 2016).

However, we cannot analyze its precise regulatory role, since auto-regulation is mainly involved in controlling levels of expression, which are not accurately captured by our semiquantitative data. We do know that in *D. melanogaster*, *gt* expression levels are governed by a temporal switch from regulatory elements that mediate broad early expression in response to maternal gradients to domain-specific elements later on (Hoermann et al., 2016).

5. Repression by terminal gap genes *tll* and *hkb* excludes the expression of gap genes from the posterior pole, restricting them to the embryo's trunk region (Fig. 3e). The terminal gap genes mediate the influence of the terminal maternal system on segmentation gene expression (Jaeger, 2011). Most of the regulatory inputs from these two genes are redundant with the exception of repression of *hb* by Hkb, which establishes the posterior boundary of the posterior *hb* domain (Ashyraliyev et al., 2009b).

4.2 Top-Down Decomposition into Dynamical Modules

Decomposing a network by tracking single regulatory interactions is but one possible way to analyze the dynamics of a complex regulatory system. We call it the bottom-up approach, as it examines how individual contributions combine to yield the overall behavior of the network. In contrast, a top-down approach considers dynamics at the level of the whole system and dissects it into modular components (see the chapter by Jaeger and Monk). This approach is based on dynamical systems theory, specifically phase space analysis (Strogatz, 2015). For our gene circuit models, this means to identify attractors, their basins, and the separatrices between them. In time-variant systems, like the gap gene network, we also need to consider the possible transitions (bifurcations) between different dynamical regimes and transient behavior along trajectories that stay far from any steady state (Verd et al., 2014).

Phase space analysis of gap gene circuits reveals two fundamentally different patterning regimes: stationary domains in the anterior, generated by multi-stability, and shifting domains in the posterior, generated by a damped oscillator (Verd et al., 2018). The presence of such different regimes allows us to identify which subsets of gap genes contribute to each of them (Fig. 3f) (Verd et al., 2019), even if the gap gene network shows no signs of modularity in its structure, being very densely connected (see Fig. 2). In the anterior trunk region, for example, only *gt*, *hb*, and *Kr* are expressed. Their interactions generate switch-like multi-stable behavior leading to stationary domain boundaries for these gap genes. In the posterior trunk region, *Kr*, *kni*, and *gt* are expressed, where they generate oscillatory behavior and shifting domain boundaries. Surprisingly, the central region of the embryo contains a third dynamical module composed of *hb*, *Kr*, and *kni*, which drives two different kinds of behavior. It straddles the bifurcation point between nuclei that show switch-like and oscillatory behavior. Thus a non-modular network produces

modular, multifunctional behavior. These subsets of gap genes are not structural but *dynamical* modules.

Strikingly, all three dynamical modules of the gap gene network exhibit exactly the same network structure (topology) (Fig. 3f), only differing in strength of regulatory interactions. This canonical structure corresponds to the AC/DC circuit, a known minimal motif for producing both switch-like and oscillatory behaviors (Panovska-Griffiths et al., 2013). This explains how a single subcircuit can produce two different dynamical behaviors depending on the maternal input it receives.

In summary, we demonstrate that one can decompose a regulatory network in multiple ways: bottom-up, by plotting the contributions of individual regulatory interactions, and top-down, by decomposing the system based on different kinds of behavior observed in the system's dynamics. Both of these approaches are valid ways to decompose a network. Both describe how specific subsets of interactions between gap genes contribute to specific aspects of gene expression. Yet, each decomposition has its peculiar focus. In the bottom-up approach, we classify contributions to specific expression features, such as the positioning, sharpening, or shift of particular domain boundaries. In the top-down approach, we focus on more general dynamical behaviors. Even though gap domains in the anterior and the posterior of the embryo look similar at first sight, they are generated by fundamentally distinct processes. These analyses may not easily add up to a unified picture of the network. Instead, they complement and contextualize each other, which makes it easier to recognize the uses and limitations of either approach. In short, these perspectives synergize to give us a deeper understanding of the system (Wimsatt, 2007).

5 Drifting Shifts: The Evolution of the Gap Gene System

A comparative analysis of the gap gene network beyond the drosophilid lineage illuminates the origin and evolution of long-germband segment determination. Moreover, it allows us to distinguish conserved aspects of the patterning mechanisms from contingent regulatory features that are evolutionarily labile. More generally, our reverse-engineering approach grounds the search for principles of network evolution in empirical evidence.

The gap gene network, in turn, is an ideal model system in which to establish methodology for comparative modeling of regulatory evolution. We can start our analysis with a common set of candidate genes, since gap genes are highly conserved across dipteran lineages (Jaeger, 2011). We can track gene expression using mRNA data, as post-transcriptional regulation is not required for the positioning of gap domains (Becker et al., 2013). We can ignore morphological changes and focus on gene expression only, because there is no growth nor tissue arrangement during the blastoderm stage. We can homologize specific developmental stages between species, as dipteran embryos are morphologically very similar to each other (Jiménez-Guri et al., 2014; Wotton et al., 2014). Moreover, we can compare

the resulting dynamic patterns at high spatiotemporal resolution (Cicin-Sain et al., 2015). And we can validate our fitted models with genetic perturbation methods, such as RNAi, that establish independent empirical evidence on gap gene regulation (Jiménez-Guri et al., 2018; Wotton et al., 2015a). All of this renders the dipteran gap gene network exceptionally tractable as a paradigm system for metazoan patterning. The combination of experimental decomposition and in silico recomposition of the gap gene system was particularly successful in the phorid scuttle fly *Megaselia abdita* (Fig. 2).

5.1 *The Scuttle Fly* Megaselia abdita*: Compensatory Evolution*

Evidence from systematic RNAi knockdown experiments and fitted gene circuit models indicates that the qualitative structure of the gap gene network is highly conserved between *M. abdita* and *D. melanogaster* (Fig. 2) (Crombach et al., 2016; Wotton et al., 2015a). Both species exhibit the same pattern of repressive interactions among gap genes, with strong double-negative feedback between complementary domains, and much weaker (and posteriorly biased) repression between gap genes with overlapping expression. All five basic regulatory mechanisms observed in *D. melanogaster* (see Fig. 3a–e) are also present in *M. abdita*. However, there are qualitative differences in the way maternal factors activate gap genes in either species, as well as quantitative differences in the strength of gap–gap cross-repressive interactions.

One experimentally observable difference between the two species is that there is no maternal expression of *cad* in *M. abdita*, while zygotic expression is present (Fig. 2) (Stauber et al., 2008). In addition, *bcd* mRNA shows a much broader anterior localization pattern than in *D. melanogaster* (Lemke et al., 2008; Stauber et al., 1999, 2000), indicating that the Bcd protein gradient extends further to the posterior in this species. Despite these expression differences, the activating role of both of these maternal factors in gap gene expression is conserved (Wotton et al., 2015b). This results in a broader initial distribution of anterior gap domains (activated by Bcd) and a delayed activation of *gt* and *hb* in the posterior of the embryo (because there is no maternal Cad) (Wotton et al., 2015a). In addition, the maternal Hb gradient does not contribute to A–P polarity in *M. abdita*, in contrast to *D. melanogaster* (Wotton et al., 2015b). Moreover, experimental evidence from the syrphid hoverfly *Episyrphus balteatus* shows no maternal expression of *hb* (Lemke et al., 2010). This suggests a qualitative rewiring of maternal inputs on the gap gene network in the evolutionary lineage leading up to *D. melanogaster* where maternal Hb acquired a role in setting up embryo polarity.

These differences in early maternal activation are later compensated by gap–gap cross-repression in *M. abdita*, such that the patterning output of the system—the position of gap domains at the onset of gastrulation—is nearly identical between the two species (Wotton et al., 2015a). In particular, *M. abdita* shows a marked anterior shift of the posterior boundary of the anterior *hb* domain, a boundary which is stationary in *D. melanogaster* and was thought to be a stable organizing center in the central region of the embryo (see Crombach et al., 2016; Wotton et al., 2015a and references therein). We explain this difference in mechanistic terms through altered repressive inputs from Kr and Kni on *hb*. While these repressors act in a redundant manner in *D. melanogaster*, they are both required for correct positioning of the *hb* boundary in *M. abdita* (Wotton et al., 2015a). Our models show that weak repression by Kr gradually diminishes the concentration of *hb* in the boundary region until *kni* can become expressed, which strongly represses *hb*, downregulating it in an irreversible manner (Fig. 3g) (Crombach et al., 2016). In summary, the *hb* boundary is controlled by a multi-stable switch in *D. melanogaster* and by a molecular ratchet in *M. abdita*.

Another difference is that the abdominal domain of *kni* and the posterior domain of *gt* form later and in a more posterior position in *M. abdita* than in *D. melanogaster*. This delayed onset of expression is caused by the absence of maternal Cad (Fig. 2). As a consequence, both domains initiate their anterior shifts with a delay, which they compensate by a higher shift velocity at later stages (Wotton et al., 2015a). The main regulatory difference between the two species lies in the effect of Gt on *hb*: in *M. abdita*, Gt weakly activates *hb*, setting the pace for the early buildup of Hb protein in the posterior of the embryo. This buildup is driven predominantly by strong *hb* auto-activation in *D. melanogaster*, where it leads to a gradual accumulation of Hb protein in the posterior of the embryo, repressing *gt* and causing its smooth shift toward the anterior. In contrast, *hb* auto-activation is much weaker in *M. abdita* and is only triggered at a later stage, once enough Hb protein has accumulated. This provides a mechanistic explanation for the accelerated domain shift at late stages. We termed this the "pull-and-trigger" mechanism for the observed biphasic shifts in *M. abdita* (Fig. 3h) (Crombach et al., 2016).

An important point to make here is that higher-level insights are rarely possible in biology without careful consideration of (mechanistic) detail. Understanding compensatory evolution in regulatory networks requires a detailed understanding of the underlying mechanisms. Only in this way can we arrive at lineage explanations—plausible scenarios for sequences of regulatory changes (Calcott, 2009)—that explain the evolution of a gene network. Such explanations are the foundation for a broader understanding of the principles of network evolution. Before we discuss such general insights, we review our work in the psychodid moth midge *C. albipunctata*.

5.2 *The Moth Midge* Clogmia albipunctata*: Shifts as Dynamic Fossils?*

For several reasons, our reverse-engineering approach was less successful in *C. albipunctata*. First of all, it was difficult to establish an efficient protocol for RNAi knockdown in this species, which hindered systematic model validation. Second, *C. albipunctata* has no maternal Bcd gradient. Instead, it uses a maternal gradient of the pair-rule gene product Odd-paired (Opa) as its anterior determinant (Yoon et al., 2019). Since this was unknown at the time, we assumed an unknown anterior gradient to be present. Finally, we strongly suspect that we miss at least one regulatory factor involved in gap gene regulation in *C. albipunctata*: Gt protein is not detectable in the posterior of the embryo (Crombach et al., 2014; García-Solache et al., 2010; Janssens et al., 2014), and a candidate gene that is expressed in the relevant region shows no gap-like phenotype upon knockdown (Jiménez-Guri et al., 2018). Therefore, a region in the posterior of the embryo lacks an expression domain at the late blastoderm stage (Fig. 2). Missing regulators are a severe problem for reverse engineering, since they can lead to defects and artifacts in models fitted to incomplete data. Together, these obstacles impeded us from extracting a consensus network. Instead, we end up with four candidate networks for *C. albipunctata* that remain to be tested against experimental evidence (Fig. 3i) (Crombach et al., 2014). This limits any detailed and robust conclusions about the mechanisms of gap gene regulation in this species, though our analysis still allowed us to gain some general insights into the evolution of long-germband segmentation.

One such insight is drawn directly from our semiquantitative gene expression data: not only posterior gap genes but also *Kr* and *hb* show extensive positional shifts toward the anterior, which are more pronounced than the shifts observed in *D. melanogaster* and *M. abdita* (Fig. 3j) (Crombach et al., 2014; García-Solache et al., 2010; Janssens et al., 2014). Gap gene patterning in *C. albipunctata* is very dynamic. Interestingly, shift mechanisms are based on weak repression with posterior bias between overlapping gap domains (where we can resolve them with our models) (Crombach et al., 2014). In this sense, they are similar in *C. albipunctata* and *M. abdita*.

Another remarkable feature of gap gene regulation in *C. albipunctata* concerns the nature of maternal regulation: our models indicate that Cad acts as a repressor of *gt*, which explains the absence of its posterior domain (Crombach et al., 2014). The absence of a posterior *gt* domain, in turn, relaxes the requirement for mutual repression between *Kr* and *gt*, as their patterns are no longer complementary. In fact, *gt* may not even be a proper gap gene in this species as it lacks a gap phenotype upon RNAi knockdown (Jiménez-Guri et al., 2018). This is probably connected to the presence of additional, yet-to-be-identified factors involved in gap gene regulation in *C. albipunctata*, but the precise role and importance of such gene recruitment and loss for gap gene network evolution remain to be determined once the missing regulators are identified.

The observation that a basally branching dipteran shows more pronounced gap domain shifts supports the view that shifts are an ancestral feature. Still, the functional significance of these shifts for segment patterning remains unclear. They may add robustness to the process (Manu et al., 2009a). In addition, they are required for the correct placement of pair-rule expression stripes—the primary regulatory targets of the gap gene system—in *D. melanogaster* (Clark, 2017). Ultimately, however, their presence may reflect evolutionary inertia more than functional conservation. Gap domain shifts—and the oscillator-based mechanisms that generate them (Verd et al., 2018, 2017)—may represent a *dynamic fossil*, a remnant of the ancestral short-germband mechanism of segment determination. This hypothesis is supported by the fact that the damped oscillatory mechanisms that produce domain shifts in the gap gene system are functionally similar to the limit-cycle oscillators of sequential short-germband segmentation (Clark, 2017; Sarrazin et al., 2012; Verd et al., 2019). But the evidence remains preliminary. Future studies based on a larger sample of evolutionary lineages are required to resolve this issue.

5.3 Evolving Mechanisms and Network Drift

What general insights into network evolution do we gain from our comparative analysis of the gap gene system? Our first conclusion involves the evolution of the five basic gap gene regulatory mechanisms. We have shown that these core principles are largely conserved across dipteran lineages: we find broad activation by maternal factors, strong mutual repression between complementary gap genes, shifts through weak repression with a posterior bias among overlapping gap domains, gap gene auto-activation, and repression by terminal gap genes in all species we have examined (Crombach et al., 2014, 2016; Wotton et al., 2015a,b). However, none of these mechanisms are perfectly conserved, and some evolved more rapidly than others. In particular, maternal inputs are more variable than any of the regulatory mechanisms specific to gap genes. This is consistent with an hourglass pattern of developmental variation in dipteran insects, where patterning becomes increasingly more canalized as we progress downward through the layers of the segmentation gene network (Duboule, 1994; Kalinka et al., 2010; Manu et al., 2009a; Perkins, 2020; Raff, 1996; Sander, 1983; Seidel, 1960; Slack et al., 1993; Wotton et al., 2015a).

The degree of conservation also varies among mechanisms at the level of gap–gap cross-regulation. This is because mutations in different mechanisms have different consequences. The core repressive feedback loops between complementary gap domains are particularly conserved. They are only partially disrupted in *C. albipunctata*, where additional regulatory factors may have acquired their function. In contrast, our work in *M. abdita* shows how alterations in maternal inputs can be buffered by alterations in the timing and extent of gap domain shifts, implicating the weak interactions between overlapping domains (Crombach et al., 2016; Wotton et al., 2015a,b). A similar explanation likely applies to the

pronounced shifts observed in *C. albipunctata* with its distinct set of maternal regulators (Crombach et al., 2014; Yoon et al., 2019). Taken together, this suggests that compensatory evolution is an important factor driving changes in gap domain shifts in dipterans.

More broadly speaking, we are providing a detailed mechanistic explanation for regulatory network evolution by developmental system drift, also called phenogenetic drift (Haag, 2007; Haag & True, 2018; Pavlicev & Wagner, 2012; True & Haag, 2001; Weiss, 2005; Weiss & Fullerton, 2000). In this mode of network evolution, regulatory interactions change without affecting the overall output of the system. In this way, regulatory systems can evolve along so-called genotype networks, which are meta-networks—networks of networks that produce the same phenotypic outcome, connected to each other through single mutational steps (Wagner, 2011). Our analysis reveals that evolution of the gap gene network occurs along such a genotype (meta-)network, predominantly driven by mutations that fine-tune the strength of regulatory interactions influencing the extent and velocity of gap domain shifts (Crombach et al., 2016).

We emphasize that the developmental system drift we observe in the gap gene system is based on quantitative changes in interaction *strengths* (Wotton et al., 2015a). This stands in stark contrast to earlier accounts of system drift, which focus on qualitative changes that alter the components, and the number or kind of interactions between them (see, e.g., True & Haag, 2001; Wagner, 2011; Weiss, 2005). Considering the highly redundant nature of eukaryotic transcriptional regulation and the high rate of evolutionary turnover for transcription factor binding sites, we expect quantitative changes in regulatory interactions to be much more widespread than qualitative changes in a system (Wotton et al., 2015a). In addition, quantitative changes tend to be less disruptive than qualitative ones and are thus less likely to be eliminated by selection. For these reasons, we predict that quantitative developmental system drift is a common mode of regulatory network evolution.

6 Outlook: *in silico* Evolution

We have presented a significant advance toward a lineage explanation—a sequence of mutational transformations—for evolution in dipteran insects (Calcott, 2009). Our new insights into system drift suggest possible evolutionary paths and transitions between lineages. However, our analysis does not provide any information about the actual sequence of regulatory changes. The main question is if there are many possible evolutionary paths or if epistatic effects in the network constrain them to a few specific sequences of changes. Of course, the actual evolutionary pathway of the gap gene network can be empirically resolved through additional sampling of dipteran lineages. This requires reverse-engineering the gap gene network in many other species, which will cost a large amount of time and effort. Meanwhile, we can test possible constraints on the sequence of transitions through *in silico* evolution.

In silico evolution is a simulation-based approach that models the evolutionary process (for reviews and example studies, see Batut et al., 2013; François, 2012, 2014; Vroomans et al., 2016, 2018; see also the chapters by Beslon et al. and by Hogeweg). It comes in many variations, yet the general idea is as follows: we simulate populations of "digital organisms" that have a genotype, which is translated into a phenotype via a G–P map. Each phenotype is assigned a fitness value based on a predefined fitness function, which is used to select individuals to create offspring. Reproduction consists in copying an individual with a certain rate of mutational changes to its genotype. In this way, populations evolve. The whole procedure is similar to optimization by evolutionary computation (see Sect. 2), except that we are less interested in the final outcome of the simulation and more in *how* the population has evolved: which evolutionary trajectories are taken? What kind of network structures are changed in the process? What intermediate stages occur? In the case of the gap gene system, the aim is to take the gap gene networks of *C. albipunctata* or *M. abdita* as initial conditions and to trace their evolution back to the common ancestor and along the phylogenetic tree to *D. melanogaster*.

In contrast to comparative empirical studies, the *in silico* approach provides several advantages for the study of evolutionary dynamics. It enables us to rapidly test and explore many scenarios, to modify any of the simulation parameters (e.g., mutational operators, rates, population sizes, etc.), and to trace parent–offspring relationships, thus creating a perfect "fossil record" (Batut et al., 2013). Especially the latter is important, as it gives us the exact sequence of mutations and their precise phenotypic effects along the lineage that leads to the fittest individual(s). Thus, by running many simulations and by searching for parallel mutational trajectories, we can separate contingency from general trends in the evolutionary process (Batut et al., 2013).

We have not yet carried out a definitive set of simulations of gap gene evolution. One reason is that the simulation setup must be designed to be robust against many of our necessarily ad hoc modeling choices. Not only is the problem underdetermined in terms of which network modeling formalism to use but also concerning population size and structure and the kind of mutations that are implemented (and at what rate). One example is whether to simulate gene duplication, deletion, and recruitment, since it is currently unknown if such events occurred in the evolution of the gap gene system (see Sect. 5). Another issue concerns the use of gene circuits: these models feed all regulatory inputs to a gene into a single sigmoid function, which means that if they are sensitive to changes in one input, they will be sensitive to all. This is clearly not an accurate representation of transcriptional regulation, which requires individual thresholds for each regulatory interaction. Hence, the latter type of model is usually preferred for *in silico* evolutionary studies (François et al., 2007; François & Siggia, 2010; ten Tusscher, 2013; ten Tusscher & Hogeweg, 2011).

Another sensitive choice is what fitness function to use, since this strongly influences if a simulation gets stuck at a local fitness peak (our unpublished results). Residual functions measuring squared differences between model and data may be useful for model optimization, but they may overconstrain the evolutionary process.

Indeed, the actual selective pressure(s) on the gap gene system may be less stringent than getting the exact spatiotemporal expression pattern right. Though we do not know what is selected for, distinct dipteran lineages show significant differences in the dynamics of gap gene expression. Thus the most relevant feature may be a regular and ordered set of segment polarity stripes by the onset of gastrulation. For this reason, we currently experiment with an indirect approach, inspired by a recent *in silico* evolution study that explores how *D. melanogaster* and the nematoceran malaria mosquito, *Anopheles gambiae*, swapped the order of their posterior *gt* and *hb* domains during evolution (Fig. 2) (Rothschild et al., 2016). Instead of defining fitness directly on gap gene expression, they define it at the next level of the segmentation gene hierarchy, basing it on the number and position of pair-rule expression stripes. In other words, fitness is defined by the output of the gap genes.

Given the above setup, we aim to answer two questions. The first concerns compensatory evolution of gap domain shifts in response to altered maternal inputs between *M. abdita* and *D. melanogaster*. The second focuses on the gain/loss of the posterior *gt* and *hb* expression domains between *C. albipunctata* and *D. melanogaster* (Fig. 2). For both questions, we take advantage of the perfect "fossil record" provided by the simulations to compare possible mutational trajectories and to search for trends or fixed sequences of mutations that lead to changes in gap gene expression across simulations. If such trends exist, they indicate the presence of epistatic constraints, which lead to a small set of key mutations being crucial for the transition from the initial to the target phenotype. On top of that, our simulations would also provide the epistatic network *context* these mutations require to exert their effects. To the best of our knowledge, this would be the first such study where evolutionary simulations are explicitly constrained by expression data.

Such simulations open up the exciting prospect of analyzing the evolution of a developmental gene regulatory network at the level of its phase space structure. We could trace changes in gene expression dynamics to the bifurcations that cause them. Such a study would actualize the pioneering insights of C. H. Waddington, René Thom, and the process structuralists, who proposed decades ago that the best way to understand the evolution of organic form, and the systems that generate it, is to understand how these systems move through the space of possible configurations (Alberch, 1991; Goodwin, 1982; Oster & Alberch, 1982; Thom, 1976; Waddington, 1957). We have never been as close to realizing this vision as we are now.

7 Conclusion

This chapter provides a progress report, following up on the research program we proposed in (Jaeger & Crombach, 2012). We have demonstrated its usefulness and its potential for evolutionary systems biology. It is an approach based on reverse engineering and *in silico* evolution, which strives to strike a compromise between the accuracy and rigor of forward modeling and the generality of the ensemble approach. We have discussed our accomplishments, the challenges overcome, and

those we still have to meet. We focused on the methodological bottlenecks of data acquisition and model fitting, on issues of network decomposition, and on our comparative analysis to understand the mechanisms underlying compensatory network drift in the dipteran gap gene system. This work is complemented by the account of dynamical network modularity presented in the chapter by Jaeger and Monk. Together with Jaeger (2018), these chapters present a comprehensive review of our efforts to understand the function and evolution of the gap gene network across dipteran lineages.

Despite a number of open challenges—such as establishing a robust setup for *in silico* evolution—we are optimistic about the prospects of our proposed approach. It enables us to elevate our piecemeal understanding of genetic regulatory mechanisms to a more integrated view of network evolution at the systems level. We have demonstrated that we can use dynamical models to recompose the orchestrated behavior of whole evolving regulatory networks and that we can do this in a rigorous, detailed, and empirically grounded way. Such recomposition is essential if we are looking for a mechanistic understanding of regulatory network evolution. The general idea is to accumulate more case studies of this kind—in different species and different developmental contexts. Ultimately, the ambition is to reveal the regularities, or even principles, underlying the evolution of regulatory networks. Here, we have documented a small but important first step on this fascinating journey.

Acknowledgments It is nearly impossible to thank all the people whose work contributed to this review. We would like to thank all members of the Jaeger lab at the CRG in Barcelona, especially Karl Wotton, Eva Jiménez-Guri, Berta Verd, Hilde Janssens, Astrid Hörmann, Damjan Cicin-Sain, and Kolja Becker for essential contributions to the project. JJ would like to thank John Reinitz and David H. Sharp, who got it all started; Michael Akam and Urs Schmidt-Ott and members of their labs for the help and support required to get the comparative work off the ground; James Sharpe for mentorship and friendship; and Nick Monk for our wonderful long-term collaboration. AC would like to thank the Sharpe lab, the EMBL/CRG Systems Biology Research Unit, and colleagues at the CRG for their support, insightful discussions, and an extraordinary postdoc experience.

References

Abdol, A. M., Cicin-Sain, D., Kaandorp, J. A., & Crombach, A. (2017). Scatter search applied to the inference of a development gene network. *Computation, 5*(2), 22.

Alberch, P. (1991). From genes to phenotype: Dynamical systems and evolvability. *Genetica, 84*(1), 5–11.

Ashyraliyev, M., Fomekong-Nanfack, Y., Kaandorp, J. A., & Blom, J. G. (2009a). Systems biology: Parameter estimation for biochemical models. *FEBS Journal, 276*(4), 886–902

Ashyraliyev, M., Siggens, K., Janssens, H., Blom, J., Akam, M., Jaeger, J. (2009b). Gene circuit analysis of the terminal gap gene *huckebein*. *PLoS Computational Biology, 5*(10), e1000548.

Ashyraliyev, M., Jaeger, J., Blom, & J. G. (2008). Parameter estimation and determinability analysis applied to *Drosophila* gap gene circuits. *BMC Systems Biology, 2*, 83.

Bailleul, R., Curantz, C., Desmarquet-Trin Dinh, C., Hidalgo, M., Touboul, J., & Manceau, M. (2019). Symmetry breaking in the embryonic skin triggers directional and sequential plumage patterning. *PLoS Biology, 17*(10), e3000448.

Batut, B., Parsons, D. P., Fischer, S., Beslon, G., & Knibbe, C. (2013). *In silico* experimental evolution: A tool to test evolutionary scenarios. *BMC Biochemistry, 14 Suppl 15*, S11.

Bechtel, W. (2011). Mechanism and biological explanation. *Philosophy in Science, 78*, 533–557.

Bechtel, W. (2012). Understanding endogenously active mechanisms: A scientific and philosophical challenge. *European Journal for Philosophy of Science, 2*, 233–248.

Bechtel, W., & Abrahamsen, A. (2005). Explanation: A mechanist alternative. *Studies in History and Philosophy of Biological and Biomedical Sciences, 36*, 421–441.

Bechtel, W., & Abrahamsen, A. (2010). Dynamic mechanistic explanation: Computational modeling of circadian rhythms as an exemplar for cognitive science. *Studies in History and Philosophy of Science, 41*, 321–333.

Becker, K., Balsa-Canto, E., Cicin-Sain, D., Hoermann, A., Janssens, H., Banga, J. R., & Jaeger, J. (2013). Reverse-engineering post-transcriptional regulation of gap genes in *Drosophila melanogaster*. *PLoS Computational Biology, 9*(10), e1003281.

Brigandt, I. (2013). Systems biology and the integration of mechanistic explanation and mathematical explanation. *Studies in History and Philosophy of Biological and Biomedical Sciences, 44*, 477–492.

Brigandt, I. (2015). Evolutionary developmental biology and the limits of philosophical accounts of mechanistic explanation. In P. A. Braillard, C. Malaterre (Eds.), *Explanation in biology* (pp. 135–173). Springer.

Burns, J. (1970). The synthetic problem and the genotype-phenotype relation in cellular metabolism. In C. H. Waddington (Ed.), *Towards a theoretical biology* (Vol. III, pp. 47–51). Edinburgh University Press.

Calcott, B. (2009). Lineage explanations: Explaining how biological mechanisms change. The British *Journal for the Philosophy of Science, 60*(1), 51–78.

Chipman, A. D. (2020). The evolution of the gene regulatory networks patterning the *Drosophila* blastoderm. *Current Topics in Developmental Biology, 139*, 297–324.

Chu, K. W., Deng, Y., & Reinitz, J. (1999). Parallel simulated annealing by mixing of states. *Journal of Computational Physics, 148*(2), 646–662.

Cicin-Sain, D., Pulido, A. H., Crombach, A., Wotton, K. R., Jiménez-Guri, E., Taly, J. F., Roma, G., & Jaeger, J. (2015). SuperFly: A comparative database for quantified spatio-temporal gene expression patterns in early dipteran embryos. *Nucleic Acids Research, 43*(Database issue), D751–755.

Clark, E. (2017). Dynamic patterning by the *Drosophila* pair-rule network reconciles long-germ and short-germ segmentation. *PLoS Biology, 15*(9), e2002439.

Clark, E., Peel, A. D., & Akam, M. (2019). Arthropod segmentation. *Development, 146*(18), dev170480.

Crombach, A., Cicin-Sain, D., Wotton, K. R., & Jaeger, J. (2012). Medium-throughput processing of whole mount in situ hybridisation experiments into gene expression domains. *PLoS One, 7*(9), e46658.

Crombach, A., García-Solache, M. A., & Jaeger, J. (2014). Evolution of early development in dipterans: Reverse-engineering the gap gene network in the moth midge *Clogmia albipunctata* (Psychodidae). *BioSystems, 123*, 74–85.

Crombach, A., Wotton, K. R., Cicin-Sain, D., Ashyraliyev, M., & Jaeger, J. (2012). Efficient reverse-engineering of a developmental gene regulatory network. *PLoS Computational Biology, 8*(7), e1002589.

Crombach, A., Wotton, K. R., Jiménez-Guri, E., & Jaeger, J. (2016). Gap gene regulatory dynamics evolve along a genotype network. *Molecular Biology and Evolution, 33*(5), 1293–1307.

Davidson, E. H., & Erwin, D. H. (2006). Gene regulatory networks and the evolution of animal body plans. *Science, 311*(5762), 796–800.

Davis, G. K., & Patel, N. H. (2002). Short, long, and beyond: Molecular and embryological approaches to insect segmentation. *Annual Review of Entomology, 47*, 669–699.

DiFrisco, J., & Jaeger, J. (2019). Beyond networks: Mechanism and process in evo-devo. *Biology and Philosophy, 34*(6), 54.

DiFrisco, J., & Jaeger, J. (2020). Genetic causation in complex regulatory systems: An integrative dynamic perspective. *BioEssays, 42*(6), 1900226.

Duboule, D. (1994). Temporal colinearity and the phylotypic progression: A basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. *Development, 1994*(Supplement), 135–142.

Fehr, D. A., Handzlik, J. E., Manu, & Loh, Y. L. (2019). Classification-based inference of dynamical models of gene regulatory networks. *G3, 9*(12), 4183–4195.

Fomekong-Nanfack, Y., Kaandorp, J. A., & Blom, J. (2007). Efficient parameter estimation for spatio-temporal models of pattern formation: Case study of *Drosophila melanogaster*. *Bioinformatics, 23*(24), 3356–3363.

François, P. (2012). Evolution *in silico*: From network structure to bifurcation theory. *Advances in Experimental Medicine and Biology, 751*, 157–182.

François, P. (2014). Evolving phenotypic networks *in silico*. *Seminars in Cell & Developmental Biology, 35*, 90–97.

François, P., Hakim, V., & Siggia, E. D. (2007). Deriving structure from evolution: Metazoan segmentation. *Molecular Systems Biology, 3*, 154.

François, P., & Siggia, E. D. (2010). Predicting embryonic patterning using mutual entropy fitness and *in silico* evolution. *Development, 137*(14), 2385–2395.

García-Solache, M., Jaeger, J., & Akam, M. (2010). A systematic analysis of the gap gene system in the moth midge *Clogmia albipunctata*. *Developmental Biology, 344*(1), 306–318.

Goodwin, B. C. (1982). Development and evolution. *Journal of Theoretical Biology, 97*(1), 43–55.

Haag, E. S. (2007). Compensatory vs. pseudocompensatory evolution in molecular and developmental interactions. *Genetica, 129*(1), 45–55.

Haag, E. S., & True, J. R. (2018). Developmental system drift. In L. Nuño de la Rosa, G. B. Müller (Eds.), *Evolutionary developmental biology*. Springer.

Hagolani, P. F., Zimm, R., Marin-Riera, M., Salazar-Ciudad, I. (2019). Cell signaling stabilizes morphogenesis against noise. *Development, 146*(20), dev179309.

Hoermann, A., Cicin-Sain, D., Jaeger, J. (2016). A quantitative validated model reveals two phases of transcriptional regulation for the gap gene *giant* in *Drosophila*. *Developmental Biology, 411*(2), 325–338.

Hopfield, J. J. (1984). Neurons with graded response have collective computational properties like those of two-state neurons. *Proceedings of the National Academy of Sciences of the United States of America, 81*(10), 3088–3092.

Jaeger, J. (2011). The gap gene network. *Cellular and Molecular Life Sciences, 68*(2), 243–274.

Jaeger, J. (2018). Shift happens: The developmental and evolutionary dynamics of the gap gene system. *Current Opinion in Systems Biology, 11*, 65–73.

Jaeger, J., Blagov, M., Kosman, D., Kozlov, K. N., Manu, Myasnikova, E., Surkova, S., Vanario-Alonso, C. E., Samsonova, M., Sharp, D. H., & Reinitz, J. (2004a). Dynamical analysis of regulatory interactions in the gap gene system of *Drosophila melanogaster*. *Genetics, 167*(4), 1721–1737.

Jaeger, J., & Crombach, A. (2012). Life's attractors: Understanding developmental systems through reverse engineering and *in silico* evolution. *Advances in Experimental Medicine and Biology, 751*, 93–119 (2012)

Jaeger, J., Irons, D., & Monk, N. (2008). Regulative feedback in pattern formation: Towards a general relativistic theory of positional information. *Development, 135*(19), 3175–3183.

Jaeger, J., & Reinitz, J. (2006). On the dynamic nature of positional information. *BioEssays, 28*(11), 1102–1111.

Jaeger, J., Sharp, D. H., & Reinitz, J. (2007). Known maternal gradients are not sufficient for the establishment of gap domains in *Drosophila melanogaster*. *Mechanisms of Development, 124*(2), 108–128.

Jaeger, J., Surkova, S., Blagov, M., Janssens, H., Kosman, D., Kozlov, K. N., Manu, Myasnikova, E., Vanario-Alonso, C. E., Samsonova, M., Sharp, D. H., & Reinitz, J. (2004b). Dynamic control of positional information in the early *Drosophila* embryo. *Nature, 430*(6997), 368–371.

Jaeger, J., & Verd, B. (2020). Dynamic positional information: Patterning mechanism versus precision in gradient-driven systems. In S. Small, J. Briscoe (Eds.), *Current topics in developmental biology, gradients and tissue patterning* (Vol. 137, pp. 219–246). Academic Press.

Janssens, H., Siggens, K., Cicin-Sain, D., Jiménez-Guri, E., Musy, M., Akam, M., & Jaeger, J. (2014). A quantitative atlas of Even-skipped and Hunchback expression in *Clogmia albipunctata* (Diptera: Psychodidae) blastoderm embryos. *EvoDevo, 5*(1), 1.

Jiménez, A., Cotterell, J., Munteanu, A., & Sharpe, J. (2015). Dynamics of gene circuits shapes evolvability. *Proceedings of the National Academy of Sciences of the United States of America, 112*(7), 2103–2108.

Jiménez, A., Cotterell, J., Munteanu, A., & Sharpe, J. (2017). A spectrum of modularity in multi-functional gene circuits. *Molecular Systems Biology, 13*(4), 925.

Jiménez-Guri, E., Huerta-Cepas, J., Cozzuto, L., Wotton, K. R., Kang, H., Himmelbauer, H., Roma, G., Gabaldón, T., & Jaeger, J. (2013). Comparative transcriptomics of early dipteran development. *BMC Genomics, 14*, 123.

Jiménez-Guri, E., Wotton, K. R., Gavilán, B., & Jaeger, J. (2014). A staging scheme for the development of the moth midge *Clogmia albipunctata*. *PLoS ONE, 9*(1), e84422.

Jiménez-Guri, E., Wotton, K. R., & Jaeger, J. (2018). *tarsal-less* is expressed as a gap gene but has no gap gene phenotype in the moth midge *Clogmia albipunctata*. *Royal Society Open Science, 5*(8), 180458.

Jostins, L., & Jaeger, J. (2010). Reverse engineering a gene network using an asynchronous parallel evolution strategy. *BMC Systems Biology, 4*(1), 17.

Kalinka, A. T., Varga, K. M., Gerrard, D. T., Preibisch, S., Corcoran, D.L., Jarrells, J., Ohler, U., Bergman, C. M., & Tomancak, P. (2010). Gene expression divergence recapitulates the developmental hourglass model. *Nature, 468*(7325), 811–814.

Kitano, H. (2002). Computational systems biology. *Nature, 420*(6912), 206–210.

Lemke, S., Busch, S. E., Antonopoulos, D. A., Meyer, F., Domanus, M. H., & Schmidt-Ott, U. (2010). Maternal activation of gap genes in the hover fly *Episyrphus*. *Development, 137*(10), 1709–1719.

Lemke, S., Stauber, M., Shaw, P.J., Rafiqi, A.M., Prell, A., & Schmidt-Ott, U. (2008). Bicoid occurrence and Bicoid-dependent *hunchback* regulation in lower cyclorrhaphan flies. *Evolution & Development, 10*(4), 413–420.

Lou, Z., & Reinitz, J. (2016). Parallel simulated annealing using an adaptive resampling interval. *Parallel Computing, 53*, 23–31.

Lynch, J. A., Brent, A. E., Leaf, D. S., Pultz, M. A., & Desplan, C. (2006). Localized maternal *orthodenticle* patterns anterior and posterior in the long germ wasp *Nasonia*. *Nature, 439*(7077), 728–732.

Manu, Surkova, S., Spirov, A. V., Gursky, V. V., Janssens, H., Kim, A. R., Radulescu, O., Vanario-Alonso, C. E., Sharp, D. H., Samsonova, M., & Reinitz, J. (2009a). Canalization of gene expression and domain shifts in the *Drosophila* blastoderm by dynamical attractors. *PLoS Computational Biology, 5*(3), e1000303.

Manu, Surkova, S., Spirov, A. V., Gursky, V. V., Janssens, H., Kim, A.R., Radulescu, O., Vanario-Alonso, C. E., Sharp, D. H., Samsonova, M., & Reinitz, J. (2009b). Canalization of gene expression in the *Drosophila* blastoderm by gap gene cross regulation. *PLoS Biology, 7*(3), e1000049.

Mjolsness, E., Sharp, D. H., & Reinitz, J. (1991). A connectionist model of development. *Journal of Theoretical Biology, 152*(4), 429–453.

Olesnicky, E. C., Brent, A. E., Tonnes, L., Walker, M., Pultz, M. A., Leaf, D., & Desplan, C. (2006). A *caudal* mRNA gradient controls posterior development in the wasp *Nasonia*. *Development 133*(20), 3973–3982.

Oster, G., & Alberch, P. (1982). Evolution and bifurcation of developmental programs. *Evolution, 36*(3), 444–459.

Panovska-Griffiths, J., Page, K. M., & Briscoe, J. (2013). A gene regulatory motif that generates oscillatory or multiway switch outputs. *Journal of the Royal Society, Interface, 10*(79), 20120826.

Pavlicev, M., & Wagner, G. P. (2012). A model of developmental evolution: Selection, pleiotropy and compensation. *Trends in Ecology & Evolution, 27*(6), 316–322.

Perkins, M. L. (2020). Dynamic positioning and precision of bistable gene expression boundaries through diffusion and morphogen decay. https://doi.org/10.1101/2020.12.17.423202

Perkins, T. J., Jaeger, J., Reinitz, J., & Glass, L. (2006). Reverse engineering the gap gene network of *Drosophila melanogaster*. *PLoS Computational Biology, 2*(5), e51.

Pigliucci, M. (2010). Genotype-phenotype mapping and the end of the 'genes as blueprint' metaphor. *Philosophical Transactions of the Royal Society B, 365*(1540), 557–566.

Pultz, M. A., Westendorf, L., Gale, S. D., Hawkins, K., Lynch, J., Pitt, J. N., Reeves, N. L., Yao, J. C. Y., Small, S., Desplan, C., & Leaf, D. S. (2005). A major role for zygotic *hunchback* in patterning the *Nasonia* embryo. *Development, 132*(16), 3705–3715.

Raff, R. A. (1996). *The shape of life: Genes, development, and the evolution of animal form*. University of Chicago Press.

Raspopovic, J., Marcon, L., Russo, L., & Sharpe, J. (2014). Modeling digits. Digit patterning is controlled by a Bmp-Sox9-Wnt Turing network modulated by morphogen gradients. *Science, 345*(6196), 566–570.

Reinitz, J., & Sharp, D. H. (1995). Mechanism of eve stripe formation. *Mechanisms of Development, 49*(1–2), 133–158

Rohr, K. B., Tautz, D., & Sander, K. (1999). Segmentation gene expression in the mothmidge *Clogmia albipunctata* (Diptera, psychodidae) and other primitive dipterans. *Development Genes and Evolution, 209*(3), 145–154.

Rosenberg, M. I., Lynch, J. A., & Desplan, C. (2009). Heads and tails: Evolution of antero-posterior patterning in insects. *Biochimica Et Biophysica Acta, 1789*(4), 333–342.

Rothschild, J. B., Tsimiklis, P., Siggia, E. D., & François, P. (2016). Predicting ancestral segmentation phenotypes from *Drosophila* to *Anopheles* using *in silico* evolution. *PLoS Genetics, 12*(5), e1006052.

Sadier, A., Twarogowska, M., Steklikova, K., Hayden, L., Lambert, A., Schneider, P., Laudet, V., Hovorakova, M., Calvez, V., & Pantalacci, S. (2019). Modeling *Edar* expression reveals the hidden dynamics of tooth signaling center patterning. *PLoS Biology, 17*(2), e3000064.

Salazar-Ciudad, I. (2006). Developmental constraints vs. variational properties: How pattern formation can help to understand evolution and development. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution, 306*(2), 107–125.

Sander, K. (1983). *Development and evolution*. Cambridge University Press.

Sarrazin, A. F., Peel, A. D., & Averof, M. (2012). A segmentation clock with two-segment periodicity in insects. *Science, 336*(6079), 338–341.

Schmidt-Ott, U., Rafiqi, A. M., & Lemke, S. (2010). Hox3/zen and the evolution of extraembryonic epithelia in insects. *Advances in Experimental Medicine and Biology, 689*, 133–144.

Seidel, F. (1960). Körpergrundgestalt und Keimstruktur. Eine Erörterung über die Grundlagen der vergleichenden und experimentellen Embryologie und deren Gültigkeit bei phylogenetischen Überlegungen. *Zoologischer Anzeiger, 164*, 245–305.

Slack, J. M., Holland, P. W., & Graham, C. F. (1993). The zootype and the phylotypic stage. *Nature, 361*(6412), 490–492.

Soyer, O. S., & O'Malley, M. A. (2013). Evolutionary systems biology: What it is and why it matters. *BioEssays, 35*(8), 696–705.

Stauber, M., Jäckle, H., & Schmidt-Ott, U. (1999). The anterior determinant *bicoid* of *Drosophila* is a derived Hox class 3 gene. *Proceedings of the National Academy of Sciences of the United States of America, 96*(7), 3786–3789.

Stauber, M., Lemke, S., & Schmidt-Ott, U. (2008). Expression and regulation of *caudal* in the lower cyclorrhaphan fly *Megaselia*. *Development Genes and Evolution, 218*(2), 81–87.

Stauber, M., Taubert, H., & Schmidt-Ott, U. (2000). Function of *bicoid* and *hunchback* homologs in the basal cyclorrhaphan fly *Megaselia* (Phoridae). *Proceedings of the National Academy of Sciences of the United States of America, 97*(20), 10844–10849.

Strogatz, S. H. (2015). *Nonlinear dynamics and chaos; with applications to physics, biology, chemistry, and engineering* (2nd ed.). Westview Press.

Surkova, S., Kosman, D., Kozlov, K., Manu, Myasnikova, E., Samsonova, A. A., M., & Reinitz, J. (2008a). Characterization of the *Drosophila* segment determination morphome. *Developmental Biology, 313*(2), 844–862.

Surkova, S., Myasnikova, E., Janssens, H., Kozlov, K. N., Samsonova, A.A., Reinitz, J., & Samsonova, M. (2008b) Pipeline for acquisition of quantitative data on segmentation gene expression from confocal images. *Fly, 2*(2), 58–66.

Thom, R. (1976). *Structural stability and morphogenesis. Advanced books classics series*. Perseus Books Group.

True, J. R., & Haag, E. S. (2001). Developmental system drift and flexibility in evolutionary trajectories. *Evolution & Development, 3*(2), 109–119.

ten Tusscher, K. H. W. J. (2013). Mechanisms and constraints shaping the evolution of body plan segmentation. *European Physical Journal E: Soft Matter and Biological Physics, 36*(5), 54.

ten Tusscher, K. H. W. J., & Hogeweg, P. (2011). Evolution of networks for body plan patterning; interplay of modularity, robustness and evolvability. *PLoS Computational Biology, 7*(10), e1002208.

Uzkudun, M., Marcon, L., & Sharpe, J. (2015). Data-driven modelling of a gene regulatory network for cell fate decisions in the growing limb bud. *Molecular Systems Biology, 11*(7), 815.

Verd, B., Clark, E., Wotton, K. R., Janssens, H., Jiménez-Guri, E., Crombach, A., & Jaeger, J. (2018). A damped oscillator imposes temporal order on posterior gap gene expression in *Drosophila*. *PLoS Biology, 16*(2), e2003174.

Verd, B., Crombach, A., & Jaeger, J. (2014). Classification of transient behaviours in a time-dependent toggle switch model. *BMC Systems Biology, 8*, 43.

Verd, B., Crombach, A., & Jaeger, J. (2017). Dynamic maternal gradients control timing and shift-rates for *Drosophila* gap gene expression. *PLoS Computational Biology, 13*(2), e1005285.

Verd, B., Monk, N. A., & Jaeger, J. (2019). Modularity, criticality, and evolvability of a developmental gene regulatory network. *Elife, 8*, e42832.

Vroomans, R. M. A., Hogeweg, P., & ten Tusscher, K. H. W. J. (2015). Segment-specific adhesion as a driver of convergent extension. *PLoS Computational Biology, 11*(2), e1004092.

Vroomans, R. M. A., Hogeweg, P., & ten Tusscher, K. H. W. J. (2016). *In silico* evo-devo: Reconstructing stages in the evolution of animal segmentation. *EvoDevo, 7*, 14.

Vroomans, R. M. A., Hogeweg, P., & ten Tusscher, K. H. W. J. (2018). Around the clock: Gradient shape and noise impact the evolution of oscillatory segmentation dynamics. *EvoDevo 9*, 24.

Waddington, C. H. (1957). *The strategy of the genes*. George Allen & Unwin.

Wagner, A. (2011). *The origins of evolutionary innovations*. Oxford University Press.

Wagner, G. P. (2014). *Homology, genes, and evolutionary innovation*. Princeton University Press.

Wagner, G. P., & Altenberg, L. (1996). Complex adaptations and the evolution of evolvability. *Evolution, 50*(3), 967–976.

Weiss, K. M. (2005). The phenogenetic logic of life. *Nature Reviews. Genetics, 6*(1), 36–45.

Weiss, K. M., & Fullerton, S. M. (2000). Phenogenetic drift and the evolution of genotype-phenotype relationships. *Theoretical Population Biology, 57*(3), 187–195.

Wiegmann, B. M., Trautwein, M. D., Winkler, I. S., Barr, N. B., Kim, J. W., Lambkin, C., Bertone, M. A., Cassel, B. K., Bayless, K. M., Heimberg, A. M., Wheeler, B. M., Peterson, K. J., Pape, T., Sinclair, B. J., Skevington, J. H., Blagoderov, V., Caravas, J., Kutty, S. N., Schmidt-Ott, U., . . . , Yeates, D. K. (2011). Episodic radiations in the fly tree of life. *Proceedings of the National Academy of Sciences of the United States of America, 108*(14), 5690–5695.

Wilson, M. J., & Dearden, P. K. (2011). Diversity in insect axis formation: Two *orthodenticle* genes and *hunchback* act in anterior patterning and influence dorsoventral organization in the honeybee (*Apis mellifera*). *Development, 138*(16), 3497–3507.

Wilson, M. J., Havler, M., & Dearden, P. K. (2010). *giant*, *krüppel*, and *caudal* act as gap genes with extensive roles in patterning the honeybee embryo. *Developmental Biology, 339*(1), 200–211.

Wimsatt, W. C. (2007). *Re-engineering philosophy for limited beings*. Harvard University Press.

Wotton, K.R., Jiménez-Guri, E., Crombach, A., Janssens, H., Alcaine-Colet, A., Lemke, S., Schmidt-Ott, U., & Jaeger, J. (2015a). Quantitative system drift compensates for altered maternal inputs to the gap gene network of the scuttle fly *Megaselia abdita*. *Elife, 4*, e04785.

Wotton, K. R., Jiménez-Guri, E., & Jaeger, J. (2015b). Maternal co-ordinate gene regulation and axis polarity in the scuttle fly *Megaselia abdita*. *PLoS Genetics, 11*(3), e1005042.

Wotton, K. R., Jiménez-Guri, E., García Matheu, B., & Jaeger, J. (2014). A staging scheme for the development of the scuttle fly *Megaselia abdita*. *PLoS ONE, 9*(1), e84421.

Yoon, Y., Klomp, J., Martin-Martin, I., Criscione, F., Calvo, E., Ribeiro, J., & Schmidt-Ott, U. (2019). Embryo polarity in moth flies and mosquitoes relies on distinct old genes with localized transcript isoforms. *Elife, 8*, e46711.

Zagorski, M., Tabata, Y., Brandenberg, N., Lutolf, M.P., Tkačik, G., Bollenbach, T., Briscoe, J., & Kicheva, A. (2017). Decoding of position in the developing neural tube from antiparallel morphogen gradients. *Science, 356*(6345), 1379–1383.

Systems Biology Approach to the Origin of the Tetrapod Limb

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Abstract It is still not understood how genome sequences are linked with diverse and spectacular forms during evolution. The difficulty to bridge genotypes and phenotypes stems from the complexity of multi-cellular systems, where thousands of genes and cells interact with each other providing developmental non-linearity. To understand how diverse morphologies have evolved, it is essential to find ways to handle such complex systems. Here, we review the fin-to-limb transition as a case study for the evolution of multi-cellular systems. We first describe the historical perspective of comparative studies between fins and limbs. Second, we introduce our approach that combines mechanistic theory, computational modeling, and in vivo experiments to provide a mechanical explanation for the morphological difference between fish fins and tetrapod limbs. This approach helps resolve a long-standing debate about anatomical homology between the skeletal elements of fins and limbs. We will conclude by proposing that due to the counter-intuitive dynamics of gene regulatory feedback, integrative approaches that combine computer modeling, theory and experiments are essential to understand the evolution of multi-cellular organisms.

1 Introduction

Multi-cellular organisms have evolved a remarkable diversity in body forms. In the last decades, thanks to the advance of DNA sequencing technologies, the genome sequence data of various species have become available. However, we still poorly

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_5

understand how genome sequences are connected to the diverse morphologies of animals. One of the causes that make genotype-phenotype mapping so difficult is that the dynamical behavior of developmental systems is not encoded in the genome (Alberch, 1991). Indeed, developmental systems involve numerous gene interactions that can generate emergent phenomena which cannot be intuitively explained by knowing just the list of genes involved—or even their interaction network. An example of such phenomena is the diffusion-driven instability proposed by Turing (1952), in which cross-regulatory interactions between multiple diffusive molecules spontaneously generate periodic spatial patterns. Therefore, in order to map genotypes into phenotypes, we need to understand the causal relationship between genetic mutations and the developmental phenomena that are not directly encoded in the genome.

The difficulty of genotype-phenotype mapping is also related to the homology problem. Homology is fundamental to evolutionary studies, since it provides the frame of reference to identify differences between species. Traditionally, homology has been defined according to two different notions (Wagner, 2014):

- "*the correspondency of a part or organ, determined by its relative position and connections, with a part or organ in a different animal*" (Owen, 1848),
- "*Attributes of two organisms are homologous when they are derived from an equivalent characteristic of the common ancestor*" (Mayr, 1982).

The first one is Richard Owen's original definition, which was proposed before the development of evolutionary theory. The second is Mayr's definition, which was supposed to replace Owen's one in the context of the modern evolutionary synthesis. Nowadays however, homology has often been associated with both notions (Wagner, 2014; Shubin & Alberch, 1986). As pointed out by Wagner (2014), although the concept of homology was first defined to systematically categorize anatomical modules, comparative analysis of coding genes is the most successful and practical application of homology. Because genes are one-dimensional sequences of nucleotides or amino acid, they are algorithmically comparable, which allows the quantitative assessment of Owen's homology. In addition, because genes are directly inherited from ancestors via replication, comparison of genes is conceptually straight forward in terms of the continuity from the common ancestor. In contrast, anatomy is often about complex three-dimensional structures, thus it is difficult to assess homology with Owen's definition. In addition, anatomy is reconstructed from inherited information, such as the genome sequence and maternal factors, at every generation through complex developmental dynamics, which makes it difficult to trace back homology by applying Mayr's definition. Without understanding of how genome information is translated into anatomical traits, homology could be concluded from superficial resemblances with ambiguous ancestral continuity (see Wagner, 2014 for further discussions on the homology concept). Therefore, a proper description of the genotype-phenotype map is required to solve the homology problem and to provide a better conceptual basis for anatomical comparisons.

In this chapter, we will use vertebrate fins and limbs as a case study for the evolution of multi-cellular systems, showing how understanding developmental mechanisms demystifies the complex relationship between gene regulatory changes and morphological diversity. Firstly, we review a historical perspective of studies of the fin-to-limb evolution. Secondly, we discuss positional information and the Turing mechanism—two key developmental concepts involved in skeletal pattern formation of the tetrapod limb. Thirdly, we will introduce a recent effort to understand molecular mechanisms that differentiate fins and limbs.

2 Homology Debates on Fins and Limbs

The tetrapod limb is often divided into three anatomical modules: stylopod (humerus/femur), zeugopod (ulna and radius/fibula and tibia), and autopod (wrist and digits), ordered from proximal to distal (Fig. 1a). Most limb variations are explained by reduction or elongation of the skeletal elements of this basic architecture, which Richard Owen called "archetype." On the other hand, comparison between fish fins and tetrapod limbs has been controversial for more than 150 years. First of all, to clarify the terminologies, fish fins are generally composed of "radials" (endoskeletal elements) and "rays" (the fluttering part of fins made of dermal bones in ray-finned fishes or stiff filaments in the case of sharks). Here, we mainly discuss fin radials, because rays are thought to have been lost in the tetrapod lineage. A German zoologist, Gegenbaur was the first to seriously tackle the homology problem between fins and limbs (Gegenbaur, 1865). Although his main hypothesis that fins/limbs originate from gill arches is no longer supported (reviewed by Jarvik, 1980), his attempt to identify homologous elements between fins and limbs is still influential in contemporary research. He categorized the adult skeletal elements of a shark pectoral fin into three modules along the anterio-posterior (AP) axis: propterygium, mesopterygium, and metapterygium, each connected to a large basal element (Fig. 1b). While fish fins generally exhibit considerable variation between species, he identified that the metapterygium was the most constant module across fish fins. The metapterygium is characterized by its unique branching pattern—multiple small skeletal elements are connected to a series of thick ones (later named as the metapterygial axis; orange line in Fig. 1b). Because of the robustness of the metapterygium, he speculated that the tetrapod limb also originated from the metapterygium, and drew a hypothetical metapterygial axis on the skeletal elements of the tetrapod limb (Fig. 1c). Since then, many researchers have attempted to identify the metapterygial axis in tetrapod limbs by incorporating embryological and fossil data, but never reached a consensus. In particular, while the majority of researchers consider that the axis "runs" through humerus and ulna (femur and fibula for the hindlimb), there was a strong disagreement regarding the autopod, which promoted a debate whether digits are the de novo structure of tetrapod limbs or just modified fin elements. For example, Watson (1913) suggested that the axis run through the digit IV, therefore digit I–III are corresponding to the

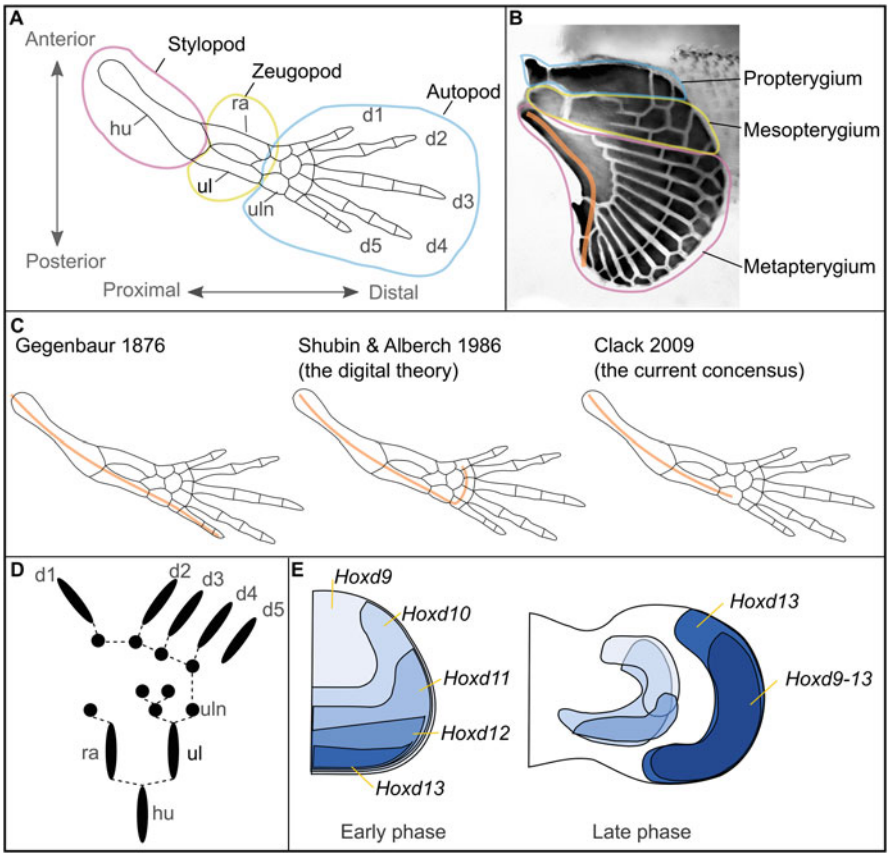

Fig. 1 The history of comparative study of fins and limbs. (**a**) The tetrapod limb. (**b**) A pectoral fin of the small-spotted catshark. (**c**) Metapterygial axes. (**d**) Shubin and Alberch's scheme of a mouse forelimb (adapted from Shubin & Alberch, 1986). (**e**) The expression patterns of *Hoxd* genes in mouse limb buds (adapted from Tarchini & Duboule, 2006). *hu* humerus, *ul* ulna, *ra* radial, *uln* ulnare, *d1-d5* digit 1–5

preaxial fin radials and digit V is the postaxial fin radial (Watson, 1913). On the other hand, Holmgren (1952) suggested the axis stops running around the wrist, so the autopod emerged as a de novo structure in the tetrapod lineage (Holmgren, 1952). This significant variation between different hypotheses raised doubt about the conceptual validity of metapterygial axis (Jarvik, 1980).

Even though all of the attempts to find homologous structures between fins and limbs failed to reach a solid conclusion, one theory became highly influential: the digital arch theory proposed by Shubin and Alberch (1986). Although this theory has been largely rejected by experimental data (Wagner & Larsson, 2007; Cohn et al., 2002), it involves a careful conceptual development of homology. Shubin and Alberch attempted to reexamine the concept of homology on the basis of

developmental mechanisms by viewing limb development as "*the product of a combination of 'global' organizers … and 'local' interactions that characterize the process of chondrogenesis.*" In general, chondrogenic condensation in limb development gradually and continuously progresses from proximal to distal. When a new element starts forming, chondrogenic condensations either branch-off or segment from the edge of already existing condensations. Shubin and Alberch considered that these local processes were necessary mechanisms for skeletal pattern formation. In addition, they assumed that the type of bifurcations that occurs (i.e., branching or segmentation) was determined by a global organizer, such as the ZPA (discussed later). Their meticulous comparison of various tetrapod limbs, lungfishes, and other fossil fishes (Fig. 1d as mouse example) led them to propose three conclusions: (a) branching events are involved only in the postaxial elements, (b) the postaxial elements also give rise to the digits via branching from the distal carpals (wrist bones), called the "digital arch," (c) the postaxial elements including the digital arch, are homologous to the metapterygial axis. Therefore, according to this theory, tetrapods acquired the autopod domain by bending metapterygial axis anteriorly during evolution (Fig. 1c).

The theory subsequently received supportive data from the analysis of genes related to positional information—*Hox* genes. During mouse limb development, 5′*Hoxd* genes (*Hoxd9*, *Hoxd10*, *Hoxd11*, *Hoxd12*, and *Hoxd13*) exhibit mainly two phases of spatial expression patterns (Tarchini & Duboule, 2006; Nelson et al., 1996; Dolle et al., 1989). In the early phase, *Hoxd9* to *Hoxd13* are transcribed in a colinear manner; *Hoxd9* is expressed in the whole limb bud, and the expression domains of d10 to d13 are restricted posteriorly in a nested manner. In the late phase (during the autopod formation), the expression domain of *Hoxd13* is expanded anteriorly to cover all the digit forming regions, and those of the others only overlap with digit II–V (Fig. 1e). Because the anterior expansion of *Hoxd* gene expression seemed correlated with the hypothesis that the axis was bent to the anterior side, this late phase of *Hoxd* gene regulation was interpreted as molecular evidence for the digital arch (Shubin et al., 1997). This interpretation was also supported by an observation that zebrafish pectoral fins lacked the late phase of Hox gene expression (Sordino et al., 1995). However, subsequent examinations of several other fish fins and re-examination of zebra fish fins showed that the anterior expansion of *Hoxd* gene expression is a deeply conserved mechanism across jawed vertebrates (Davis et al., 2007; Freitas et al., 2007; Tulenko et al., 2016; Ahn & Ho, 2008). As discussed later, these distally expressed *Hoxd* genes in fish fins seem to regulate the formation of fin rays (dermal bones) (Nakamura et al., 2016; Tulenko et al., 2017; however, this is still controversial; Freitas et al., 2012). Therefore, the late phase of *Hoxd* gene expression does not provide strong evidence for the digital arch theory.

Indeed, the theory has been often dismissed (Wagner & Larsson, 2007; Cohn et al., 2002). The weakness of this theory is that the branching and segmentation processes during skeletal pattern formation is an observed empirical regularity rather than a real developmental mechanism. A critical observation that contrasts with the digital arch theory is that the autopod can form independently of the proximal modules without branching processes (Cohn et al., 2002). Relying on

empirical regularities to identify homology is after all the same approach to Gegenbaur's analysis. However, dismissing the digital arch theory does not rule out the existence of the metapterygial axis. Indeed, current paleontological data suggest that the axis runs through at least the humerus/femur and the ulna/fibula (Clack, 2009) (Fig. 1c). On the other hand, other recent molecular evidence also suggests that the loss of the propterygium and the mesopterygium during the fin-to-limb transition resulted from a fusion of the three modules (Onimaru et al., 2015), which is roughly congruent with Jarvik's argument based on the analysis of spinal nerve distribution in fins and limbs (Jarvik, 1980). This questions the general practicality of defining homology between individual skeletal elements of fins and limbs with a traditional paleontologist framework. Instead, the concept of homology should be focused on discovering which developmental mechanisms underlie the weak identity of the skeletal elements of fins and limbs. Therefore, in the next three sections, we review the two mechanisms that have been proposed to explain skeletal pattern formation. One is the positional information model, where patterning is controlled by a global asymmetric organizer, and the other is the Turing mechanism, where patterning results from local interactions.

3 Global Asymmetric Organization in Limb Development

Embryonic limb development is a classic model of developmental biology, serving as a conceptual platform to understand morphogenesis in multicellular systems. In particular, it is a traditional model system to explain pattern formation on the basis of positional information by morphogen gradients. In the mid twentieth century, Saunders and his colleague discovered two signaling centers in chick limb development—the apical ectodermal ridge (AER; Saunders, 1948) and the zone of polarizing activity (ZPA; Saunders & Gasseling, 1968). These findings contributed to the early conceptual development of positional information, which proposed that morphogen gradients generate spatially heterogenous cell differentiation, proposed by Lewis Wolpert in 1969 (Wolpert, 1969).

The AER is a thickened ectodermal ridge, running on the tip of limb buds, and responsible for growth and pattern formation along the proximo-distal (PD) axis. In 1948, Saunders reported his discovery that removal of the AER causes terminal limb deficiencies (Saunders, 1948; Fig. 2a). Based on Saunders' experiments, Wolpert suggested a "laying-down" mechanism, in which signals from the AER lay down positional information from proximal to distal in the course of growth (Wolpert, 1969). This suggestion was later elaborated and called the "progress zone model" (Summerbell et al., 1973), in which cells in a narrow domain directly below the AER (progress zone) are kept undifferentiated by AER signals, and undergo differentiation when they become far from the AER (Fig. 2b). According to this model, the positional value of cells is determined by the duration of exposure to the AER signals, which includes in the model the assumption of a clock-like mechanism within cells to measure time. In 1990s, several fibroblast growth factors (FGFs, a

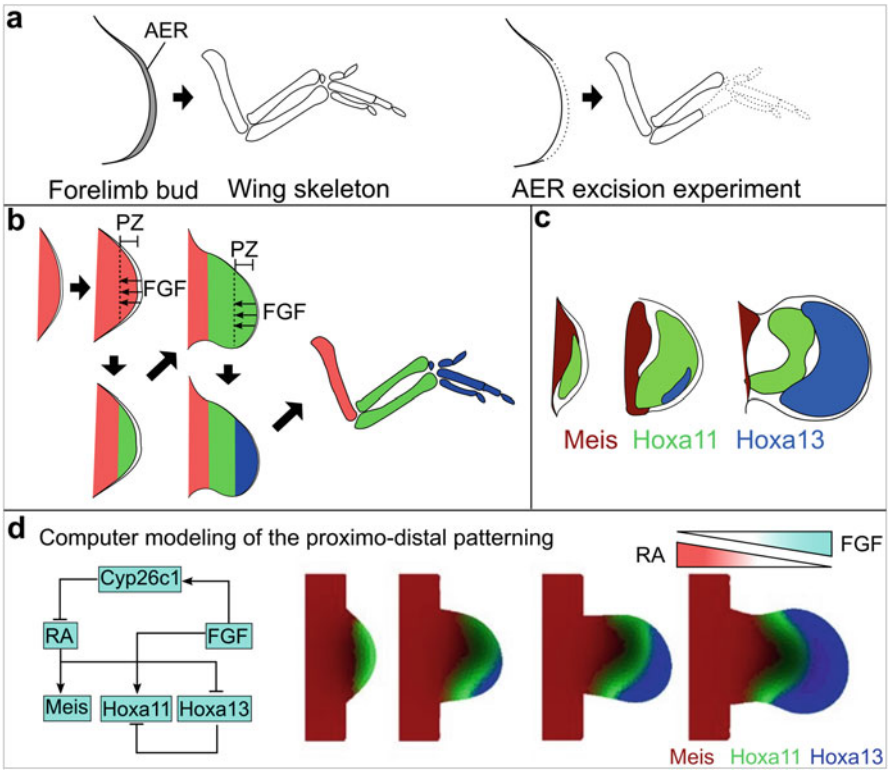

Fig. 2 PD patterning in limb development. (**a**) Saunders's AER experiments (adapted from Saunders, 1948). (**b**) The progress zone model (adapted from Tabin & Wolpert, 2007). *PZ* progress zone. (**c**) The expression pattern of *Meis1/2*, *Hoxa11*, and *Hoxa13* in mouse limb buds (based on Mercader et al., 2009). (**d**) in silico simulation of the two-signal model. (Reproduced from Uzkudun et al., 2015)

family of secreting proteins) were found to substitute the activity of the AER (Fallon et al., 1994; Niswander et al., 1993), and FGFs were indeed expressed in the AER (Niswander & Martin, 1992; Ohuchi et al., 1994; Heikinheimo et al., 1994). These series of discoveries provided the first strong evidence that morphogens controlled the pattern formation of the limb PD axis in a broad sense. Successively, alternative PD patterning models were proposed, such as the early specification model (Dudley et al., 2002) and the two-signal model (Tabin & Wolpert, 2007). In the two-signal model, a gradient of FGFs from the AER and a gradient of retinoic acid from the proximal side coordinately determine positional values. Namely, FGFs positively regulate distal genes such as *Hoxa11* (a zeugopod marker) and *Hoxa13* (an autopod marker), and RA counteracts the FGF signal to maintain proximal genes, such as *Meis1* (Fig. 2c for the expression pattern of these markers). Recently two studies provided data to show that features of both the progress zone model and the two-signal model are involved in PD patterning; FGFs are required for the activation of

the distal markers, but also act to keep cells undifferentiated (Cooper et al., 2011; Roselló-Díez et al., 2011), suggesting the requirement of a clock-like mechanism (see Delgado & Torres, 2016 for a detailed discussion). Interestingly, a data-driven computational model confirmed the validity of the two-signal model, and also indicated that the clock-like mechanism can be explained by the dynamics of gene regulatory network in the frame of the two-signal model (Uzkudun et al., 2015; Fig. 2d). Overall, several lines of evidence suggest that positional information by the FGF and RA gradients control limb PD patterning.

The case of the anterio-posterior (AP) patterning is more complicated. An important signaling region for AP patterning is the ZPA, that is located in the posterior part of limb buds and can induce a mirror-imaged duplication of the limb when grafted anteriorly (Saunders & Gasseling, 1968). From this induction ability, Wolpert suggested that the ZPA provided a gradient of "polarity potential" to specify digit identity, with the highest potential close to the ZPA and the lowest potential in the anterior side of the limb bud (Wolpert, 1969; Fig. 3a, b). More than two decades later, SHH was found to be the actual secreting molecule responsible for AP pattering because its expression domain exactly match with the ZPA, and *Shh*-expressing cells mimic the grafting experiment of the ZPA (Riddle et al., 1993). Indirect visualization of SHH gradient with its target genes, such as *Ptch1*, also helped speculate the distribution of positional values in the limb bud (Marigo et al., 1996). In addition, *Shh* null mice form only one zeugopod element (with unclear identity) and one or no digit (Chiang et al., 2001). The result is congruent with the grafting experiment, but also indicates that SHH is not only responsible for positional information along the AP axis, but it is also required for limb growth. At this point of time, perturbations of the Shh signaling pathway linearly correlated with the initial proposal by Wolpert. However, the discovery of the phenotype of the *Shh*;*Gli3* double-knockout became difficult to reconcile with the idea that SHH provides positional information. GLI3 is known to be a main factor of the SHH pathway, and to act as a transcription repressor. The repressor activity of GLI3 is inhibited by Shh, resulting in the gradient of GLI3 repressor opposite to the Shh gradient. This GLI3 repressor gradient is thought to be the actual substance of positional values in limb buds (Wang et al., 2000). The limbs of *Gli3* mutants exhibit severe polydactyly, which was interpreted to be due to the ectopic *Shh* expression observed in the anterior side of the mutant limb buds (i.e., the loss of the anterior positional value; Masuya et al., 1997). In contrast to this interpretation, the double-knockout of GLI3 and SHH also resulted in severe polydactyly with no clear identity (Te Welscher et al., 2002; Litingtung et al., 2002), showing that GLI3 and SHH are not required for digit formation, but instead that they are likely to constrain "*the polydactylous potential of the autopod*" (Litingtung et al., 2002). As we will discuss more detail in the next section, these findings are instead consistent with an alternative digit patterning mechanism based on a self-organizing Turing reaction-diffusion model (Newman, 2007; Tickle, 2006).

As we can see from the above discussions, positional information models can recapitulate some aspects of the patterning process of limb development along the PD and AP axes. However, in both cases they fail to explain important genetic

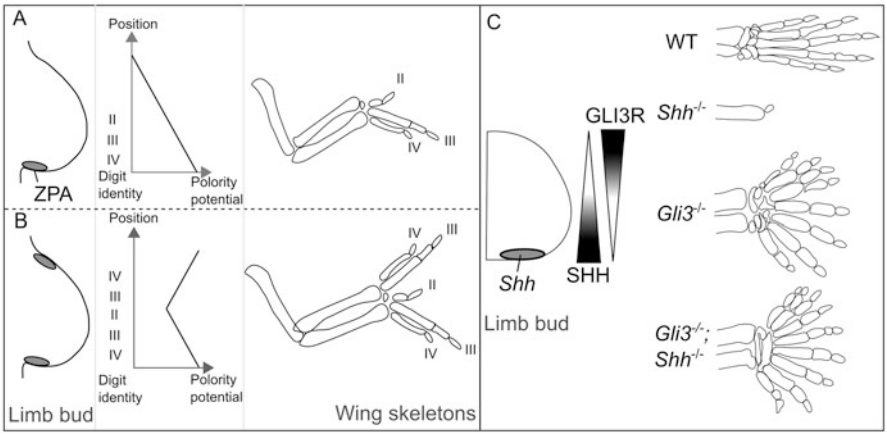

Fig. 3 Positional information theory of the ZPA. (**a**, **b**) Schemes of polarity potential in a normal chick wing (**a**) and Saunders' ZPA graft experiment (**b**) (adapted from Saunders & Gasseling, 1968 and Wolpert, 1969). (**c**) Mouse genetics on AP patterning. Left panel, a mouse limb bud with SHH and GLI3 repressor (GLI3R) gradients (based on Riddle et al., 1993 and Wang et al., 2000). Right panel, the autopods of indicated genotypes. (Adapted from Litingtung et al., 2002)

perturbations that question the relation between the genes associated with positional information and anatomical modules. In the case of AP patterning several digits can form in the absence of a SHH gradient and only a few genes have been shown to be expressed in a specific digit. For example, *Pax9* is expressed only in the digit I and is positively regulated by GLI3 (McGlinn et al., 2005), but *Pax9* mutants exhibit only a preaxial polydactyly (Peters et al., 1998), instead of loss of digit I. In the case of PD patterning, loss of function of the zeugopod markers *Hoxa11* exhibit only a subtle malformation of the forelimb wrist (Small & Potter, 1993). Similarly, while *Hoxa13* and *Hoxd13* show autopod-specific expression, the double mutants of these genes exhibit truncated polydactylous limbs rather than loss of the autopod (Fromental-Ramain et al., 1996). This nonlinear relationship between genes and phenotypes suggests that skeletal patterning cannot be fully explained by the positional information theory and advocates for alternative patterning mechanisms as discussed in the following sections.

4 Turing Mechanism as Local Interactions

Turing's reaction-diffusion mechanism is a mathematical theory that explains spontaneous pattern formation as the results of interactions between two or more diffusive molecules. The work of Alan Turing in 1952 (Turing, 1952) is one of the most important contributions in theoretical biology and yet for long time a lot of doubts were raised about its validity. In this section, we briefly introduce

the Turing reaction-diffusion model and present recent evidence that show its importance in developmental biology. Turing considered a set of simple reaction-diffusion equations to describe interaction between diffusible substances that he named morphogens:

$$\frac{\partial c}{\partial t} = f(c) + D\nabla^2 c,$$

where c is the vector of molecules or morphogens, f represents interactions between them, and D is the diagonal matrix of diffusion constants. Turing showed that under specific conditions, this system could self-organize to generate periodic spatial patterns of morphogen concentration. The profound and counterintuitive discovery of Turing is that diffusion, which usually is believe to play an equilibrating role, coupled with specific reactions could amplify random morphogen fluctuations to create spatial heterogeneity (i.e., periodic morphogen concentration patterns). The resulting patterns could be stationary periodic patterns of spots or stripes, waves or oscillations depending on parameters (Fig. 4 as an example; reviewed by Kondo & Miura, 2010). These mechanisms could recapitulate various biological pattern formation such as fish skin, seashells, and the skeletal structure of limbs (see Murray, 1993 for the further details about the Turing mechanism). However, the mere resemblance between simulated patterns and experimental data was not considered as a strong evidence for the Turing mechanism.

Rather, it was proposed that Turing systems required parameter fine-tuning (Murray, 1982), and therefore were too unreliable to control pattern formation during embryonic development (Bard & Lauder, 1974; Maini et al., 2012). This assumption was based on the observation that Turing systems could undergo the diffusion-driven instability in a very narrow parameter space region (Murray, 1982; Butler & Goldenfeld, 2011). Therefore, a major concern in biology was that these systems required large difference in diffusivities for reliable pattern formation, which was difficult to reconcile with the similar diffusion coefficients. The parameter space to obtain a Turing patterns is usually derived by performing linear stability analysis, which identifies the parameters that promote stability without diffusion and instability in the presence of diffusion. Owing to mathematical complexity, linear stability analysis was initially performed only on simple Turing systems implemented by two diffusible substances, which identified two minimal models that could generate periodic patterns: the activator-inhibitor and the substrate-depletion model. Both models required a considerable degree of differential diffusion. For example, a pervasive formulation of the activator-inhibitor model requires the inhibitor molecule to diffuse two orders of magnitude faster than the activator to form a robust pattern (Gierer & Meinhardt, 1972), which is unrealistic for most of biological molecules. Recent studies, however, indicate that differential diffusion is not a necessary condition for the majority of Turing models (White & Gilligan, 1998; Korvasová et al., 2015; Marcon et al., 2016). In particular, a recent study developed a novel method to perform an automated linear stability analysis, and demonstrated that multicomponent reaction-diffusion systems

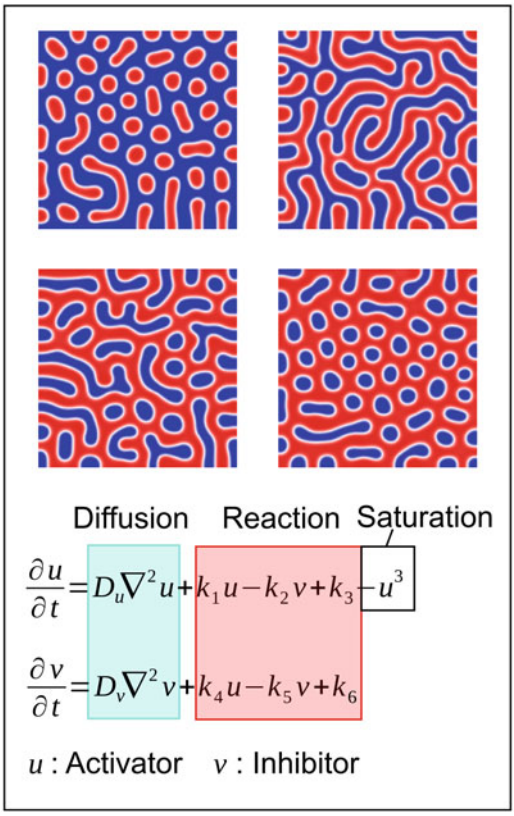

Fig. 4 An example of the Turing mechanism. The top four panels show simulation results of the bottom equations with slightly different parameters, generating spot, stripe, and anti-spot patterns. The bottom equations are a linear activator-inhibitor model

that contain nondiffusive molecules expand the parameter space of diffusion-driven instability, completely eliminating the differential diffusion requirement (Diego et al., 2018).

Another argument against Turing mechanisms was the unreliability of their patterns (Bard & Lauder, 1974). Due to sensitivity to initial conditions, simple Turing models produce patterns that can be considered unreliable following three criteria: they can have slightly different number of stripes/spots, the orientation of these periodic patterns is random and the sequence of pattern appearance cannot be easily controlled. This unreliability has been gradually solved by adding several external controls. For example, expanding boundaries at a certain speed can reliably generate patterns with a same number of periodic elements (Crampin et al., 1999). In addition, several recent studies suggested that modulation of reaction-diffusion systems with external gradients can increase the robustness of pattern formation and also control the orientation of stripes (Hiscock and Megason, 2015a; Lacalli and Harrison, 1991; Pecze, 2018; Sheth et al., 2012). However, whether or not these kinds of modulations are a biologically relevant hypothesis is still being investigated.

To summarize, the diffusion driven instability proposed by Turing is a mechanism that can create stationary periodic patterns, such as spots and stripes in a self-organizing manner. Although the theory received several criticisms, gradual progress in the theoretical understanding of the Turing mechanism has revived its biological relevance and has broadened its potential applications to developmental pattering systems. Besides the Turing mechanism, several other self-organizing patterning mechanisms based on mechanical force, cell migrations or active transport, have been studied (e.g., chemotaxis is considered to explain feather primordia pattern formation (Painter et al., 2018); see Hiscock and Megason, 2015b for a review). In the future, new self-organizing models that combine different cellular and regulatory behaviours should be explored further. Nevertheless, Turing's original model remains a seminal contribution that provides the set of theoretical conditions to explain self-organization and that could be regarded as a minimal approximation to explain pattern formation in real biological systems.

5 Intertwining the Turing Mechanism and Positional Information in Limb Development

Several attempts to build the reaction-diffusion models of limb development were already made in 1970s and 1980s (Goodwin & Trainor, 1983; Wilby & Ede, 1975; Newman & Frisch, 1979), though strictly speaking they did not involve the Turing mechanism (Othmer, 1986).

Most of these abstract models predicted that the different number of the skeletal elements in the stylopod, zeugopod, and autopod was controlled by the width of the limb bud along the anterio-posterior (AP) axis. This ruled out the requirement of a clock-like mechanism to determine positional identity along the PD axis. In particular, Wilby and Ede wisely discussed how their self-organizing model differed from Wolpert's positional information model by taking *talpid*^{3} as an example (Wilby & Ede, 1975). *talpid*^{3} is a chicken mutant that exhibits numerous extra digits and expanded limb buds (Ede & Kelly, 1964). Wilby and Ede proposed that with their reaction-diffusion model, the polydactyl *talpid*^{3} phenotype could be readily explained by the increased width of the limb bud. In contrast, Wolpert's model was not expected to produce extra digits because the expansion of limb would not change the range of the AP morphogen gradient. The only way in which Wolpert's model could explain the appearance of new digits was if a second ZPA would be initiated in the anterior part of the limb, providing a duplication of the gradient similar to grafting experiment. Just 20 years later, without noticing Wilby and Ede's idea, the *talpid*^{3} mutant was indeed found to have a normal *Shh* expression pattern and no duplication of the ZPA (Francis-West et al., 1995), which should have been supportive evidence for reaction-diffusion models. In the following decades, more sophisticated Turing mechanisms were proposed by several groups to explain limb skeletal patterning (Chaturvedi et al., 2005; Hentschel et al., 2004; Zhu et al., 2010;

Miura et al., 2006; Miura & Shiota, 2000a, b; Miura & Maini, 2004). However, there was seemingly a tremendous gap between theoretical and experimental biologists, which was probably due to the fact that the models were rather abstract and they lacked clear experimental data to support their assumptions.

Although the Turing mechanism was not a mainstream of investigation for a long time, it was gradually realized that the patterning mechanism of limb development could not be understood only on the basis of the positional information theory (Tickle, 2006). As mentioned in the previous section, the critical turning point was the surprising discovery of the polydactylous phenotype of *Gli3;Shh* double mutants (Te Welscher et al., 2002; Litingtung et al., 2002), in which the signaling center connected to AP patterning by positional information was completely abolished. These mutants suggested that digits formed spontaneously by a self-organizing mechanism, that produced more digits in larger limbs. Almost a decade later, three studies confirmed this hypothesis (Sheth et al., 2007, 2012, 2013). These studies showed that not only more digits were created in expanded limbs that lacked GLI3, but that in this genetic background, the number of digits could be further increased by removing *Hoxd* and *Hoxa* genes. These genes were originally thought to encode positional values for digit specification. However, in Sheth et al. (2012), a quantitative comparison between combinatorial mutations of *Hoxa* and *Hoxd* genes and an abstract Turing model showed that *Hox* genes act as negative regulators of the wavelength of a Turing mechanism responsible for periodic digit patterning. Interestingly, the authors went further to speculate that the pentadactyl limb evolved by a gradual increase in the dose of distal *Hox* expression to reduce the numerous skeletal elements of ancestral fish fins. As discussed later, this speculation seems to agree with experimental observations of catshark fin buds. This work provided a new interpretation of the function of *Hox* genes and a mechanical explanation of polydactylous phenotypes in the absence of positional information. This was also the first study to provide evidence that Turing models combined external modulations such as *Hox* gene dose and gradients from the AER, thereby robustly reproducing digit patterning.

The molecular candidates responsible for the Turing mechanism (Turing molecules), however, were still unknown. A previous study proposed *Tgfb2* as a strong Turing molecule candidate, because it enhances chondrogenic differentiation and it is localized with prechondrogenic condensation in micromass cultures (Miura & Shiota, 2000b). However, *Tgfb2* expression in limb development starts after the digit pattern appears (Raspopovic et al., 2014). In addition, *Tgfb2* null mutants show only a minor patterning defect (Sanford et al., 1997). FGFs are also often mentioned as a candidate (Hentschel et al., 2004), but there is no evidence for the co-localization of their signal with condensations. Although an FGF receptor, *Fgfr2* (more precisely its IIIc isoform) is expressed in the prechondrogenic mesenchyme (Szebenyi et al., 1995; Sheeba et al., 2010), the loss of *Fgfr2* in the limb mesenchyme results in a minor growth defect (Eswarakumar et al., 2002). Similarly, no patterning defects are also observed in the limbs lacking galectins (e.g., Georgiadis et al., 2007), which were proposed as Turing molecules to explain digit patterning in chick embryos.

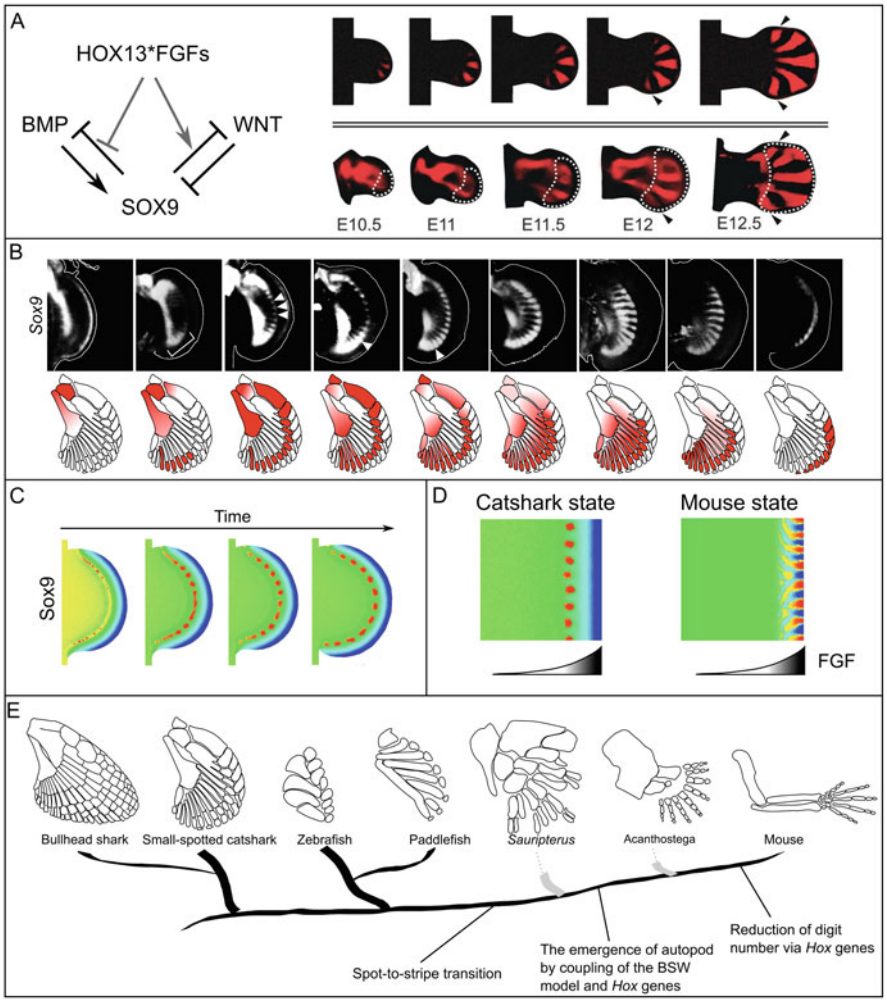

Fig. 5 Computer modeling of limb and fin development. (**a**) The BSW network (left) and comparison between a simulation result (top right) and real *Sox9* expression (right bottom). From Raspopovic et al., Science 345:566 (2014). Reprinted with permission from AAAS. (**b**) Time-series of *Sox9* expression of the catshark pectoral fin buds. Bottoms are corresponding skeletal elements. (**c**) A simulation of *Sox9* expression of the catshark fin buds. (**d**) The difference between fins and limbs (**b**–**d** are reproduced from Onimaru et al., 2016). (**e**) The diverse morphology of fins. Note that distal nodular radials are widely recognized in fish fins, but *Sauripterus* (a fossil fish) may have had different type of fins. An autopod-like structure is seen in *Acanthostega* (a stem fossil tetrapod), but it shows polydactyly. (*Sauripterous* and *Acanthostega* are adapted from Davis et al., 2004 and Coates, 1996, respectively)

Strong experimental evidence in support of specific Turing molecules was provided only more recently (Raspopovic et al., 2014). This study sought to find molecules that show either in-phase or out-of-phase periodic patterns as seen in typical Turing models such as the activator-inibitor and subtrate-depletion models. By performing a detailed spatio-temporal analysis of candidates with comparative microarray analysis, in situ hybridization and analysis of protein distributions, this study identified three promising molecules as Turing candidates: BMP, SOX9, and WNT (referred to as the BSW model; Fig. 5a). SOX9 is the earliest chondrogenic marker (therefore in-phase with digit primordia), and is required for prechondrogenic condensation (Liu et al., 2018). This study showed that *Bmp2* expression and WNT signal exhibited out-of-phase distributions with respect to Sox9 that was congruent with the requirement for Turing molecules. In addition, perturbations of the BMP and WNT pathways are consisted with their roles as Turing molecules that regulate Sox9. It is worth emphasizing that in this study, three technical advances played a central role to test the molecular candidates. Firstly, the development of a computational model based on accurate quantification of 2D limb bud morphology, including information about growth, tissue movement, and spatial gene expression dynamics (Marcon et al., 2011). This allowed the authors to compare realistic simulations of digit patterning with experimental expression patterns. Secondly, the use of an ex utero limb culture method, which utilizes the air liquid interface cell culture system, to perform perturbation and test model predictions in a near in vivo environment. Thirdly, the development of a novel Turing model with three components that include a non-diffusing reactant corresponding to SOX9. This last point was a major difference with previous models, which considered only two diffusible substances. In general, multicomponent models with non-diffusible elements relax the parameter constraint required to form Turing patterns and increase the robustness of pattern formation. This extended Turing model, known as the BSW model, captures the essential dynamics of skeletal pattern formation showed by *Sox9* expression patterns. In addition, the model integrates a Turing network with positional signals, such as FGFs and HOX genes that help to generate the properly aligned digit primordia in a reproducible manner. The theoretical idea that external gradients could modulate Turing systems to obtain specific patterns was already proposed previously (Gierer & Meinhardt, 1972). However, the BSW model represents the first biologically relevant realization of this idea supported by experimental data. Taken together, this study does not only represent a major step forward to understand limb development, but it also invokes a conceptual innovation by showing that Turing mechanisms and positional information can act simultaneously, and should not be considered as alternative mechanisms (Green & Sharpe, 2015; Miura, 2013).

Indeed, positional information and the Turing mechanism have often been seen as contrasting patterning strategies. The former has a hierarchical nature, because morphogen gradients are upstream and genes for differentiation are downstream. Moreover, it requires a signaling center that imposes a global asymmetry, where identity is uniquely determined as a function of distance from the signaling source. Turing models on the contrary are self-organizing, and are based on local molecular

interactions that amplify random fluctuations to generate a periodic pattern. This model generates identical elements that are regularly distributed in space. Positional information and the Turing mechanism, however, can act together to form intermediate structures, e.g., periodic pattern with slightly different wavelength (Green & Sharpe, 2015). In such intermediate systems, the identity of the resulting anatomical modules can be vague and quantitative rather than qualitative. As seen by *Gli3* and *Hox* gene mutants, the loss of genes related to positional information by genetic perturbations may accentuate the nature of the Turing mechanism.

6 Emergence of Homology by Coupling Turing Mechanism and Positional Information

As we have seen in the previous sections, an integrative approach is required to understand the evolution of fins and limbs. We covered a wide range of topics such as classical comparative anatomy, developmental biology, and mathematical models. However, the above discussion can be summarized into two main points: (1) the homology problem between fins and limbs is likely due to poor understandings of the developmental mechanism that underlies skeletal pattern formation; (2) Turing mechanism and positional information control together limb development. In this section, we discuss how both mechanisms are also involved in the development of fish fins.

Molecular studies of the fin-to-limb transition have suffered from similar problems to the ones faced in limb development that we mentioned in the previous section. Although key genes responsible for positional information, such as HOX genes, SHH, and FGFs, were analyzed to investigate fin development in several species including sharks, rays and ray-finned fishes, the observed differences were not readily interpretable. For example, the overlapping expression domains of *Hoxa11* and *Hoxa13* seem to be a common feature in pectoral fin buds, while in tetrapod limbs, *Hoxa11* and *Hoxa13* are expressed in the zeugopod and autopod domains separately (Kherdjemil & Kmita, 2018; Leite-Castro et al., 2016). However, as discussed earlier, current data shows that these genes alone do not affect the skeletal patterning process in mouse limb buds, therefore this regulatory difference alone cannot fully explain the anatomical difference between fins and limbs. Secondly, a study (Onimaru et al., 2015) showed that the gross expression domains of genes related to the AP positional information (or SHH targets) are shifted to the posterior side of catshark pectoral fin buds. Experimentally shifting of these expression domains anteriorly caused a fusion of the anterior radials to metapterygium, which is correlated with the pro- and mesopterygium loss during the fin-to-limb transition. However, owing to the lack of the one-to-one correspondence between gene expression domains and skeletal elements, it remains unclear how these genes regulate skeletal patterning.

Fish fins exhibit a greater variety of skeletal patterns than tetrapod limbs (Fig. 5e). Most fins seem to have an affinity to the basic characteristic of the Turing pattern, because they are composed of numerous and regular periodic elements, but also show a weak asymmetric trend along both of the AP and PD axes. Therefore, given the aforementioned nature of limb development, the pattern formation of fish fins strongly points to a Turing mechanism that is still weakly controlled by positional information. This idea was recently tested in (Onimaru et al., 2016), where a combination of data driven in silico modeling and in vivo experiments was used to investigate catshark pectoral fin development. This study showed that the Turing mechanism driven by the interactions of BMP, SOX9, and WNT seems to be conserved at least between mouse digits and the distal fin elements of the catshark (Fig. 5b for *Sox9* expression data and Fig. 5c for simulated *Sox9* expression with the BSW model). However, there are several differences that are likely responsible for their different anatomical structures: (1) in the catshark, the distal fin radials arise from a periodic spot pattern of *Sox9* expression (Fig. 5b), in contrast to the stripe pattern in mouse digit formation, highlighting one of the major parameters that cause the anatomical difference between fins and limbs. (2) In catshark fins, the BSW model seems to be decoupled from the distal Hox genes (yet still regulated by FGFs). *Sox9* expression in catshark pectoral fin buds is located proximal to the distal *Hoxa13* expression without an overlapping domain, which is congruent with the reverse correlation between the amount of *Hox* genes and the number of skeletal elements (Sheth et al., 2012). Interestingly, two independent groups concurrently suggested that HOX13s in fish fins regulate the differentiation of fin rays, but not radials (Nakamura et al., 2016; Tulenko et al., 2017). In addition, the computational model predicts that modulation of WNT-related parameters shifts the *Sox9* expression domain distally, and changes the spot pattern into a stripe pattern (Fig. 5d). Therefore, it is tempting to hypothesize that a distal shift of *Sox9* expression and a pattern change from spots to stripes were critical events prior to the de novo acquisition of digits. Subsequently, the acquisition of wavelength regulation by distal Hox genes (Sheth et al., 2012) may have resulted in the emergence of the homologous autopod and a gradual reduction of the number of digits until the current pentadactyl state (Fig. 5e).

It is worth noting that there is an earlier attempt to simulate the diverse morphologies of fins with the Turing mechanism (Zhu et al., 2010). In this study, a single computational framework simulated overall skeletal patterns of fins and limbs of various species including the catshark, obtaining a good degree of resemblance between known skeletal patterns and simulations. The models developed in this study however were not constrained by experimental data. Instead they arbitrarily modulated the AP width and wavelength without comparison with real data, which limits the predictive power of the model. In fact, the *Sox9* expression pattern of catshark fin buds (Onimaru et al., 2016) does not fit with the catshark simulation presented in this study. This highlights that to draw a biologically meaningful conclusion, computational models should be constrained with real data.

In this section, we discussed how the underling developmental patterning mechanisms play a central role to understand the morphological difference between

fins and limb. Evidence indicates that a Turing mechanism modulated by positional information is very likely to underlie skeletal pattern formation in both fins and limbs. The diverse morphologies of fins radials and digits can be traced to changes in the modulations of a Turing mechanism to generate topologically different patterns, such as spots and stripes with slight changes in parameters. Therefore, continuous changes in genotype space can trigger a drastic or even discontinuous change in the phenotype, causing a disparity between homology at gene regulatory and morphological levels. This is a major conceptual shift to understand fin-to-limb evolution, and it provides a new framework to infer homology and similarity between appendages. Nevertheless, many aspects of the fin-to-limb transition remain unclear. For example, the biological significance of the metapterygium is not yet solved. *Sox9* expression data (Fig. 5b; Onimaru et al., 2016) suggest that the metapterygium at least in the catshark, is not formed by a branching process that can be inferred from the adult skeletal pattern. Instead, the distal elements appear separately from the proximal ones and they are connected later. Therefore, a branching-like pattern, which is thought to be the characteristic of the metapterygium, may not represent a common developmental mechanism derived from the last common ancestor of jawed vertebrates. Another untouched issue is the actual genetic changes that are responsible for the different dynamics of fin and limb pattern formation. Because several shark genome sequences were recently released (Hara et al., 2018), comparative genome analysis is now possible and will help us draw a more detail genotype-phenotype map of fins and limbs.

7 Evolutionary Systems Biology Toward the Genotype-Phenotype Mapping

Three general conclusions:

1. Anatomical homology can be recognized when the elements of an organ exhibit global asymmetry (possibly induced by morphogen gradients).
2. The non-linear and periodic nature of self-organizing mechanisms obscures the homologous relationship of elements between species.
3. Because global asymmetries and self-organizing mechanisms can work as a single system, there could be intermediate states where a homologous relationship becomes vague.

These conclusions may not be applicable to every case of evolutionary studies, but they will work as precaution by showing that some homology problems may not be solvable because of the nature of developmental mechanisms. The avian digit identity problem—a debate if avian digits should be counted as “I, II, III” or “II, III, IV”—might be the case of such “overdiagnosis”. Indeed, evolutionary drifts between periodic and identity-containing patterns appear to be observed in other systems, such as body segmentation in insects and computational experiments

(Jiménez et al., 2015; Salazar-Ciudad et al., 2001; Verd et al., 2018). The very essence of our argument is that homologous gene regulatory networks can generate distinct morphologies. In other words, even if a morphological part or a gene regulatory network was derived from a common ancestor, the homologous relation can disappear by changes in the developmental mechanism responsible for the morphological identity.

In this chapter, we focused on the problems surrounding the homology concept that emerges from the comparison between fins and limbs. Our discussion can be re-examined under the light of two alternative theories. The first is the Developmental Systems Drift theory (DSD), which emphasizes the idea that "*many characters known to be homologous between taxa have diverged in their morphogenetic or gene regulatory underpinnings*" (True and Haag, 2001). In other words, this theory highlights that developmental mechanisms can vary while the phenotype remains unchanged. In this picture, investigating the evolutionary continuity of developmental mechanisms does not provide reliable clues for morphological evolution. Although the DSD theory is dominant in some cases (e.g., sex determination), interpreting a shared developmental mechanism as a signature of homology is at least partially verified by another theory, the "character identity networks (ChINs)" (Wagner, 2007, 2014). This theoretical framework states that if two characters are homologous, they are likely to be within the phylogenetic continuity of the conserved core gene regulatory network (i.e., ChIN) responsible for the identity of the characters. ChINs are described as the interface of two evolutionary variable processes: positional information (or inductive) signals and downstream genes responsible for species-specific morphological characters. The "ChIN model" was introduced to explain highly variable (yet partially conserved) developmental programs that underlie homologous organs between species. In this chapter, we discussed how the development of the distal elements of fins and limbs may be interpreted within the phylogenetic continuity of a conserved ChIN implemented by the BSW Turing network under the influence of external positional information signals. In this case, unlike the assumption of the classical ChIN model, the Turing mechanism exhibits a strong developmental plasticity and does not require highly variable species-specific mechanism to produce different characters. Instead, relatively small changes in regulation strength of the Turing gene regulatory network can drive the emergence of substantially different characters making morphological homology disappear.

By reviewing computational methods, mathematical theories, and experimental biology, we have attempted to create an integral understanding of the evolution of fins and limbs. One of the critical factors that we did not cover in this chapter is genome information. Because evolution is driven by genetic mutations, approaches to integrate genome information into models are needed to draw a more realistic (if not whole) picture of the genotype-phenotype map. The recent advancement in DNA-sequencing technologies has improved the availability of genome sequences from various species. However, currently, owing to the complicated organization of the genome, the ability to extract functional information such as protein structures and gene regulatory networks from genome sequences remains limited. Several

recent studies indicate that deep learning-based methods are promising to interpret genome information (e.g., Onimaru et al., 2018; Quang and Xie, 2016; Zhou and Troyanskaya, 2015). Developing methods to understand what is encoded in genomes will facilitate closing the gap between genotypes and phenotypes.

In conclusion, we have discussed how the nature of morphological evolution depends on developmental mechanisms. Because of the counter-intuitive dynamics of gene regulatory feedbacks like those present in Turing systems, a multi-disciplinary approach that combines computer modeling, theoretical and experimental biology is essential to understand the evolution of multi-cellular organisms. Therefore, finding a good collaboration between researchers from different fields or educational opportunities to learn different fields will be a key to stimulate the growth of evolutionary systems biology.

Acknowledgments We thank Dr. Tatsuya Hirasawa, Dr. Xavier Diego, and Dr. James Sharpe for fruitful discussions. This work was supported in part by JSPS KAKENHI grant number 17K15132, a Special Postdoctoral Researcher Program of RIKEN, and a research grant from MEXT to RIKEN Center for Biosystems Dynamics Research to K.O., and by the María de Maeztu Unit of Excellence MDM-2016-0687 (MINECO, Spain) to L.M.

References

Ahn, D., & Ho, R. K. (2008). Tri-phasic expression of posterior Hox genes during development of pectoral fins in zebrafish: Implications for the evolution of vertebrate paired appendages. *Developmental Biology, 322*, 220–233.

Alberch, P. (1991). From genes to phenotype: Dynamical systems and evolvability. *Genetica, 84*, 5.

Bard, J., & Lauder, I. (1974). How well does Turing's theory of morphogenesis work? *Journal of Theoretical Biology, 45*, 501–531. https://doi.org/10.1016/0022-5193(74)90128-3

Butler, T., & Goldenfeld, N. (2011). Fluctuation-driven Turing patterns. *Physical Review E: Statistical, Nonlinear, and Soft Matter Physics, 84*, 011112. https://doi.org/10.1103/PhysRevE.84.011112

Chaturvedi, R., Huang, C., Kazmierczak, B., et al. (2005). On multiscale approaches to three-dimensional modelling of morphogenesis. *Journal of The Royal Society Interface, 2*, 237–253. https://doi.org/10.1098/rsif.2005.0033

Chiang, C., Litingtung, Y., Harris, M. P., et al. (2001). Manifestation of the limb prepattern: Limb development in the absence of sonic hedgehog function. *Developmental Biology, 236*, 421–435. https://doi.org/10.1006/dbio.2001.0346

Clack, J. A. (2009). The fin to limb transition: New data, interpretations, and hypotheses from paleontology and developmental biology. *Annual Review of Earth and Planetary Sciences, 37*, 163–179.

Coates, M. I. (1996). The Devonian tetrapod Acanthostega gunnari Jarvik: Postcranial anatomy, basal interrelationships and patterns of skeletal evolution. *Transactions of the Royal Society of Edinburgh: Earth Sciences, 87*, 363–421.

Cohn, M. J., Lovejoy, C. O., Wolpert, L., & Coates, M. I. (2002). Branching, segmentation and the metapterygial axis: Pattern versus process in the vertebrate limb. *BioEssays, 24*, 460–465. https://doi.org/10.1002/bies.10088

Cooper, K. L., Hu, J. K.-H., ten Berge, D., et al. (2011). Initiation of proximal-distal patterning in the vertebrate limb by signals and growth. *Science, 332*, 1083–1086.

Crampin, E. J., Gaffney, E. A., & Maini, P. K. (1999). Reaction and diffusion on growing domains: Scenarios for robust pattern formation. *Bulletin of Mathematical Biology, 61*, 1093–1120. https://doi.org/10.1006/bulm.1999.0131

Davis, M. C., Dahn, R. D., & Shubin, N. H. (2007). An autopodial-like pattern of Hox expression in the fins of a basal actinopterygian fish. *Nature, 447*, 473–476. https://doi.org/10.1038/nature05838

Davis, M. C., Shubin, N. H., & Daeschler, E. B. (2004). A new specimen of Sauripterus taylori (Sarcopterygii, Osteichthyes) from the Famennian Catskill Formation of North America. *Journal of Vertebrate Paleontology, 24*, 26–40.

Delgado, I., & Torres, M. (2016). Gradients, waves and timers, an overview of limb patterning models. In *Seminars in cell and developmental biology* (pp. 109–115). Elsevier.

Diego, X., Marcon, L., Müller, P., & Sharpe, J. (2018). Key features of Turing systems are determined purely by network topology. *Physics Review X, 8*, 021071. https://doi.org/10.1103/PhysRevX.8.021071

Dolle, P., Izpisua-Belmonte, J. C., Falkenstein, H., Renucci, A., et al. (1989). Coordinate expression of the murine Hox-5 complex homoeobox-containing genes during limb pattern formation. *Nature, 342*, 767–772.

Dudley, A. T., Ros, M. A., & Tabin, C. J. (2002). A re-examination of proximodistal patterning during vertebrate limb development. *Nature, 418*, 539–544. https://doi.org/10.1038/nature00945

Ede, D. A., & Kelly, W. A. (1964). Developmental abnormalities in the trunk and limbs of the talpid^3 mutant of the fowl. *Journal of Embryology and Experimental Morphology, 12*, 339–356.

Eswarakumar, V. P., Monsonego-Ornan, E., Pines, M., et al. (2002). The IIIc alternative of Fgfr2 is a is a positive regulator of bone formation. *Development, 129*, 3783–3793.

Fallon, J. F., López, A., Ros, M. A., et al. (1994). FGF-2: Apical ectodermal ridge growth signal for chick limb development. *Science, 264*, 104–107. https://doi.org/10.1126/science.7908145

Francis-West, P. H., Robertson, K. E., Ede, D. A., et al. (1995). Expression of genes encoding bone morphogenetic proteins and sonic hedgehog in talpid (ta3) limb buds: Their relationships in the signalling cascade involved in limb patterning. *Developmental Dynamics, 203*, 187–197. https://doi.org/10.1002/aja.1002030207

Freitas, R., Gómez-Marín, C., Wilson, J. M., et al. (2012). Hoxd13 contribution to the evolution of vertebrate appendages. *Developmental Cell, 23*, 1219–1229. https://doi.org/10.1016/j.devcel.2012.10.015

Freitas, R., Zhang, G., & Cohn, M. J. (2007). Biphasic Hoxd gene expression in shark paired fins reveals an ancient origin of the distal limb domain. *PLoS One, 2*, e754.

Fromental-Ramain, C., Warot, X., Messadecq, N., et al. (1996). Hoxa-13 and Hoxd-13 play a crucial role in the patterning of the limb autopod. *Development, 122*, 2997–3011.

Gegenbaur, C. (1865). *Untersuchungen zur vergleichenden anatomie der wirbeltiere* (Vol. II). Wilhelm Engelmann.

Georgiadis, V., Stewart, H. J. S., Pollard, H. J., et al. (2007). Lack of galectin-1 results in defects in myoblast fusion and muscle regeneration. *Developmental Dynamics, 236*, 1014–1024. https://doi.org/10.1002/dvdy.21123

Gierer, A., & Meinhardt, H. (1972). A theory of biological pattern formation. *Kybernetik, 12*, 30–39. https://doi.org/10.1007/BF00289234

Goodwin, B. C., & Trainor, L. E. H. (1983). The ontogeny and phylogeny of the pendactyl limb. In *Development and evolution* (pp. 75–98). Springer.

Green, J. B. A., & Sharpe, J. (2015). Positional information and reaction-diffusion: Two big ideas in developmental biology combine. *Development, 142*, 1203–1211. https://doi.org/10.1242/dev.114991

Hara, Y., Yamaguchi, K., Onimaru, K., et al. (2018). Shark genomes provide insights into elasmobranch evolution and the origin of vertebrates. *Nature Ecology & Evolution, 2*, 1761. https://doi.org/10.1038/s41559-018-0673-5

Heikinheimo, M., Lawshé, A., Shackleford, G. M., et al. (1994). Fgf-8 expression in the post-gastrulation mouse suggests roles in the development of the face, limbs and central nervous system. *Mechanisms of Development, 48*, 129–138. https://doi.org/10.1016/0925-4773(94)90022-1

Hentschel, H. G. E., Glimm, T., Glazier, J. A., & Newman, S. A. (2004). Dynamical mechanisms for skeletal pattern formation in the vertebrate limb. *Proceedings of the Royal Society B: Biological Sciences, 271*, 1713–1722. https://doi.org/10.1098/rspb.2004.2772

Hiscock, T. W., & Megason, S. G. (2015a). Orientation of Turing-like patterns by morphogen gradients and tissue anisotropies. *Cell Systems, 1*, 408–416. https://doi.org/10.1016/j.cels.2015.12.001

Hiscock, T. W., & Megason, S. G. (2015b). Mathematically guided approaches to distinguish models of periodic patterning. *Development, 142*, 409–419. https://doi.org/10.1242/dev.107441

Holmgren, N. (1952). An embryological analysis of the mammalian carpus and its bearing upon the question of the origin of the tetrapod limb. *Acta Zoologica, 33*, 1–115.

Jarvik, E. (1980). *Basic structure and evolution of vertebrates* (Vol. 2). Academic Press Inc.

Jiménez, A., Cotterell, J., Munteanu, A., & Sharpe, J. (2015). Dynamics of gene circuits shapes evolvability. *Proceedings of the National Academy of Sciences, 112*, 2103–2108. https://doi.org/10.1073/pnas.14110651

Kherdjemil, Y., & Kmita, M. (2018). Insights on the role of hox genes in the emergence of the pentadactyl ground state. *Genesis, 56*, e23046.

Kondo, S., & Miura, T. (2010). Reaction-diffusion model as a framework for understanding biological pattern formation. *Science, 329*, 1616–1620.

Korvasová, K., Gaffney, E. A., Maini, P. K., et al. (2015). Investigating the Turing conditions for diffusion-driven instability in the presence of a binding immobile substrate. *Journal of Theoretical Biology, 367*, 286. https://doi.org/10.1016/j.jtbi.2014.11.024

Lacalli, T. C., & Harrison, L. G. (1991). From gradients to segments: Models for pattern formation in early *Drosophila* embryogenesis. *Seminars in Developmental Biology, 2*, 107–117.

Leite-Castro, J., Beviano, V., Rodrigues, P., & Freitas, R. (2016). HoxA genes and the fin-to-limb transition in vertebrates. *Journal of Developmental Biology, 4*, 10. https://doi.org/10.3390/jdb4010010

Litingtung, Y., Li, Y., Fallon, J. F., & Chiang, C. (2002). Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. *Nature, 418*, 979–983. https://doi.org/10.1038/nature01033

Liu, C.-F., Angelozzi, M., Haseeb, A., & Lefebvre, V. (2018). SOX9 is dispensable for the initiation of epigenetic remodeling and the activation of marker genes at the onset of chondrogenesis. *Development, 145*, dev164459. https://doi.org/10.1242/dev.164459

Maini, P. K., Woolley, T. E., Baker, R. E., et al. (2012). Turing's model for biological pattern formation and the robustness problem. *Interface Focus, 2*, 487–496.

Marcon, L., Arqués, C. G., Torres, M. S., & Sharpe, J. (2011). A computational clonal analysis of the developing mouse limb bud. *PLoS Computational Biology, 7*, e1001071.

Marcon, L., Diego, X., Sharpe, J., & Müller, P. (2016). High-throughput mathematical analysis identifies Turing networks for patterning with equally diffusing signals. *eLife, 5*, e14022. https://doi.org/10.7554/eLife.14022

Marigo, V., Scott, M. P., Johnson, R. L., et al. (1996). Conservation in hedgehog signaling: Induction of a chicken patched homolog by Sonic hedgehog in the developing limb. *Development, 122*, 1225–1233.

Masuya, H., Sagai, T., Moriwaki, K., & Shiroishi, T. (1997). Multigenic control of the localization of the zone of polarizing activity in limb morphogenesis in the mouse. *Developmental Biology, 182*, 42–51.

Mayr, E. (1982). *The growth of biological thought: Diversity, evolution, and inheritance*. Belknap Press of Harvard University Press.

McGlinn, E., Van Bueren, K. L., Fiorenza, S., et al. (2005). Pax9 and Jagged1 act downstream of Gli3 in vertebrate limb development. *Mechanisms of Development, 122*, 1218–1233. https://doi.org/10.1016/j.mod.2005.06.012

Mercader, N., Selleri, L., Criado, L. M., et al. (2009). Ectopic Meis1 expression in the mouse limb bud alters P-D patterning in a Pbx1-independent manner. *The International Journal of Developmental Biology, 53*, 1483–1494.

Miura, T. (2013). Turing and Wolpert work together during limb development. *Science Signaling, 6*, pe14. https://doi.org/10.1126/scisignal.2004038

Miura, T., & Maini, P. K. (2004). Speed of pattern appearance in reaction-diffusion models: Implications in the pattern formation of limb bud mesenchyme cells. *Bulletin of Mathematical Biology, 66*, 627–649. https://doi.org/10.1016/j.bulm.2003.09.009

Miura, T., & Shiota, K. (2000a). Extracellular matrix environment influences chondrogenic pattern formation in limb bud micromass culture: Experimental verification of theoretical models. *The Anatomical Record, 258*, 100–107. https://doi.org/10.1002/(SICI)1097-0185(20000101)258:1<100::AID-AR11>3.0.CO;2-3

Miura, T., & Shiota, K. (2000b). TGFβ2 acts as an "activator" molecule in reaction-diffusion model and is involved in cell sorting phenomenon in mouse limb micromass culture. *Developmental Dynamics, 217*, 241–249. https://doi.org/10.1002/(SICI)1097-0177(200003)217:3<241::AID-DVDY2>3.0.CO;2-K

Miura, T., Shiota, K., Morriss-Kay, G., & Maini, P. K. (2006). Mixed-mode pattern in Doublefoot mutant mouse limb-turing reaction-diffusion model on a growing domain during limb development. *Journal of Theoretical Biology, 240*, 562–573.

Murray, J. D. (1982). Parameter space for Turing instability in reaction diffusion mechanisms: A comparison of models. *Journal of Theoretical Biology, 98*, 143–163. https://doi.org/10.1016/0022-5193(82)90063-7

Murray, J. D. (1993). *Mathematical biology*. Springer.

Nakamura, T., Gehrke, A. R., Lemberg, J., et al. (2016). Digits and fin rays share common developmental histories. *Nature, 537*, 225–228. https://doi.org/10.1038/nature19322

Nelson, C. E., Morgan, B. A., Burke, A. C., et al. (1996). Analysis of Hox gene expression in the chick limb bud. *Development, 122*, 1449–1466. https://doi.org/10.1038/342767a0

Newman, S. A. (2007). The Turing mechanism in vertebrate limb patterning. *Nature Reviews. Molecular Cell Biology, 8*, 1. https://doi.org/10.1038/nrm1830-c1

Newman, S. A., & Frisch, H. L. (1979). Dynamics of skeletal pattern formation in developing chick limb. *Science, 205*, 662–668.

Niswander, L., & Martin, G. R. (1992). Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development, 114*, 755–768.

Niswander, L., Tickle, C., Vogel, A., et al. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell, 75*, 579–587. https://doi.org/10.1016/0092-8674(93)90391-3

Ohuchi, H., Yoshioka, H., Tanaka, A., et al. (1994). Involvement of androgen-induced growth factor (FGF-8) gene in mouse embryogenesis and morphogenesis. *Biochemical and Biophysical Research Communications, 204*, 882–888. https://doi.org/10.1006/bbrc.1994.2542

Onimaru, K., Kuraku, S., Takagi, W., et al. (2015). A shift in anterior–posterior positional information underlies the fin-to-limb evolution. *eLife, 4*, e07048. https://doi.org/10.7554/eLife.07048.001

Onimaru, K., Marcon, L., Musy, M., et al. (2016). The fin-to-limb transition as the re-organization of a Turing pattern. *Nature Communications, 7*, 11582. https://doi.org/10.1038/ncomms11582

Onimaru, K., Nishimura, O., & Kuraku, S. (2018). A regulatory-sequence classifier with a neural network for genomic information processing. *bioRxiv*, 355974. https://doi.org/10.1101/355974

Othmer, H. G. (1986). On the Newman-Frisch model of limb chondrogenesis. *Journal of Theoretical Biology, 121*, 505–508. https://doi.org/10.1016/S0022-5193(86)80105-9

Owen, R. (1848). *On the archetype and homologies of the vertebrate skeleton*. John van Voorst.

Painter, K. J., Ho, W., & Headon, D. J. (2018). A chemotaxis model of feather primordia pattern formation during avian development. *Journal of Theoretical Biology, 437*, 225–238. https://doi.org/10.1016/j.jtbi.2017.10.026

Pecze, L. (2018). A solution to the problem of proper segment positioning in the course of digit formation. *Bio Systems, 173*, 266. https://doi.org/10.1016/j.biosystems.2018.04.005

Peters, H., Neubuser, A., Kratochwil, K., & Balling, R. (1998). Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes & Development, 49*, 2735–2747.

Quang, D., & Xie, X. (2016). DanQ: A hybrid convolutional and recurrent deep neural network for quantifying the function of DNA sequences. *Nucleic Acids Research, 44*, e107. https://doi.org/10.1093/nar/gkw226

Raspopovic, J., Marcon, L., Russo, L., & Sharpe, J. (2014). Digit patterning is controlled by a Bmp-Sox9-Wnt Turing network modulated by morphogen gradients. *Science, 345*, 566–570.

Riddle, R. D., Johnson, R. L., Laufer, E., & Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell, 75*, 1401–1416. https://doi.org/10.1016/0092-8674(93)90626-2

Roselló-Díez, A., Ros, M. A., & Torres, M. (2011). Diffusible signals, not autonomous mechanisms, determine the main proximodistal limb subdivision. *Science, 332*, 1086–1088. https://doi.org/10.1126/science.1199489

Salazar-Ciudad, I., Newman, S. A., & Solé, R. V. (2001). Phenotypic and dynamical transitions in model genetic networks I. Emergence of patterns and genotype-phenotype relationships. *Evolution & Development, 3*, 84–94. https://doi.org/10.1046/j.1525-142X.2001.003002084.x

Sanford, L. P., Ormsby, I., Groot, A. C., et al. (1997). TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. *Development, 124*, 2659–2670. https://doi.org/10.1086/597422.Tumor

Saunders, J. W. (1948). The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *The Journal of Experimental Zoology, 108*, 363–403. https://doi.org/10.1002/jez.1401080304

Saunders, J. W., & Gasseling, M. T. (1968). Ectodermal-mesenchymal interactions in the origin of limb symmetry. In *Epithelial mesenchymal interactions* (pp. 78–97). Springer.

Sheeba, C. J., Andrade, R. P., Duprez, D., & Palmeirim, I. (2010). Comprehensive analysis of fibroblast growth factor receptor expression patterns during chick forelimb development. *The International Journal of Developmental Biology, 54*, 1515–1524. https://doi.org/10.1387/ijdb.092887cs

Sheth, R., Bastida, M. F., & Ros, M. (2007). Hoxd and Gli3 interactions modulate digit number in the amniote limb. *Developmental Biology, 310*, 430–441. https://doi.org/10.1016/j.ydbio.2007.07.023

Sheth, R., Grégoire, D., Dumouchel, A., et al. (2013). Decoupling the function of Hox and Shh in developing limb reveals multiple inputs of Hox genes on limb growth. *Development, 140*, 2130–2138. https://doi.org/10.1242/dev.089409

Sheth, R., Marcon, L., Bastida, M. F., et al. (2012). Hox genes regulate digit patterning by controlling the wavelength of a Turing-type mechanism. *Science, 338*, 1476–1480. https://doi.org/10.1126/science.1226804

Shubin, N., Tabin, C., & Carroll, S. (1997). Fossils, genes and the evolution of animal limbs. *Nature, 388*, 639–648.

Shubin, N. H., & Alberch, P. (1986). A morphogenetic approach to the origin and basic organization of the tetrapod limb. *Evolutionary Biology, 20*, 318–390.

Small, K. M., & Potter, S. S. (1993). Homeotic transformations and limb defects in Hox A11 mutant mice. *Genes & Development, 7*, 2318–2328. https://doi.org/10.1101/gad.7.12a.2318

Sordino, P., van der Hoeven, F., & Duboule, D. (1995). Hox gene expression in teleost fins and the origin of vertebrate digits. *Nature, 375*, 678–681.

Summerbell, D., Lewis, J. H., & Wolpert, L. (1973). Positional Information in chick limb morphogenesis. *Nature, 244*, 492–496. https://doi.org/10.1038/244492a0

Szebenyi, G., Savage, M. P., Olwin, B. B., & Fallon, J. F. (1995). Changes in the expression of fibroblast growth factor receptors mark distinct stages of chondrogenesis in vitro and during chick limb skeletal patterning. *Developmental Dynamics, 204*, 446–456. https://doi.org/10.1002/aja.1002040410

Tabin, C., & Wolpert, L. (2007). Rethinking the proximodistal axis of the vertebrate limb in the molecular era. *Genes & Development, 21*, 1433–1442. https://doi.org/10.1101/gad.1547407

Tarchini, B., & Duboule, D. (2006). Control of Hoxd genes' collinearity during early limb development. *Developmental Cell, 10*, 93–103. https://doi.org/10.1016/j.devcel.2005.11.014

Te Welscher, P., Zuniga, A., Kuijper, S., et al. (2002). Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. *Science, 298*, 827–830. https://doi.org/10.1126/science.1075620

Tickle, C. (2006). Making digit patterns in the vertebrate limb. *Nature Reviews. Molecular Cell Biology, 7*, 45–53.

True, J. R., & Haag, E. S. (2001). Developmental system drift and flexibility in evolutionary trajectories. *Evolution & Development*. https://doi.org/10.1046/j.1525-142X.2001.003002109.x

Tulenko, F. J., Augustus, G. J., Massey, J. L., et al. (2016). HoxD expression in the fin-fold compartment of basal gnathostomes and implications for paired appendage evolution. *Scientific Reports, 6*, 22720. https://doi.org/10.1038/srep22720

Tulenko, F. J., Massey, J. L., Holmquist, E., et al. (2017). Fin-fold development in paddlefish and catshark and implications for the evolution of the autopod. *Proceedings of the Royal Society B: Biological Sciences, 284*, 20162780. https://doi.org/10.1098/rspb.2016.2780

Turing, A. M. (1952). The chemical basis of morphogenesis. *Philosophical Transactions of the Royal Society B, 237*, 37–72.

Uzkudun, M., Marcon, L., & Sharpe, J. (2015). Data-driven modelling of a gene regulatory network for cell fate decisions in the growing limb bud. *Molecular Systems Biology, 11*, 815. https://doi.org/10.15252/msb.20145882

Verd, B., Clark, E., Wotton, K. R., Janssens, H., Jiménez-Guri, E., Crombach, A., & Jaeger, J. (2018). A damped oscillator imposes temporal order on posterior gap gene expression in *Drosophila*. *PLoS Biology, 16*, e2003174. https://doi.org/10.1371/journal.pbio.2003174

Wagner, G. P. (2007). The developmental genetics of homology. *Nature Review Genetics*. https://doi.org/10.1038/nrg2099

Wagner, G. P. (2014). *Homology, genes and evolutionary innovation*. Princeton university press.

Wagner, G. P., & Larsson, H. C. E. (2007). Fins and limbs in the study of evolutionary novelties. In B. K. Hall (Ed.), *Fins into limbs: Evolution, development, and transformation* (pp. 49–61). The University of Chicago Press.

Wang, B., Fallon, J., & Beachy, P. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell, 100*, 423–434. https://doi.org/10.1016/S0092-8674(00)80678-9

Watson, D. M. S. (1913). On the primitive tetrapod limb. *Anatomischer Anzeiger, 44*, 24–27.

White, K. A. J., & Gilligan, C. A. (1998). Spatial heterogeneity in three-species, plant-parasite-hyperparasite, systems. *Philosophical Transactions of the Royal Society B: Biological Sciences, 353*, 543–557.

Wilby, O. K., & Ede, D. A. (1975). A model generating the pattern of cartilage skeletal elements in the embryonic chick limb. *Journal of Theoretical Biology, 52*, 199–217. https://doi.org/10.1016/0022-5193(75)90051-X

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *Journal of Theoretical Biology, 25*, 1–47.

Zhou, J., & Troyanskaya, O. G. (2015). Predicting effects of noncoding variants with deep learning–based sequence model. *Nature Methods, 12*, 931–934. https://doi.org/10.1038/nmeth.3547

Zhu, J., Zhang, Y. T., Alber, M. S., & Newman, S. A. (2010). Bare bones pattern formation: A core regulatory network in varying geometries reproduces major features of vertebrate limb development and evolution. *PLoS One, 5*, e10892.

Experimental Evolution to Understand the Interplay Between Genetics and Adaptation

Jana Helsen and Rob Jelier

Abstract Experimental evolution has grown to be a powerful and versatile tool to study the evolutionary dynamics of genetics. The principle is simple: cells are grown under a specific selective pressure, mutations arise over time, and lineages carrying mutations that increase fitness can outcompete others. Here we discuss how experimental evolution has allowed us to study various evolutionary processes, from clonal interference to diminishing-returns epistasis and genetic hitchhiking. Next, we discuss how experimental evolution can reveal how an organism's genotype affects evolutionary processes, how adaptation can sometimes fix even the most severe fitness defects, and how the approach can be used to learn more about the genetic architecture of complex traits. Finally, we look ahead at how experimental evolution can be used to study genetic networks and, conversely, how the structure of such networks influences evolution.

1 Understanding Adaptation by Experimental Evolution

Adaptation is the process by which organisms adjust to their environment and improve their chance of producing fecund offspring. During adaptation, mutations that increase fitness can get selected and fix themselves in the evolving population, a process that is fundamental in shaping the genetic architecture of complex traits. The

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_6

historical view of adaptation starts with Darwin himself, and was one of gradualism, where adaptation takes place through many small mutations of minor effect. Fisher consolidated this view in 1930 in the form of a coherent but simplified model: the geometric model of adaptation (Fisher, 1930). In this model, Fisher argued that the probability that a random mutation has a beneficial effect drops quickly when the effect size of the mutation increases, according to $1-\phi(x)$, with x proportional to the effect size and ϕ the cumulative distribution function of a normal distribution. He concluded that small-effect mutations would dominate adaptation. Kimura pointed out in the 1960s that fitness effects that were too small would not spread fast enough to fix efficiently in a population, pointing to the importance of intermediate-effect mutations (Kimura, 1964; Kimura et al., 1968). While these models have proven to be very useful, they were in fact based on indirect observations. This changed in the 1980s, when experimental evolution gained traction and allowed us to directly study and observe the dynamics of adaptation. Since then, the method has yielded many important insights into how adaptation proceeds and what a theory of adaptation should represent (Orr, 2005). Below we will concisely review seven of the most crucial findings that have been made using experimental evolution.

First, adaptation to a new environment or challenge is typically swift, with a rapid initial fitness increase that depends on a few large-effect mutations (Holder & Bull, 2001). For example, Lenski's famous Long Term Evolution Experiment (LTEE) (see Box 1) showed a power-law adaptation curve across 12 populations. Fitness improved rapidly over the first ~1000 generations, caused by few mutations of large effect, after which a phase of ever slower adaptation started with mutations showing decreasing fitness benefit (Wiser et al., 2013). The rate of adaptation, quantified by the increase in fitness over time, therefore depends on how far adaptation has proceeded and is reflected by the current fitness in the given condition. Three factors contribute to explain this phenomenon: (a) the overall distribution of fitness effects of mutations (see second point below); (b) the mutations that are present at any given time in a population (in terms of both quantity and quality), which in turn depends on population size and mutation rate (see third and seventh points); and (c) diminishing-returns epistasis, the phenomenon whereby beneficial mutations have smaller fitness effects in fitter backgrounds (see fourth point). The potential for rapid adaptation is quite general and has been observed in experimental evolution studies across many conditions, species, and genetic backgrounds (Liu et al., 2015; Rainey & Travisano, 1998; Rancati et al., 2008; Szamecz et al., 2014).

Box 1 The Long Term Evolution Experiment

In the Long Term Evolution Experiment, 12 evolving *E. coli* populations were started from the same ancestral strain in 1988 and have been continuously propagated to this day. The populations grow in a minimal medium (DM25)

(continued)

Box 1 (continued)

with glucose as the limiting factor and are passed to fresh medium with a 100-fold dilution every day. Periodically, population samples are frozen, so they can be examined in detail at a later time. As of 2020, the experiment has run for more than 73,000 generations. The experiment is remarkable for its duration but also for the many phenomena observed and studied as the evolutionary process unfolded in the populations (reviewed in Lenski, 2017). For example, the study has demonstrated how the pace of adaptation changes over time, as well as the repeatability of adaptation between populations and the competition between beneficial mutations in a population (clonal interference). More surprising was the observation that ecological interaction took place between stably co-existing strains. Two distinct lineages arose in 1 population and co-existed for over 50,000 generations. One strain specialized in rapid growth on glucose, beneficial after transfer to fresh medium, whereas the other specialized in fast growth on acetate, the product of the former's metabolism and beneficial as glucose supply is limiting. Both strains stably co-existed in a cross-feeding relationship driven by the particular growth conditions during the experiment (Le Gac et al., 2012).

Second, though there are many mutations that provide a positive fitness effect, variants of large effect are rare. A crucial part of understanding how adaptation proceeds comes from examining the distribution of fitness effects of genetic variants (reviewed in Eyre-Walker & Keightley, 2007). Experimental evolution makes it possible for a direct measurement of the distribution of such fitness effects. By following how fast variants rise in frequency in a population, we can estimate their relative fitness as well. Sequencing populations allows to follow variants as they rise and fall during selection (Lang et al., 2013; Voordeckers et al., 2015), but at limited resolution. To overcome this limitation, Levy et al. created a barcoded budding yeast library, where each lineage contained 1 of ~500,000 random DNA barcodes (Levy et al., 2015). Using this system, the evolutionary dynamics could be followed in much higher detail (see Box 2). By measuring the fitness of many lineages using this method, though limited to a single genotype and condition, it was shown that large-effect variants are rare, but many variants provide a modest fitness advantage. About 0.04% of the genome's bases (~5000 bases) confer a larger than 5% fitness benefit when mutated. It is clear, however, that the distribution of fitness effects heavily depends on the condition and current fitness of the strain, as strains closer to the optimum growth rate will by necessity have fewer options for rapid fitness increase.

Box 2 Focus on Adaptation Dynamics with Barcoding

Due to cost considerations, whole genome sequencing of populations is normally limited in sequencing depth, i.e., the number of reads that cover a genomic position. This means that the detail and resolution for tracking lineages as they rise and fall in a population are limited, especially for new lineages when their frequency is still low. One trick to improve on this limitation is to introduce a highly variable but short barcode into the population, which can then be sequenced at very high coverage. In the paper that introduced this approach, a random 20-nucleotide barcode was synthesized as random primers and then ligated into a plasmid backbone (Levy et al., 2015). The plasmid library was subsequently transformed into yeast cells, and a Cre-loxP recombination system was used to insert the barcodes into an engineered genome location (Levy et al., 2015). The method allowed the researchers to follow lineages arising and competing in a population in unprecedented detail.

Third, clonal interference is the norm in large asexually reproducing populations. In a more or less typical microbial experimental evolution setting, the mutation rate will be on the order of $\sim 10^{-10}$ per base per generation (Zhu et al., 2014), effective population sizes can be in the order of $\sim 10^{7}$, and (for example) the baker's yeast genome has about $\sim 10^{7}$ bases, so we can expect $\sim 10^{4}$ new mutations to arise every generation within the population, of which a handful (say $\sim 0.04\%$; see point 2) should be beneficial. Many of these mutations will be lost by chance before they can expand their occurrence rate in the population, but we can expect several beneficial mutations to co-exist and rise in frequency at the same time and so cause competition between alleles. The term clonal interference refers to the effects of this competition, in that some beneficial mutations do not fix, or fix more slowly, because other fit alleles are expanding in the population at the same time. In the absence of recombination by sexual reproduction, only one of the concurrent beneficial alleles can fix, and this will slow down adaptation (Gerrish & Lenski, 1998; Good et al., 2012). This process will also ensure that only large-effect variants can fix early in adaptation. Further, the stochastic nature of the mutation rate as well as the chance that additional beneficial mutations are acquired in an expanding lineage can give rise to complex patterns of clonal expansion and shrinkage (Lang et al., 2013).

A fourth defining feature of adaptation is epistasis. This is the phenomenon whereby the effect of one mutation depends on the presence or absence of other mutations. One particular recurring observation during adaptation is that a beneficial allele will tend to cause less fitness gain when introduced into a strain that is fitter than when introduced into a less fit strain (Chou et al., 2011; Khan et al., 2011; Kryazhimskiy et al., 2014, 2009; MacLean et al., 2010; Moore et al., 2000; Wünsche et al., 2017). This is called diminishing-returns epistasis and can also be described as a generalized negative epistasis between beneficial mutations (i.e.,

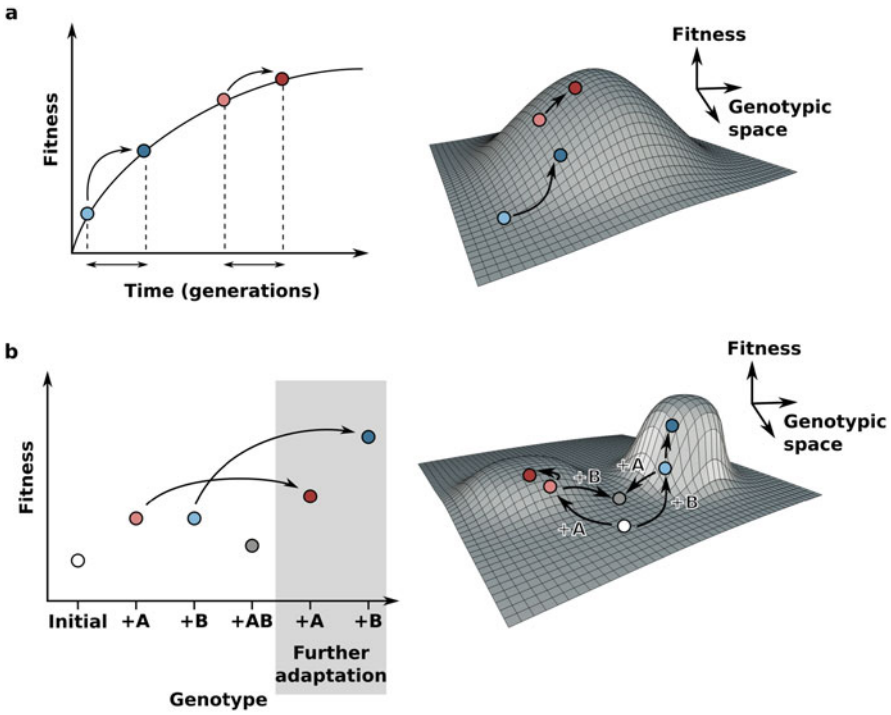

Fig. 1 Fitness landscapes and epistasis. (**a**) Diminishing-returns epistasis. The beneficial effect of a mutation depends on the stage of adaptation of the strain. The same beneficial mutation tends to cause smaller fitness gains when introduced in a fitter strain than in a less fit strain. (**b**) Reciprocal (negative) sign epistasis. This causes a rugged fitness landscape and implies that the order in which beneficial mutations occur in a lineage is important. In this hypothetical example, mutations A and B are equally beneficial when introduced into the initial unfit strain. However, due to negative epistasis between the mutations, their combined effect is worse than their individual effects. As a result, strains that first acquired mutation A will never acquire mutation B and vice versa. Even though they initially have the same beneficial effect, the two mutations direct strains toward different peaks within the fitness landscape. In the illustrated example, strains that first acquired mutation B have the potential to achieve higher fitness values after further adaptation than strains that first acquired mutation A

when mutations occur together, their combined effect is worse than what would be predicted by the sum of their individual effects) (see Fig. 1a). One implication of this is that the order in which beneficial mutations occur in a lineage is important. For example, one particular beneficial mutation that causes a strain to grow twice as fast during early adaptation can have only a marginal beneficial effect on fitness during the later stages of adaptation. At that point, the strain will have already acquired a whole other set of beneficial mutations, and negative epistasis between these mutations and the additional beneficial mutation will result in the new mutation having a smaller effect on growth. This implies that the mutation has a much higher chance of being fixed during early adaptation compared to later on in the

evolutionary process. A second observation is that beneficial mutations can show strong sign epistasis when combined. Sign epistasis between mutations occurs when the presence of one mutation (A) causes another beneficial mutation (B) to become deleterious, such that either $f(B) > f(-), f(B, A) < f(A)$ (where $f(B)$ represents fitness in the presence of B, negative epistasis shown here) or, the other way around, $f(B) < f(-), f(B, A) > f(A)$ (positive epistasis). In the context of adaptation, reciprocal sign epistasis is of particular interest, as it can lead to a complex multi-peaked fitness landscape (Poelwijk et al., 2011). In this special case of sign epistasis, the effect of both mutations is reversed when they are combined: $f(A) < f(-), f(B) < f(-), f(B, A) > max(f(B), f(A))$. If two beneficial mutations show reciprocal sign epistasis, the lower fitness combination is unlikely to remain in the population. The available mutational routes toward higher fitness will become conditional on existing mutations and hence constrained (see Box 3 and Fig. 1b). Reciprocal negative sign epistasis has been observed and studied in several experimental evolution experiments (Chiotti et al., 2014; Kvitek and Sherlock, 2011) and was even found to be common among initial adaptive mutations in a biosynthetic pathway (Ono et al., 2017). In In their large-scale study, Szamecz et al. (2014) found two mutations that decreased fitness when they occurred on their own: deleting MDM34 and mutating MGA2. However, the combination of the two mutations resulted in a higher fitness than either of the single mutations, an example of reciprocal positive sign epistasis. Indeed, mutations in MGA2 were selected during evolution in mdm34Δ strains (Szamecz et al., 2014). Rojas Echenique et al. aimed to investigate the effect of epistasis on a larger scale (Rojas Echenique et al., 2019). After evolving a set of gene deletion mutants, they tried to reintroduce the originally deleted gene in the evolved strains. For over half of these strains, they did not succeed in reverting the deletion, possibly due to strong epistasis between the deletion and the acquired compensatory mutations. In other words, the compensatory mutations showed reciprocal positive sign epistasis and were strongly detrimental in the absence of the gene deletion.

Box 3 Reciprocal Sign Epistasis and Fitness Landscapes

A useful concept to get an intuitive grasp of epistasis and its role in evolution is the evolutionary fitness landscape (see schematic example in Fig. 1) (Wright, 1932, 1988). In an adaptive fitness landscape, each coordinate represents a point in the genotypic space, and the value on the z-axis indicates the corresponding fitness of the genotype. In this way, evolution can be visualized as an exploration of the landscape, where selection favors genotypes that climb a fitness peak. Epistasis has a large effect on determining the shape of the landscape. Reciprocal sign epistasis, where the effect of

(continued)

Box 3 (continued)

individual mutations is reversed when combined, is the reason why fitness landscapes can show not just one single peak but multiple local peaks, what is called "ruggedness" (Poelwijk et al., 2007, 2011). This ruggedness has been observed in studies using experimental evolution. For example, Kvitek and Sherlock recurrently observed mutations in two loci, MTH1 and HXT6/HXT7, in yeast populations evolving under glucose limitation (Kvitek & Sherlock, 2011). Individually, these mutations are both beneficial for fitness. However, the double mutant has a fitness that is lower than even that of the wild-type strain. As a result, strains that carry the MTH1 mutation are unlikely to achieve the slightly higher fitness peak of those with mutations in HXT6/HXT7. In fitness landscape parlance, the two peaks are separated by a deep fitness valley, with a mutational path unlikely to be taken in the face of natural selection.

Fifth, parallel adaptive evolution can be common (Anderson et al., 2003; Riehle et al., 2001). Identical mutations or mutations within the same pathway occur much more often during experimental evolution than would be expected by chance. For example, in the adaptation of a cell polarization mutant, Laan et al. found the same gene to be inactivated in all ten replicate adapting populations (Laan et al., 2015). One factor, as noted above, is that large-effect mutations tend to be rare and may be efficiently selected for in a large enough population. Another good example of studies where parallel trajectories are often observed is in those that studied the development of antibiotic resistance in *E. coli*. The parallelism of evolved antibiotic resistance was found to be high enough at the level of mutated molecular pathways so that simple linear models could be trained to successfully predict the level of antibiotic resistance, using the gene expression of only a few genes (Suzuki et al., 2014). Other large-scale studies found more modest but still elevated rates of parallel mutation (Rojas Echenique et al., 2019; Szamecz et al., 2014), potentially indicating less rare large-effect mutations being available. A possible explanation is the relatively high fitness of the adapting strains. When the growth speed approximates optimality, there is limited potential for a large fitness increase with a single mutation (see also fourth point above). Sign epistasis can be another contributing factor. If strong epistasis is present between beneficial variants, it can increase diversity between adaptation experiments, as the mutational trajectory becomes dependent on which epistatic mutation arises first in the lineage.

Sixth, hitchhiking mutations are common and highlight the benefit of sex. In asexual populations, all variants in an individual's genome are passed on to its offspring, and their frequency will rise and fall together in the population according to the individual's fitness. This implies that a variant showing a rapid fixation during adaptation may not be beneficial but is merely hitchhiking with the fixation of a beneficial variant elsewhere in the genome. This complicates analyses of

experimental evolution studies, as the fitness effects of fixed mutations need to be confirmed experimentally (Lang et al., 2013; Voordeckers et al., 2015). One way to get around this is to observe patterns of parallel adaptive evolution, as a hitchhiking mutant should only occur at background rates in independent experiments. The phenomena of hitchhiking and clonal interference differ between asexually and sexually reproducing populations. As budding yeast can grow both asexually and sexually, it can be used to make a direct comparison of adaptation in both scenarios. For example, McDonald et al. compared the adaptation rate between asexually growing populations and those that were forced to reproduce solely by sexual reproduction (McDonald et al., 2016). They found that sex can indeed allow for faster adaptation, as predicted by theoretical work. It allows beneficial mutations to recombine, which in turn avoids clonal interference, and allows beneficial mutations to dissociate from neutral or mildly detrimental hitchhiking mutations (McDonald et al., 2016).

The seventh and last point is that adaptation can proceed rapidly through relatively crude alterations that only have positive effects within a narrow range of conditions. One such alteration that is often observed during experimental evolution is loss of protein function (Kvitek and Sherlock, 2013; Laan et al., 2015; Szamecz et al., 2014). This is counterintuitive given that genes have to be subject to positive selection to be maintained in a genome at all. Adaptation by gene loss does occur in nature (see, e.g., D'Souza et al., 2014), but it may be observed more frequently in experimental evolution due to the typically controlled and predictable conditions. Indeed, loss-of-function mutations tend to come with negative fitness consequences in conditions other than the controlled environment under which experimental evolution proceeds (Kvitek and Sherlock, 2013; Szamecz et al., 2014). For example, in a study to better understand how fitness increased during adaptation using *S. cerevisiae*, mutations were found to be beneficial in only a narrow range of conditions (Li et al., 2018). During the experiment, strains were serially transferred, and the population went through a lag, fermentation, and finally a limited respiration phase after every transfer. The adapted strains had improved respiration and a shortened lag phase, which improved fitness in the experimental conditions. However, there was a trade-off with survivability, which dramatically limited the conditions in which the adaptations were beneficial (Li et al., 2018). Trade-offs are also of crucial importance when strains evolve in fluctuating environments, i.e., where the selecting environment fluctuates over time. Since mutations that enable a strain to grow faster in one environment quite often come with trade-offs for growth in other environments, the extent and speed of evolution when evolving in such conditions can be severely constrained. A related phenomenon is the cost of generalism, where generalists (organisms that are fit in a broad niche) usually have a lower fitness under specific constant conditions than specialists (organisms that are fit in a small niche) (Bono et al., 2020). Next to loss of gene function, another common crude adaptation route is aneuploidy (Rancati et al., 2008; Sunshine et al., 2015). The adaptive aneuploidies tend to have big fitness effects, but only strongly affect the transcription of relatively few genes (Sunshine et al., 2015). As with the loss-of-function mutations, the fitness effects are mostly

condition-specific (Sunshine et al., 2015). Both aneuploidies and loss-of-function mutations occur de novo relatively frequently when compared to gain-of-function mutations or promoter mutations that specifically alter gene expression responses. Combined with their large, but specific, fitness effects, this may explain their frequent appearance during experimental evolution.

2 Experimental Evolution to Study Genes and Traits

Experimental evolution can be used to study complex traits and to also improve our understanding of how the genotype of an organism affects evolutionary processes. In classical experimental evolution experiments such as the Long Term Evolution Experiment, genetic variation arises spontaneously by mutation. However, to be able to systematically explore the interplay between genotype and evolution, genetic variation can also be explicitly introduced at the start of the experiment (Fig. 2). By crossing and backcrossing divergent strains, we can acquire a population with a controlled set of genetic variants. After growing such populations under selection, the frequency of the variants can then be correlated with fitness. Alternatively, different offspring from such crosses can be evolved separately, to explore how their differences in genotype influence adaptation. Another way of inducing genetic variation is to use recombinant DNA technology. This allows us to, for example, evaluate the potential for adaptation under near-fatal gene perturbations. Further, we can study whether and how a specific mutation in an adapting strain can constrain the mutational trajectory during adaptation. A constrained (i.e., parallel) trajectory in a strain that suffered a deleterious gene perturbation could inform us on which cellular processes can alleviate the stress experienced by the cell during adaptation. We will expand upon these two techniques to induce genetic variation and the most important insights they have provided in the three sections below.

2.1 Evolving the Progeny of Crosses to Understand the Genetic Structure of Complex Traits

Experimental evolution can be used to identify beneficial variants from standing genetic variation. This can be effectively employed to explain the genetic origins of phenotypic differences between individuals. By repeatedly crossing two strains, a synthetic population can be made in which the alleles of the strains segregate in different combinations. Imposing a selective pressure can then increase the relative frequency of beneficial variants (see Fig. 3). For this approach to work, the population can even be grown for only a handful of generations, just long enough to see that the differences in fitness between lineages are reflected in occurrence frequency in the population. Parts et al. studied the genetics underlying

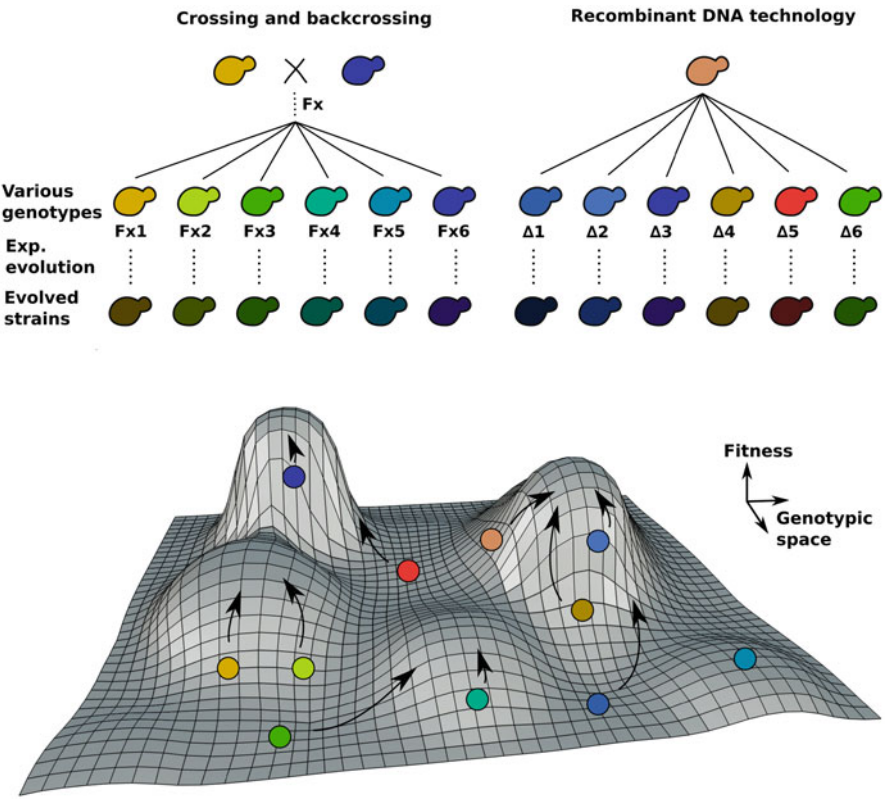

Fig. 2 Evolving various genotypes to explore the link between genotype and evolution. Variation in genotype can be generated in a controlled manner by crossing and backcrossing divergent strains or by using recombinant DNA technology to, for example, systematically knock down genes. By next evolving the diverse array of genotypes, we can measure the effect of genetic variation on the speed of adaptation and observe how adaptation proceeds when starting from different parts in the fitness landscape

heat sensitivity in *S. cerevisiae* (Parts et al., 2011). They crossed a heat-tolerant North American oak tree bark strain and a heat-sensitive West African palm wine strain and generated between 10 and 100 million random segregants. They then grew a pool of these segregants at normal and high temperatures for 12 days, to enrich for lineages with beneficial alleles. After sequencing the enriched pools, 21 genomic regions were enriched in the population. They found that the resolution of the regions and their number compared favorably to standard genomic linkage studies. However, this method is limited to selecting for haplotypes that arose during the original crosses, which is only a small fraction of all possible combinations of segregating alleles in the population. Burke et al. (2014) started from a synthetic population with genetic variants stemming from a four-way cross of diploid budding yeast strains from different geographic origins (Burke et al., 2014). They switched

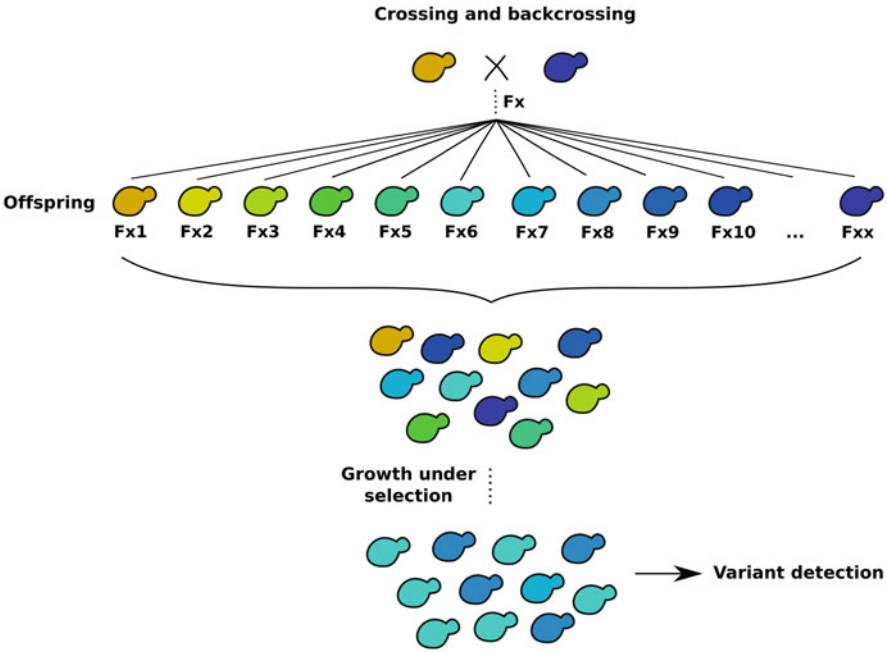

Fig. 3 Growing the offspring of crosses under selection to understand complex traits. By repeatedly crossing two strains, a synthetic population can be made in which the alleles of the strains segregate in different combinations. Imposing a selective pressure can then increase the relative frequency of beneficial variants

between mitotic and sexual propagation to break the linkage between alleles and evolved for over 500 generations with 18 rounds of recombination. This regime enriches the most beneficial alleles efficiently, causing a convergence between independently evolving populations. In theory, this should be a more powerful and better resolved way to identify the functional variants that contribute additively to the phenotypic differences between the original strains.

The genetic variability in the offspring of a cross can also be used in combination with experimental evolution to study the genetic basis of adaptability itself. Jerison et al. independently evolved 230 individuals descended from a cross between lab and wine yeast strains for 500 generations (see Fig. 2; here they evolve individual offspring, not the population) (Jerison et al., 2017). The aim of the study was to use quantitative genetic tools to identify the genetic basis of adaptability, defined as the speed at which a strain can improve its fitness during evolution. In line with previous work, the authors confirmed that adaptability is highly heritable. A large part of the differences in heritability between strains could be explained by the loci that affect the initial fitness of the strain and indeed the fitness of the founder strains. Strains that are less fit initially will improve their fitness more quickly during evolution, which can be explained by the phenomenon of diminishing-returns

epistasis as discussed above (Sect. 1, fourth point). In addition to this, a few genomic regions were found to be specifically associated with adaptability, beyond the effect of founder growth rate (Jerison et al., 2017). The locus with the strongest signal was explored in more detail. The wine strain allele of a gene within this locus, KRE33, is involved in the biogenesis of the small ribosomal subunit and was detrimental to growth in the experimental conditions. However, further mutations in the same gene or functionally related genes could compensate for the allele's effect to improve fitness relatively quickly, and the locus was hence associated with increased adaptability.

2.2 Gene Essentiality and Evolvability

In rich medium, a large portion of yeast genes can be removed without having a big effect on fitness. In fact, the deletion of some genes can actually increase fitness or decrease fitness with a wide range of effect sizes (Giaever et al., 2002). Essential genes are traditionally defined as being critical for survival irrespective of the growth condition. However, the notion of gene essentiality has been found to be dependent on genotype. For example, many synthetic lethal interactions were found in a screen of millions of double gene knockouts in baker's yeast. Costanzo et al. identified around 3300 "conditionally essential" genes, about half of the total number of genes, that are essential when they occur in a particular deletion background (Costanzo et al., 2010, 2016). Further, Dowell et al. compared gene essentiality between two commonly used *S. cerevisiae* lab strains (Dowell et al., 2010). They found that both strains contain genes that are essential in one strain but not in the other. They concluded that the essentiality of some genes depends entirely on the genetic background in which they occur (Dowell et al., 2010).

Experimental evolution has contributed to changing how the concept of essentiality is viewed. Liu et al. were able to recover viable deletion strains by using adaptive evolution for around 9% of the yeast genes that were originally tagged as "essential" (Liu et al., 2015). They sporulated heterologous diploid strains, in which one copy of the essential gene was knocked out, and screened a large number of spores. If a strain could yield viable offspring—even if only a diminutive fraction—and contained the gene deletion, that strain would be included in an evolution experiment. The authors showed that these strains could improve their growth rates substantially by adaptation. One perspective on this finding is that there is a problem with the definition of essential genes, and indeed the yeast deletion collection has technical limitations, and phenotypic annotations should be interpreted with caution (Giaever and Nislow, 2014). However, a more striking insight is that even some of the most serious growth defects can be overcome, or suppressed, by acquiring compensatory mutations.

2.3 Compensatory Trajectories After Gene Loss

One way of exploring the effect of genotype on evolution is to evolve strains that only differ in the deletion of one particular gene in parallel and then compare how these strains adapt. Adaptation may be specific to the deleted gene or general for the condition and follow a single or various adaptive routes. Further, the way the strain adapts may reveal what has been corrected at a molecular level. If deleting the gene results in a growth deficit, one could expect that only a specific set of mutations is able to reverse the strain's fitness loss.

Various small-scale experiments have shown that gene deletion strains can have a characteristic mutational trajectory and a straightforward way of adapting (see also the fifth point in Sect. 1 about parallel evolution). Wild-type *Saccharomyces cerevisiae* cells repeatedly amplify the sulfate transporter SUL1 when they are evolved in sulfate-limiting conditions (Gresham et al., 2008) (introducing aneuploidies; see also the seventh point in Sect. 1). Strains lacking the SUL1 transporter predictably duplicate the paralogous transporter SUL2. Similarly, variants elsewhere in the genome can change which mutation has the largest beneficial effect, as *Saccharomyces uvarum*, a close relative of *S. cerevisiae*, amplifies SUL2 when it is evolved under the same sulfate-limiting conditions (Sanchez et al., 2017). In other cases, however, adaptation can be complex. Deleting MYO1, the only myosin II gene in budding yeast, results in cells that are unable to divide properly (Watts et al., 1987). After serially passaging these myo1Δ yeast populations for around 400 generations, the majority of them can adapt and significantly restore cytokinesis even though the evolved strains are still less fit than their ancestor wild type (Rancati et al., 2008). In this case, though despite evidence of parallel evolution at the level of chromosomal aneuploidies, the populations show a variety of morphologies, mutational and phenotypic profiles, indicating they have followed diverse compensatory trajectories. At least three modes were identified to restore cytokinesis, two of which by mechanisms distinct from the original damaged system.

Studies where a larger number of deletion strains are evolved allow for a general study into the phenomenon of adaptation after gene loss. Teng et al. cleverly made use of the fact that the yeast deletion collection has already undergone several rounds of selection and as such carries compensatory mutations (Teng et al., 2013). They first screened the haploid deletion collection for phenotypic heterogeneity within individual deletion strains, under the assumption that heterogeneous populations contain clones with unfixed secondary mutations. After this, they dissected the mutations that were responsible for this variation and concluded that the gene that was originally deleted is indeed a key predictor for the secondary mutations in a strain. Szamecz et al. performed a more controlled wide-scale study by evolving a large set of deletion mutants in the lab (Szamecz et al., 2014). They generated 180 haploid yeast gene deletion strains with growth defects on rich medium and evolved them in quadruplicate in this medium for around 400 generations; 65% of the strains recovered $\geq$50% of the fitness lost due to the gene deletion (Szamecz et al., 2014).

They sequenced a subset of evolved clones and observed that the mutations that were found after evolution tended to be specific to the function of the deleted gene. However, replicate lineages of the same deletion strain did not generally acquire mutations within the same genes. Rojas Echenique et al. selected a set of 37 genes with functions across diverse functional modules and evolved deletion mutants of these genes on rich medium for about 500 generations (Rojas Echenique et al., 2019). In this case, the majority of compensatory mutations seemed to be nonspecific to the original genotype, even though a small but significant correlation pattern was observed between the functional module of the deleted gene and the functional module of the mutated genes.

In a recent and elegant study involving *Escherichia coli*, McCloskey et al. knocked out five different core metabolic genes and evolved the resulting strains in quintuplicate on glucose minimal medium (McCloskey et al., 2018). By characterizing the mutations that were acquired during evolution, as well as measuring gene expression levels, intracellular metabolite levels, and metabolic fluxes, they showed that each deletion strain followed specific adaptive paths to correct their growth-limiting molecular phenotypes. For example, cells lacking a functional succinate dehydrogenase, which converts succinate into fumarate in the TCA cycle, specifically acquired mutations to reduce the flux through the TCA cycle (McCloskey et al., 2018). In contrast, strains without phosphoglucose isomerase, the catalyst of the second step in glycolysis, experienced increased redox and sugar phosphate stress before evolution. During experimental evolution, cells reproducibly follow mutational paths that result in a reduced flux in those pathways (McCloskey et al., 2018).

In conclusion, a strain lacking a specific gene tends to evolve in a genotype-specific way, though this can still be quite variable. If a clear mutational trajectory can be identified, the adaptations can provide insight into the processes perturbed by the gene deletion.

3 Outlook: Using Experimental Evolution for Systems Genetics

As introduced in the previous section, the genetic background of an evolving organism can influence the available mutational trajectories. One way of looking at this is to think of the fitness landscape (see Box 3 and Fig. 2) as being shaped by the genotype, which determines through epistatic interactions which variants will be most adaptive. Obviously genes are part of an integrated system and interact with each other at different scales; locally at the level of protein interactions or gene transcription and globally as cellular processes that jointly define organismal properties such as fitness. The solutions found by evolution will always be successful trade-offs given the genotype and environment and will reflect how different parts of the cellular machinery work together. This implies that adaptation experiments have the potential to give us a better general understanding of cellular processes. Below, we

will discuss early results and perspectives on learning about genetic networks from experimental evolution experiments.

As mentioned above, recent large-scale assays have systematically mapped genetic interactions between gene deletions. This has resulted in a detailed map of the genetic wiring within a cell (Costanzo et al., 2010, 2016). When we look at the average degree of connectivity in this genetic network, the majority of genes has only interactions with few genes, whereas a small fraction has a very large number of interactions. The genes with the highest number of interactions, the so-called network hubs, usually perform central cellular functions, and many of them are essential (Jeong et al., 2001). As the perturbation of a network hub tends to have large fitness effects (Batada et al., 2006), and given that genes with more interaction partners tend to show a slower rate of evolution (Alvarez-Ponce et al., 2017; Fraser et al., 2002; Krylov et al., 2003), it was thought that changes during evolution primarily occur away from hubs and at the periphery of the network (Kim et al., 2007). Through experimental evolution, it has been possible to test the effect of perturbing a hub gene on adaptation. For example, Koubkova-Yu et al. investigated the role in evolution of Hsp90, a molecular chaperone and a well-known network hub (Koubkova-Yu et al., 2018). They replaced the native *S. cerevisiae* HSP90 with a homolog from *Yarrowia lipolytica* and used experimental evolution to allow the strain to adapt. They showed that perturbing a hub does not preclude adaptation: instead, the strains evolved in a remarkably diverse manner by acquiring mutations in different parts of the genetic network. If we project this situation onto a fitness landscape, then we can say that the perturbation of the hub gene brought the strain into a deep fitness valley from which many distinct evolutionary paths could provide a way out (Helsen et al., 2019).

Further research is needed to test if the finding is generalizable and holds for other highly interacting genes. Specifically, is it just the number of genetic interactions that is important, or is it actually the function of the protein which determines how adaptation proceeds? Does it matter in evolution if a perturbed protein, causing a drop in fitness, is a transcription factor, a chromatin factor, a core metabolic enzyme, or a structural protein? Further, accepting that knocking out a hub gene does not inhibit adaptation, can we identify parts in the network where perturbations are poorly compensated? For example, if heritability itself is affected by a perturbation, does that affect adaptability?

Another feature of genetic and physical interaction networks is that they have a clustered structure, where clusters are groups of genes with a high density of interactions. Such clusters tend to overlap with the so-called functional modules, groups of genes that work together to perform a cellular function and whose activity is (partly) self-regulated, such as metabolic pathways or protein complexes (Tong et al., 2004). An interesting question is how this modular structure comes into play when organisms evolve. For example, earlier we discussed how generalized diminishing-returns epistasis slows down adaptation as fitness increases (fourth point of Sect. 1). The deeper cause of this widespread phenomenon is not really understood. Clearly, growth speed can increase only up to a point, for example, because basic physical processes, such as diffusion, limit the maximal speed

of chemical reactions. It is not straightforward, however, to link such ideas to actual biological gene networks. One framework for this is the model of "modular epistasis" (Silander et al., 2007; Tenaillon et al., 2012; Wei and Zhang, 2019), which asserts that the fitness of an individual depends on the action of several functional modules. Mutations can improve fitness by improving the functioning of the modules, but only until they reach a state close to their optimal performance. Experimental support for this model is limited. The previously mentioned studies by Szamecz et al. and Rojas Echenique et al. offer a first clue to the role of modular network structure in adaptation (Rojas Echenique et al., 2019; Szamecz et al., 2014). They both show that strains with deletions in the same functional module tend to evolve in a more similar way than strains lacking genes from other modules. Still, most of the acquired mutations appear not specific to the deleted gene, and only a limited amount of parallel evolution is observed. In retrospect, these studies had limited power because of their generic setup and limited repeats but also because there was little follow-up on leads to identify exactly what was corrected during adaptation. Nonetheless, these types of experiments can reveal the molecular phenotypes that govern a complex trait and show which functional modules interact and how they interact.

4 Conclusions

Experimental evolution is a versatile method that can be applied to answer many biological questions, and its applications extend beyond what we have discussed here. For example, from an ecological point of view, the method can be used to explore how diverse strains can stably co-exist and how this phenomenon can be emulated and controlled. Experimental evolution is now also being applied in multicellular organisms, such as fruit flies and nematodes, which bring their own set of research questions. The method is also frequently being used now in biotechnology, to optimize strains for the production of industrially relevant compounds such as ethanol and to evolve specific proteins for desired properties, such as brighter and more stable fluorescent protein tags.

In this chapter, we discussed how experimental evolution has allowed us to directly study many factors related to the process of adaptation, which has aided in firmly establishing phenomena such as clonal interference and diminishing-returns epistasis. The approach has also shed light on how an organism's genotype influences adaptation. Additionally, it has proved a useful tool in finding the genetic basis of complex traits. The crucial power of the method lies in the fact that it can efficiently select the most beneficial genetic changes out of millions of variants. Even though the method also comes with a number of caveats, such as the fact that adaptation using experimental evolution can be highly specific to the chosen growth regime, with important trade-offs to growth in other conditions, the method holds considerable potential to ask system-level questions about complex traits and genetic architecture.

References

Alvarez-Ponce, D., Feyertag, F., & Chakraborty, S. (2017). Position matters: Network centrality considerably impacts rates of protein evolution in the human protein-protein interaction network. *Genome Biology and Evolution, 9*, 1742–1756. https://doi.org/10.1093/gbe/evx117

Anderson, J. B., Sirjusingh, C., Parsons, A. B., Boone, C., Wickens, C., Cowen, L.E., & Kohn, L.M. (2003). Mode of selection and experimental evolution of antifungal drug resistance in *Saccharomyces cerevisiae*. *Genetics, 163*, 1287–1298.

Batada, N. N., Hurst, L. D., & Tyers, M. (2006). Evolutionary and physiological importance of hub proteins. *PLoS Computational Biology, 2*, e88 (2006). https://doi.org/10.1371/journal.pcbi.0020088

Bono, L. M., Draghi, J. A., & Turner, P. E. (2020). Evolvability costs of niche expansion. *Trends in Genetics, 36*, 14–23 (2020). https://doi.org/10.1016/j.tig.2019.10.003

Burke, M. K., Liti, G., & Long, A. D. (2014). Standing genetic variation drives repeatable experimental evolution in outcrossing populations of *Saccharomyces cerevisiae*. *Molecular Biology and Evolution, 31*(12), 3228–3239.

Chiotti, K. E., Kvitek, D. J., Schmidt, K. H., Koniges, G., Schwartz, K., Donckels, E.A., & Sherlock, G. (2014). The valley-of-death: Reciprocal sign epistasis constrains adaptive trajectories in a constant, nutrient limiting environment. *Genomics, 104*(6), 431–437.

Chou, H. H., Chiu, H. C., Delaney, N. F., Segrè, D., & Marx, C. J. (2011). Diminishing returns epistasis among beneficial mutations decelerates adaptation. *Science, 332*, 1190–1192. https://doi.org/10.1126/science.1203799

Costanzo, M., Baryshnikova, A., Bellay, J., Kim, Y., Spear, E. D., Sevier, C. S., Ding, H., Koh, J. L.Y., Toufighi, K., Mostafavi, S., Prinz, J., St Onge, R. P., VanderSluis, B., Makhnevych, T., Vizeacoumar, F. J., Alizadeh, S., Bahr, S., Brost, R. L., Chen, Y., …, Boone, C. (2010). The genetic landscape of a cell. *Science, 327*, 425–431. https://doi.org/10.1126/science.1180823

Costanzo, M., VanderSluis, B., Koch, E.N., Baryshnikova, A., Pons, C., Tan, G., Wang, W., Usaj, M., Hanchard, J., Lee, S. D., Pelechano, V., Styles, E. B., Billmann, M., van Leeuwen, J., van Dyk, N., Lin, Z. Y., Kuzmin, E., Nelson, J., Piotrowski, J. S., …, Boone, C. (2016). A global genetic interaction network maps a wiring diagram of cellular function. *Science, 353*. https://doi.org/10.1126/science.aaf1420

Dowell, R. D., Ryan, O., Jansen, A., Cheung, D., Agarwala, S., Danford, T., Bernstein, D. A., Rolfe, P. A., Heisler, L. E., Chin, B., Nislow, C., Giaever, G., Phillips, P. C., Fink, G. R., Gifford, D. K., & Boone, C. (2010). Genotype to phenotype: A complex problem. *Science, 328*, 469. https://doi.org/10.1126/science.1189015

D'Souza, G., Waschina, S., Pande, S., Bohl, K., Kaleta, C., & Kost, C. (2014). Less is more: Selective advantages can explain the prevalent loss of biosynthetic genes in bacteria. *Evolution, 68*(9), 2559–2570.

Eyre-Walker, A., & Keightley, P. D. (2007). The distribution of fitness effects of new mutations. *Nature Reviews. Genetics, 8*, 610–618. https://doi.org/10.1038/nrg2146

Fisher, R. (1930). *The genetical theory of natural selection*. Oxford Univ. Press.

Fraser, H. B., Hirsh, A. E., Steinmetz, L. M., Scharfe, C., & Feldman, M. W. (2002). Evolutionary rate in the protein interaction network. *Science, 296*, 750–752. https://doi.org/10.1126/science.1068696

Gerrish, P. J., & Lenski, R. E. (1998). The fate of competing beneficial mutations in an asexual population. *Genetica, 102–103*, 127–144.

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Véronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., André, B., Arkin, A. P., Astromoff, A., El-Bakkoury, M., Bangham, R., Benito, R., Brachat, S., Campanaro, S., Curtiss, M., Davis, K.,…, Johnston, M. (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature, 418*, 387–391. https://doi.org/10.1038/nature00935

Giaever, G., & Nislow, C. (2014). The yeast deletion collection: A decade of functional genomics. *Genetics, 197*, 451–465. https://doi.org/10.1534/genetics.114.161620

Good, B. H., Rouzine, I. M., Balick, D. J., Hallatschek, O., & Desai, M. M. (2012). Distribution of fixed beneficial mutations and the rate of adaptation in asexual populations. *Proceedings of the National Academy of Sciences of the United States of America, 109*, 4950–4955. https://doi.org/10.1073/pnas.1119910109

Gresham, D., Desai, M. M., Tucker, C. M., Jenq, H. T., Pai, D. A., Ward, A., DeSevo, C. G., Botstein, D., & Dunham, M. J. (2008). The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. *PLoS Genetics, 4*, e1000303.https://doi.org/10.1371/journal.pgen.1000303

Helsen, J., Frickel, J., Jelier, R., & Verstrepen, K. J. (2019). Network hubs affect evolvability. *PLoS Biology, 17*, e3000111. https://doi.org/10.1371/journal.pbio.3000111

Holder, K. K., & Bull, J. J. (2001). Profiles of adaptation in two similar viruses. *Genetics, 159*, 1393–1404.

Jeong, H., Mason, S. P., Barabási, A. L., & Oltvai, Z. N. (2001). Lethality and centrality in protein networks. *Nature, 411*, 41–42. https://doi.org/10.1038/35075138

Jerison, E. R., Kryazhimskiy, S., Mitchell, J. K., Bloom, J. S., Kruglyak, L., & Desai, M. M. (2017). Genetic variation in adaptability and pleiotropy in budding yeast. *eLife, 6*, e27167. https://doi.org/10.7554/eLife.27167

Khan, A. I., Dinh, D. M., Schneider, D., Lenski, R. E., & Cooper, T. F. (2011). Negative epistasis between beneficial mutations in an evolving bacterial population. *Science, 332*, 1193–1196. https://doi.org/10.1126/science.1203801

Kim, P. M., Korbel, J. O., & Gerstein, M. B. (2007). Positive selection at the protein network periphery: Evaluation in terms of structural constraints and cellular context. *Proceedings of the National Academy of Sciences of the United States of America, 104*, 20274–20279. https://doi.org/10.1073/pnas.0710183104

Kimura, M. (1964). Diffusion models in population genetics. *Journal of Applied Probability, 1*(2), 177–232.

Kimura, M., et al. (1968). Evolutionary rate at the molecular level. *Nature, 217*(5129), 624–626.

Koubkova-Yu, T. C. T., Chao, J. C., & Leu, J. Y. (2018). Heterologous Hsp90 promotes phenotypic diversity through network evolution. *PLoS Biology, 16*, e2006450. https://doi.org/10.1371/journal.pbio.2006450

Kryazhimskiy, S., Rice, D. P., Jerison, E. R., & Desai, M. M. (2014). Microbial evolution. Global epistasis makes adaptation predictable despite sequence-level stochasticity. *Science, 344*, 1519–1522. https://doi.org/10.1126/science.1250939

Kryazhimskiy, S., Tkacik, G., & Plotkin, J. B. (2009). The dynamics of adaptation on correlated fitness landscapes. *Proceedings of the National Academy of Sciences of the United States of America 106*, 18638–18643. https://doi.org/10.1073/pnas.0905497106

Krylov, D. M., Wolf, Y. I., Rogozin, I. B., & Koonin, E. V. (2003). Gene loss, protein sequence divergence, gene dispensability, expression level, and interactivity are correlated in eukaryotic evolution. *Genome Research, 13*, 2229–2235. https://doi.org/10.1101/gr.1589103

Kvitek, D. J., & Sherlock, G. (2011). Reciprocal sign epistasis between frequently experimentally evolved adaptive mutations causes a rugged fitness landscape. *PLoS Genetics, 7*, e1002056. https://doi.org/10.1371/journal.pgen.1002056

Kvitek, D. J., & Sherlock, G. (2013). Whole genome, whole population sequencing reveals that loss of signaling networks is the major adaptive strategy in a constant environment. *PLoS Genetics, 9*(11), e1003972.

Laan, L., Koschwanez, J. H., & Murray, A. W. (2015). Evolutionary adaptation after crippling cell polarization follows reproducible trajectories. *Elife, 4*, e09638.

Lang, G. I., Rice, D. P., Hickman, M. J., Sodergren, E., Weinstock, G. M., Botstein, D., & Desai, M. M. (2013). Pervasive genetic hitchhiking and clonal interference in forty evolving yeast populations. *Nature 500*, 571–574. https://doi.org/10.1038/nature12344

Le Gac, M., Plucain, J., Hindré, T., Lenski, R. E., & Schneider, D. (2012). Ecological and evolutionary dynamics of coexisting lineages during a long-term experiment with *Escherichia coli*. *Proceedings of the National Academy of Sciences, 109*(24), 9487–9492.

Lenski, R. E. (2017). Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations. *The ISME Journal, 11*(10), 2181.

Levy, S. F., Blundell, J. R., Venkataram, S., Petrov, D. A., Fisher, D. S., & Sherlock, G. (2015). Quantitative evolutionary dynamics using high-resolution lineage tracking. *Nature, 519*, 181–186. https://doi.org/10.1038/nature14279

Li, Y., Venkataram, S., Agarwala, A., Dunn, B., Petrov, D. A., Sherlock, G., & Fisher, D. S. (2018). Hidden complexity of yeast adaptation under simple evolutionary conditions. *Current Biology, 28*, 515–525.e6. https://doi.org/10.1016/j.cub.2018.01.009

Liu, G., Yong, M. Y. J., Yurieva, M., Srinivasan, K. G., Liu, J., Lim, J. S. Y., Poidinger, M., Wright, G. D., Zolezzi, F., Choi, H., Pavelka, N., & Rancati, G. (2015). Gene essentiality is a quantitative property linked to cellular evolvability. *Cell, 163*, 1388–1399. https://doi.org/10.1016/j.cell.2015.10.069

MacLean, R. C., Perron, G. G., & Gardner, A. (2010). Diminishing returns from beneficial mutations and pervasive epistasis shape the fitness landscape for rifampicin resistance in *Pseudomonas aeruginosa*. *Genetics, 186*, 1345–1354. https://doi.org/10.1534/genetics.110.123083

McCloskey, D., Xu, S., Sandberg, T.E., Brunk, E., Hefner, Y., Szubin, R., Feist, A. M., & Palsson, B. O. (2018). Evolution of gene knockout strains of *E. coli* reveal regulatory architectures governed by metabolism. *Nature Communications 9*, 3796. https://doi.org/10.1038/s41467-018-06219-9

McCloskey, D., Xu, S., Sandberg, T. E., Brunk, E., Hefner, Y., Szubin, R., Feist, A. M., & Palsson, B. O. (2018a). Growth adaptation of *gnd* and *sdhCB Escherichia coli* deletion strains diverges from a similar initial perturbation of the transcriptome. *Frontiers in Microbiology, 9*, 1793. https://doi.org/10.3389/fmicb.2018.01793

McCloskey, D., Xu, S., Sandberg, T. E., Brunk, E., Hefner, Y., Szubin, R., Feist, A. M., & Palsson, B. O. (2018b). Multiple optimal phenotypes overcome redox and glycolytic intermediate metabolite imbalances in *Escherichia coli pgi* knockout evolutions. *Applied and Environmental Microbiology, 84*. https://doi.org/10.1128/AEM.00823-18

McDonald, M. J., Rice, D. P., & Desai, M. M. (2016). Sex speeds adaptation by altering the dynamics of molecular evolution. *Nature, 531*(7593), 233

Moore, F. B., Rozen, D. E., & Lenski, R. E. (2000). Pervasive compensatory adaptation in *Escherichia coli*. *Proceedings. Biological Sciences, 267*, 515–522. https://doi.org/10.1098/rspb.2000.1030

Ono, J., Gerstein, A. C., & Otto, S. P. (2017). Widespread genetic incompatibilities between first-step mutations during parallel adaptation of *Saccharomyces cerevisiae* to a common environment. *PLoS Biology, 15*(1), e1002591.

Orr, H. A. (2005). The genetic theory of adaptation: A brief history. *Nature Reviews. Genetics 6*, 119–127. https://doi.org/10.1038/nrg1523

Parts, L., Cubillos, F. A., Warringer, J., Jain, K., Salinas, F., Bumpstead, S. J., Molin, M., Zia, A., Simpson, J. T., Quail, M. A., Moses, A., Louis, E. J., Durbin, R., & Liti, G. (2011). Revealing the genetic structure of a trait by sequencing a population under selection. *Genome Research, 21*, 1131–1138. https://doi.org/10.1101/gr.116731.110

Poelwijk, F. J., Kiviet, D. J., Weinreich, D. M., & Tans, S. J. (2007). Empirical fitness landscapes reveal accessible evolutionary paths. *Nature 445*, 383–386. https://doi.org/10.1038/nature05451

Poelwijk, F. J., Tănase-Nicola, S., Kiviet, D. J., & Tans, S. J. (2011). Reciprocal sign epistasis is a necessary condition for multi-peaked fitness landscapes. *Journal of Theoretical Biology 272*, 141–144. https://doi.org/10.1016/j.jtbi.2010.12.015

Rainey, P. B., & Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. *Nature, 394*, 69–72. https://doi.org/10.1038/27900

Rancati, G., Pavelka, N., Fleharty, B., Noll, A., Trimble, R., Walton, K., Perera, A., Staehling-Hampton, K., Seidel, C. W., & Li, R. (2008). Aneuploidy underlies rapid adaptive evolution of yeast cells deprived of a conserved cytokinesis motor. *Cell, 135*, 879–893. https://doi.org/10.1016/j.cell.2008.09.039

Riehle, M. M., Bennett, A. F., & Long, A. D. (2001). Genetic architecture of thermal adaptation in escherichia coli. *Proceedings of the National Academy of Sciences of the United States of America 98*, 525–530. https://doi.org/10.1073/pnas.021448998

Rojas Echenique, J. I., Kryazhimskiy, S., Nguyen Ba, A. N., & Desai, M. M. (2019). Modular epistasis and the compensatory evolution of gene deletion mutants. *PLoS Genetics, 15*, e1007958. https://doi.org/10.1371/journal.pgen.1007958

Sanchez, M. R., Miller, A. W., Liachko, I., Sunshine, A. B., Lynch, B., Huang, M., Alcantara, E., DeSevo, C. G., Pai, D. A., Tucker, C. M., Hoang, M. L., & Dunham, M. J. (2017). Differential paralog divergence modulates genome evolution across yeast species. *PLoS Genetics 13*, e1006585. https://doi.org/10.1371/journal.pgen.1006585

Silander, O. K., Tenaillon, O., & Chao, L. (2007). Understanding the evolutionary fate of finite populations: The dynamics of mutational effects. *PLoS Biology, 5*(4), e94.

Sunshine, A. B., Payen, C., Ong, G. T., Liachko, I., Tan, K. M., & Dunham, M. J. (2015). The fitness consequences of aneuploidy are driven by condition-dependent gene effects. *PLoS Biology 13*, e1002155. https://doi.org/10.1371/journal.pbio.1002155

Suzuki, S., Horinouchi, T., & Furusawa, C. (2014). Prediction of antibiotic resistance by gene expression profiles. *Nature Communications, 5*, 5792. https://doi.org/10.1038/ncomms6792

Szamecz, B., Boross, G., Kalapis, D., Kovács, K., Fekete, G., Farkas, Z., Lázár, V., Hrtyan, M., Kemmeren, P., Groot Koerkamp, M. J. A., Rutkai, E., Holstege, F. C. P., Papp, B., & Pál, C. (2014). The genomic landscape of compensatory evolution. *PLoS Biology, 12*, e1001935. https://doi.org/10.1371/journal.pbio.1001935

Tenaillon, O., Rodríguez-Verdugo, A., Gaut, R. L., McDonald, P., Bennett, A. F., Long, A. D., & Gaut, B. S. (2012). The molecular diversity of adaptive convergence. *Science, 335*(6067), 457–461.

Teng, X., Dayhoff-Brannigan, M., Cheng, W. C., Gilbert, C. E., Sing, C. N., Diny, N. L., Wheelan, S. J., Dunham, M. J., Boeke, J. D., Pineda, F. J., & Hardwick, J. M. (2013). Genome-wide consequences of deleting any single gene. *Molecular Cell, 52*, 485–494. https://doi.org/10.1016/j.molcel.2013.09.026

Tong, A. H. Y., Lesage, G., Bader, G. D., Ding, H., Xu, H., Xin, X., et al. (2004). Global mapping of the yeast genetic interaction network. *Science 303*(5659), 808–813.

Voordeckers, K., Kominek, J., Das, A., Espinosa-Cantú, A., De Maeyer, D., Arslan, A., Van Pee, M., van der Zande, E., Meert, W., Yang, Y., Zhu, B., Marchal, K., DeLuna, A., Van Noort, V., Jelier, R., & Verstrepen, K. J. (2015). Adaptation to high ethanol reveals complex evolutionary pathways. *PLoS Genetics, 11*, e1005635. https://doi.org/10.1371/journal.pgen.1005635

Watts, F. Z., Shiels, G., & Orr, E. (1987). The yeast MYO1 gene encoding a myosin-like protein required for cell division. *The EMBO Journal, 6*, 3499–3505.

Wei, X., & Zhang, J. (2019). Patterns and mechanisms of diminishing returns from beneficial mutations. *Molecular Biology and Evolution, 36*(5), 1008–1021.

Wiser, M. J., Ribeck, N., & Lenski, R. E. (2013). Long-term dynamics of adaptation in asexual populations. *Science, 342*(6164), 1364–1367.

Wright, S. (1932). The roles of mutations, inbreeding, crossbreeding and selection in evolution. In *Proc Sixth Int Congr Genet*.

Wright, S. (1988). Surfaces of selective value revisited. *The American Naturalist, 131*(1), 115–123.

Wünsche, A., Dinh, D. M., Satterwhite, R. S., Arenas, C. D., Stoebel, D.M., & Cooper, T. F. (2017). Diminishing-returns epistasis decreases adaptability along an evolutionary trajectory. *Nature Ecology & Evolution, 1*, 61. https://doi.org/10.1038/s41559-016-0061

Zhu, Y. O., Siegal, M. L., Hall, D. W., & Petrov, D. A. (2014). Precise estimates of mutation rate and spectrum in yeast. *Proceedings of the National Academy of Sciences, 111*(22), E2310–E2318.

Addressing Evolutionary Questions with Synthetic Biology

Florian Baier and Yolanda Schaerli

Abstract Synthetic biology emerged as an engineering discipline to design and construct artificial biological systems. Synthetic biological designs aim to achieve specific biological behavior, which can be exploited for biotechnological, medical, and industrial purposes. In addition, mimicking natural systems using well-characterized biological parts also provides powerful experimental systems to study evolution at the molecular and systems level. A strength of synthetic biology is to go beyond nature's toolkit, to test alternative versions and to study a particular biological system and its phenotype in isolation and in a quantitative manner. Here, we review recent work that implemented synthetic systems, ranging from simple regulatory circuits, rewired cellular networks to artificial genomes and viruses, to study fundamental evolutionary concepts. In particular, engineering, perturbing or subjecting these synthetic systems to experimental laboratory evolution provides a mechanistic understanding on important evolutionary questions, such as: Why did particular regulatory network topologies evolve and not others? What happens if we rewire regulatory networks? Could an expanded genetic code provide an evolutionary advantage? How important is the structure of genome and number of chromosomes? Although the field of evolutionary synthetic biology is still in its teens, further advances in synthetic biology provide exciting technologies and novel systems that promise to yield fundamental insights into evolutionary principles in the near future.

1 Introduction

Evolutionary biology traditionally studies past or present organisms to reconstruct past evolutionary events with the aim to explain and predict their evolution. However, understanding evolution and why life evolved the way it did might require

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_7

going beyond solutions found in nature. As the evolutionist, J. M. Smith states in 1992 in respect to the development of computational models of artificial evolving systems: "*So far, we have been able to study only one evolving system, and we cannot wait for interstellar flight to provide us with a second. If we want to discover generalizations about evolving systems, we will have to look at artificial ones.*" (J. M. Smith, 1992). Now, with advances in synthetic, systems and computational biology it is actually possible to design, create, and study artificial (synthetic) biological systems (Cameron et al., 2014). The interdisciplinary field of synthetic biology essentially started with the publication of the first synthetic regulatory networks in 2000 (Elowitz & Leibler, 2000; Gardner et al., 2000; Becskei & Serrano, 2000) and since then expanded to the design and construction of more complex genetic circuits (Bashor & Collins, 2018), organelles (Lee et al., 2018), and even whole genomes (Hutchison et al., 2016) and cells (Blain & Szostak, 2014). The design and construction of synthetic biological systems is usually focused on new and desirable metabolic, sensory, regulatory, and physical capabilities, which are of particular interest for biotechnological and medical applications (Xie & Fussenegger, 2018; Nielsen & Keasling, 2016; Tang et al., 2020). Synthetic systems are built by combining, co-opting, and modifying biological parts that are implemented in a biological host "chassis" (Y.-H. Wang et al., 2013). A desirable feature of synthetic systems is their (at least partial) orthogonality, meaning that their functionality is not affecting the host's regulation and fitness (C. C. Liu et al., 2018a). This allows manipulating, tuning and recording the function of synthetic biological systems independently and without causing much undesirable side effects. In contrast, studying natural systems is often challenging because many biological phenotypes are difficult to disentangle, quantify and characterize. Even for the well-studied single-cell model organisms such as *E. coli* and yeast, we often poorly understand how genes are functionally interconnected and contribute to particular phenotypes (Paaby & Rockman, 2013).

The ability to design and build synthetic biological systems that achieve a specific desired phenotype already demonstrates a significant knowledge about its functionality, which is summarized by Richard Feynman's famous quote: "*What I cannot create, I do not understand*" (written on his blackboard at the time of his death in February 1988). One step further is to not only build a system with a particular purpose in mind, but also to study it and learn how it behaves when it is perturbed and/or evolving. Directed evolution mimics the process of diversification and natural selection that resembles Darwinian evolution under well-defined conditions and is frequently used to optimize proteins and enzymes toward specific functions (Zeymer & Hilvert, 2018). Beyond its applied side, the ability to control each parameter in directed evolution experiments has substantially contributed toward a more fundamental understanding of the dynamics and constraints of molecular evolution (Kaltenbach & Tokuriki, 2014; Arnold, 2010; Dean & Thornton, 2007). Similarly, the well-defined properties of synthetic biological systems and the ability to manipulate and report the system's behavior have motivated researchers to explore fundamental biological and evolutionary questions with synthetic biology (Peisajovich, 2012; Davidson et al., 2012; de Lorenzo, 2018; Bashor & Collins,

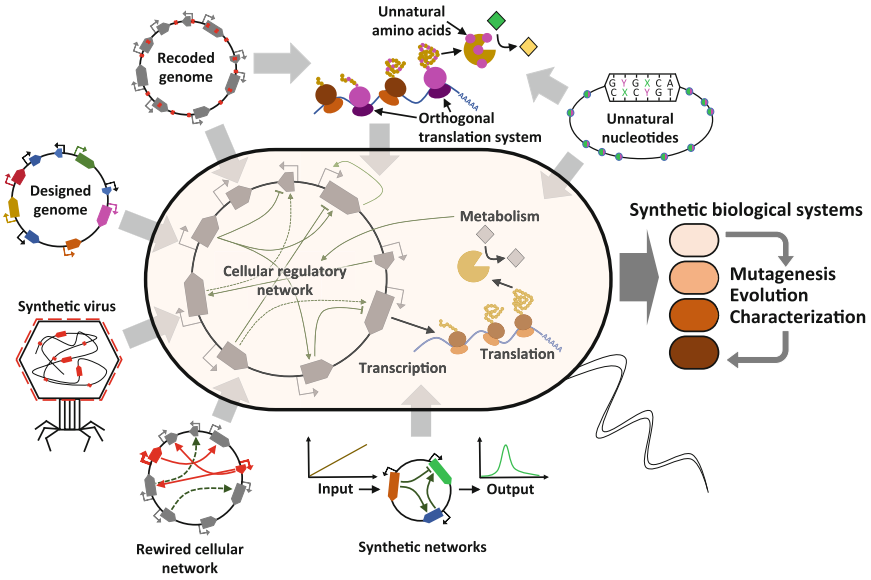

Fig. 1 Overview of synthetic biological systems discussed in this chapter. Bottom-up designed and built synthetic biological systems are implemented into a host cell (center) that executes their function using its internal cellular machinery and resources. Synthetic biological systems are amenable for characterization, perturbations, and evolution

2018; Simon et al., 2019). Although the exciting synthesis of evolutionary and synthetic biology is still young, it benefits from existing technologies that have been used to debug and optimize imperfect designs of synthetic systems (Yokobayashi et al., 2002; Cobb et al., 2012; Haseltine & Arnold, 2007).

In the following, we will highlight a selection of studies that apply synthetic biology tools to understand evolutionary dynamics at the systems level. In particular, we focus on examples of synthetic systems, ranging from simple synthetic regulatory networks (Sect. 2), rewired gene regulatory networks (Sect. 3), and extended genetic codes to synthetic viruses and genomes (Sect. 4) (summarized in Fig. 1 as a graphical overview). In Sect. 5, we end with a discussion on how further advances in synthetic biology will pave the way for a deeper understanding of life and evolutionary principles and how synthetic biology and evolutionary systems biology can benefit from each other in the future.

2 Synthetic Regulatory Networks

Regulatory networks control the spatial and temporal expression of downstream genes through interactions between DNA, RNA, proteins and/or metabolites (Rockman & Kruglyak, 2006; Hill et al., 2020). The first synthetic gene regulatory

networks (synGRNs) that were constructed are the repressilator and the toggle switch in 2000 (Gardner et al., 2000; Elowitz & Leibler, 2000). Since then, many diverse operational behaviors have been achieved with synGRNs, such as logic gates (Guet et al., 2002), counting events (Friedland et al., 2009), cellular memory (Ajo-Franklin et al., 2007), pattern formation (Schaerli et al., 2014; Barbier et al., 2020; Santos-Moreno et al., 2020), cell polarization (Chau et al., 2012), and light-sensing (Tabor et al., 2009) (reviewed in Xie & Fussenegger, 2018; Santos-Moreno & Schaerli, 2018; Bashor & Collins, 2018). SynGRNs have been successfully implemented in prokaryotic and eukaryotic systems (Xie & Fussenegger, 2018). In addition to the numerous potential applications of synGRNs in biotechnology, biomedicine and other fields (Xie & Fussenegger, 2018; Ruder et al., 2011; Weber & Fussenegger, 2011), synGRNs are also great model systems to study function and evolution of cellular regulation (Crocker & Ilsley, 2017; Bashor & Collins, 2018; Davies, 2017; Santos-Moreno & Schaerli, 2018). In the following, we will highlight several studies that explored design principles and evolutionary dynamics with synGRNs.

2.1 Exploring Network Design Space with Synthetic Regulatory Networks

A certain biological function can be achieved with different networks varying in their topologies, i.e., in their type and number of regulatory nodes and interactions (Cotterell & Sharpe, 2010). Explorations of distinct networks topologies can be highly insightful in regard to their functional properties and evolutionary potential. Chau et al. explored the design space of simple gene regulatory networks that achieve cell polarization (Chau et al., 2012). Spatial organization within cells through polarization is crucial for many cellular behaviors such as motility, asymmetric cell division and establishing polarity in epithelial cells and neurons (Raman et al., 2018). The team first computationally predicted all one- and two-node network topologies capable of cell polarization. All functional solutions contained one of three minimal motifs: positive feedback, mutual inhibition, or inhibition with positive feedback. Combinations of two or three minimal motifs increased the robustness of cell polarization, i.e., it was achieved over a larger range of parameters. To test their predictions experimentally, Chau et al. built an elegant synthetic system to study cell polarization in budding yeast using a toolkit of well-characterized biological parts including promoters, kinases, phosphatases, and localization tags, most of which are not naturally found in budding yeast. Using this strategy, the authors were able to generate the three minimal motifs predicted to provide cell polarization and explored the parameter range under which the networks are functional. In agreement with their theoretical predictions, the minimal motifs alone gave rise to cell polarization, although within a limited parameter range. However, when the minimal motifs were combined into more complex networks, robust cell

polarization could be achieved over a wide parameter range. Thus, in this case, network robustness can be achieved by combining multiple minimal motifs, which might explain why combinations of multiple network motifs occur frequently in nature (Chau et al., 2012). In summary, this work not only demonstrated that it is possible to predict, design and synthetically build cell polarization networks, but also how multiple interactions contribute to network robustness and consequently evolutionary dynamics.

In a similar approach, Schaerli and colleagues explored the design space of three-node gene regulatory networks that translate a morphogen concentration gradient into a stripe-like gene expression pattern, i.e., a low-high-low gene expression along a morphogen gradient (Schaerli et al., 2014). The ability of GRNs to convert a gradient input into spatial information is crucial during development, for example during axial patterning of the *Drosophila* embryo (Wolpert, 1969). Similar to the study above, Schaerli et al. first explored the design space computationally. Interestingly, the identified networks can be divided into four groups and the simplest network of each group corresponds to one of the four types of incoherent feed-forward loops (Mangan & Alon, 2003). Each of the four groups employs a distinct dynamical mechanism (spatiotemporal course of gene expression) to form a stripe (Cotterell & Sharpe, 2010). The four simple networks were constructed by combining well-characterized regulatory components with a fluorescent reporter and expressed in *E. coli*. Indeed, a stripe pattern was experimentally achieved with all four network topologies. Based on the experimental and model results, they also designed and built a two-node stripe-forming network, representing the archetype of the four minimal three-node network topologies. Conclusively, this study experimentally demonstrated that stripe formation can be achieved with various network topologies and dynamical mechanisms, some of which have not yet been discovered in nature.

2.2 *Exploring Evolutionary Dynamics with Synthetic Regulatory Networks*

In a follow-up study, Schaerli et al. used two of the stripe-forming synthetic networks (the incoherent feed-forward loops type 2 (I2) and 3 (I3)) to investigate whether and how the underlying dynamical regulatory mechanism of a network biases and affects its evolutionary potential (Fig. 2a) (Schaerli et al., 2018). To this end, they introduced random mutations into each network and used a combination of experimental measurements, DNA sequencing, and mathematical modeling to understand how mechanisms of GRNs affect the ability to evolve novel phenotypes, i.e., phenotypes that are different from a stripe (Fig. 2a). Remarkably, each network could only access a limited set of novel phenotypes and the accessible phenotypes differed for each network. The study provides thus the first empirical evidence that the underlying regulatory mechanisms of a GRN can cause constrained variation, as

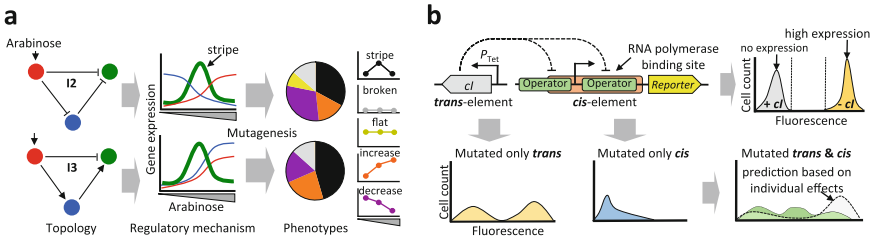

Fig. 2 Using synthetic circuits to understand evolutionary constraints and epistasis in gene regulatory networks. (**a**) Investigating evolutionary biases of two synthetic networks (incoherent feed-forward loops type 2 (I2, top) and 3 (I3, bottom)) that achieve stripe formation with distinct topologies and regulatory mechanisms (Schaerli et al., 2018). The networks were implemented in *E. coli* cells with a "morphogen" (arabinose) input receiver gene (red), an intermediate loop gene (blue) and an output gene (green) with GFP expression as readout. Arrows represent activation and bars indicate repression. Mutations were introduced into the regulatory regions of each node of both networks and resulting phenotypic changes were characterized at three arabinose concentrations (low, medium and high). The two circuits produce a different spectrum of novel gene expression phenotypes, e.g., only I2 achieves a flat phenotype. (**b**) A synthetic transcriptional regulatory system to study how mutations in single and multiple components affect gene expression phenotypes (Lagator et al., 2017). In this system, an inducible *trans*-element (lambda repressor protein *cI*) represses expression of a fluorescent reporter gene by binding to the operator sites in a *cis*-element that overlaps with the promoter region (RNA polymerase binding site). Presence of the *trans*-element (+cI) results in a low fluorescence phenotype, whereas its absence (−cI) results in a high fluorescence phenotype. Introducing mutations only in *trans* yields a bimodal fluorescence distribution, whereas mutations only in *cis* yield low and intermediate fluorescence phenotypes. Combined mutagenesis of *trans* and *cis* sequences produces more intermediate fluorescence phenotypes than expected from each separately

was previously proposed (Jiménez et al., 2015). Consequently, GRNs with the same phenotype, but different underlying topology or regulatory mechanism, may not be equally evolvable and may constrain an organism's ability to evolve innovative and/or adaptive properties.

Using a similar approach, the Guet lab aimed at understanding how mutations in multiple components of a regulatory system interact and potentially yield new expression phenotypes (Lagator et al., 2017). The group built a simple synthetic regulatory system in *E. coli* consisting of three interacting molecular components: two *trans*-regulatory elements, namely a repressor (cI from lambda phage) and an endogenous RNA polymerase, and a *cis*-regulatory element consisting of the promoter and overlapping repressor binding sites (Fig. 2b). The three components regulate the expression of a fluorescent protein that can be quantitatively measured. To understand how mutations in two components alter the regulatory behavior and function of the system, they introduced mutations in the *cis*-regulatory element and in the repressor. They analyzed the effect of the mutations independently and in combination. Surprisingly, when introducing mutations in both components the regulatory system produced gene expression phenotypes that were not observed when mutating only one component. The authors attribute these emerging phenotypes to epistatic interactions between the transcription factor and its DNA binding site.

Epistasis means that the combined functional effect of two or more mutations differs from the expected value based on the individual effects (Lehner, 2011; Nghe et al., 2020). In this case, epistasis increased phenotypic variation that selection can act on and thus might facilitate subsequent adaptive evolution. This seems to be true also for other transcriptional regulatory systems, such as for the above-described stripe-forming three-node synGRN of Schaerli et al. (2018). Here, mutations in multiple network nodes gave rise to a wider spectrum of phenotypes compared to mutations in only one of three network nodes.

Phenotypic heterogeneity, e.g., due to stochasticity in gene expression, can be a beneficial property for microorganisms in a changing or fluctuating environment by providing some individuals within a population with a survival advantage (reviewed in Ackermann, 2015; Payne & Wagner, 2019). How does phenotypic heterogeneity influence evolutionary dynamics? To address this question experimentally the Pál lab used an elegant combination of synthetic biology and experimental evolution by designing and implementing two synGRNs that control the expression of an antifungal resistance gene in *S. cerevisiae* with different degrees of gene expression heterogeneity (Bódi et al., 2017). They found that synGRNs with higher heterogeneity not only provided a higher initial resistance to the antifungal drug, but also allowed the yeast cells to evolve a higher resistance after several rounds of evolution under gradually increasing concentrations of the antifungal drug. Also, beneficial mutations in the synGRN with high heterogeneity were contingent on this high gene expression stochasticity, meaning that their adaptive effects were substantially reduced in a background with low gene expression stochasticity. Thus, gene expression stochasticity can influence evolutionary trajectories by widening the spectrum of available beneficial mutations during evolutionary adaptation. Remarkably, in the synGRN with initial low stochasticity elevated phenotypic heterogeneity evolved as a direct response to the antifungal stress. However, at the same time, the benefit of high phenotypic heterogeneity trades-off with reduced fitness in the drug-free medium. Thus, gene expression stochasticity might be an evolvable trait that is selected for in fluctuating and changing environments (Sánchez-Romero & Casadesús, 2013; Arnoldini et al., 2014; Holland et al., 2014; Salathé et al., 2009; Acar et al., 2008; Kuwahara & Soyer, 2012; Sato et al., 2003).

3 Rewired Regulatory Networks

Understanding why natural GRNs evolved a particular topology and which components and connections of a network are either necessary or dispensable for functionality is a difficult question to address. Classical genetic approaches usually delete individual network components or interactions to decipher their functional role within the network. However, alternative topologies with new and rewired interactions are difficult to study in this way. Instead, with a synthetic biology approach network components and interactions cannot only be deleted but also added, rewired, and fine-tuned (Mukherji & van Oudenaarden, 2009; Bashor et al.,

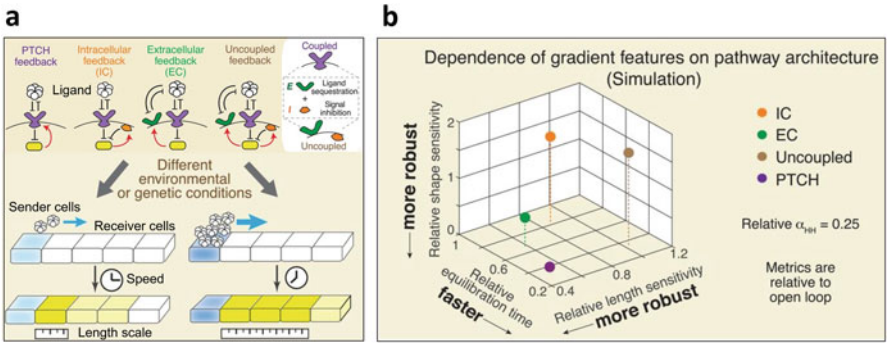

Fig. 3 Rewiring of cellular circuits to understand design principles. (**a**) Hedgehog pathway topologies studied by Li et al. The natural bifunctional PTCH receptor provides intracellular (IC) and extracellular (EC) feedback simultaneously. The IC feedback inhibits the signaling pathway only intracellular, whereas the EC feedback does so only extracellular by sequestering the ligand. The PTCH feedback was uncoupled through separating the functional parts of the PTCH receptor. The cell-culture system with sender and receiver cells allowed quantitative analysis of the spatiotemporal patterning dynamics resulting from the different circuit wirings. (**b**) Summary of robustness and speed properties of different feedback architectures for Hedgehog (HH) signaling gradient formation. (Reproduced with permission Li et al., 2018)

2010). Additionally, a networks' output, which might be difficult to observe and quantify, can be linked to an additional measurable output such as expression of a fluorescent protein.

An interesting question is why a particular topology was favored during evolution over alternative topologies. A study by the Elowitz lab investigated the network topology of the Hedgehog signaling pathway by reconstituting a developmental morphogen gradient in vitro, with a tunable synthetic rewiring of regulatory interactions and a fluorescent readout in a cell-culture model, combined with in silico modeling (Fig. 3a) (Li et al., 2018). The Hedgehog pathway is crucial in establishing positional information for proper patterning during embryonic development and is composed of a double-negative regulatory logic and an additional negative feedback (Briscoe & Thérond, 2013). The results revealed that the natural negative feedback architecture shows the most robust behavior in length scale and amplitude of the hedgehog signaling gradient compared to alternative architectures. In addition, it reaches steady state more rapidly and over a wider range of signaling molecule concentration than alternative topologies (Fig. 3b). However, it remains an open question of whether the rapid response and robustness of the system to changes in the rate of morphogen production has been directly selected for during evolution or rather resulted as a by-product of other evolutionary forces (Kaneko, 2007; Ciliberti et al., 2007).

Isalan and colleagues went beyond individual networks and instead rewired large parts of the *E. coli* genome (Isalan et al., 2008). The authors rewired a set of transcription factor and σ-factor genes with different unrelated regulatory regions, thus creating almost 600 reconnected networks on top of an otherwise

unchanged *E. coli* genome. Surprisingly, only a few of the synthetic rewirings did considerably affect growth, although a follow-up study showed that perturbations of gene expression span up to four orders of magnitude and changed up to ~70% of the transcriptome (Baumstark et al., 2015). In fact, some of the rewirings were actually beneficial under stressful conditions such as heat-shock and prolonged stationary phase. Thus, substantial rewiring of transcriptional networks, at least in *E. coli*, is tolerable to some extent and may even be advantageous under stressful environmental conditions.

4 Synthetic Genomics

The ability to do large-scale recoding and the emergence of computationally (re-)designed synthetic genes and genomes opened many possibilities for applied and fundamental research. Synthetic genomics advanced drastically since the first synthetic gene was synthesized in 1970 (Agarwal et al., 1970). In particular the cost, quality and speed of de novo DNA synthesis improved significantly, as well as assembly technologies that allow the de novo synthesis of whole chromosomes and genomes (H. Wang et al., 2016; Haimovich et al., 2015). Here, we will focus on the evolutionary perspective of recoded and designed synthetic genomes (see also Pál et al., 2014) and refer to other excellent reviews that cover technological aspects and potential applications of this exciting topic (Mukai et al., 2017; Chari & Church, 2017; Haimovich et al., 2015; W. Zhang et al., 2020).

4.1 Extending the Alphabet of Life

In the early 2000s, researchers first demonstrated that the genetic code can be engineered and expanded to incorporate nonnatural amino acids (nnAA) with distinct chemical and structural properties (Chin et al., 2003; L. Wang et al., 2001) (reviewed in C. C. Liu & Schultz, 2010; Chin, 2014; Santos-Moreno & Schaerli, 2020a). To encode a nnAA within a gene or genome, an unassigned free codon (usually the rare TAG amber stop codon) is reassigned to encode a nnAA (Chin, 2017). Second, incorporation of a nnAA into proteins requires an orthogonal tRNA-synthetase pair that specifically recognizes the assigned codon and the nnAA, but not any other codon or AA (Chin, 2017). Protein engineers have used nnAAs to generate proteins and enzymes with new biophysical and chemical properties that would not be possible with standard AAs (W. H. Zhang et al., 2013; C. C. Liu & Schultz, 2010). Now, with the development of genome-wide editing tools researchers have recoded whole genomes in order to allow incorporation of nnAAs into the proteome (Arranz-Gibert et al., 2018; Fredens et al., 2019). The Isaacs and Church labs engineered an *E. coli* strain (named C321.ΔA) that has all 321 TAG stop codons replaced with the synonymous TAA stop codon (Lajoie et al.,

2013). The complete codon substitution allowed the deletion of the release factor 1 (RF1, which terminates translation at UAG and UAA). Therefore, UAG codons are unassigned in C321.ΔA while RF2 still terminates translation at UGA and UAA. The evolutionary consequence of the complete codon substitution and RF1 deletion is that horizontally acquired DNA containing TAG codons cannot correctly be translated, meaning that the organism is genetically isolated (Lajoie et al., 2013). On the one hand, this provides resistance to phages that contain TAG codons but it also hinders the acquisition of potentially beneficial DNA, such as plasmids containing antibiotic resistance genes. Jing Ma and Isaacs performed an interesting evolution experiment with phage populations infecting a mixture of standard and recoded *E. coli* strains at various ratios (N. J. Ma & Isaacs, 2016). The study showed that phages adapted toward C321.ΔA by reducing their TAG codon usage. This experiment provides compelling evidence that phages and viruses adapt rapidly their genetic code to achieve compatibility with their host.

Another intriguing question is how an expanded genetic code beyond the generic 20 amino acids alters the evolution of an organism. Does the availability of new chemistry promote novel opportunities for beneficial mutations? A study by the Barrick lab addressed this question experimentally by evolving T7 bacteriophages with a host *E. coli* in which the amber stop codon (TAG) was reassigned to incorporate 3-iodotyrosine (IdioY) as a 21st amino acid (Fig. 4a) (Hammerling et al., 2014). The T7 phage populations improved their lysis times (fitness proxy) during several rounds of laboratory evolution and adapted specifically to the IdioY incorporating *E. coli* host. At the end of the experiment several improved phage mutants also incorporated IdioY into their genome and their improved fitness was dependent on the unnatural amino acid. For example, a Tyr39-to-IodoY mutation in the T7 Type II holin gene was more beneficial than having a Tyr or Trp at this position. Although other mutants in this study evolved to have the same fitness using only the generic 20 amino acids, the readiness to which the phage population adapted to an alternative genetic code and incorporated a non-natural amino acid in its proteins is quite remarkable. Since incorporation of nnAA can generate proteins with novel functionalities and properties (see reviews Chin, 2014; C. C. Liu & Schultz, 2010), an expanded genetic code could potentially facilitate evolution toward higher fitness beyond what is feasible with the universal 20 amino acids.

Recently, systems have been developed that allow the incorporation of more than one nnAA at the same time in one organism. This is achieved by freeing up and reassigning existing triplet codons (Fredens et al., 2019) or by using quadruplet codons (K. Wang et al., 2012), both of which require modified and orthogonal translation systems in the host cell. For example, the Chin lab recoded the entire *E. coli* genome to use only 61 (instead of 64) codons, which will eventually allow the reassignment of three codons to distinct nnAAs (Fredens et al., 2019). To encode even more nnAAs simultaneously, orthogonal quadruplet-decoding ribosome and tRNA-synthetase have been evolved in the laboratory, which theoretically allows the incorporation of more than 200 different nnAAs in recombinant proteins or recoded organisms (K. Wang et al., 2012). However, the approach is challenging and so far

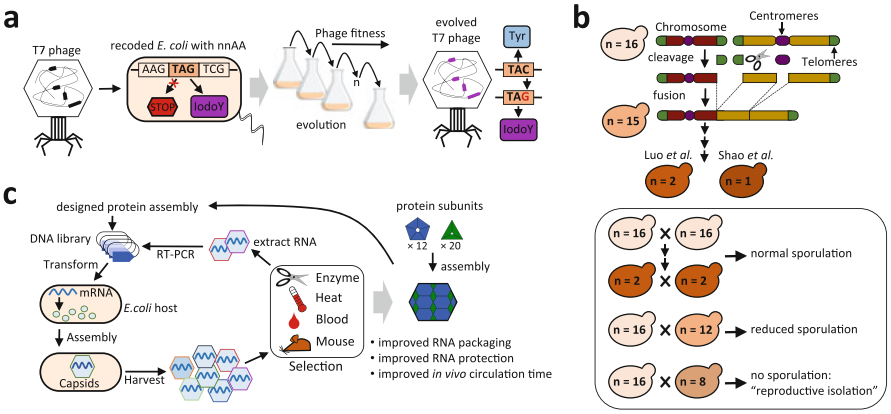

Fig. 4 Evolutionary insights with modified, recoded, and designed synthetic genomes. (**a**) Adaptive evolution experiment of T7 bacteriophages infecting a recoded *E. coli* strain with an expanded genetic code that incorporates 3-idiotyrosine (IodoY) at the amber stop codon TAG (Hammerling et al., 2014). Phages adapt by incorporating IdioY in their proteome, which provides higher fitness than other amino acids at the same positions. (**b**) Two independent studies explore the consequences of step-wise fusing the 16 chromosomes ($n = 16$) in *S. cerevisiae* down to $n = 2$ (J. Luo et al., 2018a) and $n = 1$ (Shao et al., 2019). (**Box**) Mating strains with equal numbers of chromosomes produces viable spores. Increasing the difference in chromosome numbers between mating strains results in reduced spore viable and eventually in reproductive isolation. (**c**) Evolution of a computationally designed protein capsid that encapsulates its own RNA (Butterfield et al., 2017; Terasaka et al., 2018). A DNA mutant library is transformed into *E. coli* hosts that produce the encoding capsids, which are subsequently harvested and selected for improved properties, such as RNase and heat stability as well as blood and mouse circulation times (small box). Isolated RNA is reverse-transcribed to DNA with RT-PCR (reverse-transcription polymerase chain reaction) to start a new round of evolution. Several rounds of evolution resulted in improved RNA packaging and stability within the capsid

only a few studies were able to encode nnAAs with quadruplet codons (Neumann et al., 2010; Niu et al., 2013).

Not only have nnAAs been incorporated into proteins, but also nonnatural nucleotides into the DNA and RNA of living organisms (Pinheiro & Holliger, 2012; Y. Zhang & Romesberg, 2018). In 2014, the Romesberg lab reported the first successful incorporation of an unnatural base pair (UBP) dNaM-dTPT3 into an *E. coli* plasmid (Malyshev et al., 2014). The UBP forms through hydrophobic interactions instead of hydrogen bonding present in the natural base pairs. However, cells with an expanded genetic code grew poorly and very easily lost the UBP. The stable maintenance of the six-letter/three-base-pair code required tuning of the nucleotide uptake system and the implementation of an elegant Cas9-based control system that eliminates DNA that had lost the UBP (Y. Zhang et al., 2017a). Subsequently, the team extended their system to code for nnAAs with the UBP (Y. Zhang et al., 2017b). This required transcription by T7 RNA polymerase and translation involving a tRNA containing the unnatural anticodon. As a proof-of-principle, the Romesberg lab successfully incorporated a nnAA into a fluorescent

protein using two codons that contain the UBP (Y. Zhang et al., 2017b). In a recent follow-up study, the Romesberg lab systematically generated and studied the functionality of unnatural codons using the dNaM-dTPT3 UBP (Fischer et al., 2020). Out of the 152 theoretical possible codons they identified nine functional unnatural codons that are stably integrated in DNA, transcribed into mRNA and tRNA, and finally can be decoded into nnAAs in proteins. Out of the nine, three unnatural codons also function orthogonal and can be used for simultaneously incorporating of multiple nnAAs into a single protein, thus allowing the decoding of 67 codons in a living semisynthetic organism (Fischer et al., 2020).

Together, this body of work demonstrates that the central dogma of life is not limited to four DNA bases and 20 amino acids. This in turn may allow us to design and construct new synthetic life forms that are different from natural ones at the molecular level. The work on synthetic genetic polymers (XNA) with different backbone chemistries than DNA and RNA also brings us closer to this goal (Pinheiro & Holliger, 2012; Pinheiro et al., 2012; Anosova et al., 2016; Hoshika et al., 2019). Not only can such synthetic life forms be exploited for applications such as biocontainment, therapeutics, and novel chemistry (Sun et al., 2014), but also to better understand the evolutionary constraints and benefits of the natural genetic code and the canonical nucleic and amino acids (Bacher et al., 2004; Koonin & Novozhilov, 2017).

4.2 Synthetic Karyotyping

The number of chromosomes varies widely in eukaryotic species. What happens if the number of chromosomes changes? Comparative studies between related species with different chromosome numbers are difficult to interpret due to the simultaneous presence of other changes in the genome, such as sequence divergence and genomic rearrangements. This makes it difficult to assign a given phenotypic feature to the difference in chromosome number. Synthetic biology allows us to change only the chromosome number, while maintaining the genetic content. Two studies successfully fused the 16 chromosomes of the budding yeast in successive rounds of chromosome fusion down to two chromosomes ($n = 2$) (J. Luo et al., 2018a) or a single giant chromosome ($n = 1$) (Shao et al., 2019) (Fig. 4b). Both groups used the CRISPR–Cas9 technology to remove the telomers and centromeres of the chromosomes and took advantage of the endogenous DNA repair machinery for chromosome fusion. Surprisingly, chromosome fusion had little impact on cell fitness, with only small fitness defects becoming apparent at $n = 2$ and $n = 1$. Also, the mating efficiency of strains with the same number of chromosomes was unaffected, e.g., $n = 2 \times n = 2$ were not different in sporulation efficiency compared to $n = 16 \times n = 16$. However, the larger the difference in chromosome number between the two mating partners, the fewer viable spores resulted from crossing. For example, $n = 16$ could only produce viable spores with strains having at least ten chromosomes, but not less. Therefore, despite having identical sequences,

a reduction to eight chromosomes is enough to isolate strains reproductively. This is interesting because the phenomenon of "reproductive isolation" is usually associated with sequence divergence rather than with the number of chromosomes (Greig, 2009). One avenue for future work with these strains might be to perform laboratory evolution experiments in order to investigate how they adapt to a reduced number of chromosomes (Liti, 2018).

4.3 Synthetic Viruses

Viruses and phages are one of the simplest biological systems in which genotype and phenotype are directly linked through encapsulation. However, they require a host to proliferate. Given the small genomes of viruses, it is not surprising that the first genomes to be chemically synthesized were that of viruses, namely that of the poliovirus in 2002 (Cello et al., 2002) and of the bacteriophage φX174 in 2003 (H. O. Smith et al., 2003). Building a synthetic virus only requires chemically synthesizing its genome and injecting it into the right host cells, which will then produce viral particles that are infectious. The synthesis of viral genomes has allowed reconstructing and characterizing viruses from past and current pandemics, such as the Spanish Flu virus from 1918 (Tumpey et al., 2005) and SARS-CoV-2 (Thao et al., 2020), and studying the effect of genome modifications on pathology as well as to test potential vaccine candidates (Wimmer & Paul, 2011).

The emergence of protein cages capable of encapsulating its DNA or RNA genome was probably the critical step in the evolution of viruses. Two studies describe the design, construction and evolution of viral-like capsids from nonviral proteins that encapsulate their own genetic information (Butterfield et al., 2017; Terasaka et al., 2018) (Fig. 4c). In both cases, the genome packaging and protection properties of the starting capsids were improved by carrying out several rounds of in vitro directed evolution. This approach quickly yielded mutants that could compete with recombinant virus vectors and thus established simple evolutionary pathways by which virus-like genome packaging can emerge. In the future, such synthetic capsids might be endowed with further properties such as cell recognition and infection, unloading and even self-replication (Lemire et al., 2018). Moreover, synthetic viruses and phages offer interesting alternatives to natural viruses and phages as vectors in drug delivery in therapeutics and vaccines or as platforms for phage display (Citorik et al., 2014).

4.4 Designing a Synthetic Minimal Genome

The minimum number of genes to sustain life is a fundamental question in biology. Researchers have approached this question theoretically by hypothesizing that a common set of genes shared between species with small genomes might be

a good approximation of how many genes are essential for life (Mushegian & Koonin, 1996). Complementary, scientists performed transposon mutagenesis on the *Mycoplasma genitalium* genome, which has the smallest genome of any organism that can be grown in pure culture, to identify essential genes for bacterial growth under laboratory conditions (Glass et al., 2006). Synthetic biology now offers tools to address this question by designing and synthesizing a minimal synthetic genome. In 2010, the team of Craig Venter generated the first bacterial cell controlled by a chemically synthesized *M. mycoides* genome (1077 kb), named JCVI-syn1.0 (Gibson et al., 2010). Although the JCVI-syn1.0 genome is nearly identical to the natural *M. mycoides* genome, it was an important technical milestone toward bottom-up genome designs. In 2016, the Venter team released JCVI-syn3.0, a reduced version of the JCVI-syn1.0 synthetic genome: JCVI-syn3.0 contains only 473 genes encoded on only 531 kb, which makes it the smallest genome of any autonomously replicating cell known so far. In addition to essential genes, it also contains quasi-essential genes that are required for robust growth with a doubling time of around 180 min (Hutchison et al., 2016). Thus, JCVI-syn3.0 is an approximation of a minimal cellular genome. Intriguingly, even in this simple organism the cellular function of 149 genes (≈31%) is still unknown. Thus, much has still to be learnt about what functions are required for life. Once the functions of all these genes are known, the genome size may potentially even be further reduced. In addition, knowing all the biochemical, structural and cellular functions essential for supporting life, one can start to design new organisms from these basic principles and learn much about the origin and evolution of life (Göpfrich et al., 2018; Forster & Church, 2006).

4.5 Synthetic Self-Replicating Systems in Cell-Like Compartments

Studying the factors that facilitated the emergence of life from chemical molecules is an exciting, but obviously also a challenging task. In the last decade, several groups have built simple self-replicating systems based on RNA molecules, which are hypothesized to have stored genetic information and at the same time catalyzed chemical reactions in primitive cells (Joyce & Szostak, 2018). A particular interesting question is how compartmentalization facilitated the early evolution of self-replicating molecules such as RNA and dealt with the emergence of parasitic mutants, which are replicated, but themselves are not replicating and thus would cause eventual collapse of the system (Ichihashi et al., 2013). Matsumura et al. (2016) evolved self-replicating RNA molecules in a scenario of repeated mixing and compartmentalization in nonbiological material, using a droplet-based microfluidic system, which provided protection from emerging parasitic mutants. The study supports the hypothesis that transient compartmentalization, e.g., in aerosols, micro-compartments in hydrothermal vents or on mineral surfaces, has facilitated evolution

before the first cell-like structures emerged (Ichihashi et al., 2013). In another study, Mizuuchi & Ichihashi extended a synthetic RNA self-replicating system to depend on cooperation, which is a necessary requirement for the evolution of higher complexity (Mizuuchi & Ichihashi, 2018). Emerging parasitic RNA molecules plagued self-replication and cooperation, but compartmentalization protected the system and supported cooperation. Interestingly, evolutionary adaptation through mutations toward higher replication efficiency was characterized by improved "selfish" replication and at the same time coevolution of the cooperators.

5 Outlook

Since its foundation in 2000, synthetic biology has undergone dramatic growth into a vibrant research discipline that is poised to provide fundamental insights into biological questions as well as to revolutionize many aspects of our lives, for example by producing smart materials, sustainable biofuels and personalized therapeutics (Cameron et al., 2014; Purnick & Weiss, 2009; Tang et al., 2020). This progress was enabled by improved and novel technologies, including better computational models, cheaper DNA sequencing, improved DNA synthesis, high-precision DNA editing tools such as CRISPR, novel tools for gene expression control, microfluidic devices, as well as high-throughput assembly and screening methods (Gach et al., 2017; H. Wang et al., 2016; Cobb et al., 2013; Santos-Moreno & Schaerli, 2020b). As reviewed here, synthetic biological systems also started to improve our understanding of fundamental evolutionary concepts. We predict that this process will continue, and we highlight here some research avenues where we expect interesting results in the near future.

The improvement in quality and speed of de novo DNA synthesis and assembly technologies and the accompanying reduced costs allowed the de novo synthesis of whole chromosomes and genomes, as discussed above for bacterial genomes. The Synthetic Yeast 2.0 consortium (www.syntheticyeast.org) is on its way to build the first synthetic eukaryotic genome, a synthetic version of *S. cerevisiae* genome, called Sc2.0. So far, the synthesis of six (out of 16) synthetic Sc2.0 chromosomes has been published and we expect soon the publication of the whole genome (Richardson et al., 2017; Kannan & Gibson, 2017). The next version of a synthetic yeast genome (Sc3.0) is also already in planning (Dai et al., 2020) with the aim for further compacting the synthetic chromosomes (Z. Luo et al., 2021). For now, the Sc2.0 features the following changes compared to the natural yeast genome: all TAG stop codons are changed to TAA, loxP sites are introduced after nonessential genes to allow increased evolutionary diversification using SCRaMbLE (synthetic chromosome rearrangement and modification by loxP-mediated evolution), and enhanced genome stability is achieved through removal of repeat elements, introns and relocation of all transfer RNAs to a new chromosome (Richardson et al., 2017). These design features not only increase its potential for biotechnology applications, but will also allow us to address fundamental

questions. For example, the SCRaMbLE technology, with its different versions, such as L-SCRaMbLE (Hochrein et al., 2018) (light-inducible and completely reversible), in vitro DNA SCRaMbLE (Wu et al., 2018), SCRaMbLE-in (W. Liu et al., 2018b) (combination of in vitro and in vivo recombination) and ReSCuES (Z. Luo et al., 2018b) (reporter of SCRaMbLEd cells using efficient selection), allows large-scale genome reshuffling to expand the evolutionary potential of budding yeast (Blount et al., 2018; L. Ma et al., 2019; Wightman et al., 2020). Evolution experiments with SCRaMbLEd synthetic genomes might provide crucial insights into speciation, minimal genome requirements and genome evolution (Szymanski & Calvert, 2018). The Genome Project-write (http://engineeringbiologycenter.org), aiming to synthesize the human and other genomes, is also poised to significantly advance genome-engineering technologies and provide new exciting platforms to study evolutionary questions (Boeke et al., 2016).

Another area that promises to yield fundamental insights into evolutionary systems biology questions is the building of a synthetic cell. Several interdisciplinary consortia, such as BaSyC (Building a Synthetic Cell, www.basyc.nl), MaxSynBio (www.maxsynbio.mpg.de) and Synthetic Cell Initiative (www.syntheticcell.eu) started to work on the ambitious goal to create a completely synthetic cell-like system that has characteristics of life, such as reproduction, metabolism, growth, compartmentalization, homeostasis, heredity, adaptation and communication. Although we are still far away from a synthetic cell that is truly "alive," simple cell-like systems that exhibit some of these characteristics have already been built (Vogele et al., 2018; van Nies et al., 2018; Lavickova et al., 2020; Buddingh & van Hest, 2017).

So far, we discussed how evolutionary systems biology can benefit from synthetic biology. However, the combination of the two disciplines is not a one-way road. Despite impressive progress, building synthetic systems rationally is often still not straightforward. Therefore, synthetic biologists increasingly choose to select or screen a library of different variants to obtain systems that function as desired (de Lorenzo, 2018; Szymanski & Calvert, 2018). By adopting the powerful method of directed evolution, which originates from protein engineering (Packer & Liu, 2015), functional synthetic systems can be obtained through screening or selecting from randomized or combinatorial libraries (Schaerli & Isalan, 2013; Duarte et al., 2017; Cobb et al., 2013). In fact, initial designs of computationally designed proteins (Giger et al., 2013; Blomberg et al., 2013), synthetic circuits (Yokobayashi et al., 2002), metabolic pathways (Bachmann, 2016), synthetic genomes (Wannier et al., 2018) and virus-like nucleocapsids (Butterfield et al., 2017; Terasaka et al., 2018) have been optimized with directed evolution. In many of these cases, rational design and modeling alone could not have identified the necessary modifications to optimize the systems. Indeed, various techniques based on evolutionary principles have been developed such as MAGE (multiplex automated genome engineering) (H. H. Wang et al., 2009), SEER (serial enrichment for efficient recombineering) (Wannier et al., 2020) DIvERGE (directed evolution with random genomic mutations) (Nyerges et al., 2018), CAGE (conjugative assembly genome engineering) (Isaacs et al., 2011), PACE (phage-assisted continuous evolution) (Esvelt et al., 2011) and

eVOLVER (automated high-throughput growth experiments) (Wong et al., 2018) and help to achieve ambitious goals in synthetic biology.

In conclusion, the combination of synthetic and evolutionary systems biology is proving to be a successful partnership. As showcased in this book chapter, the application of synthetic biology to address evolutionary questions has already produced promising results. In the future, the combination of synthetic systems and evolutionary experiments promises to deliver further exciting fundamental insights into the principles of molecular evolution.

Acknowledgments We thank Sara Mitri, Nienke Jager, and members of the Schaerli group for critical reading and valuable feedback. We acknowledge support by the Swiss National Science Foundation grant 31003A_175608.

References

Acar, M., Mettetal, J. T., & van Oudenaarden, A. (2008). Stochastic switching as a survival strategy in fluctuating environments. *Nature Genetics, 40*(4), 471–475.

Ackermann, M. (2015). A functional perspective on phenotypic heterogeneity in microorganisms. *Nature Reviews Microbiology, 13*(8), 497–508.

Agarwal, K. L., et al. (1970). Total synthesis of the gene for an alanine transfer ribonucleic acid from yeast. *Nature, 227*(5253), 27–34.

Ajo-Franklin, C. M., et al. (2007). Rational design of memory in eukaryotic cells. *Genes & Development, 21*(18), 2271–2276.

Anosova, I., et al. (2016). The structural diversity of artificial genetic polymers. *Nucleic Acids Research, 44*(3), 1007–1021.

Arnold, F. H. (2010). How proteins adapt: Lessons from directed evolution. *Cold Spring Harbor Symposia on Quantitative Biology, 74*(0), 41–46.

Arnoldini, M., et al. (2014). Bistable expression of virulence genes in *Salmonella* leads to the formation of an antibiotic-tolerant subpopulation. *PLoS Biology, 12*(8), e1001928.

Arranz-Gibert, P., Vanderschuren, K., & Isaacs, F. J. (2018). Next-generation genetic code expansion. *Current Opinion in Chemical Biology, 46*, 203–211.

Bacher, J. M., et al. (2004). Evolving new genetic codes. *Trends in Ecology & Evolution, 19*(2), 69–75.

Bachmann, B. O. (2016). Applied evolutionary theories for engineering of secondary metabolic pathways. *Current Opinion in Chemical Biology, 35*, 133–141.

Barbier, I., Perez-Carrasco, R., & Schaerli, Y. (2020). Controlling spatiotemporal pattern formation in a concentration gradient with a synthetic toggle switch. *Molecular Systems Biology, 16*(6), 611.

Bashor, C. J., & Collins, J. J. (2018). Understanding biological regulation through synthetic biology. *Annual Review of Biophysics, 47*, 399–423.

Bashor, C. J., et al. (2010). Rewiring cells: Synthetic biology as a tool to interrogate the organizational principles of living systems. *Annual Review of Biophysics, 39*, 515–537.

Baumstark, R., et al. (2015). The propagation of perturbations in rewired bacterial gene networks. *Nature Communications, 6*, 10105.

Becskei, A., & Serrano, L. (2000). Engineering stability in gene networks by autoregulation. *Nature, 405*, 590–593.

Blain, J. C., & Szostak, J. W. (2014). Progress toward synthetic cells. *Annual Review of Biochemistry, 83*, 615–640.

Blomberg, R., et al. (2013). Precision is essential for efficient catalysis in an evolved Kemp eliminase. *Nature, 503*(7476), 418–421.

Blount, B. A., et al. (2018). Rapid host strain improvement by in vivo rearrangement of a synthetic yeast chromosome. *Nature Communications, 9*(1), 1932.

Bódi, Z., et al. (2017). Phenotypic heterogeneity promotes adaptive evolution. *PLoS Biology, 15*(5), e2000644.

Boeke, J. D., et al. (2016). The genome project-write. *Science, 353*(6295), 126–127.

Briscoe, J., & Thérond, P. P. (2013). The mechanisms of Hedgehog signalling and its roles in development and disease. *Nature Reviews Molecular Cell Biology, 14*(7), 416–429.

Buddingh, B. C., & van Hest, J. C. M. (2017). Artificial cells: Synthetic compartments with life-like functionality and adaptivity. *Accounts of Chemical Research, 50*(4), 769–777.

Butterfield, G. L., et al. (2017). Evolution of a designed protein assembly encapsulating its own RNA genome. *Nature, 552*(7685), 415–420.

Cameron, D. E., Bashor, C. J., & Collins, J. J. (2014). A brief history of synthetic biology. *Nature Reviews Microbiology, 12*(5), 381–390.

Cello, J., Paul, A. V., & Wimmer, E. (2002). Chemical synthesis of poliovirus cDNA: Generation of infectious virus in the absence of natural template. *Science, 297*(5583), 1016–1018.

Chari, R., & Church, G. M. (2017). Beyond editing to writing large genomes. *Nature Reviews Genetics, 18*(12), 749–760.

Chau, A. H., et al. (2012). Designing synthetic regulatory networks capable of self-organizing cell polarization. *Cell, 151*(2), 320–332.

Chin, J. W. (2014). Expanding and reprogramming the genetic code of cells and animals. *Annual Review of Biochemistry, 83*(1), 379–408.

Chin, J. W. (2017). Expanding and reprogramming the genetic code. *Nature, 550*(7674), 53–60.

Chin, J. W., et al. (2003). An expanded eukaryotic genetic code. *Science, 301*(5635), 964–967.

Ciliberti, S., Martin, O. C., & Wagner, A. (2007). Robustness can evolve gradually in complex regulatory gene networks with varying topology. *PLoS Computational Biology, 3*(2), e15.

Citorik, R. J., Mimee, M., & Lu, T. K. (2014). Bacteriophage-based synthetic biology for the study of infectious diseases. *Current Opinion in Microbiology, 19*, 59–69.

Cobb, R. E., Si, T., & Zhao, H. (2012). Directed evolution: An evolving and enabling synthetic biology tool. *Current Opinion in Chemical Biology, 16*(3-4), 285–291.

Cobb, R. E., Sun, N., & Zhao, H. (2013). Directed evolution as a powerful synthetic biology tool. *Methods, 60*(1), 81–90.

Cotterell, J., & Sharpe, J. (2010). An atlas of gene regulatory networks reveals multiple three-gene mechanisms for interpreting morphogen gradients. *Molecular Systems Biology, 6*, 1–14.

Crocker, J., & Ilsley, G. R. (2017). Using synthetic biology to study gene regulatory evolution. *Current Opinion in Genetics & Development, 47*, 91–101.

Dai, J., et al. (2020). Sc3.0: Revamping and minimizing the yeast genome. *Genome Biology, 21*(1), 205.

Davidson, E. A., Windram, O. P. F., & Bayer, T. S. (2012). Building synthetic systems to learn nature's design principles. *Advances in Experimental Medicine and Biology, 751*, 411–429.

Davies, J. (2017). Using synthetic biology to explore principles of development. *Development, 144*(7), 1146–1158.

de Lorenzo, V. (2018). Evolutionary tinkering vs. rational engineering in the times of synthetic biology. *Life Sciences, Society and Policy, 14*(1), 18.

Dean, A. M., & Thornton, J. W. (2007). Mechanistic approaches to the study of evolution: The functional synthesis. *Nature Reviews Genetics, 8*(9), 675–688.

Duarte, J. M., Barbier, I., & Schaerli, Y. (2017). Bacterial microcolonies in gel beads for high-throughput screening of libraries in synthetic biology. *ACS Synthetic Biology, 6*(11), 1988–1995.

Elowitz, M. B., & Leibler, S. (2000). A synthetic oscillatory network of transcriptional regulators. *Nature, 403*(6767), 335–338.

Esvelt, K. M., Carlson, J. C., & Liu, D. R. (2011). A system for the continuous directed evolution of biomolecules. *Nature, 472*(7344), 499–503.

Fischer, E. C., et al. (2020). New codons for efficient production of unnatural proteins in a semisynthetic organism. *Nature Chemical Biology, 16*, 570–576.
Forster, A. C., & Church, G. M. (2006). Towards synthesis of a minimal cell. *Molecular Systems Biology, 2*, 45.
Fredens, J., et al. (2019). Total synthesis of *Escherichia coli* with a recoded genome. *Nature, 569*(7757), 514–518.
Friedland, A. E., et al. (2009). Synthetic gene networks that count. *Science, 324*(5931), 1199–1202.
Gach, P. C., et al. (2017). Droplet microfluidics for synthetic biology. *Lab on a Chip, 17*(20), 3388–3400.
Gardner, T. S., Cantor, C. R., & Collins, J. J. (2000). Construction of a genetic toggle switch in *Escherichia coli*. *Nature, 403*(6767), 339–342.
Gibson, D. G., et al. (2010). Creation of a bacterial cell controlled by a chemically synthesized genome. *Science, 329*(5987), 52–56.
Giger, L., et al. (2013). Evolution of a designed retro-aldolase leads to complete active site remodeling. *Nature Chemical Biology, 9*(8), 494–498.
Glass, J. I., et al. (2006). Essential genes of a minimal bacterium. *Proceedings of the National Academy of Sciences, 103*(2), 425–430.
Göpfrich, K., Platzman, I., & Spatz, J. P. (2018). Mastering complexity: Towards bottom-up construction of multifunctional eukaryotic synthetic cells. *Trends in Biotechnology, 36*(9), 938–951.
Greig, D. (2009). Reproductive isolation in *Saccharomyces*. *Heredity, 102*(1), 39–44.
Guet, C. C., et al. (2002). Combinatorial synthesis of genetic networks. *Science, 296*(5572), 1466–1470.
Haimovich, A. D., Muir, P., & Isaacs, F. J. (2015). Genomes by design. *Nature Reviews Genetics, 16*(9), 501–516.
Hammerling, M. J., et al. (2014). Bacteriophages use an expanded genetic code on evolutionary paths to higher fitness. *Nature Chemical Biology, 10*(3), 178–180.
Haseltine, E. L., & Arnold, F. H. (2007). Synthetic gene circuits: Design with directed evolution. *Annual Review of Biophysics and Biomolecular Structure, 36*(1), 1–19.
Hill, M. S., Zande, P. X. T. V., & Wittkopp, P. J. (2020). Molecular and evolutionary processes generating variation in gene expression. *Nature Reviews Genetics, 22*, 203–215.
Hochrein, L., et al. (2018). L-SCRaMbLE as a tool for light-controlled Cre-mediated recombination in yeast. *Nature Communications, 9*(1), 1931.
Holland, S. L., et al. (2014). Phenotypic heterogeneity is a selected trait in natural yeast populations subject to environmental stress. *Environmental Microbiology, 16*(6), 1729–1740.
Hoshika, S., et al. (2019). Hachimoji DNA and RNA: A genetic system with eight building blocks. *Science, 363*, 884–887.
Hutchison, C. A., et al. (2016). Design and synthesis of a minimal bacterial genome. *Science, 351*(6280), aad6253.
Ichihashi, N., et al. (2013). Darwinian evolution in a translation-coupled RNA replication system within a cell-like compartment. *Nature Communications, 4*, 2494.
Isaacs, F. J., et al. (2011). Precise manipulation of chromosomes in vivo enables genome-wide codon replacement. *Science, 333*(6040), 348–353.
Isalan, M., et al. (2008). Evolvability and hierarchy in rewired bacterial gene networks. *Nature, 452*(7189), 840–845.
Jiménez, A., et al. (2015). Dynamics of gene circuits shapes evolvability. *Proceedings of the National Academy of Sciences of the United States of America, 112*(7), 2103–2108.
Joyce, G. F., & Szostak, J. W. (2018). Protocells and RNA self-replication. *Cold Spring Harbor Perspectives in Biology, 10*(9), a034801.
Kaltenbach, M., & Tokuriki, N. (2014). Dynamics and constraints of enzyme evolution. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 322*(7), 468–487.
Kaneko, K. (2007). Evolution of robustness to noise and mutation in gene expression dynamics. *PLoS One, 2*(5), e434.
Kannan, K., & Gibson, D. G. (2017). Yeast genome, by design. *Science, 355*(6329), 1024–1025.

Koonin, E. V., & Novozhilov, A. S. (2017). Origin and evolution of the universal genetic code. *Annual Review of Genetics, 51*(1), 45–62.

Kuwahara, H., & Soyer, O. S. (2012). Bistability in feedback circuits as a byproduct of evolution of evolvability. *Molecular Systems Biology, 8*, 564.

Lagator, M., et al. (2017). Regulatory network structure determines patterns of intermolecular epistasis. *eLife, 6*, 1–22.

Lajoie, M. J., et al. (2013). Genomically recoded organisms expand biological functions. *Science, 342*(6156), 357–360.

Lavickova, B., Laohakunakorn, N., & Maerkl, S. J. (2020). A partially self-regenerating synthetic cell. *Nature Communications, 11*, 1–11.

Lee, K. Y., et al. (2018). Photosynthetic artificial organelles sustain and control ATP-dependent reactions in a protocellular system. *Nature Biotechnology, 36*(6), 530–535.

Lehner, B. (2011). Molecular mechanisms of epistasis within and between genes. *Trends in Genetics, 27*(8), 323–331.

Lemire, S., Yehl, K. M., & Lu, T. K. (2018). Phage-based applications in synthetic biology. *Annual Review of Virology, 5*(1), 453–476.

Li, P., et al. (2018). Morphogen gradient reconstitution reveals Hedgehog pathway design principles. *Science, 360*(6388), 543–548.

Liti, G. (2018). Yeast chromosome numbers minimized using genome editing. *Nature, 560*(7718), 317–318.

Liu, C. C., & Schultz, P. G. (2010). Adding new chemistries to the genetic code. *Annual Review of Biochemistry, 79*, 413–444.

Liu, C. C., et al. (2018a). Toward an orthogonal central dogma. *Nature Chemical Biology, 14*(2), 103–106.

Liu, W., et al. (2018b). Rapid pathway prototyping and engineering using in vitro and in vivo synthetic genome SCRaMbLE-in methods. *Nature Communications, 9*, 1–12.

Luo, J., et al. (2018a). Karyotype engineering by chromosome fusion leads to reproductive isolation in yeast. *Nature, 560*(7718), 392–396.

Luo, Z., et al. (2018b). Identifying and characterizing SCRaMbLEd synthetic yeast using ReSCuES. *Nature Communications, 9*(1), 1930.

Luo, Z., et al. (2021). Compacting a synthetic yeast chromosome arm. *Genome Biology, 22*, 1–18.

Ma, L., et al. (2019). SCRaMbLE generates evolved yeasts with increased alkali tolerance. *Microbial Cell Factories, 18*, 1–11.

Ma, N. J., & Isaacs, F. J. (2016). Genomic recoding broadly obstructs the propagation of horizontally transferred genetic elements. *Cell Systems, 3*(2), 199–207.

Malyshev, D. A., et al. (2014). A semi-synthetic organism with an expanded genetic alphabet. *Nature, 509*(7500), 385–388.

Mangan, S., & Alon, U. (2003). Structure and function of the feed-forward loop network motif. *Proceedings of the National Academy of Sciences, 100*(21), 11980–11985.

Matsumura, S., et al. (2016). Transient compartmentalization of RNA replicators prevents extinction due to parasites. *Science, 354*(6317), 1293–1296.

Mizuuchi, R., & Ichihashi, N. (2018). Sustainable replication and coevolution of cooperative RNAs in an artificial cell-like system. *Nature Ecology & Evolution, 2*(10), 1654–1660.

Mukai, T., et al. (2017). Rewriting the genetic code. *Annual Review of Microbiology, 71*, 557–577.

Mukherji, S., & van Oudenaarden, A. (2009). Synthetic biology: Understanding biological design from synthetic circuits. *Nature Reviews Genetics, 10*(12), 859–871.

Mushegian, A. R., & Koonin, E. V. (1996). A minimal gene set for cellular life derived by comparison of complete bacterial genomes. *Proceedings of the National Academy of Sciences, 93*(19), 10268–10273.

Neumann, H., et al. (2010). Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome. *Nature, 464*(7287), 441–444.

Nghe, P., et al. (2020). Predicting evolution using regulatory architecture. *Annual Review of Biophysics, 49*(1), 181–197.

Nielsen, J., & Keasling, J. D. (2016). Engineering cellular metabolism. *Cell, 164*(6), 1185–1197.

Niu, W., Schultz, P. G., & Guo, J. (2013). An expanded genetic code in mammalian cells with a functional quadruplet codon. *ACS Chemical Biology, 8*(7), 1640–1645.

Nyerges, Á., et al. (2018). Directed evolution of multiple genomic loci allows the prediction of antibiotic resistance. *Proceedings of the National Academy of Sciences, 115*(25), E5726–E5735.

Paaby, A. B., & Rockman, M. V. (2013). The many faces of pleiotropy. *Trends in Genetics, 29*(2), 66–73.

Packer, M. S., & Liu, D. R. (2015). Methods for the directed evolution of proteins. *Nature Reviews Genetics, 16*(7), 379–394.

Pál, C., Papp, B., & Pósfai, G. (2014). The dawn of evolutionary genome engineering. *Nature Reviews Genetics, 15*(7), 504–512.

Payne, J. L., & Wagner, A. (2019). The causes of evolvability and their evolution. *Nature Reviews Genetics, 20*(1), 24–38.

Peisajovich, S. G. (2012). Evolutionary synthetic biology. *ACS Synthetic Biology, 1*(6), 199–210.

Pinheiro, V. B., & Holliger, P. (2012). The XNA world: Progress towards replication and evolution of synthetic genetic polymers. *Current Opinion in Chemical Biology, 16*(3-4), 245–252.

Pinheiro, V. B., et al. (2012). Synthetic genetic polymers capable of heredity and evolution. *Science, 336*(6079), 341–344.

Purnick, P. E. M., & Weiss, R. (2009). The second wave of synthetic biology: From modules to systems. *Nature Reviews Molecular Cell Biology, 10*(6), 410–422.

Raman, R., Pinto, C. S., & Sonawane, M. (2018). Polarized organization of the cytoskeleton: Regulation by cell polarity proteins. *Journal of Molecular Biology, 430*(19), 3565–3584.

Richardson, S. M., et al. (2017). Precise, automated control of conditions for high-throughput growth of yeast and bacteria with eVOLVER. *Science, 355*(6329), 1040–1044.

Rockman, M. V., & Kruglyak, L. (2006). Genetics of global gene expression. *Nature Reviews Genetics, 7*(11), 862–872.

Ruder, W. C., Lu, T., & Collins, J. J. (2011). Synthetic biology moving into the clinic. *Science, 333*(6047), 1248–1252.

Salathé, M., Van Cleve, J., & Feldman, M. W. (2009). Evolution of stochastic switching rates in asymmetric fitness landscapes. *Genetics, 182*(4), 1159–1164.

Sánchez-Romero, M. A., & Casadesús, J. (2013). Contribution of phenotypic heterogeneity to adaptive antibiotic resistance. *Proceedings of the National Academy of Sciences of the United States of America, 111*(1), 355–360.

Santos-Moreno, J., & Schaerli, Y. (2018). Using synthetic biology to engineer spatial patterns. *Advanced Biosystems, 12*, 1800280.

Santos-Moreno, J., & Schaerli, Y. (2020a). Changing the biological Rosetta stone: The (commercial) potential of recoded microbes. *Microbial Biotechnology, 13*(1), 11–13.

Santos-Moreno, J., & Schaerli, Y. (2020b). CRISPR-based gene expression control for synthetic gene circuits. *Biochemical Society Transactions, 48*(5), 1979–1993.

Santos-Moreno, J., et al. (2020). Multistable and dynamic CRISPRi-based synthetic circuits. *Nature Communications, 11*, 1–8.

Sato, K., et al. (2003). On the relation between fluctuation and response in biological systems. *Proceedings of the National Academy of Sciences, 100*(24), 14086–14090.

Schaerli, Y., & Isalan, M. (2013). Building synthetic gene circuits from combinatorial libraries: Screening and selection strategies. *Molecular BioSystems, 9*(7), 1559–1567.

Schaerli, Y., et al. (2014). A unified design space of synthetic stripe-forming networks. *Nature Communications, 5*, 4905.

Schaerli, Y., et al. (2018). Synthetic circuits reveal how mechanisms of gene regulatory networks constrain evolution. *Molecular Systems Biology, 14*(9), e8102.

Shao, Y., et al. (2019). Creating a functional single-chromosome yeast. *Nature, 560*(7718), 331–335.

Simon, A. J., d'Oelsnitz, S., & Ellington, A. D. (2019). Synthetic evolution. *Nature Biotechnology, 37*(7), 730–743.

Smith, H. O., et al. (2003). Generating a synthetic genome by whole genome assembly: phiX174 bacteriophage from synthetic oligonucleotides. *Proceedings of the National Academy of Sciences, 100*(26), 15440–15445.

Smith, J. M. (1992). Evolutionary biology - Byte-sized evolution. *Nature, 355*(6363), 772–773.

Sun, S. B., Schultz, P. G., & Kim, C. H. (2014). Therapeutic applications of an expanded genetic code. *ChemBioChem, 15*(12), 1721–1729.

Szymanski, E., & Calvert, J. (2018). Designing with living systems in the synthetic yeast project. *Nature Communications, 9*(1), 2950.

Tabor, J. J., et al. (2009). A synthetic genetic edge detection program. *Cell, 137*(7), 1272–1281.

Tang, T.-C., et al. (2020). Materials design by synthetic biology. *Nature Reviews Materials*, 1–19.

Terasaka, N., Azuma, Y., & Hilvert, D. (2018). Laboratory evolution of virus-like nucleocapsids from nonviral protein cages. *Proceedings of the National Academy of Sciences of the United States of America, 115*(21), 5432–5437.

Thao, T., et al. (2020). Rapid reconstruction of SARS-CoV-2 using a synthetic genomics platform. *Nature, 582*, 561–565.

Tumpey, T. M., et al. (2005). Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science, 310*(5745), 77–80.

van Nies, P., et al. (2018). Self-replication of DNA by its encoded proteins in liposome-based synthetic cells. *Nature Communications, 9*(1), 1583.

Vogele, K., et al. (2018). Towards synthetic cells using peptide-based reaction compartments. *Nature Communications, 9*(1), 3862.

Wang, H., La Russa, M., & Qi, L. S. (2016). CRISPR/Cas9 in genome editing and beyond. *Annual Review of Biochemistry, 85*, 227–264.

Wang, H. H., et al. (2009). Programming cells by multiplex genome engineering and accelerated evolution. *Nature, 460*(7257), 894–898.

Wang, K., Schmied, W. H., & Chin, J. W. (2012). Reprogramming the genetic code: From triplet to quadruplet codes. *Angewandte Chemie International Edition in English, 51*(10), 2288–2297.

Wang, L., et al. (2001). Expanding the genetic code of *Escherichia coli*. *Science, 292*, 498–500.

Wang, Y.-H., Wei, K. Y., & Smolke, C. D. (2013). Synthetic biology: Advancing the design of diverse genetic systems. *Annual Review of Chemical and Biomolecular Engineering, 4*(1), 69–102.

Wannier, T. M., et al. (2018). Adaptive evolution of genomically recoded *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America, 115*(12), 3090–3095.

Wannier, T. M., et al. (2020). Improved bacterial recombineering by parallelized protein discovery. *Proceedings of the National Academy of Sciences of the United States of America, 117*(24), 13689–13698.

Weber, W., & Fussenegger, M. (2011). Emerging biomedical applications of synthetic biology. *Nature Reviews Genetics, 13*(1), 21–35.

Wightman, E. L. I., et al. (2020). Rapid optimisation of cellulolytic enzymes ratios in *Saccharomyces cerevisiae* using in vitro SCRaMbLE. *Biotechnology for Biofuels, 13*, 1–10.

Wimmer, E., & Paul, A. V. (2011). Synthetic poliovirus and other designer viruses: What have we learned from them? *Annual Review of Microbiology, 65*(1), 583–609.

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *Journal of Theoretical Biology, 25*(1), 1–47.

Wong, B. G., et al. (2018). Precise, automated control of conditions for high-throughput growth of yeast and bacteria with eVOLVER. *Nature Biotechnology, 36*(7), 614–623.

Wu, Y., et al. (2018). In vitro DNA SCRaMbLE. *Nature Communications, 9*(1), 1935.

Xie, M., & Fussenegger, M. (2018). Designing cell function: Assembly of synthetic gene circuits for cell biology applications. *Nature Reviews Molecular Cell Biology, 19*, 507–525.

Yokobayashi, Y., Weiss, R., & Arnold, F. H. (2002). Directed evolution of a genetic circuit. *Proceedings of the National Academy of Sciences, 99*(26), 16587–16591.

Zeymer, C., & Hilvert, D. (2018). Directed evolution of protein catalysts. *Annual Review of Biochemistry, 87*, 131–157.

Zhang, W., Mitchell, L. A., Bader, J. S., & Boeke, J. D. (2020). Synthetic genomes. *Annual Review of Biochemistry, 89*(1), 77–101.

Zhang, W. H., Otting, G., & Jackson, C. J. (2013). Protein engineering with unnatural amino acids. *Current Opinion in Structural Biology, 23*(4), 581–587.

Zhang, Y., & Romesberg, F. E. (2018). Semisynthetic organisms with expanded genetic codes. *Biochemistry, 57*(15), 2177–2178.

Zhang, Y., et al. (2017a). A semisynthetic organism engineered for the stable expansion of the genetic alphabet. *Proceedings of the National Academy of Sciences of the United States of America, 114*(6), 1317–1322.

Zhang, Y., et al. (2017b). A semi-synthetic organism that stores and retrieves increased genetic information. *Nature, 551*(7682), 644–647.

An Evolutionary Systems Biology View on Metabolic System Structure and Dynamics

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Abstract Cellular metabolism consists of many interconnected reactions that present feedbacks through cyclic reaction motifs and through metabolite regulation of enzyme kinetics. In addition, metabolism is interlinked with gene regulation and other cellular, energy-driven processes such as division and motility. While many important insights have been gained on metabolism in the last decades, we are still far from a complete, predictive understanding of it. This is reflected in our current, limited ability to pinpoint the drivers of metabolic system dynamics and devising ways to engineer it.

In this review paper, we argue that the study of metabolism through the lens of evolutionary biology can provide further insights into its structure and dynamics. By structure, we mean the composing reactions of a metabolic system, and how these reactions are connected with each other through shared metabolites, while by dynamics, we mean the temporal behaviour and responses of the resulting metabolic system. Following an introductory section, we summarise the key findings on the structure and dynamics of cellular metabolism within an evolutionary systems

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_8

perspective in Sects. 2 and 3. In doing so, we highlight two key ways of thinking about metabolic systems, one based on considering metabolism optimised for biomass production, and another one based on considering metabolism as a self-regulating emergent system for maintaining nonequilibrium metabolic fluxes. From this second consideration, we then expand to discuss the possible biophysical drivers that could have played a key role in shaping metabolic systems in Sect. 4. Finally, in Sect. 5, we call for an evolutionary perspective on metabolism that takes into account both of the above considerations. We conclude by highlighting key areas of future research where this combined view can provide valuable insights.

1 Introduction

Metabolism is the collective biochemical reaction set through which cells convert available chemicals from the environment into energy and cell components. Cells cannot and could not have existed without metabolism, thus, metabolic systems must have been very early inventions in the evolution of life, possibly emerging before cells. Such an ancient character of metabolism is evident in the fact that many metabolic reactions and systems are conserved across a range of uni- and multicellular organisms (Yamada et al., 2006). These highly conserved aspects of metabolism, as well as variations across organisms may provide a clue back to the chemical conditions during early origins of life and the current environments of organisms.

Discussions regarding the origin of life usually emphasise the need for self-replication and information carrier molecules. The emergence of such molecules must have been linked tightly with the emergence of early metabolic systems that provided the building blocks needed for their synthesis. Such systems might have had an abiotic nature initially (Branscomb & Russell, 2013; Keller et al., 2014, 2017; Messner et al., 2017), but it is possible that their structure and tight linkage with reproduction came to be embedded into the fabric of current-day biological systems. Indeed, it is difficult to separate metabolism from the other aspects of cell physiology, including membrane potential (Merrins et al., 2016), gene expression and regulation (Chubukov et al., 2014; You et al., 2013), motility (Egbert et al., 2010), and cell division (Papagiannakis et al., 2017). It is this linkage that makes an understanding of cell metabolism a prerequisite for a complete understanding of cell physiology. Besides its importance as a fundamental research topic, metabolism and its connections to cell physiology relate to biotechnological applications such as bioproduction in microbial or mammalian cells, and to medicine, including possible cancer treatment (Jain et al., 2012; Locasale, 2013).

Evolutionary considerations in the study of metabolism are not rare (Nam et al., 2011; Papp et al., 2009) but do commonly take an adaptive stance that views metabolism as optimised for biomass production. While there have been cases where laboratory evolution experiments resulted in metabolic adaptations matching such optimality predictions (Ibarra et al., 2002; Fong & Palsson, 2004), there are

also many instances where solely adaptive explanations cannot capture observations from metabolic systems (Papp et al., 2009; Schuster et al., 2008). In this review, we advocate the importance of considering also the possibility of metabolic system features emerging through nonadaptive mechanisms, as by-products of biophysical and biochemical requirements on maintaining metabolic fluxes out of equilibrium. In particular, the first emergence of metabolic systems and their early evolution must have been driven by achieving nonequilibrium flux across system boundaries, and the drivers for achieving such a state must have influenced system structure and dynamics in ways that is still visible in current-day metabolism. We argue that such an extended, evolutionary view on metabolism can provide better insights on '*why the metabolism is the way it is*', and in turn, lead to new ways of understanding and engineering current metabolic systems.

In the following two sections, we first review the current knowledge on the structure (i.e. composition and connectedness) and dynamics (i.e. temporal behaviour of metabolic fluxes) of metabolic systems and discuss them from the perspective of evolutionary systems biology. Rather than providing an exhaustive summary of the vast literature on metabolism, we highlight findings that we believe are representative or of key importance to our current thinking and understanding of metabolism and its evolution. In Sect. 4, we consider the possible key biological and biophysical factors that might drive and constrain the evolution of metabolic systems. Finally, in Sect. 5, we provide a future outlook that evaluates how taking an evolutionary systems biology approach to metabolism can open up new ways of understanding and engineering metabolic systems.

2 Structural Features of Metabolic Systems

The elucidation of the structure of metabolic systems starts with the pioneering biochemical studies of early 1900s. These Nobel-winning studies mapped key metabolic conversions within cells onto specific enzymes and organised these into so-called 'pathways' (Gottschalk, 1986). Known today mostly through the names of their discoverers, these include the Entner–Doudoroff (ET), Embden–Meyerhof–Parnas (EMP) and pentose-phosphate (PP) pathways involved in glucose uptake and conversion into pyruvate, and the Krebs pathway (a.k.a. tricarboxylic acid cycle) involved in the conversion of pyruvate into biomass precursors (Neidhardt et al., 1990). As biochemical studies continue to define new enzymatic reactions and pathways, organised databases of enzyme function (e.g. KEGG: Kanehisa, 2013; BRENDA: Jeske et al., 2019) allow us to increasingly achieve biochemical annotation through evolutionary relatedness among enzymes. This, together with increasing sequencing ability allows obtaining the lists of enzymatic reactions from genome sequences and derive insights on how cellular metabolism is organised in different organisms. It must be noted, however, that any analysis of reaction maps will be limited by the accuracy of such maps, which will relate to the quality of enzyme function annotations (derived biochemically or from genomic sequences by

homology). Setting aside such limitations, the study of metabolic reaction maps has flourished in the last few decades with the application of graph theory.

2.1 Metabolic Maps as Graphs (Networks)

Conceptually, it is easy to make the transition from a biochemist's drawing of a metabolic reaction pathway to a mathematically well-defined graph or network representation (see Fig. 1). Indeed, metabolites can readily be envisioned as nodes in a network connected through enzymatic reactions. The actual practice of mapping a biochemical reaction set into a network, however, can be done in several different ways that preserve or lose different types of information (Sandefur et al., 2012; Montañez et al., 2010; Zhou & Nakhleh, 2011; Arita, 2004; Beber et al., 2012) (Fig. 1).

In one approach, networks are defined such that there are two sets of nodes in the network; the molecular species in one set and the reactions themselves in the other set. This approach defines a bipartite graph, where the edges connect nodes from elements of one set (species nodes) to elements of the other set (reaction nodes) only (Fig. 1b). To make this network representation simpler, a logical first step is to combine the set of reaction nodes with their corresponding edges across the network. This results in a unipartite graph representation with only one type of node, which corresponds to the metabolites. Edges in this representation correspond to reactions that involve (or connect) the associated nodes (metabolites) (Fig. 1c). While some information on reaction mechanisms is lost in the unipartite graph representation, it is commonly used in databases and many graph theoretical network analyses. The latter aspect is important, because the representation of a metabolic system as a uni- or bipartite graph can have direct impact on the results of common network analyses such as degree distribution and modularity (Montañez et al., 2010; Zhou & Nakhleh, 2011; Arita, 2004; Beber et al., 2012) (see legend of Fig. 1).

2.2 Connectivity Within Metabolic Networks

Notwithstanding the importance of choices associated with the abstraction of a metabolic system as a network, the analyses of the resulting networks using graph theory can provide insights into their large-scale properties. An early, key finding in this regard was that the connectivity distributions of metabolic systems are more aligned with a scale-free distribution. For this distribution the probability of finding a node with connectivity k, scales with $k^{-\gamma}$, with γ being a constant degree exponent. This contrasts with a Poisson distribution expected from a random network (Jeong et al., 2000). The scale-free like distribution indicates the presence

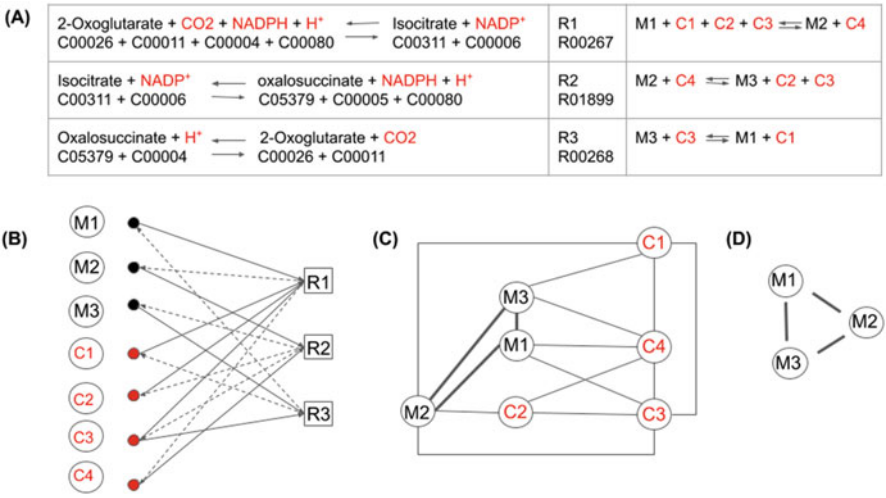

Fig. 1 Cartoon diagram showing a selection of approaches to capture biochemical reaction information as a graph, and possible discrepancies that can arise from this choice. We consider a small section of the central metabolism as an illustrative example. (**a**). The system contains three enzymatic reactions labelled as R1, R2 and R3, as well as with their KEGG reaction IDs (R00267, R01899, and R00268). The main metabolites {M1, M2 and M3} and additional ones {C1, C2, C3 and C4} are highlighted in black and red font respectively. (**b**) A directed bipartite graph corresponding to the reaction system given in (**a**). The enzyme information can be encoded within the reaction class (rectangular boxes), while substrates and products are represented by a metabolite class (circles). Solid and dashed lines represent involvement of substrates and products respectively in each reaction. (**c**). An undirected unipartite graph representing the same system. A reduced graphical complexity has been obtained by omitting the enzymatic information (i.e. the reaction class). Note that this results in the loss of the specificity of products and substrates towards reactions. (**d**) Another alternative graph representation with further reduction of complexity. Additional metabolites C1, C2, C3 and C4 have been absorbed into the graph. A comparison between these different graphs shows differences in graph-based statistics such as centrality and node degree; for example metabolites M1, M2 and M3 have different degrees {4, 5, 4}, in (**c**), but the same degree of 2 in (**d**)

of highly connected nodes, so-called hubs, in the system. It has been argued that these highly connected metabolites, that include the well-known energy carriers (such as ATP and GTP) and reductive equivalents (such as NADH and NADPH), represent evolutionarily ancient parts of the system (Wagner & Fell, 2001).

In order to better understand if any specific connectivity distribution could infer a functional (or evolutionary) benefit to a network, it is necessary to consider possible mechanisms that can generate such distribution. A general, empirical model for network expansion, which can lead to a scale-free connectivity distribution has been proposed and involves the preferential attachment of new nodes onto existing ones based on their connectivity (Ravasz et al., 2002). While the general nature of this model is useful to construct random networks with scale-free connectivity distribution, it has been shown that alternative, and biochemically more realistic

network expansion schemes can also lead to networks that display such connectivity distributions (Keller, 2005; Salathé et al., 2005; Takemoto & Akutsu, 2008; Pastor-Satorras et al., 2003). Additionally, *in silico* evolution of toy metabolic systems of enzymatic reactions, under selection for supporting growth, has shown to lead to the emergence of networks with scale-free connectivity distribution and hubs (Pfeiffer et al., 2005; Hintze & Adami, 2008). In one such study, the initial system that was composed of broad-specificity enzymes evolved, under selection for faster growth, into one that is composed of enzymes with high specificity, some of which highly connected, i.e. enzyme specificity and hubs have emerged (Pfeiffer et al., 2005). However, whether hubs emerged or not was dependent on the presence/absence of group transfer reactions in the toy metabolism, suggesting that the observed connectivity distributions in metabolic networks might be a by-product of the nature of biochemical reactions involved. An alternative theory suggests that the observed connectivity distributions (and in particular the presence of hubs) have emerged from selection for increased robustness of metabolic systems against loss of enzymes, since presence of hubs can infer to the system such robustness (Jeong et al., 2000). This argument, however, is not fully supported by subsequent analyses. In particular, it is indicated that the robustness of metabolic systems to enzyme loss is *apparent*; many of the enzymes that can be seemingly dispensable under a metabolically rich environment (suggesting high robustness to enzyme loss) are actually required in some other, metabolically limited environment (Papp et al., 2004). This kind of apparent robustness has emerged under *in silico* evolution experiments, where toy metabolic models were evolved under selection for growth under fluctuating environments (Soyer & Pfeiffer, 2010).

In summary, these findings show that the general connectivity distribution of metabolic networks is different from random ones, but might not have been directly selected for during evolution and rather resulted as a by-product of other evolutionary forces acting on metabolic systems. More broadly, they highlight the need for any adaptive arguments proposed for the possible evolutionary origins and significance of any structural network features to be evaluated carefully against simpler and possibly nonadaptive explanations (Papp et al., 2009; Zhou & Nakhleh, 2011; Basler et al., 2012).

2.3 Modules in Metabolic Networks

Another structural feature observed in graph representation of metabolic networks is the presence of clusters, or modules, where member nodes in a module present higher connectivity among themselves compared to the rest of the network (Ravasz et al., 2002; Guimerà & Amaral, 2005). It is shown that many of these modules correspond to known pathways that are described based on biochemical studies (Guimerà & Amaral, 2005). As with connectivity distributions, several evolutionary mechanisms have been proposed that can lead to the emergence of modular networks, including selection for increased enzyme specialisation (Soyer, 2007;

Espinosa-Soto & Wagner, 2010) or robustness to gene loss (Ravasz et al., 2002), or growth under static (Takemoto, 2012), randomly fluctuating (Hintze & Adami, 2008), or structured environments (Lipson et al., 2002; Kashtan & Alon, 2005). Modularity being the result of selection for robustness, is not supported by a subsequent study, which found no relation between robustness against genetic and metabolic perturbations and modularity in computationally generated toy models of metabolic systems (Holme, 2011). Modularity emerging from selection for growth under fluctuating or modular environments had a mixed support from subsequent analyses. Analysis of network modularity in different organisms that were grouped according to the environmental variability that they experience suggested some correlation between the two (Parter et al., 2007). Analysis of randomly generated metabolic networks also suggested a relation between modularity (based on flux distributions) and ability of a metabolic system to sustain growth in different simulated environments (Samal et al., 2011). Several other analyses found distinct levels of modularity for organisms living in different habitats or living different lifestyles (e.g. auto- vs. heterotrophic) (Kreimer et al., 2008; Takemoto & Borjigin, 2011; Mazurie et al., 2010), however there was no simple correlation between possible indicators of environmental variability (for example number of transport enzymes) and network modularity (Kreimer et al., 2008). Few studies found instead a strong correlation between habitat temperature and the level of metabolic modularity (Takemoto et al., 2007; Takemoto & Akutsu, 2008). *In silico* evolution of a toy metabolic system under fluctuating environments has found no positive relation between the level of modularity emerging over an evolutionary period and the level of environmental fluctuation over that time (Hintze & Adami, 2008). Taken together, these findings leave it inconclusive at the moment if the nature and frequency of environmental fluctuations had a strong influence on the selection of the level of network modularity.

It must be noted that any analysis of modularity in a network will depend on the definition of modularity and its quantification, as well as the choice of system abstraction used for the network representation. Methods that are alternative to those based on simple graph representation of metabolic systems have been attempted to define and measure modules (Kanehisa, 2013; Yamada et al., 2006; Muto et al., 2013; Sorokina et al., 2015). Perhaps the most straightforward of these is to define metabolic modules based on sets of reactions that are associated with each other according to the information in the literature. The KEGG database for example employs this strategy to define 'metabolic modules', which contain reactions resulting from literature-based pathway definitions, or are associated to a set of enzymes that are shown to either form a larger complex or are organised together on the genome (Kanehisa, 2013). The latter aspect is further developed into a 'phylogenetically' motivated metabolic module description, where modules are identified from the phylogenetic distribution profiles of individual enzymes and their 'connectedness' within metabolic reaction maps (Yamada et al., 2006). This approach indicated that enzymes that are closely connected (in terms of how many reactions separate their substrates) share more similar phylogenetic distributions across sequenced genomes. While plausible, this finding needs to be reevaluated in

light of phylogenetic distributions of modules corrected for phylogenetic relatedness of the organisms that they are found in, since such a correction is shown to impact the level of 'true' modularity in all biological networks analysed (Snel & Huynen, 2004).

Another alternative approach is to define modules based on reaction similarities. In this case, all known reactions are grouped into categories based on the similarity of the atomic conversions that they enable and then are used to analyse existing, known metabolic systems for common reaction patterns. This approach has allowed identification of so-called reaction modules, which represent patterns of specific reaction groups reoccurring in different parts of metabolism (Muto et al., 2013; Sorokina et al., 2015). While some of these reaction modules correspond to aforementioned, enzyme-based metabolic modules, some of them represent unique modules (Kanehisa, 2013). The existence of such modules suggests that despite the differences in the enzymes employed, different parts of metabolism employ similar reactions and that metabolic system evolution might be driven by adaptation of existing reactions to 'work' on new metabolites (Schmidt et al., 2003). Supporting this suggestion, simulating the evolution of metabolic systems through a reaction-based expansion algorithm, where new reactions are added to a network when their corresponding metabolites are made available by previous reactions or by the environment, showed that the resulting expanded networks display similar properties as those observed in nature (Ebenhoh et al., 2004; Ebenhöh et al., 2005; Raymond & Segrè, 2006). These findings can be interpreted as metabolic systems having emerged as a core set of chemical reactions, which are then reused in slightly different biochemical contexts when that network expanded to accommodate new molecules (Raymond & Segrè, 2006; Muto et al., 2013). This reaction-based view of metabolic evolution would be in agreement with an enzyme-based evolutionary scenario that considers early enzymes of low specificity subsequently diverging and specialising on different substrates, while maintaining a core set of reaction types (Jensen, 1976; Schmidt et al., 2003; Pfeiffer et al., 2005). It is important to note, in this context, that many of the current-day enzymes display promiscuous (low specificity) functions that are shown to facilitate the evolution of new pathways within the metabolic system (Kim et al., 2010; Soo et al., 2011).

2.4 Network Motifs

An alternative structural analysis of metabolic systems abstracted as networks, is to search for small interaction patterns within them that are overrepresented in the original network compared to randomised networks serving as null models. These patterns, or so-called motifs, were first identified in signalling and transcription networks (Milo et al., 2002). These two types of networks are found to display different motif prevalence, suggestive of a link between network function and type of motifs present (Milo et al., 2004). Indeed, subsequent studies have shown

that several of the motifs found in signalling and transcription networks can embed specific functions in a dynamical context, including noise filtering in signal transduction, and decoupling of the expression speed and level, in gene regulation (Goentoro et al., 2009; Mangan & Alon, 2003; Mangan et al., 2006; Alon, 2007; Lipshtat et al., 2008).

These findings, where overrepresented motifs from specific networks display specific dynamical and functional properties that are relevant to the overall function of those networks, suggest that motifs could provide the link between structural and dynamical (or functional) analyses of networks. This possibility, however, is called into question both in terms of statistical significance of overrepresented motifs and in terms of how specific their functional and dynamical properties are. The identification of overrepresented motifs is directly influenced by the choice of null models that the original, analysed networks are compared to (Artzy-Randrup, 2004). This raises the issue of identifying a suitable null model for the network being analysed, with alternative null models giving rise to different motif significance results (Konagurthu & Lesk, 2008; Avetisov et al., 2010). Even if overrepresented motifs are correctly identified, their functional significance is difficult to assess. For example, the bi-fan motif, identified in gene regulatory motifs and suggested to display specific dynamical properties, displays a range of dynamics under different parameter sets and modelling choices (Ingram et al., 2006). Similarly, an analysis of all three-node signalling network motifs indicated dependence of their response dynamics on the specifics of biochemical implementation choices in the model used (Soyer et al., 2006).

Identification and analysis of network motifs in metabolic systems are subject to these same issues as well. While specific metabolic network motifs were identified as significant (Eom et al., 2006), it was subsequently shown that this result is dependent both on the original network representation used and the randomised networks used for comparison (Beber et al., 2012).

3 Dynamics of Metabolic Systems

Cell metabolism is a dynamical process that converts an initial set of environmentally available metabolites into a set of end products that are released into the environment or incorporated into biomass (Fig. 2). While many reactions take part in this process, an overall chemical reaction can be written to describe the full conversion from substrates to end products. This overall reaction takes the form of a redox reaction, indicating that cell metabolism enables the flux of electrons across many reactions and between an initial electron donor and a final electron acceptor (Gottschalk, 1986) (Fig. 2a). It is shown that these intercoupled reactions, the involvement of conserved moieties in many of them, and metabolite-mediated allosteric regulation of enzymes (Fig. 2b) can all lead to rich temporal dynamics including oscillations and multi-stability (Reich & Sel'kov, 1981). These

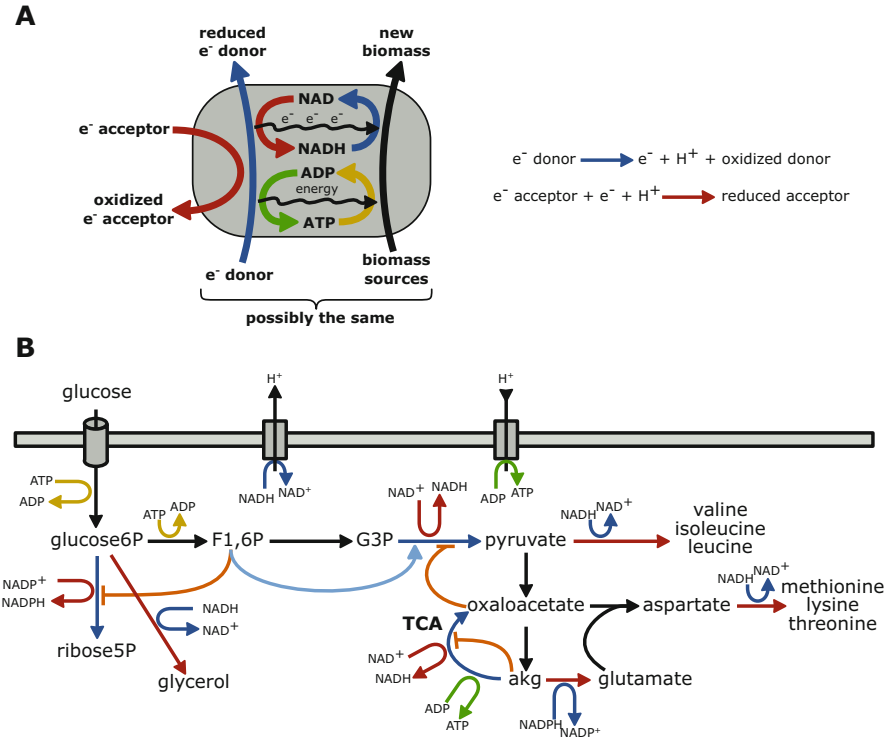

Fig. 2 Schematic representation of the chemical transformations carried out by a cell. (**a**) Representation of metabolic reactions at cell scale, considering the cell itself as a black box. From this standpoint, the phenomenon of cell growth appears as the combination of a catabolic and an anabolic reaction, as well as energy extraction from redox reactions that transfer electrons from an initial electron donor to a final electron acceptor. (**b**) More detailed representation of metabolic reactions, where the focus is shifted from the cell to its metabolic network, and highlighting both the many redox reactions and other types of chemical conversions. The metabolites are linked by chemical reactions (black arrows), sometimes involving the conversion of conserved moieties that are involved in redox (e.g. NAD/NADH, yellow for reduction and green for oxidation) and energy (e.g. ADP/ATP, red for energy investment and blue for energy extraction) balances. The upkeep of conserved moieties' balances can involve interaction with protein complexes embedded in the membrane (shown in grey). Some of the enzymes catalysing the reactions of the metabolic network are regulated by the concentration of metabolites, through allosteric regulations (shown as light blue and orange arrows)

nonlinear dynamics, on their own, or in combination with gene regulation, can then give rise to dynamic cellular behaviours. One of the key, open challenges in metabolic research is to decipher these higher-level metabolic behaviours and pinpoint structural features in the network that can be used as their explanatory and predictive indicators.

3.1 Overflow Metabolism and the Respiration-Fermentation Switch

Perhaps one of the earliest dynamical observation on higher-level behaviour of metabolism is a shift from pure respiration into fermentation or respiro-fermentation with changing conditions. This shift, known as contre-effet Pasteur, Warburg, or, Crabtree effect, is described initially in yeast and mammalian cells (De Deken, 1966). In a respiratory mode, metabolism utilises a strong electron acceptor such as oxygen and the associated, membrane bound electron transport chain. In fermentative mode, instead, metabolism utilises weak organic acids as electron acceptors and associated pathways, which provide a stoichiometric balance between reactions oxidising and reducing the key electron carriers (such as NAD^+ and NADH) (Gottschalk, 1986). Cells are found to display a shift from respiration to fermentation with changing availability of electron acceptors or carbon source, and with increasing growth rate (De Deken, 1966; Christen & Sauer, 2011; Meyer et al., 1984; Nanchen et al., 2006; Schulze & Lipe, 1964; Valgepea et al., 2010; Luli & Strohl, 1990; Postma et al., 1989; Rieger et al., 1983; Dauner et al., 2001). While a shift into fermentative pathways due to lack of strong electron acceptors can be intuitively understood as the only route to sustain electron flow, a similar shift due to increased carbon availability or growth rate are nonintuitive as they occur under the continued presence of strong electron acceptors such as oxygen and despite higher energy efficiency of respiration.

It has been suggested that a switch into fermentative pathways in presence of oxygen, happens due to limitations on the respiratory chain and associated pathways (Postma et al., 1989; Rieger et al., 1983). In this argument, the increasing carbon flow cannot be sustained by respiration alone, and any overflow needs to be directed into fermentative pathways to maintain stoichiometric balances and an assumed optimal growth (Majewski & Domach, 1990; Varma et al., 1993). This 'limitation-based' view, is extended by recent studies, which have argued for cellular space (Szenk et al., 2017; Zhuang et al., 2011) or protein content being key limiting factors that can favour fermentation over respiration, because fermentation is more efficient with regards to these features compared to respiration (i.e. higher energy produced per protein or space investment) (Molenaar et al., 2009; Basan et al., 2015; Schuster et al., 2011; Goelzer & Fromion, 2017; Wortel et al., 2018). The idea that overall protein amounts can be limiting is linked to the observation that increasing growth rates, where a switch to fermentation happens, results in an increased investment from the shared proteome pool into ribosomes (Klumpp et al., 2009) and that this can cause a shift into the more protein efficient fermentation (Basan et al., 2015; Schuster et al., 2011). Recent temporal measurements on proteome allocation, however, do not necessarily show a shift in the expression of the enzymes involved in central metabolism vs. respiration when cells undergo a respiration-to-fermentation switch (Goel et al., 2015; Metzl-Raz et al., 2017). Moreover, the fact that not all yeast species exhibit the Crabtree effect (De Deken, 1966) indicates that

the extent and dynamics of the Crabtree effects can be tuned, instead of being the result of an insurmountable limitation arising from resource allocation.

A plausible alternative explanation for the onset of a respiration-fermentation shift is the dynamics of the oxidised and reduced forms of key redox carriers within metabolism. These forms are involved in many of the reactions of the central metabolism, including glycolysis, TCA cycle, and pathways branching from these, as well as the respiratory chain (Fig. 2b). Thus, it is possible that shifts in the NAD^+/NADH balance can directly affect the flux distribution across these different pathways (Hatakeyama & Furusawa, 2017; Reich & Sel'kov, 1981). It is observed, both in yeast and *E. coli*, that altering NADH/NAD^+ dynamics with synthetically incorporated oxidases alters the critical level of glucose, at which the respiration-to-fermentation switch happens (Vemuri et al., 2006, 2007). Additionally, it is found that changes in the activity of pyruvate kinase, a key enzyme implicated in cancer cells' increased capacity for the respiration-fermentation switch (Diaz-Ruiz et al., 2009), can cause a feedback onto the dynamics of NADPH, which acts both as an electron carrier and a neutraliser of reactive oxygen species through redox reactions (Grüning et al., 2011). It is also possible to envision similar limitations, and impacts on metabolic fluxes, arising from ATP/ADP balances. Indeed, ATP balance is implicated to affect metabolic fluxes under different conditions in *B. subtilis*, including conditions favouring a respiration-fermentation switch (Dauner et al., 2001). That limitations arising from ATP/ADP and NAD^+/NADH balances can cause changes in overall metabolic fluxes, and lead to metabolic switches and overflows, is an attractive mechanistic explanation that could explain several additional metabolic overflows such as of amino acids and vitamins (Dauner et al., 2001; Jiang et al., 2018; Ponomarova et al., 2017).

The proposition that metabolic shifts are due to certain cellular limitations is an interesting concept to consider through the lens of evolution. It can be argued that any limitations on cellular resources can be overcome under appropriate selective pressures. If such limitations have not been overcome, then this is suggestive that they are either linked to physical hard bounds that evolution cannot surpass, or relate to trade-offs between different selective pressures. One such trade-off is proposed between growth rate and yield (Novak et al., 2006; Schuster et al., 2011; Bachmann et al., 2013; Wortel et al., 2018). For example, increasing the number of ATP producing reactions in a linear pathway would slow its overall flux rate, presenting a simple mechanism of a rate-yield trade-off (Pfeiffer et al., 2001; Heinrich et al., 1991). Such a thermodynamic basis for a rate-yield trade-off, combined with the higher energy yield of respiration, is used to argue for it driving the respiration-to-fermentation shift (Pfeiffer et al., 2001).

The respiration-to-fermentation shift, or overflow metabolism, results in the excretion of organic acids from cells, such as acetate and lactate. These organics can be used as a carbon source by other cells and result in a so-called cross-feeding interaction. It has been theoretically shown that trade-offs among uptake efficiencies of different carbon sources can lead to cells evolve into specialists on one of such carbon sources, creating the basis for the emergence of cross-feeding even within a single population (Doebeli, 2002). Indeed, long-term evolution experiments with

Escherichia coli resulted in the emergence of different clones that are shown to interact metabolically through cross-feeding (Le Gac et al., 2008; Rozen & Lenski, 2000; Grosskopf & Soyer, 2016).

3.2 Carbon Preference and Catabolic Pathway Switching

Another phenomenon observed with overall metabolic dynamics is the 'carbon preference' or 'carbon catabolic repression'. It was found that when microbes are cultivated in the presence of a mix of different substrates that they can catabolise, consumption follows a sequential pattern (Görke & Stülke, 2008; Monod, 1949), with an order of carbon sources specific to the microbial species (Collier et al., 1996; Van Den Bogaard et al., 2000; Parche et al., 2006). While it is shown that such carbon source switching can involve genetic regulatory networks with possible bistable dynamics (Ozbudak et al., 2004; Solopova et al., 2014; van Hoek & Hogeweg, 2006), it is less clear how the initial sensing of different carbon sources is achieved and conveyed to the regulatory level. One simplistic explanation is that carbon sources are directly sensed to trigger activation and inhibition of the associated downstream catabolic pathways to enforce the carbon preference hierarchy. This mechanism has been shown to be implemented in a few bacteria, but only for a small subset of their possible substrates (Aidelberg et al., 2014). Recent studies suggest that the adaptation of the microbial metabolic networks is not modulated by direct detection of the concentration of the entry-point substrates, but rather by the temporal dynamics of the concentration of key metabolites inside the network. It is proposed that there could be sensing of the metabolic fluxes through these key metabolites, which can react with transcription factors and thus affect the expression of catabolic enzymes to create feedback systems (Kotte et al., 2010; Görke & Stülke, 2008; Aidelberg et al., 2014).

A kinetic model of metabolism and gene regulation featuring central carbon pathways and several regulatory interactions between specific metabolites and transcription factors has allowed successful simulation of the carbon preference hierarchy (Kotte et al., 2010). Analysis of this model suggested that a given metabolite can become a 'flux-reporting', key metabolite, either because it is only produced under specific environmental conditions, or because it sits between two low-energy reactions, where its concentration can act as a reporter of the flux direction at this point in the metabolic system (Kotte et al., 2010). For example, in the *E. coli* metabolic system, fructose-1,6-bisphosphate, an intermediate of glycolysis, and its interaction with the transcription factor Cra is implicated as a flux-sensing regulatory system (Kochanowski et al., 2013).

Allosteric regulation of enzymes, that is regulation of enzyme activity through binding of substrates and products or additional metabolites, is another possible route to altering metabolic fluxes. This is possibly an evolutionary more ancient route to regulating metabolic systems, as it would not require gene regulation and the intermediary role of transcription factors. Additionally, allosteric regulation could allow for a quicker response to changing environmental conditions. Information on

the allosteric regulation of many enzymes is available and catalogued in databases (Jeske et al., 2019; Huang et al., 2011), and its regulatory role is implicated in several computational and experimental studies. The dynamic variations of many fluxes in the metabolic network of *B. subtilis* were not correlated to the expression level of its enzymes, suggesting that other means of regulation were at play, including allosteric regulation (Chubukov et al., 2013). Recent experimental work shows that allosteric regulation is required in addition to transcriptional control to explain the observed flux dynamics during catabolic repression and co-utilisation in *B. subtilis* (Buffing et al., 2018). Similarly, in *E. coli*, explaining metabolic flux shifts in response to changes in the nature of the carbon source required multiple regulatory layers including allosteric regulation (Link et al., 2013; Gerosa et al., 2015). A metabolic network model implementing such candidate allosteric regulations was able to predict flux dynamics under changing substrate availability (Machado et al., 2015).

3.3 Oscillations and Bistability

The high-level observations of pathway switching and catabolic hierarchy indicate that metabolic system fluxes can be abruptly altered upon changes in conditions. Such dynamics are suggestive of multistable, nonlinear dynamics, which could be expected from any system that displays high interconnectedness as seen in metabolic systems; many metabolites are acted upon by many different enzymes, individual enzymes can form dimers and heteromers that can bind multiple substrates and additional, nonsubstrate metabolites, and multiple reactions can connect through their metabolites to form cyclic or feedback reaction systems. These features provide a significant potential for metabolic systems to implement nonlinear dynamics such as bistability, oscillation, and homeostasis (Reich & Sel'kov, 1981).

Among these, bistability refers to a dynamical system that can attain two different steady states depending on initial conditions. Changes from one of these steady states to the other can be caused through perturbations in parameters or concentrations of system components. In the context of metabolic systems, two steady states would manifest themselves as different flux rates across reactions and perturbations can arise from changes in enzyme or metabolite concentrations, or through changes in catalytic rates of enzymes (induced for example through allosteric regulation). Bistability in metabolic system dynamics has been implicated in the context of respiration-to-fermentation switch (Lei et al., 2003), and when carbon metabolism is initiated on glucose (van Heerden et al., 2014) or switches from glucose to other carbon sources (Kotte et al., 2014; Şimşek & Kim, 2018; Ozbudak et al., 2004; Solopova et al., 2014). In particular, the latter studies found subpopulations, within isogenic populations, that show different metabolic behaviours not caused by mutations. In glucose-shift experiments, additional experiments with isotope labelled carbon indicated that these subpopulations emerged at the time of the shift, i.e. in response to changing conditions, and in a manner dependent on the concentrations of the different carbon source (Kotte et al., 2014). This suggests

that the metabolic system implements bistable dynamics, such that changes in external glucose concentrations can lead some cells to shift to a new metabolic steady-state flux distribution. Indeed, mathematical models implementing bistability are proposed to explain these experimental observations, in some cases involving transcriptional feedback in addition to metabolic dynamics (Kotte et al., 2014; Ozbudak et al., 2004; Solopova et al., 2014), and in other cases just the metabolic dynamics (Planqué et al., 2014).

In terms of bistability arising solely from metabolic system dynamics, there have been many theoretical studies indicating the possibility of bistability within simple enzymatic reaction systems (Fig. 3a). For example, bistability is shown to

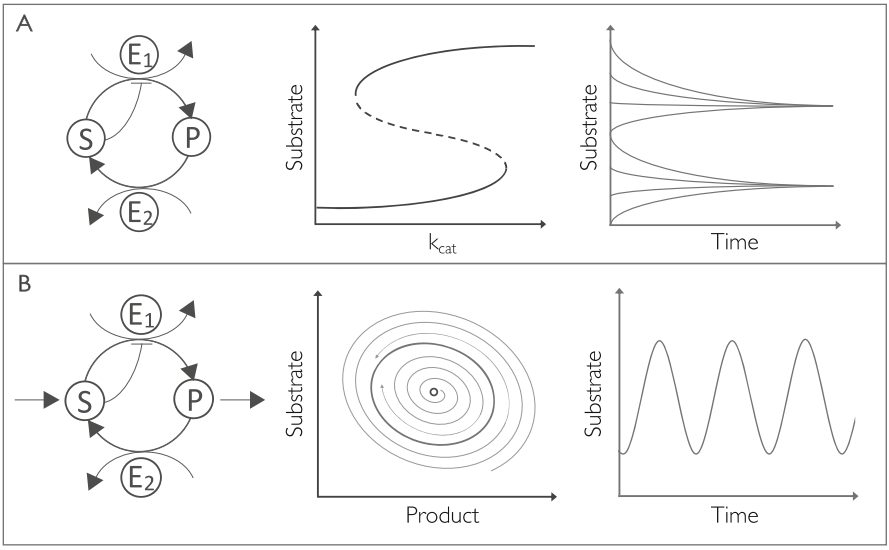

Fig. 3 Two cyclic reaction motifs and their corresponding dynamics. Note that such cyclic reaction motifs are readily found in natural metabolic systems, and in particular for reactions involving NAD^+/NADH-linked dehyrogenases (e.g. isocitrate and dihydrolipoyl dehydrogenases) and kinase-phosphatase pairs (e.g. phosphofructokinase—fructosebiphosphate pair). Reaction motifs are shown as cartoons with labelled circles representing substrate (S), product (P) and enzymes (E_1 and E_2). (**a**) Substrate (S) is converted into product (P) and then back again by two different enzymes (E_1 and E_2). These two enzymatic reactions can involve additional and different substrates and products (not shown) such that thermodynamic feasibility of the cycle is ensured. The activity of E_1 is substrate inhibited, that is, high concentrations of substrate reduce the rate of substrate to product conversion. Such a motif can display bistability under certain parameter conditions as shown in the subsequent panels. The middle panel shows the steady-state concentration of the substrate against the catalytic constant for E_1 (k_{cat}), while the last panel shows the time evolution of the substrate concentration, simulated from different starting conditions. (**b**) The same reaction motif as in (**a**), with the addition of flux into substrate from an external reservoir or upstream reaction (not shown) and flux out from the product into a downstream reaction (not shown). In this case, substrate concentration may oscillate within certain parameter regimes as shown in the subsequent panels. The first panel shows the steady-state concentration of product against the steady-state concentration of substrate, while the second panel shows the evolution of substrate concentration over time

be possible in systems of few coupled enzymatic reactions (Edelstein, 1971; Reich & Sel'kov, 1981). A particular 'reaction motif' that has been studied extensively is a two-enzyme cyclic reaction system, where a substrate is converted into a product and then back again, with both forward and backward reactions usually involving different co-substrates. It is common that the enzyme catalysing the forward reaction is regulated through substrate inhibition or substrate inhibition coupled with product activation (Hervagault & Canu, 1987; Cimino & Hervagault, 1990; Simonet et al., 1996; Guidi & Goldbeter, 1998; Mulukutla et al., 2014). This motif is found in several locations within metabolism, particularly around dehydrogeneases such as lactate dehydrogenase (Simonet et al., 1996), and kinase/phosphatase pairs such as those involved around fructose-6-phosphate (Mulukutla et al., 2014). These reactions convert different metabolites back and forth, using the NAD^+/NADH or ADP/ATP pairs as reaction partners. The theoretical findings from such cyclic reaction models were further supported by several in vitro re-constitution experiments that confirmed bistability experimentally and that were performed with different pyruvate kinase, lactate dehydrogenase, and isocitrate dehydrogenase enzymes and their corresponding partners in creating cyclic reaction schemes (Cimino & Hervagault, 1990; Simonet et al., 1996; Guidi et al., 1998).

In addition to bistability, threshold dynamics known as ultrasensitivity can arise from metabolic branching points (LaPorte et al., 1984), and can also lead to heterogeneities in metabolic phenotypes. Both ultrasensitivity and bistability are manifested by nonlinear 'input–output' relations, where the output of a system can change its steady-state value abruptly at a threshold value of a specific parameter of the system (Fig. 3a). Thus, if these dynamics are coupled with intrinsic or external noise in a relevant parameter across a population of cells, heterogenous metabolic outputs and cellular phenotypes can be observed. In this context, it is notable that significant level of noise or variance is seen in several metabolic parameters, including sugar uptake (Nikolic et al., 2013, 2017), ATP levels (Yaginuma et al., 2014), and expression levels of the enzymes involved in glycolysis and the TCA cycle (Rosenthal et al., 2018).

The same basic models that show bistable behaviour (as discussed above) can readily be extended with in- and out-fluxes of involved metabolites, to display oscillations (Fig. 3b) (Higgins, 1964; Sel'kov, 1968; Guidi & Goldbeter, 1998, 2000; Goldbeter & Guilmot, 1996). While these theoretical demonstrations of specific enzymatic schemes leading to oscillations have not been explored in detail in vitro, metabolic oscillations are readily observed both in vivo (Satroutdinov et al., 1992; Richard et al., 1993, 1996; Keulers et al., 1996; Sohn et al., 2000; Wittmann et al., 2005; Dodd & Kralj, 2017; Papagiannakis et al., 2017) and in situ, with cell extracts (Boiteux et al., 1975; Frenkel, 1968; Chance et al., 1964). In the latter case, both damped and sustained oscillations are observed, usually with a phase ranging from few to tens of minutes. It is possible that these oscillations relate to artificial changes in ATP dynamics arising from cell extract preparations (Frenkel, 1968), however, the fact that oscillations could be entrained by controlled glucose additions (Boiteux et al., 1975), show that there is an inherent ability for oscillatory dynamics in the underpinning enzymatic system. This ability is suggested to be linked

to the enzyme phosphofructokinase (PFK), which catalyses the phosphorylation of fructose-6-phosphate into fructose-diphosphate (Laurent et al., 1979). Several mathematical models of this enzymatic reaction, and incorporating the observed allosteric regulation of PFK both by its substrates and products, confirm the possibility of sustained oscillations (Higgins, 1964; Sel'kov, 1968; Goldbeter & Lefever, 1972).

In the case of intact cells, oscillatory dynamics are observed to occur within the central carbon pathways and displaying a phase of tens of minutes (Satroutdinov et al., 1992) up to several hours (Wittmann et al., 2005; Papagiannakis et al., 2017). Metabolic oscillations were demonstrated at single-cell level and are found to be autonomous but coupled with cell cycle oscillations (Papagiannakis et al., 2017). Additional studies across cell populations found that cells can synchronise metabolic oscillations under some conditions (Satroutdinov et al., 1992; Richard et al., 1993), and proposed several possible mediators including acetaldehyde, hydrogen sulphide, carbon dioxide, and pH (Richard et al., 1996; Keulers et al., 1996; Sohn et al., 2000; Dodd & Kralj, 2017). Models, involving some of these proposed synchronisation molecules, were also developed (Wolf & Heinrich, 2000; Wolf et al., 2001) and could reproduce experimental findings.

4 Evolutionary and Physical Drivers (and Constraints) on Metabolic Systems

We have highlighted, so far, a diverse range of structural and dynamical features of metabolism. We would argue that despite this accumulated wealth of information, we still lack a predictive understanding of metabolism at a systems level. For example, for many of the observed dynamics, it is not clear what their causative mechanisms are and how they could be influenced with external and internal perturbations. Additionally, for many of the observed structural features, we do not know what their functional significance are. Answering these open questions, as well as better conceptualising metabolic systems and devising new means to influencing their behaviour can benefit from identification of evolutionary drivers and constraints. While we have alluded to specific evolutionary arguments and studies in the above sections on observed properties, here we would like to summarise additional evolutionary and biophysical drivers and constraints relating to metabolism.

4.1 Thermodynamics

As collections of chemical reactions, metabolic systems must obey the laws of thermodynamics (Alberty, 2005). An active metabolic system remains away from

thermodynamic equilibrium through interactions of cells with external energy sources including molecular, thermal or pH gradients, and photo radiation, and as such, metabolism and its link to cellular growth can be described through the formalisms of nonequilibrium thermodynamics (Cannon & Baker, 2017; Westerhoff et al., 1983; Hellingwerf et al., 1982; Desmond-Le Quéméner & Bouchez, 2014; Goldbeter, 2018). Thus, considering thermodynamics-based ideas and criteria can provide insights into the evolution and present organisation of metabolic systems. For example, it has been shown that the utilisation of low-energy reactions that are more prone to thermodynamic inhibition due to product accumulation can allow relaxation of the ecological competitive-exclusion principle, and lead to co-existence of diverse species implementing different metabolic conversions, starting from a single substrate (Grosskopf & Soyer, 2016). Such low-energy reactions are present within cellular metabolism itself (Fig. 4), suggesting that metabolic pathway diversification could have evolved as a way to overcome thermodynamic bottlenecks. Other studies made similar suggestions for thermodynamics playing a key role in determining the overall organisation of cellular metabolism (Bar-Even et al., 2012a, b) and metabolically interacting multiple species (Vallino, 2010). In the latter case, computational simulations showed that applying the theory of 'entropy maximisation' in a way such that entropy production is maximised in a toy metabolic model over a time span and across a varying environment, results in a system behaviour similar to that observed from experimental microbial microcosms (Vallino, 2010). It remains to be seen if this type of optimisation can also explain the organisation of cellular metabolism or not.

Besides its possible role in shaping metabolic system organisation, thermodynamic constraints could also be directly influencing their temporal dynamics. Evidence for this possibility comes from the observation that many reactions within central carbon metabolism have free energies of reaction close to zero (Miller & Smith-Magowan, 1990; see also Fig. 4 legend). These reactions can become thermodynamic bottlenecks or reverse pathway flux direction under certain conditions (González-Cabaleiro et al., 2013; Dauner et al., 2001), thereby becoming influencing points for metabolic system dynamics. Thus, a combination of measuring metabolic concentrations and assessing reaction thermodynamics can allow an understanding of metabolic fluxes within a system, or conditions for enabling a certain flux distribution (Kümmel et al., 2006; Bennett et al., 2009; Noor et al., 2014). It is also possible that thermodynamic limitations under certain conditions can serve a regulatory or feedback role. For example, it is indicated that some proportion of observed flux shifts with changing carbon sources are explained by changes in reaction thermodynamics (Gerosa et al., 2015) and the excretion or consumption of acetate can be thermodynamically controlled by external acetate concentration (Enjalbert et al., 2017).

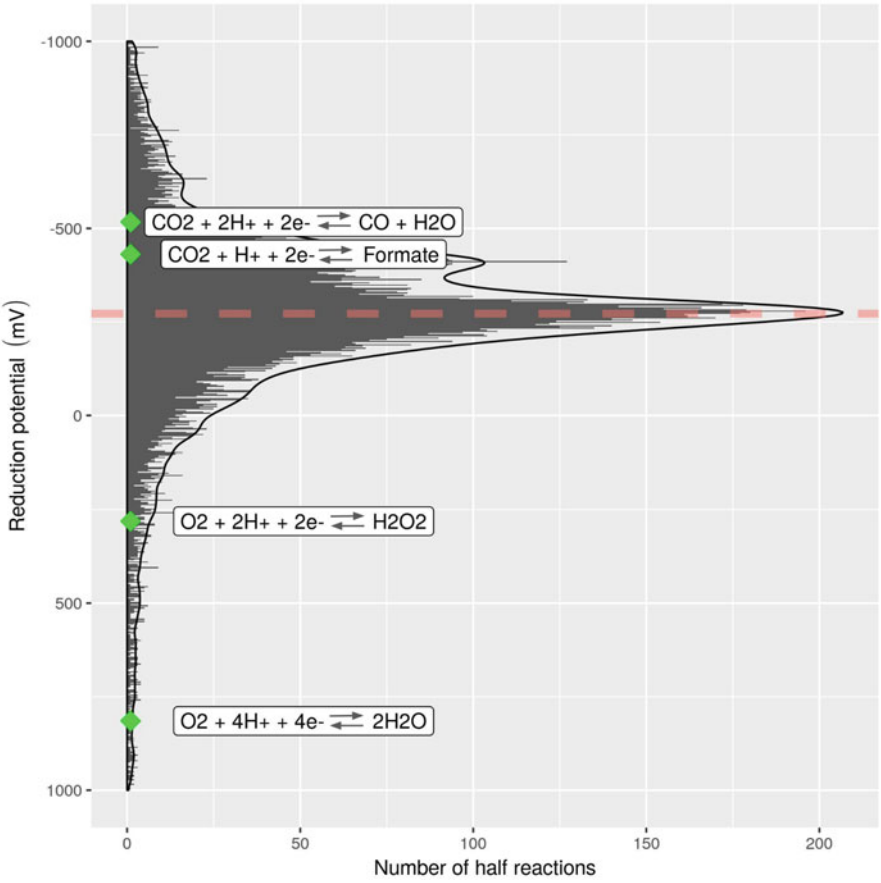

Fig. 4 Frequency histogram showing the standard reduction potentials (at pH = 7) of a set of metabolic redox half-reactions among a select set of 140 metabolites. This set of metabolites was compiled by Thauer (1977) along with their standard free energies of formation (at pH = 7). Using this set, we have computationally generated all possible atom balanced reduction half-reactions among the 140 metabolites (a total of 14,563 reactions) and computed the standard reduction potential for each reaction from the standard free energies of formation of the constituting metabolites and the number of electrons involved. The mean reduction potential of these half-reactions was found to be −272.03 mV (red dashed line). While the presented results contain both biologically realised and unrealised reactions, it is interesting to note that the mean of the distribution is close to the reduction potential of the NAD^+/NADH pair at −320 mV. Four examples of biologically observed reactions are indicated on the distribution, at their corresponding standard reduction potentials; calculated values according to the presented approach are {−517, −431, 281, 815 mV}, while corresponding values from the literature are {−500, −420, 300, 820 mV} (Voet et al., 2013). Note that higher (more positive) values of standard reduction potentials indicate metabolites affinity for electrons, i.e. their tendency to be reduced in a redox reaction. Thus, half-reactions with more negative (positive) reduction potentials have a tendency to run in the oxidation (reduction) direction

4.2 Biomass and Energy Production

The connection between metabolism and the production of cell constituents (i.e. biomass) is clear and well-formulated (Neidhardt et al., 1990). This clear connection has led to a dominant evolutionary view on metabolism that puts it as a servant to the cell and argues that selection for cells' proliferation dominates the evolution of metabolism. This view has led to the development of the commonly used stoichiometric modelling through flux balance analysis (FBA), which uses optimisation for biomass yield (on a given substrate) as its basis (Price et al., 2004). Alternative optimality criteria for FBA has also been formulated, but these also assume links to biomass formation; maximisation of ATP yield (Schuster et al., 2008; Schuetz et al., 2007) and minimisation of enzyme investment (i.e. total flux in the FBA context) along with biomass maximisation (Holzhütter, 2004). The latter idea has recently given rise to the Resource Balance Analysis (RBA), where the focus is still the optimisation of biomass production but considering not only metabolic flux constraints but also constraints arising from protein allocation to different cellular processes including transcription and house-keeping (Goelzer & Fromion, 2017).

Optimality ideas are also used to explore the biochemically feasible space of reactions using both kinetic simulations and graph theoretical approaches and to see if observed metabolic pathways are superior to alternatives in terms of supporting cellular growth. Both kinetic studies using optimality analysis and the simulation of feasible possible pathways indicated that glycolysis represents an optimal solution for maximising ATP flux (Heinrich et al., 1997; Court et al., 2015). Graph theoretical approaches suggested that the central carbon pathways represent enzymatically minimal routes among the different metabolites that act as precursors to biomass (Noor et al., 2010), and that the pentose-phosphate pathway represent the most enzyme-efficient solution to the sugar conversion it implements (Meléndez-Hevia & Isidoro, 1985). Enzyme cost minimisation with biomass optimisation, as used in the RBA approach, is also used to explain the presence of enzymatically different, but seemingly functionally redundant pathways in central metabolism, in particular, the glycolytic Embden–Meyerhoff–Parnass and Entner–Doudoroff pathways (Flamholz et al., 2013). These pathways are shown to require different levels of enzyme investment for achieving the same flux, a point that is used to argue that their evolution was driven for a requirement to sustain efficient biomass formation under environmental conditions that can support different levels of protein production (Flamholz et al., 2013). Following on from these findings, it was shown that when a metabolic system is simultaneously optimised for maximum flux and minimal enzyme investment, the resulting flux distributions correspond to so-called elementary flux modes (Wortel et al., 2014; Müller et al., 2014), which are paths through the metabolic system that have minimal enzymatic steps and that can sustain the required metabolic conversion under steady-state conditions (Schuster et al., 2000). In a recent study, these elementary flux modes are enumerated and analysed for their biomass yield and the growth rate that they can sustain when assuming minimal enzyme investment (Wortel et al., 2018).

4.3 Maintenance of Metabolic Gradients and Physicochemical Constraints

Selection alone for more or faster biomass formation could not have been a key driver of early metabolic systems that must have predated cells (or at least cells as we know them today). Instead, these systems must have been directly born out of nonequilibrium thermodynamics emerging from chemical gradients (Branscomb & Russell, 2013). Thus, the presence and maintenance of these gradients, as well as the chemicophysical properties of associated metabolites can still be relevant and informative for the current-day metabolic systems.

Stabilisation of mechanisms that can generate extracellular entropic gradients on which metabolism can operate is considered an important prerequisite for the emergence of metabolism (Branscomb & Russell, 2013). Extracellular spatial organisations that can allow metabolic systems to operate on chemical gradients are argued not to readily form in a well-mixed 'primordial soups' based on considerations of diffusion rates (Barge et al., 2017; Branscomb & Russell, 2013). A possible solution to this problem is mounds of hydrothermal vents that can sustain metabolic gradients (Branscomb & Russell, 2013; Martin & Russell, 2007). There is also a possible role for self-forming coacervates, a type of phase separation driven by charged molecules, that could maintain short-scale metabolic gradients due to different diffusion rates through the bulk and coacervate phases (Oparin, 1965). In this context, it is interesting to note that synthetic coacervates are shown to be able to harbour enzymatic processes (Nakashima et al., 2018) and coacervate-like phase separations are observed in current-day cells (Nott et al., 2016).

Adaptation of metabolites into these early cell-like formations (or liquid phases) and ultimately into cellular metabolism could have been driven by their physicochemical properties. For example, metabolites that are more readily involved in the formation of coacervates or that are readily trapped in them, might end up being locked-in into later metabolic systems. From this perspective, it is interesting to note that the analysis of physicochemical properties of current-day metabolites indicate some trends in terms of reactivity, solubility, and diffusion across membranes (Morowitz et al., 2002; Srinivasan & Morowitz, 2009; Bar-Even et al., 2012a, b), there seems to be relations between metabolites' connectivity in graph representations of metabolism and their polarity (Zhu et al., 2011), and that simple physicochemical pruning rules applied on the available chemical space can lead to biologically relevant subsets of molecules (Morowitz et al., 2002).

If the early evolution of metabolic systems involved a phase-separated, cell-like environment, than there must have been mechanisms to ensure maintaining a metabolic gradient across such an environment. It has been suggested that maintaining such a gradient in light of fluctuating external metabolite concentrations can be ensured by cyclic reaction systems (Hatakeyama & Furusawa, 2017; Reich & Sel'kov, 1981). Such cyclic systems are highly prevalent in current-day metabolism in form many coupled reactions that use the same conserved moieties such as NAD/NADH and ADP/ATP in opposing directions (see Fig. 3).

5 Conclusion and Future Outlook

The extensive biochemical and genetic study of metabolism provided us with detailed information on metabolic systems. While this information can and has been condensed to graphical representations, graph theoretical analyses of metabolic systems have not necessarily yielded clear insights to structure–function relations and predictive capabilities. It is indeed possible that many of the structural features of metabolic networks are by-products of biochemical and biophysical drivers. Indeed, when using biochemically appropriate representation and randomisation schemes, the significance of the gross structural properties of metabolic networks are highly dependent on the exact null model utilised (Basler et al., 2012; Zhou & Nakhleh, 2011). When compared to similarly complex abiotic chemical systems, many structural features of metabolism are found not to be unique (Holme et al., 2011), suggesting that there is not necessarily any functional (or evolutionary) significance to these properties.

In terms of dynamics, most focus has been on modelling pathway dynamics, while considering them as isolated entities. This divide-and-conquer type approach might be justified due to the curse of dimensionality and lack of detailed kinetic parameters associated with large-scale metabolic models, yet, we argue that it will eventually be limited because metabolism is so highly interconnected. Thus, we advocate a push for analysis of models that can take into account the connected nature of metabolism, especially through conserved moieties such as redox and energy carriers. It is possible that simulations with tractable toy models (e.g. combining metabolism with other cellular processes; Molenaar et al., 2009; Weisse et al., 2015), or new approaches, such as statistical thermodynamics applied to metabolic dynamics (Cannon, 2014; Thomas et al., 2014), can provide headways in this direction.

It has been a common practice to conceptualise metabolism within an adaptive evolutionary framework and see it as a servant to achieving optimal biomass production. Within this view, it is proposed that pathway dynamics can be understood through supply–demand type relations (van Heerden et al., 2015; Hofmeyr & Cornish-Bowden, 2000), where cell growth determines demand for biomass precursors (and ATP), which is then delivered through pathways such as glycolysis. This adaptive view has also given rise to the development of whole-genome scale stoichiometric models of metabolism and their study through FBA and biomass optimisation. In our view, and as noted also by others (Schuetz et al., 2007; Schuster et al., 2008), this strong reliance on an adaptive evolutionary argument and ad hoc constraints limits the FBA approach. Efforts are now being made to improve FBA's predictive power with the development of flux rate constraints that are based on biophysical arguments (Mori et al., 2016), and with the development of approaches that enable sampling of larger number of possible flux distributions rather than using linear optimisation on a single objective to obtain a single flux distribution (Binns et al., 2015). The former point is also being addressed with the recently developed RBA, which assumes metabolism to be simultaneously optimised for maximum

biomass production at minimum enzyme investment (Wortel et al., 2018; Goelzer & Fromion, 2017; Flamholz et al., 2013; Schuster et al., 2011; Molenaar et al., 2009; Holzhütter, 2004).

It remains to be verified if selection for fast or high-yield biomass production is, or was, a dominant force on the evolution of metabolism and if the issue of enzyme allocation is a widespread and evolutionarily relevant limitation. For example, while unicellular organisms are shown to evolve rapidly under selection for faster growth, selection for individual cell proliferation is certainly not the dominant factor in the case of multicellular organisms, where cell collectives have to limit their growth to achieve developmental constraints and requirements. Even in the case of unicellular organisms, many environments only support extremely slow growth (Jørgensen & Marshall, 2015) and selection for fast growth might never become relevant. Similarly, the argument that minimisation of enzyme investment being a strong factor shaping metabolic system evolution needs to be considered carefully. While it is likely that cellular protein content has an upper bound, it is less clear if cells are close to this upper bound to the extent that limits on enzyme amount would directly influence metabolic fluxes, or if such effects would operate equally for every enzyme or under different conditions (Brown, 1991). While some studies indicate control architectures for transcriptional regulation of enzyme levels to confer to optimality criteria (Chubukov et al., 2012), these were confined to linear, biosynthesis pathways within metabolism. In contrast, global analyses of metabolic systems suggest that enzyme levels are not necessarily tightly regulated as metabolic fluxes change with changing conditions. For example, proteomics studies have found that levels of different enzymes do not change as metabolic fluxes shift (Goel et al., 2015; Metzl-Raz et al., 2017) and several experimental analysis pointed to the importance of allosteric and thermodynamic regulation (Rossell et al., 2006; Chubukov et al., 2013; Link et al., 2013; Gerosa et al., 2015; Machado et al., 2015; Buffing et al., 2018) rather than transcriptional regulation of enzyme levels.

It has also been shown that the commonly pre-assumed tight linkage between energy harvesting through catabolic pathways and biomass formation through anabolic pathways is not necessarily observed and there are many instances of significant ‘energy spilling’ (Russell & Cook, 1995; Dauner et al., 2001). In its simplest form, such spilling can happen, and some of the harvested energy is ‘lost’ by the cell to drive chemical reactions. It has been largely documented that the amount of harvested energy not converted into biomass is correlated to some properties of the anabolic pathways, across many taxa and growth conditions (Heijnen et al., 1992; von Stockar et al., 2006; Roden & Jin, 2011; Smeaton & Van Cappellen, 2018) and this observed link has been used to couple the stoichiometry of energy harvest and biomass synthesis, and successfully predict population growth yields (González-Cabaleiro et al., 2015). However, a mechanistic understanding of this correlation remains elusive.

Considering links to the pre-cellular metabolic systems, an alternative evolutionary view can be formulated that considers metabolic evolution as primarily being shaped and constrained by thermodynamics and physicochemical factors (Vallino, 2010; Branscomb & Russell, 2013). Thus, these factors need to be included in

formulating both evolutionary ideas and dynamical models of metabolism. While steady-state, stoichiometric models tried to include reaction thermodynamics as additional constraints in their optimisation formulations (Flamholz et al., 2013; Henry et al., 2007; Hoppe et al., 2007), dynamical simulations incorporating thermodynamics are only recently being started to be explored in the context of metabolism (Cannon, 2014; Cannon & Baker, 2017; Cannon et al., 2018; González-Cabaleiro et al., 2013, 2015; Thomas et al., 2014).

We argue that it will be productive to reconcile the two evolutionary views on metabolism; metabolism as a highly regulated system optimised solely for cell growth vs. a self-organising system governed by thermodynamics and biophysical factors. It is possible, for example, to consider that thermodynamically driven and self-organising, early metabolic systems could have been stabilised by cell-like structures, which could have then helped stabilise and perpetuate those metabolic systems. There could be remnants of such feedbacks still evident in current-day metabolic systems, where features that have emerged (and maintained) solely due to thermodynamic and other physicochemical drivers are intertwined with those that resulted from selection for increased biomass production rate or yield. To this end, we note that considering metabolism as composed of interlinked catabolic and anabolic pathways within a thermodynamic framework allows a successful empirical description of cell growth and biomass yield (von Stockar et al., 2006), and in certain cases, growth dynamics (González-Cabaleiro et al., 2015). Similarly, explaining metabolic regulation seems to benefit from the synthesis of regulatory mechanisms based solely on metabolite-driven effects, and those based on transcriptional control of enzyme levels (Chubukov et al., 2014).

While many ideas and studies about metabolism concerns steady-state fluxes, it is clear that metabolic dynamics are highly nonlinear and can readily give rise to bistability and non-steady-state dynamics such as oscillations. Both of these dynamical features are linked to higher functionalities, with bistability implicated in dynamic switching of metabolic fluxes and oscillations linked to the regulation of cell cycle (Lloyd et al., 2003; Papagiannakis et al., 2017), management of superoxide generation during growth (Murray et al., 2007), and resilience and communication in multicellular structures such as biofilms (Liu et al., 2015). Thus, further study of metabolic dynamics, and their molecular driving mechanisms, can provide important insights on how higher-level cellular and multicellular behaviours arise and are maintained through metabolism. While it is possible that the emergence of oscillations is intertwined with bistability (Martinez-Corral et al., 2018), one can already note that the same metabolic 'motif' that can mediate bistability can also readily be extended with additional features to mediate oscillations (as discussed above). Interestingly, and as a side note, the association of simple metabolic motifs with potentially complex nonlinear dynamics led to the suggestion that engineering of enzymatic dynamics could be an ideal route for implementing specific dynamics with biological systems (Arkin & Ross, 1994). Subsequent focus in the then emerging field of synthetic biology, however, focused on engineering of transcriptional regulation. It would be useful, in our view, to now reconsider enzymatic systems from an engineering perspective, to use them to implement

specific system dynamics (such as bistability). This would be facilitated by new mathematical and experimental tools (such as ability to create multidomain proteins) and by further exploring the biochemical and kinetic determinants of enzyme reaction motifs that enable in them specific dynamics. In this context, identifying allosteric interactions, as being done with emerging proteomic approaches (Li et al., 2010; Piazza et al., 2018), and considering the resulting reaction motifs from these interactions (Reich & Sel'kov, 1981) can allow us to better understand the role of allosteric regulation in metabolic system evolution, either as a positive factor or as something to be avoided (Alam et al., 2017). Finally, an electrochemical view on metabolism (Berry, 1981; Zerfass et al., 2018) can allow interfacing and controlling metabolism through such reaction motifs, for example, by controlling redox states of conserved moieties to drive the dynamics of interlinked reaction cycles.

Experimental analysis of metabolic systems is constantly benefiting from the application of omics and biophysical techniques, including metabolomics and imaging. We argue that, as these new techniques develop, old ideas need also be revisited with new tools. For example, thermodynamic information on many reactions within metabolism are not available, and measurement of key physiological parameters such as pH, respiration rate, or ATP/ADP ratio are still mostly lacking at the single-cell level. Such single-cell measurements can be essential to develop better understanding of cellular metabolism, which is implicated to show heterogeneities within clonal populations (Nikolic et al., 2013, 2017; Yaginuma et al., 2014; Rosenthal et al., 2018) and discover the key trade-offs arising from metabolic system structure and dynamics. Single-cell analyses can also allow identifying metabolic interactions within populations (Rosenthal et al., 2018), especially in populations with an inherent structure, such as biofilms and tissue. This, in turn, can allow us to make connections between metabolic dynamics and emergence of division of labour and multicellularity (Liu et al., 2015). Within spatially organised systems, the role of diffusion of metabolites, especially charged ones, across or within membranes needs to be considered, as they can give rise to the formulation of new modes of communication such as metabolite-driven electrochemical signalling (Prindle et al., 2015). Finally, the analysis of metabolic systems under conditions of no growth, but sustained viability, is another under-studied area, which can give better insights into the connections between metabolism and other physiological processes, and in particular membrane potential and cell division.

Evolutionary thinking can provide a canvas on which to evaluate findings from metabolic systems and draw up new experiments. More specifically, the use of evolutionary thinking and experiments for the identification of selective trade-offs, physicochemical constraints, and ecology-evolutionary feedbacks can provide insights into current-day metabolic systems. For example, the consideration of possible feedbacks between ecological and evolutionary dynamics can help us better understand the emergence of metabolic interactions within microbial communities (Grosskopf et al., 2016). The consideration of trade-offs between different selectable traits, on the other hand, can allow proposition of multistable metabolic behaviours that might become embedded in the metabolism of different

cell types in multicellular organisms. The advancement of an evolutionary thinking in metabolic research can thus bear important insights into the future.

Acknowledgements We thank Marco Polin, Munehiro Asally, and Christian Zerfass for comments on an earlier version of this manuscript.

References

Aidelberg, G., Towbin, B. D., Rothschild, D., Dekel, E., Bren, A., & Alon, U. (2014). Hierarchy of non-glucose sugars in *Escherichia coli*. *BMC Systems Biology, 8*, 133. https://doi.org/10.1186/s12918-014-0133-z

Alam, M. T., Olin-Sandoval, V., Stincone, A., Keller, M. A., Zelezniak, A., Luisi, B. F., & Ralser, M. (2017). The self-inhibitory nature of metabolic networks and its alleviation through compartmentalization. *Nature Communications, 8*, 16018. https://doi.org/10.1038/ncomms16018

Alberty, R. A. (2005). *Thermodynamics of biochemical reactions*. John Wiley & Sons, Inc.

Alon, U. (2007). Network motifs: Theory and experimental approaches. *Nature Reviews. Genetics, 8*(6), 450–461. https://doi.org/10.1038/nrg2102

Arita, M. (2004). The metabolic world of *Escherichia coli* is not small. *Proceedings of the National Academy of Sciences, 101*(6), 1543–1547. https://doi.org/10.1073/pnas.0306458101

Arkin, A., & Ross, J. (1994). Computational functions in biochemical reaction networks. *Biophysical Journal, 67*(2), 560–578. https://doi.org/10.1016/S0006-3495(94)80516-8

Artzy-Randrup, Y. (2004). Comment on "Network motifs: Simple building blocks of complex networks" and "superfamilies of evolved and designed networks". *Science, 305*, 1107. https://doi.org/10.1126/science.1099334

Avetisov, V. A., Nechaev, S. K., & Shkarin, A. B. (2010). On the motif distribution in random block-hierarchical networks. *Physica A: Statistical Mechanics and its Applications, 389*, 5895. https://doi.org/10.1016/j.physa.2010.09.016

Bachmann, H., Fischlechner, M., Rabbers, I., Barfa, N., Branco Dos Santos, F., Molenaar, D., & Teusink, B. (2013). Availability of public goods shapes the evolution of competing metabolic strategies. *Proceedings of the National Academy of Sciences of the United States of America, 110*(35), 14302–14307. https://doi.org/10.1073/pnas.1308523110

Bar-Even, A., Flamholz, A., Noor, E., & Milo, R. (2012a). Rethinking glycolysis: On the biochemical logic of metabolic pathways. *Nature Chemical Biology, 8*(6), 509–517. https://doi.org/10.1038/nchembio.971

Bar-Even, A., Flamholz, A., Noor, E., & Milo, R. (2012b). Thermodynamic constraints shape the structure of carbon fixation pathways. *Biochimica et Biophysica Acta - Bioenergetics, 1817*(9), 1646–1659. https://doi.org/10.1016/j.bbabio.2012.05.002

Barge, L. M., Branscomb, E., Brucato, J. R., Cardoso, S. S. S., Cartwright, J. H. E., Danielache, S. O., et al. (2017). Thermodynamics, disequilibrium, evolution: Far-from-equilibrium geological and chemical considerations for origin-of-life research. *Origins of Life and Evolution of Biospheres, 47*(1), 39–56. https://doi.org/10.1007/s11084-016-9508-z

Basan, M., Hui, S., Okano, H., Zhang, Z., Shen, Y., Williamson, J. R., & Hwa, T. (2015). Overflow metabolism in *Escherichia coli* results from efficient proteome allocation. *Nature, 528*(7580), 99–104. https://doi.org/10.1038/nature15765

Basler, G., Grimbs, S., Ebenhöh, O., Selbig, J., & Nikoloski, Z. (2012). Evolutionary significance of metabolic network properties. *Journal of the Royal Society Interface, 9*(71), 1168–1176. https://doi.org/10.1098/rsif.2011.0652

Beber, M. E., Fretter, C., Jain, S., Müller-Hannemann, M., & Hütt, M.-T. (2012). Artefacts in statistical analyses of network motifs: General framework and application to metabolic networks. *Journal of the Royal Society Interface, 9*, 3426–3435.

Bennett, B. D., Kimball, E. H., Gao, M., Osterhout, R., Van Dien, S. J., & Rabinowitz, J. D. (2009). Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nature Chemical Biology, 5*, 593. https://doi.org/10.1038/nchembio.186

Berry, M. N. (1981). An electrochemical interpretation of metabolism. *FEBS Letters, 134*(2), 133–138.

Binns, M., de Atauri, P., Vlysidis, A., Cascante, M., & Theodoropoulos, C. (2015). Sampling with poling-based flux balance analysis: Optimal versus sub-optimal flux space analysis of *Actinobacillus succinogenes*. *BMC Bioinformatics, 16*, 49. https://doi.org/10.1186/s12859-015-0476-5

Boiteux, A., Goldbeter, A., & Hess, B. (1975). Control of oscillating glycolysis of yeast by stochastic periodic and steady source of substrate: A model and experimental study. *Proceedings of the National Academy of Sciences, 72*(10), 3829–3833. https://doi.org/10.1073/pnas.72.10.3829

Branscomb, E., & Russell, M. J. (2013). Turnstiles and bifurcators: The disequilibrium converting engines that put metabolism on the road. *Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1827*(2), 62–78. https://doi.org/10.1016/j.bbabio.2012.10.003

Brown, G. C. (1991). Total cell protein concentration as an evolutionary constraint on the metabolic control distribution in cells. *Journal of Theoretical Biology, 153*, 195. https://doi.org/10.1016/S0022-5193(05)80422-9

Buffing, M. F., Link, H., Christodoulou, D., & Sauer, U. (2018). Capacity for instantaneous catabolism of preferred and non-preferred carbon sources in *Escherichia coli* and *Bacillus subtilis*. *Scientific Reports, 8*, 11760. https://doi.org/10.1038/s41598-018-30266-3

Cannon, W., Zucker, J., Baxter, D., Kumar, N., Baker, S., Hurley, J., & Dunlap, J. (2018). Prediction of metabolite concentrations, rate constants and post-translational regulation using maximum entropy-based simulations with application to central metabolism of *Neurospora crassa*. *Processes, 6*(6), 63. https://doi.org/10.3390/pr6060063

Cannon, W. R. (2014). Simulating metabolism with statistical thermodynamics. *PLoS One, 9*(8), e103582. https://doi.org/10.1371/journal.pone.0103582

Cannon, W. R., & Baker, S. E. (2017). Non-steady state mass action dynamics without rate constants: Dynamics of coupled reactions using chemical potentials. *Physical Biology, 14*, 055003. https://doi.org/10.1088/1478-3975/aa7d80

Chance, B., Eastbrook, R. W., & Ghosh, A. (1964). Damped sinusoidal oscillations of cytoplasmic reduced pyridine nucleotide in yeast cells. *The Journal of Biological Chemistry, 51*, 1244–1251.

Christen, S., & Sauer, U. (2011). Intracellular characterization of aerobic glucose metabolism in seven yeast species by13C flux analysis and metabolomics. *FEMS Yeast Research, 11*(3), 263–272. https://doi.org/10.1111/j.1567-1364.2010.00713.x

Chubukov, V., Gerosa, L., Kochanowski, K., & Sauer, U. (2014). Coordination of microbial metabolism. *Nature Reviews. Microbiology, 12*, 327. https://doi.org/10.1038/nrmicro3238

Chubukov, V., Uhr, M., Le Chat, L., Kleijn, R. J., Jules, M., Link, H., et al. (2013). Transcriptional regulation is insufficient to explain substrate-induced flux changes in *Bacillus subtilis*. *Molecular Systems Biology, 9*, 709. https://doi.org/10.1038/msb.2013.66

Chubukov, V., Zuleta, I. A., & Li, H. (2012). Regulatory architecture determines optimal regulation of gene expression in metabolic pathways. *Proceedings of the National Academy of Sciences, 109*, 5127. https://doi.org/10.1073/pnas.1114235109

Cimino, A., & Hervagault, J. F. (1990). Irreversible transitions in a model substrate cycle An experimental illustration. *FEBS Letters, 263*(2), 199–205. https://doi.org/10.1016/0014-5793(90)81373-V

Collier, D. N., Hager, P. W., & Phibbs, P. V. (1996). Catabolite repression control in the *Pseudomonads*. *Research in Microbiology, 147*, 551. https://doi.org/10.1016/0923-2508(96)84011-3

Court, S. J., Waclaw, B., & Allen, R. J. (2015). Lower glycolysis carries a higher flux than any biochemically possible alternative. *Nature Communications, 6*, 1–8. https://doi.org/10.1038/ncomms9427

Dauner, M., Storni, T., & Sauer, U. (2001). *Bacillus subtilis* metabolism and energetics in carbon-limited and excess-carbon chemostat culture. *Journal of Bacteriology, 183*, 7308. https://doi.org/10.1128/JB.183.24.7308-7317.2001

De Deken, R. H. (1966). The Crabtree effect: A regulatory system in yeast. *Journal of General Microbiology, 44*(2), 149–156. https://doi.org/10.1099/00221287-44-2-149

Desmond-Le Quéméner, E., & Bouchez, T. (2014). A thermodynamic theory of microbial growth. *The ISME Journal, 8*(8), 1747–1751. https://doi.org/10.1038/ismej.2014.7

Diaz-Ruiz, R., Uribe-Carvajal, S., Devin, A., & Rigoulet, M. (2009). Tumor cell energy metabolism and its common features with yeast metabolism. *BBA - Reviews on Cancer, 1796*(2), 252–265. https://doi.org/10.1016/j.bbcan.2009.07.003

Dodd, B. J. T., & Kralj, J. M. (2017). Live cell imaging reveals pH oscillations in *Saccharomyces cerevisiae* during metabolic transitions. *Scientific Reports, 7*, 13922. https://doi.org/10.1038/s41598-017-14382-0

Doebeli, M. (2002). A model for the evolutionary dynamics of cross-feeding polymorphisms in microorganisms. *Population Ecology, 44*(2), 59–70. https://doi.org/10.1007/s101440200008

Ebenhoh, O., Handorf, T., & Heinrich, R. (2004). Structural analysis of expanding metabolic networks. *Genome Informatics, 15*, 35. https://doi.org/10.11234/GI1990.15.35

Ebenhöh, O., Handorf, T., & Heinrich, R. (2005). A cross species comparison of metabolic network functions. *Genome Informatics, 16*, 203. https://doi.org/10.11234/GI1990.16.203

Edelstein, B. B. (1971). Autocatalysis in a biological system. *Journal of Theoretical Biology, 32*, 191. https://doi.org/10.1016/0022-5193(71)90146-9

Egbert, M. D., Barandiaran, X. E., & di Paolo, E. A. (2010). A minimal model of metabolism-based chemotaxis. *PLoS Computational Biology, 6*, e1001004. https://doi.org/10.1371/journal.pcbi.1001004

Enjalbert, B., Millard, P., Dinclaux, M., Portais, J. C., & Létisse, F. (2017). Acetate fluxes in *Escherichia coli* are determined by the thermodynamic control of the Pta-AckA pathway. *Scientific Reports, 7*, 42135. https://doi.org/10.1038/srep42135

Eom, Y. H., Lee, S., & Jeong, H. (2006). Exploring local structural organization of metabolic networks using subgraph patterns. *Journal of Theoretical Biology, 241*, 823. https://doi.org/10.1016/j.jtbi.2006.01.018

Espinosa-Soto, C., & Wagner, A. (2010). Specialization can drive the evolution of modularity. *PLoS Computational Biology, 6*(3), e1000719. https://doi.org/10.1371/journal.pcbi.1000719

Flamholz, A., Noor, E., Bar-Even, A., Liebermeister, W., & Milo, R. (2013). Glycolytic strategy as a tradeoff between energy yield and protein cost. *Proceedings of the National Academy of Sciences of the United States of America, 110*(24), 10039–10044. https://doi.org/10.1073/pnas.1215283110

Fong, S. S., & Palsson, B. (2004). Metabolic gene-deletion strains of *Escherichia coli* evolve to computationally predicted growth phenotypes. *Nature Genetics, 36*, 1056. https://doi.org/10.1038/ng1432

Frenkel, R. (1968). Control of reduced oscillations diphosphopyridine in beef heart extracts nucleotide II. Oscillations of glycolytic intermediates and adenine nucleotides. *Archives of Biochemistry and Biophysics, 125*, 157–165.

Gerosa, L., Haverkorn Van Rijsewijk, B. R. B., Christodoulou, D., Kochanowski, K., Schmidt, T. S. B., Noor, E., & Sauer, U. (2015). Pseudo-transition analysis identifies the key regulators of dynamic metabolic adaptations from steady-state data. *Cell Systems, 1*, 270. https://doi.org/10.1016/j.cels.2015.09.008

Goel, A., Eckhardt, T. H., Puri, P., de Jong, A., Branco dos Santos, F., Giera, M., et al. (2015). Protein costs do not explain evolution of metabolic strategies and regulation of ribosomal content: Does protein investment explain an anaerobic bacterial Crabtree effect? *Molecular Microbiology, 97*, 77. https://doi.org/10.1111/mmi.13012

Goelzer, A., & Fromion, V. (2017). Resource allocation in living organisms. *Biochemical Society Transactions, 45*, 945. https://doi.org/10.1042/bst20160436

Goentoro, L., Shoval, O., Kirschner, M. W., & Alon, U. (2009). The incoherent feedforward loop can provide fold-change detection in gene regulation. *Molecular Cell, 36*(5), 894–899. https://doi.org/10.1016/j.molcel.2009.11.018

Goldbeter, A. (2018). Dissipative structures in biological systems: Bistability, oscillations, spatial patterns and waves. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences, 376*, 20170376. https://doi.org/10.1098/rsta.2017.0376

Goldbeter, A., & Guilmot, J. M. (1996). Thresholds and oscillations in enzymatic cascades. *Journal of Physical Chemistry, 100*(49), 19174–19181. https://doi.org/10.1021/jp962298e

Goldbeter, A., & Lefever, R. (1972). Dissipative structures for an allosteric model: Application to glycolytic oscillations. *Biophysical Journal, 12*(10), 1302–1315. https://doi.org/10.1016/S0006-3495(72)86164-2

González-Cabaleiro, R., Lema, J. M., Rodríguez, J., & Kleerebezem, R. (2013). Linking thermodynamics and kinetics to assess pathway reversibility in anaerobic bioprocesses. *Energy & Environmental Science, 6*(12), 3780. https://doi.org/10.1039/c3ee42754d

González-Cabaleiro, R., Ofieru, I. D., Lema, J. M., & Rodríguez, J. (2015). Microbial catabolic activities are naturally selected by metabolic energy harvest rate. *The ISME Journal, 9*(12), 2630–2641. https://doi.org/10.1038/ismej.2015.69

Görke, B., & Stülke, J. (2008). Carbon catabolite repression in bacteria: Many ways to make the most out of nutrients. *Nature Reviews. Microbiology, 6*, 613. https://doi.org/10.1038/nrmicro1932

Gottschalk, G. (1986). *Bacterial metabolism*. Springer. https://doi.org/10.1007/978-1-4612-1072-6

Grosskopf, T., Consuegra, J., Gaffé, J., Willison, J. C., Lenski, R. E., Soyer, O. S., & Schneider, D. (2016). Metabolic modelling in a dynamic evolutionary framework predicts adaptive diversification of bacteria in a long-term evolution experiment. *BMC Evolutionary Biology, 16*(1), 163. https://doi.org/10.1186/s12862-016-0733-x

Grosskopf, T., & Soyer, O. S. (2016). Microbial diversity arising from thermodynamic constraints. *The ISME Journal, 10*, 2725. https://doi.org/10.1038/ismej.2016.49

Grüning, N. M., Rinnerthaler, M., Bluemlein, K., Mülleder, M., Wamelink, M. M. C., Lehrach, H., et al. (2011). Pyruvate kinase triggers a metabolic feedback loop that controls redox metabolism in respiring cells. *Cell Metabolism, 14*(3), 415–427. https://doi.org/10.1016/j.cmet.2011.06.017

Guidi, G. M., Carlier, M. F., & Goldbeter, A. (1998). Bistability in the isocitrate dehydrogenase reaction: An experimentally based theoretical study. *Biophysical Journal, 74*(3), 1229–1240. https://doi.org/10.1016/S0006-3495(98)77837-3

Guidi, G. M., & Goldbeter, A. (1998). From bistability to oscillations in a model for the isocitrate dehydrogenase reaction. *Biophysical Chemistry, 72*(1–2), 201–210. https://doi.org/10.1016/S0301-4622(98)00134-3

Guidi, G. M., & Goldbeter, A. (2000). Oscillations and bistability predicted by a model for a cyclical bienzymatic system involving the regulated isocitrate dehydrogenase reaction. *Biophysical Chemistry, 83*(2), 153–170. https://doi.org/10.1016/S0301-4622(99)00136-2

Guimerà, R., & Amaral, L. A. N. (2005). Functional cartography of complex metabolic networks. *Nature, 433*, 895. https://doi.org/10.1038/nature03288

Hatakeyama, T. S., & Furusawa, C. (2017). Metabolic dynamics restricted by conserved carriers: Jamming and feedback. *PLoS Computational Biology, 13*, e1005847. https://doi.org/10.1371/journal.pcbi.1005847

Heijnen, J. J., van Loosdrecht, M. C., & Tijhuis, L. (1992). A black box mathematical model to calculate auto- and heterotrophic biomass yields based on Gibbs energy dissipation. *Biotechnology and Bioengineering, 40*(10), 1139–1154. https://doi.org/10.1002/bit.260401003

Heinrich, R., Montero, F., Klipp, E., Waddell, T. G., & Melendez-Hevia, E. (1997). Theoretical approaches to the evolutionary optimization of glycolysis: Thermodynamic and kinetic constraints. *European Journal of Biochemistry/FEBS, 243*(1–2), 191.

Heinrich, R., Schuster, S., & Holzhütter, H. G. (1991). Mathematical analysis of enzymic reaction systems using optimization principles. *European Journal of Biochemistry, 201*(1), 1–21.

Hellingwerf, K. J., Lolkema, J. S., Otto, R., Neijssel, O. M., Stouthamer, A. H., Harder, W., et al. (1982). Energetics of microbial growth: An analysis of the relationship between growth and its mechanistic basis by mosaic non-equilibrium thermodynamics. *FEMS Microbiology Letters, 15*, 7–17.

Henry, C. S., Broadbelt, L. J., & Hatzimanikatis, V. (2007). Thermodynamics-based metabolic flux analysis. *Biophysical Journal, 92*(5), 1792–1805. https://doi.org/10.1529/biophysj.106.093138

Hervagault, J. F., & Canu, S. (1987). Bistability and irreversible transitions in a simple substrate cycle. *Journal of Theoretical Biology, 127*(4), 439–449. https://doi.org/10.1016/S0022-5193(87)80141-8

Higgins, J. (1964). A chemical mechanism for oscillation of glycolytic intermediates in yeast cells. *Proceedings of the National Academy of Sciences U S A, 51*, 989. https://doi.org/10.1073/pnas.51.6.989

Hintze, A., & Adami, C. (2008). Evolution of complex modular biological networks. *PLoS Computational Biology, 4*(2), e23.

Hofmeyr, J. H. S., & Cornish-Bowden, A. (2000). Regulating the cellular economy of supply and demand. *FEBS Letters, 476*, 47. https://doi.org/10.1016/S0014-5793(00)01668-9

Holme, P. (2011). Metabolic robustness and network modularity: A model study. *PLoS One, 6*(2), e16605. https://doi.org/10.1371/journal.pone.0016605

Holme, P., Huss, M., & Lee, S. H. (2011). Atmospheric reaction systems as null-models to identify structural traces of evolution in metabolism. *PLoS One, 6*, e19759. https://doi.org/10.1371/journal.pone.0019759

Holzhütter, H. G. (2004). The principle of flux minimization and its application to estimate stationary fluxes in metabolic networks. *European Journal of Biochemistry, 271*(14), 2905–2922. https://doi.org/10.1111/j.1432-1033.2004.04213.x

Hoppe, A., Hoffmann, S., & Holzhütter, H.-G. G. (2007). Including metabolite concentrations into flux balance analysis: Thermodynamic realizability as a constraint on flux distributions in metabolic networks. *BMC Systems Biology, 1*, 23. https://doi.org/10.1186/1752-0509-1-23

Huang, Z., Zhu, L., Cao, Y., Wu, G., Liu, X., Chen, Y., et al. (2011). ASD: A comprehensive database of allosteric proteins and modulators. *Nucleic Acids Research, 39*, D663. https://doi.org/10.1093/nar/gkq1022

Ibarra, R. U., Edwards, J. S., & Palsson, B. O. (2002). *Escherichia coli* K-12 undergoes adaptive evolution to achieve in silico predicted optimal growth. *Nature, 420*, 186. https://doi.org/10.1038/nature01149

Ingram, P. J., Stumpf, M. P. H., & Stark, J. (2006). Network motifs: Structure does not determine function. *BMC Genomics, 7*, 108. https://doi.org/10.1186/1471-2164-7-108

Jain, M., Nilsson, R., Sharma, S., Madhusudhan, N., Kitami, T., Souza, A. L., et al. (2012). Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. *Science, 336*(6084), 1040–1044. https://doi.org/10.1126/science.1218595

Jensen, R. A. (1976). Enzyme recruitment in evolution of new function. *Annual Review of Microbiology, 30*, 409. https://doi.org/10.1146/annurev.mi.30.100176.002205

Jeong, H., Tombor, B., Albert, R., Oltvai, Z. N., & Barabási, A. L. (2000). The large-scale organization of metabolic networks. *Nature, 407*(6804), 651–654.

Jeske, L., Placzek, S., Schomburg, I., Chang, A., & Schomburg, D. (2019). BRENDA in 2019: A European ELIXIR core data resource. *Nucleic Acids Research, 47*, D542. https://doi.org/10.1093/nar/gky1048

Jiang, X., Zerfaß, C., Feng, S., Eichmann, R., Asally, M., Schäfer, P., & Soyer, O. S. (2018). Impact of spatial organization on a novel auxotrophic interaction among soil microbes. *The ISME Journal, 12*, 1443–1456. https://doi.org/10.1038/s41396-018-0095-z

Jørgensen, B. B., & Marshall, I. P. G. (2015). Slow microbial life in the seabed. *Annual Review of Marine Science, 8*, 311. https://doi.org/10.1146/annurev-marine-010814-015535

Kanehisa, M. (2013). Automated interpretation of metabolic capacity from genome and metagenome sequences. In *Quantitative biology*. Springer. https://doi.org/10.1007/s40484-013-0019-x

Kashtan, N., & Alon, U. (2005). Spontaneous evolution of modularity and network motifs. *Proceedings of the National Academy of Sciences of the United States of America, 102*(39), 13773–13778. https://doi.org/10.1073/pnas.0503610102

Keller, E. F. (2005). Revisiting "scale-free" networks. *BioEssays, 27*(10), 1060–1068. https://doi.org/10.1002/bies.20294

Keller, M. A., Kampjut, D., Harrison, S. A., & Ralser, M. (2017). Sulfate radicals enable a non-enzymatic Krebs cycle precursor. *Nature Ecology and Evolution., 1*, 83. https://doi.org/10.1038/s41559-017-0083

Keller, M. A., Turchyn, A. V., & Ralser, M. (2014). Non-enzymatic glycolysis and pentose phosphate pathway-like reactions in a plausible Archean ocean. *Molecular Systems Biology, 10*, 725. https://doi.org/10.1002/msb.20145228

Keulers, M., Satroutdinov, A. D., Suzuki, T., & Kuriyama, H. (1996). Synchronization affector of autonomous short-period-sustained oscillation of *Saccharomyces cerevisiae*. *Yeast, 12*, 673. https://doi.org/10.1002/(SICI)1097-0061(19960615)12:7<673::AID-YEA958>3.0.CO;2-C

Kim, J., Kershner, J. P., Novikov, Y., Shoemaker, R. K., & Copley, S. D. (2010). Three serendipitous pathways in *E. coli* can bypass a block in pyridoxal-5g-2-phosphate synthesis. *Molecular Systems Biology, 6*, 436. https://doi.org/10.1038/msb.2010.88

Klumpp, S., Zhang, Z., & Hwa, T. (2009). Growth rate-dependent global effects on gene expression in bacteria. *Cell, 139*(7), 1366–1375. https://doi.org/10.1016/j.cell.2009.12.001

Kochanowski, K., Volkmer, B., Gerosa, L., Haverkorn van Rijsewijk, B. R., Schmidt, A., & Heinemann, M. (2013). Functioning of a metabolic flux sensor in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America, 110*(3), 1130–1135. https://doi.org/10.1073/pnas.1202582110

Konagurthu, A. S., & Lesk, A. M. (2008). On the origin of distribution patterns of motifs in biological networks. *BMC Systems Biology, 2*, 73. https://doi.org/10.1186/1752-0509-2-73

Kotte, O., Volkmer, B., Radzikowski, J. L., & Heinemann, M. (2014). Phenotypic bistability in Escherichia coli's central carbon metabolism. *Molecular Systems Biology, 10*(7), 736–736. https://doi.org/10.15252/msb.20135022

Kotte, O., Zaugg, J. B., & Heinemann, M. (2010). Bacterial adaptation through distributed sensing of metabolic fluxes. *Molecular Systems Biology, 6*, 355. https://doi.org/10.1038/msb.2010.10

Kreimer, A., Borenstein, E., Gophna, U., & Ruppin, E. (2008). The evolution of modularity in bacterial metabolic networks. *Proceedings of the National Academy of Sciences of the United States of America, 105*(19), 6976–6981. https://doi.org/10.1073/pnas.0712149105

Kümmel, A., Panke, S., & Heinemann, M. (2006). Putative regulatory sites unraveled by network-embedded thermodynamic analysis of metabolome data. *Molecular Systems Biology, 2*, 20060034. https://doi.org/10.1038/msb4100074

LaPorte, D. C., Walsh, K., & Koshland, D. E. (1984). The branch point effect. Ultrasensitivity and subsensitivity to metabolic control. *The Journal of Biological Chemistry, 259*(22), 14068–14075.

Laurent, M., Seydoux, F. J., & Dessen, P. (1979). Allosteric regulation of yeast phosphofructokinase. Correlation between equilibrium binding, spectroscopic and kinetic data. *Journal of Biological Chemistry, 254*, 7515.

Le Gac, M., Brazas, M. D., Bertrand, M., Tyerman, J. G., Spencer, C. C., Hancock, R. E. W., & Doebeli, M. (2008). Metabolic changes associated with adaptive diversification in *Escherichia coli*. *Genetics, 178*(2), 1049–1060. https://doi.org/10.1534/genetics.107.082040

Lei, F., Olsson, L., & Jørgensen, S. B. (2003). Experimental investigations of multiple steady states in aerobic continuous cultivations of *Saccharomyces cerevisia*. *Biotechnology and Bioengineering, 82*, 766. https://doi.org/10.1002/bit.10624

Li, X., Gianoulis, T. A., Yip, K. Y., Gerstein, M., & Snyder, M. (2010). Extensive in vivo metabolite-protein interactions revealed by large-scale systematic analyses. *Cell, 143*, 639. https://doi.org/10.1016/j.cell.2010.09.048

Link, H., Kochanowski, K., & Sauer, U. (2013). Systematic identification of allosteric protein-metabolite interactions that control enzyme activity in vivo. *Nature Biotechnology, 31*, 357. https://doi.org/10.1038/nbt.2489

Lipshtat, A., Purushothaman, S. P., Iyengar, R., & Ma'ayan, A. (2008). Functions of bifans in context of multiple regulatory motifs in signaling networks. *Biophysical Journal, 94*, 2566. https://doi.org/10.1529/biophysj.107.116673

Lipson, H., Pollack, J. B., & Suh, N. P. (2002). On the origin of modular variation. *Evolution, 56*(8), 1549–1556.

Liu, J., Prindle, A., Humphries, J., Gabalda-Sagarra, M., Asally, M., Lee, D. D. Y., et al. (2015). Metabolic co-dependence gives rise to collective oscillations within biofilms. *Nature, 523*(7562), 550–554. https://doi.org/10.1038/nature14660

Lloyd, D., Lemar, K. M., Salgado, L. E. J., Gould, T. M., & Murray, D. B. (2003). Respiratory oscillations in yeast: Mitochondrial reactive oxygen species, apoptosis and time; a hypothesis. *FEMS Yeast Research, 3*(4), 333–339. https://doi.org/10.1016/S1567-1356(03)00071-0

Locasale, J. W. (2013). Serine, glycine and one-carbon units: Cancer metabolism in full circle. *Nature Reviews Cancer, 13*(8), 572–583. https://doi.org/10.1038/nrc3557

Luli, G. W., & Strohl, W. R. (1990). Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *Applied and Environmental Microbiology, 56*, 1004.

Machado, D., Herrgård, M. J., & Rocha, I. (2015). Modeling the contribution of allosteric regulation for flux control in the central carbon metabolism of E. coli. *Frontiers in Bioengineering and Biotechnology, 3*, 154. https://doi.org/10.3389/fbioe.2015.00154

Majewski, R. A., & Domach, M. M. (1990). Simple constrained-optimization view of acetate overflow in E. coli. *Biotechnology and Bioengineering, 35*(7), 732–738.

Mangan, S., & Alon, U. (2003). Structure and function of the feed-forward loop network motif. *Proceedings of the National Academy of Sciences of the United States of America, 100*(21), 11980.

Mangan, S., Itzkovitz, S., Zaslaver, A., & Alon, U. (2006). The incoherent feed-forward loop accelerates the response-time of the gal system of *Escherichia coli*. *Journal of Molecular Biology, 356*(5), 1073–1081. https://doi.org/10.1016/j.jmb.2005.12.003

Martin, W., & Russell, M. J. (2007). On the origin of biochemistry at an alkaline hydrothermal vent. *Philosophical Transactions of the Royal Society, B: Biological Sciences, 362*, 1887. https://doi.org/10.1098/rstb.2006.1881

Martinez-Corral, R., Liu, J., Süel, G. M., & Garcia-Ojalvo, J. (2018). Bistable emergence of oscillations in growing *Bacillus subtilis* biofilms. *Proceedings of the National Academy of Sciences, 115*, E8333. https://doi.org/10.1073/pnas.1805004115

Mazurie, A., Bonchev, D., Schwikowski, B., & Buck, G. A. (2010). Evolution of metabolic network organization. *BMC Systems Biology, 4*, 59. https://doi.org/10.1186/1752-0509-4-59

Meléndez-Hevia, E., & Isidoro, A. (1985). The game of the pentose phosphate cycle. *Journal of Theoretical Biology, 117*, 251. https://doi.org/10.1016/S0022-5193(85)80220-4

Merrins, M. J., Poudel, C., McKenna, J. P., Ha, J., Sherman, A., Bertram, R., & Satin, L. S. (2016). Phase analysis of metabolic oscillations and membrane potential in pancreatic islet β-cells. *Biophysical Journal, 110*, 691. https://doi.org/10.1016/j.bpj.2015.12.029

Messner, C. B., Driscoll, P. C., Piedrafita, G., De Volder, M. F. L., & Ralser, M. (2017). Nonenzymatic gluconeogenesis-like formation of fructose 1,6-bisphosphate in ice. *Proceedings of the National Academy of Sciences, 114*, 7403. https://doi.org/10.1073/pnas.1702274114

Metzl-Raz, E., Kafri, M., Yaakov, G., Soifer, I., Gurvich, Y., & Barkai, N. (2017). Principles of cellular resource allocation revealed by condition-dependent proteome profiling. *eLife, 6*, e28034. https://doi.org/10.7554/eLife.28034

Meyer, H. P., Leist, C., & Fiechter, A. (1984). Acetate formation in continuous culture of *Escherichia coli* K12 D1 on defined and complex media. *Journal of Biotechnology, 1*(5–6), 355–358. https://doi.org/10.1016/0168-1656(84)90027-0

Miller, S. L., & Smith-Magowan, D. (1990). The thermodynamics of the Krebs cycle and related compounds. *Journal of Physical and Chemical Reference Data, 19*(4), 1049–1073.

Milo, R., Itzkovitz, S., Kashtan, N., Levitt, R., Shen-Orr, S., Ayzenshtat, I., et al. (2004). Superfamilies of evolved and designed networks. *Science, 303*(5663), 1538.

Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D., & Alon, U. (2002). Network motifs: Simple building blocks of complex networks. *Science, 298*(5594), 824.

Molenaar, D., van Berlo, R., de Ridder, D., & Teusink, B. (2009). Shifts in growth strategies reflect tradeoffs in cellular economics. *Molecular Systems Biology, 5*, 323. https://doi.org/10.1038/msb.2009.82

Monod, J. (1949). The growth of bacterial cultures. *Annual Review of Microbiology, 3*, 371–394.

Montañez, R., Medina, M. A., Solé, R. V., & Rodríguez-Caso, C. (2010). When metabolism meets topology: Reconciling metabolite and reaction networks. *BioEssays, 32*, 246. https://doi.org/10.1002/bies.200900145

Mori, M., Hwa, T., Martin, O. C., De Martino, A., & Marinari, E. (2016). Constrained allocation flux balance analysis. *PLoS Computational Biology, 12*(6), e1004913. https://doi.org/10.1371/journal.pcbi.1004913

Morowitz, H. J., Kostelnik, J. D., Yang, J., & Cody, G. D. (2002). The origin of intermediary metabolism. *Proceedings of the National Academy of Sciences, 97*, 7704. https://doi.org/10.1073/pnas.110153997

Müller, S., Regensburger, G., & Steuer, R. (2014). Enzyme allocation problems in kinetic metabolic networks: Optimal solutions are elementary flux modes. *Journal of Theoretical Biology, 347*, 182–190. https://doi.org/10.1016/j.jtbi.2013.11.015

Mulukutla, B. C., Yongky, A., Daoutidis, P., & Hu, W. S. (2014). Bistability in glycolysis pathway as a physiological switch in energy metabolism. *PLoS One, 9*, e98756. https://doi.org/10.1371/journal.pone.0098756

Murray, D. B., Beckmann, M., & Kitano, H. (2007). Regulation of yeast oscillatory dynamics. *Proceedings of the National Academy of Sciences, 104*, 2241. https://doi.org/10.1073/pnas.0606677104

Muto, A., Kotera, M., Tokimatsu, T., Nakagawa, Z., Goto, S., & Kanehisa, M. (2013). Modular architecture of metabolic pathways revealed by conserved sequences of reactions. *Journal of Chemical Information and Modeling, 53*, 613. https://doi.org/10.1021/ci3005379

Nakashima, K. K., Baaij, J. F., & Spruijt, E. (2018). Reversible generation of coacervate droplets in an enzymatic network. *Soft Matter, 14*(3), 361–367. https://doi.org/10.1039/c7sm01897e

Nam, H., Conrad, T. M., & Lewis, N. E. (2011). The role of cellular objectives and selective pressures in metabolic pathway evolution. *Current Opinion in Biotechnology, 22*, 595. https://doi.org/10.1016/j.copbio.2011.03.006

Nanchen, A., Schicker, A., & Sauer, U. (2006). Nonlinear dependency of intracellular fluxes on growth rate in miniaturized continuous cultures of *Escherichia coli*. *Applied and Environmental Microbiology, 72*(2), 1164–1172. https://doi.org/10.1128/AEM.72.2.1164-1172.2006

Neidhardt, C. F., Ingraham, L. J., & Schaechter, M. (1990). *Physiology of the bacterial cell: A molecular approach*. Sinauer Associates Inc.

Nikolic, N., Barner, T., & Ackermann, M. (2013). Analysis of fluorescent reporters indicates heterogeneity in glucose uptake and utilization in clonal bacterial populations. *BMC Microbiology, 13*(1), 1–13. https://doi.org/10.1186/1471-2180-13-258

Nikolic, N., Schreiber, F., Dal Co, A., Kiviet, D. J., Bergmiller, T., Littmann, S., et al. (2017). Cell-to-cell variation and specialization in sugar metabolism in clonal bacterial populations. *PLoS Genetics, 13*(12), 1–24. https://doi.org/10.1371/journal.pgen.1007122

Noor, E., Bar-Even, A., Flamholz, A., Reznik, E., Liebermeister, W., & Milo, R. (2014). Pathway thermodynamics highlights kinetic obstacles in central metabolism. *PLoS Computational Biology, 10*(2), e1003483. https://doi.org/10.1371/journal.pcbi.1003483

Noor, E., Eden, E., Milo, R., & Alon, U. (2010). Central carbon metabolism as a minimal biochemical walk between precursors for biomass and energy. *Molecular Cell, 39*(5), 809–820. https://doi.org/10.1016/j.molcel.2010.08.031

Nott, T. J., Craggs, T. D., & Baldwin, A. J. (2016). Membraneless organelles can melt nucleic acid duplexes and act as biomolecular filters. *Nature Chemistry, 8*, 569. https://doi.org/10.1038/nchem.2519

Novak, M., Pfeiffer, T., Lenski, R. E., Sauer, U., & Bonhoeffer, S. (2006). Experimental tests for an evolutionary trade-off between growth rate and yield in *E. coli*. *The American Naturalist, 168*(2), 242. https://doi.org/10.2307/3844729

Oparin, A. I. (1965). *Origin of life*. Dover Publications.

Ozbudak, E. M., Thattai, M., Lim, H. N., Shraiman, B. I., & Van Oudenaarden, A. (2004). Multistability in the lactose utilization network of *Escherichia coli*. *Nature, 427*(6976), 737–740. https://doi.org/10.1038/nature02298

Papagiannakis, A., Niebel, B., Wit, E. C., & Heinemann, M. (2017). Autonomous metabolic oscillations robustly gate the early and late cell cycle. *Molecular Cell, 65*(2), 285–295. https://doi.org/10.1016/j.molcel.2016.11.018

Papp, B., Pál, C., & Hurst, L. D. (2004). Metabolic network analysis of the causes and evolution of enzyme dispensability in yeast. *Nature, 429*(6992), 661–664. https://doi.org/10.1038/nature02636

Papp, B., Teusink, B., & Notebaart, R. A. (2009). A critical view of metabolic network adaptations. *Human Frontier Science Program, 3*, 24–35.

Parche, S., Beleut, M., Rezzonico, E., Jacobs, D., Arigoni, F., Titgemeyer, F., & Jankovic, I. (2006). Lactose-over-glucose preference in *Bifidobacterium longum* NCC2705: *glcP*, encoding a glucose transporter, is subject to lactose repression. *Journal of Bacteriology, 188*, 1260. https://doi.org/10.1128/JB.188.4.1260-1265.2006

Parter, M., Kashtan, N., & Alon, U. (2007). Environmental variability and modularity of bacterial metabolic networks. *BMC Evolutionary Biology, 7*, 169. https://doi.org/10.1186/1471-2148-7-169

Pastor-Satorras, R., Smith, E., & Solé, R. V. (2003). Evolving protein interaction networks through gene duplication. *Journal of Theoretical Biology, 222*, 199–210. https://doi.org/10.1016/S0022-5193(03)00028-6

Pfeiffer, T., Schuster, S., & Bonhoeffer, S. (2001). Cooperation and competition in the evolution of ATP-producing pathways. *Science, 292*(5516), 504–507. https://doi.org/10.1126/science.1058079

Pfeiffer, T., Soyer, O. S., & Bonhoeffer, S. (2005). The evolution of connectivity in metabolic networks. *PLoS Biology, 3*(7), e228. https://doi.org/10.1371/journal.pbio.0030228

Piazza, I., Kochanowski, K., Cappelletti, V., Fuhrer, T., Noor, E., Sauer, U., & Picotti, P. (2018). A map of protein-metabolite interactions reveals principles of chemical communication. *Cell, 172*, 358. https://doi.org/10.1016/j.cell.2017.12.006

Planqué, R., Bruggeman, F. J., Teusink, B., & Hulshof, J. (2014). Understanding bistability in yeast glycolysis using general properties of metabolic pathways. *Mathematical Biosciences, 255*(1), 33–42. https://doi.org/10.1016/j.mbs.2014.06.006

Ponomarova, O., Gabrielli, N., Sauer, U., Se, D. C., Ralser, M., Patil, K. R., et al. (2017). Yeast creates a niche for symbiotic lactic acid bacteria through nitrogen overflow article yeast creates a niche for symbiotic lactic acid bacteria through nitrogen overflow. *Cell Systems, 5*, 345–357. https://doi.org/10.1016/j.cels.2017.09.002

Postma, E., Verduyn, C., Scheffers, W. A., & Van Dijken, J. P. (1989). Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology, 55*, 468.

Price, N. D., Reed, J. L., & Palsson, B. Ø. (2004). Genome-scale models of microbial cells: Evaluating the consequences of constraints. *Nature Reviews. Microbiology, 2*(11), 886–897. https://doi.org/10.1038/nrmicro1023

Prindle, A., Liu, J., Asally, M., Ly, S., Garcia-Ojalvo, J., & Süel, G. M. (2015). Ion channels enable electrical communication in bacterial communities. *Nature, 527*, 59. https://doi.org/10.1038/nature15709

Ravasz, E., Somera, A. L., Mongru, D. A., Oltvai, Z. N., & Barabási, A. L. (2002). Hierarchical organization of modularity in metabolic networks. *Science, 297*(5586), 1551–1555. https://doi.org/10.1126/science.1073374

Raymond, J., & Segrè, D. (2006). The effect of oxygen on biochemical networks and the evolution of complex life. *Science, 311*(5768), 1764–1767. https://doi.org/10.1126/science.1118439

Reich, J. G., & Sel'kov, E. E. (1981). *Energy metabolism of the cell: A theoretical treatise*. Academic Press.

Richard, P., Bakker, B. M., Teusink, B., Van Dam, K., & Westerhoff, H. V. (1996). Acetaldehyde mediates the synchronization of sustained glycolytic oscillations in populations of yeast cells. *European Journal of Biochemistry, 235*, 238. https://doi.org/10.1111/j.1432-1033.1996.00238.x

Richard, P., Teusink, B., Westerhoff, H. V., & van Dam, K. (1993). Around the growth phase transition *S. cerevisiae's* make-up favours sustained oscillations of intracellular metabolites. *FEBS Letters, 318*, 80. https://doi.org/10.1016/0014-5793(93)81332-T

Rieger, M., Kaeppeli, O., & Fiechter, A. (1983). The role of limited respiration in the incomplete oxidation of glucose by *Saccharomyces cerevisiae*. *Journal of General Microbiology, 129*, 653. https://doi.org/10.1099/00221287-129-3-653

Roden, E. E., & Jin, Q. (2011). Thermodynamics of microbial growth coupled to metabolism of glucose, ethanol, short-chain organic acids, and hydrogen. *Applied and Environmental Microbiology, 77*(5), 1907–1909. https://doi.org/10.1128/AEM.02425-10

Rosenthal, A. Z., Qi, Y., Hormoz, S., Park, J., Li, S. H.-J., & Elowitz, M. B. (2018). Metabolic interactions between dynamic bacterial subpopulations. *eLife, 7*, 1–18. https://doi.org/10.7554/eLife.33099

Rossell, S., van der Weijden, C. C., Lindenbergh, A., van Tuijl, A., Francke, C., Bakker, B. M., & Westerhoff, H. V. (2006). Unraveling the complexity of flux regulation: A new method demonstrated for nutrient starvation in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences, 103*, 2166. https://doi.org/10.1073/pnas.0509831103

Rozen, D. E., & Lenski, R. E. (2000). Long-term experimental evolution in *Escherichia coli*. VIII. Dynamics of a balanced polymorphism. *The American Naturalist, 155*(1), 24–35. https://doi.org/10.1086/303299

Russell, J. B., & Cook, G. M. (1995). Energetics of bacterial growth: Balance of anabolic and catabolic reactions. *Microbiological Reviews, 59*, 48.

Salathé, M., May, R. M., & Bonhoeffer, S. (2005). The evolution of network topology by selective removal. *Journal of the Royal Society of Interface, 2*(5), 533–536. https://doi.org/10.1098/rsif.2005.0072

Samal, A., Wagner, A., & Martin, O. C. (2011). Environmental versatility promotes modularity in genome-scale metabolic networks. *BMC Systems Biology, 5*, 135. https://doi.org/10.1186/1752-0509-5-135

Sandefur, C., Mincheva, M., & Schnell, S. (2012). Network representations and methods for the analysis of chemical and biochemical pathways. *Changes, 29*(9), 997–1003. https://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

Satroutdinov, A. D., Kuriyama, H., & Kobayashi, H. (1992). Oscillatory metabolism of *Saccharomyces cerevisiae* in continuous culture. *FEMS Microbiology Letters, 77*, 261. https://doi.org/10.1111/j.1574-6968.1992.tb05525.x

Schmidt, S., Sunyaev, S., Bork, P., & Dandekar, T. (2003). Metabolites: A helping hand for pathway evolution? *Trends in Biochemical Sciences, 28*, 336. https://doi.org/10.1016/S0968-0004(03)00114-2

Schuetz, R., Kuepfer, L., & Sauer, U. (2007). Systematic evaluation of objective functions for predicting intracellular fluxes in *Escherichia coli*. *Molecular Systems Biology, 3*, 119. https://doi.org/10.1038/msb4100162

Schulze, K. L., & Lipe, R. S. (1964). Relationship between substrate concentration, growth rate, and respiration rate of *Escherichia coli* in continuous culture. *Archiv fuer Mikrobiologie, 48*(1), 1–20. https://doi.org/10.1007/BF00406595

Schuster, S., Fell, D. A., & Dandekar, T. (2000). A general definition of metabolic pathways useful for systematic organization and analysis of complex metabolic networks. *Nature Biotechnology, 18*, 326. https://doi.org/10.1038/73786

Schuster, S., de Figueiredo, L. F., Schroeter, A., & Kaleta, C. (2011). Combining metabolic pathway analysis with Evolutionary Game Theory: Explaining the occurrence of low-yield pathways by an analytic optimization approach. *Biosystems, 105*(2), 147–153. https://doi.org/10.1016/j.biosystems.2011.05.007

Schuster, S., Pfeiffer, T., & Fell, D. A. (2008). Is maximization of molar yield in metabolic networks favoured by evolution? *Journal of Theoretical Biology, 252*(3), 497–504. https://doi.org/10.1016/j.jtbi.2007.12.008

Sel'kov, E. E. (1968). Self-oscillations in glycolysis. 1. A simple kinetic model. *European Journal of Biochemistry, 4*(1), 79–86.

Simonet, E., Bourdillon, C., Hervagault, J. F., & Gervais, M. (1996). Bistability in coupled open substrate cycles: Numerical and experimental approaches. *Journal of Physical Chemistry, 100*(49), 19148–19152. https://doi.org/10.1021/jp961734u

Şimşek, E., & Kim, M. (2018). The emergence of metabolic heterogeneity and diverse growth responses in isogenic bacterial cells. *The ISME Journal, 12*, 1199. https://doi.org/10.1038/s41396-017-0036-2

Smeaton, C. M., & Van Cappellen, P. (2018). Gibbs energy dynamic yield method (GEDYM): Predicting microbial growth yields under energy-limiting conditions. *Geochimica et Cosmochimica Acta, 241*, 1. https://doi.org/10.1016/j.gca.2018.08.023

Snel, B., & Huynen, M. A. (2004). Quantifying modularity in the evolution of biomolecular systems. *Genome Research, 14*, 391. https://doi.org/10.1101/gr.1969504

Sohn, H. Y., Murray, D. B., & Kuriyama, H. (2000). Ultradian oscillation of *Saccharomyces cerevisiae* during aerobic continuous culture: Hydrogen sulphide mediates population synchrony. *Yeast, 16*, 1185. https://doi.org/10.1002/1097-0061(20000930)16:13<1185::AID-YEA619>3.0.CO;2-W

Solopova, A., van Gestel, J., Weissing, F. J., Bachmann, H., Teusink, B., Kok, J., & Kuipers, O. P. (2014). Bet-hedging during bacterial diauxic shift. *Proceedings of the National Academy of Sciences, 111*, 7427. https://doi.org/10.1073/pnas.1320063111

Soo, V. W. C., Hanson-Manful, P., & Patrick, W. M. (2011). Artificial gene amplification reveals an abundance of promiscuous resistance determinants in *Escherichia coli*. *Proceedings of the National Academy of Sciences, 108*, 1424. https://doi.org/10.1073/pnas.1012108108

Sorokina, M., Medigue, C., & Vallenet, D. (2015). A new network representation of the metabolism to detect chemical transformation modules. *BMC Bioinformatics, 16*, 385. https://doi.org/10.1186/s12859-015-0809-4

Soyer, O. S. (2007). Emergence and maintenance of functional modules in signaling pathways. *BMC Evolutionary Biology, 7*, 205. https://doi.org/10.1186/1471-2148-7-205

Soyer, O. S., & Pfeiffer, T. (2010). Evolution under fluctuating environments explains observed robustness in metabolic networks. *PLoS Computational Biology, 6*(8), e1000907. https://doi.org/10.1371/journal.pcbi.1000907

Soyer, O. S., Salathé, M., & Bonhoeffer, S. (2006). Signal transduction networks: Topology, response and biochemical processes. *Journal of Theoretical Biology, 238*(2), 416–425. https://doi.org/10.1016/j.jtbi.2005.05.030

Srinivasan, V., & Morowitz, H. J. (2009). Analysis of the intermediary metabolism of a reductive chemoautotroph. *The Biological Bulletin, 217*, 222. https://doi.org/10.1086/BBLv217n3p222

Szenk, M., Dill, K. A., & de Graff, A. M. R. (2017). Why do fast-growing bacteria enter overflow metabolism? Testing the membrane real estate hypothesis. *Cell Systems, 5*(2), 95–104. https://doi.org/10.1016/j.cels.2017.06.005

Takemoto, K. (2012). Metabolic network modularity arising from simple growth processes. *Physical Review E: Statistical, Nonlinear, and Soft Matter Physics, 86*, 036107. https://doi.org/10.1103/PhysRevE.86.036107

Takemoto, K., & Akutsu, T. (2008). Origin of structural difference in metabolic networks with respect to temperature. *BMC Systems Biology, 2*, 82. https://doi.org/10.1186/1752-0509-2-82

Takemoto, K., & Borjigin, S. (2011). Metabolic network modularity in archaea depends on growth conditions. *PLoS One, 6*, e25874. https://doi.org/10.1371/journal.pone.0025874

Takemoto, K., Nacher, J. C., & Akutsu, T. (2007). Correlation between structure and temperature in prokaryotic metabolic networks. *BMC Bioinformatics, 8*, 303. https://doi.org/10.1186/1471-2105-8-303

Thauer, R., Jungermann, K., & Decker, K. (1977). Energy conversation in chemotrophic anaerobic bacteria. *Bacteriological Reviews, 41*, 100–180.

Thomas, D. G., Jaramillo-Riveri, S., Baxter, D. J., & Cannon, W. R. (2014). Comparison of optimal thermodynamic models of the tricarboxylic acid cycle from heterotrophs, cyanobacteria, and green sulfur bacteria. *The Journal of Physical Chemistry. B, 118*(51), 14745–14760. https://doi.org/10.1021/jp5075913

Valgepea, K., Adamberg, K., Nahku, R., Lahtvee, P.-J., Arike, L., & Vilu, R. (2010). Systems biology approach reveals that overflow metabolism of acetate in *Escherichia coli* is triggered by carbon catabolite repression of acetyl-CoA synthetase. *BMC Systems Biology, 4*(1), 166. https://doi.org/10.1186/1752-0509-4-166

Vallino, J. J. (2010). Ecosystem biogeochemistry considered as a distributed metabolic network ordered by maximum entropy production. *Philosophical Transactions of the Royal Society, B: Biological Sciences, 365*(1545), 1417–1427. https://doi.org/10.1098/rstb.2009.0272

van den Bogaard, P. T. C., Kleerebezem, M., Kuipers, O. P., & De Vos, W. M. (2000). Control of lactose transport, β-galactosidase activity, and glycolysis by CcpA in *Streptococcus thermophilus*: Evidence for carbon catabolite repression by a non-phosphoenolpyruvate-dependent phosphotransferase system sugar. *Journal of Bacteriology, 182*, 5982. https://doi.org/10.1128/JB.182.21.5982-5989.2000

van Heerden, J. H., Bruggeman, F. J., & Teusink, B. (2015). Multi-tasking of biosynthetic and energetic functions of glycolysis explained by supply and demand logic. *BioEssays, 37*(1), 34–45. https://doi.org/10.1002/bies.201400108

van Heerden, J. H., Wortel, M. T., Bruggeman, F. J., Heijnen, J. J., Bollen, Y. J. M., Planqué, R., et al. (2014). Lost in transition: Start-up of glycolysis yields subpopulations of nongrowing cells. *Science, 343*, 1245114. https://doi.org/10.1126/science.1245114

van Hoek, M. J. A., & Hogeweg, P. (2006). In silico evolved *lac* operons exhibit bistability for artificial inducers, but not for lactose. *Biophysical Journal, 91*(8), 2833–2843. https://doi.org/10.1529/biophysj.105.077420

Varma, A., Boesch, B. W., & Palsson, B. O. (1993). Stoichiometric interpretation of *Escherichia coli* glucose catabolism under various oxygenation rates. *Applied and Environmental Microbiology, 59*(8), 2465–2473. https://doi.org/10.1016/0009-2509(89)85169-3

Vemuri, G. N., Altman, E., Sangurdekar, D. P., Khodursky, A. B., & Eiteman, M. A. (2006). Overflow metabolism in *Escherichia coli* during steady-state growth: Transcriptional regulation and effect of the redox ratio. *Applied and Environmental Microbiology, 72*(5), 3653–3661. https://doi.org/10.1128/AEM.72.5.3653-3661.2006

Vemuri, G. N., Eiteman, M. A., McEwen, J. E., Olsson, L., & Nielsen, J. (2007). Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America, 104*(7), 2402–2407. https://doi.org/10.1073/pnas.0607469104

Voet, D., Voet, J. G., & Pratt, C. W. (2013). *Fundamentals of biochemistry* (4th ed.). John Wiley & Sons.

von Stockar, U., Maskow, T., Liu, J., Marison, I. W., & Patiño, R. (2006). Thermodynamics of microbial growth and metabolism: An analysis of the current situation. *Journal of Biotechnology, 121*(4), 517–533. https://doi.org/10.1016/j.jbiotec.2005.08.012

Wagner, A., & Fell, D. A. (2001). The small world inside large metabolic networks. *Proceedings of the Royal Society B: Biological Sciences, 268*, 1803. https://doi.org/10.1098/rspb.2001.1711

Weisse, A. Y., Oyarzún, D. A., Danos, V., & Swain, P. S. (2015). Mechanistic links between cellular trade-offs, gene expression, and growth. *Proceedings of the National Academy of Sciences of the United States of America, 112*(9), E1038–E1047. https://doi.org/10.1073/pnas.1416533112

Westerhoff, H. V., Hellingwerf, K. J., & Van Dam, K. (1983). Thermodynamic efficiency of microbial growth is low but optimal for maximal growth rate. *Proceedings of the National Academy of Sciences, 80*(1), 305–309.

Wittmann, C., Hans, M., Van Winden, W. A., Ras, C., & Heijnen, J. J. (2005). Dynamics of intracellular metabolites of glycolysis and TCA cycle during cell-cycle-related oscillation in *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering, 89*, 839. https://doi.org/10.1002/bit.20408

Wolf, J., & Heinrich, R. (2000). Effect of cellular interaction on glycolytic oscillations in yeast: A theoretical investigation. *Biochemical Journal, 345*, 321–334. https://doi.org/10.1042/0264-6021:3450321

Wolf, J., Sohn, H. Y., Heinrich, R., & Kuriyama, H. (2001). Mathematical analysis of a mechanism for autonomous metabolic oscillations in continuous culture of *Saccharomyces cerevisiae*. *FEBS Letters, 499*(3), 230–234. https://doi.org/10.1016/S0014-5793(01)02562-5

Wortel, M. T., Noor, E., Ferris, M., Bruggeman, F. J., & Liebermeister, W. (2018). Metabolic enzyme cost explains variable trade-offs between microbial growth rate and yield. *PLoS Computational Biology, 14*, e1006010. https://doi.org/10.1371/journal.pcbi.1006010

Wortel, M. T., Peters, H., Hulshof, J., Teusink, B., & Bruggeman, F. J. (2014). Metabolic states with maximal specific rate carry flux through an elementary flux mode. *The FEBS Journal, 281*(6), 1547–1555. https://doi.org/10.1111/febs.12722

Yaginuma, H., Kawai, S., Tabata, K. V., Tomiyama, K., Kakizuka, A., Komatsuzaki, T., et al. (2014). Diversity in ATP concentrations in a single bacterial cell population revealed by quantitative single-cell imaging. *Scientific Reports, 4*, 6522. https://doi.org/10.1038/srep06522

Yamada, T., Kanehisa, M., & Goto, S. (2006). Extraction of phylogenetic network modules from the metabolic network. *BMC Bioinformatics, 7*, 130. https://doi.org/10.1186/1471-2105-7-130

You, C., Okano, H., Hui, S., Zhang, Z., Kim, M., Gunderson, C. W., et al. (2013). Coordination of bacterial proteome with metabolism by cyclic AMP signalling. *Nature, 500*, 301. https://doi.org/10.1038/nature12446

Zerfass, C., Asally, M., & Soyer, O. S. (2018). Interrogating metabolism as an electron flow system. *Current Opinion in Systems Biology, 13*, 59–67. https://doi.org/10.1016/j.coisb.2018.10.001

Zhou, W., & Nakhleh, L. (2011). Properties of metabolic graphs: Biological organization or representation artifacts? *BMC Bioinformatics, 12*, 132. https://doi.org/10.1186/1471-2105-12-132

Zhu, Q., Qin, T., Jiang, Y. Y., Ji, C., Kong, D. X., Ma, B. G., & Zhang, H. Y. (2011). Chemical basis of metabolic network organization. *PLoS Computational Biology, 7*(10), 1–8. https://doi.org/10.1371/journal.pcbi.1002214

Zhuang, K., Vemuri, G. N., & Mahadevan, R. (2011). Economics of membrane occupancy and respiro-fermentation. *Molecular Systems Biology, 7*, 500. https://doi.org/10.1038/msb.2011.34

Robustness and Evolvability in Transcriptional Regulation

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Abstract The relationship between genotype and phenotype is central to our understanding of development, evolution, and disease. This relationship is known as the genotype-phenotype map. Gene regulatory circuits occupy a central position in this map, because they control when, where, and to what extent genes are expressed, and thus drive fundamental physiological, developmental, and behavioral processes in living organisms as different as bacteria and humans. Mutations that affect these gene expression patterns are often implicated in disease, so it is important that gene regulatory circuits are robust to mutation. Such mutations can also bring forth beneficial phenotypic variation that embodies or leads to evolutionary adaptations or innovations. Here, we review recent theoretical and experimental work that sheds light on the robustness and evolvability of gene regulatory circuits.

1 Introduction

Two of the most fundamental properties of living systems are robustness and evolvability (Wagner, 2005; Masel & Trotter, 2010). Robustness is the invariance of a phenotype in the presence of environmental or genetic change. Evolvability is the ability of a living system to generate phenotypic variation that is both heritable and adaptive (Payne & Wagner, 2019). A large number of studies have focused on elucidating the molecular mechanisms of both robustness and evolvability, and

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_9

establishing the relationship between these two properties, at multiples scales of biological organization, ranging from the structural and functional properties of RNA and proteins to the ability of a metabolic network to create biomass from nutrients. The evidence from these studies suggests that robustness can facilitate evolvability. Here, we review a subset of this large body of work, specifically highlighting studies that have focused on the molecular mechanisms of robustness and evolvability in transcriptional regulation. Specifically, we discuss mechanisms of robustness against perturbations caused by genetic mutations (mutational robustness), rather than mechanisms of robustness against nongenetic perturbations (environmental robustness), although mutational robustness is correlated with environmental robustness in many instances (de Visser et al., 2003; Lehner, 2010).

Most of the early studies on the robustness and evolvability of transcriptional regulation were theoretical or computational (Kauffman, 1969; Wagner, 1996; Bergman & Siegal, 2003). The reason for this is the enormous complexity of the gene circuits (and their individual molecular components) that control when, where, and to what extent genes are expressed. Advances in high-throughput technologies are changing this picture, providing mechanistic insight into how transcriptional regulation is robust to mutational change, yet able to bring forth new and beneficial phenotypes. These studies, which are the focus of our chapter, are also validating a long-standing body of theoretical work on the relationship between robustness and evolvability in transcriptional regulation.

While there are multiple mechanisms of gene regulation acting at different stages of information transmission from DNA to protein (Alonso & Wilkins, 2005; Keren et al., 2010; Pauli et al., 2011; Guttman & Rinn, 2012; Pelechano & Steinmetz, 2013; Smith & Meissner, 2013; Tian & Manley, 2016; Zhao et al., 2016), the fundamental spatiotemporal control of gene expression occurs at the level of gene transcription. Transcriptional regulation drives essential physiological processes—e.g., how cells respond to their environment (Ptashne & Gann, 2002)—, behavioral processes—e.g., mating in yeast (Tsong et al., 2006)—, and developmental processes—e.g., embryonic patterning in diptera (Lawrence, 1992). Transcriptional regulation is mediated by sequence-specific DNA-binding proteins known as transcription factors (TFs). They regulate gene transcription by binding short DNA sequences (6–12 base pairs) known as TF binding sites in the promoters or enhancers of genes. The binding of a TF to a gene's regulatory region may activate or repress the transcription of that gene by promoting or blocking the recruitment of the RNA polymerase to the transcription start site. The strength of this regulatory effect is partly determined by the TF's affinity for its site. Genes coding for TFs typically represent 5–10% of the total number of genes in a given genome (Madan Babu et al., 2006; Vaquerizas et al., 2009; Stormo & Zhao, 2010), and their products can regulate the expression of other TFs, forming transcriptional regulatory circuits that control gene expression in space and time. These circuits occupy a central position in the mapping from genotype to phenotype, and drive fundamental physiological, developmental, and behavioral processes in all living organisms from bacteria to humans.

In this chapter, we first review the mechanisms of mutational robustness and evolvability in (*trans*-acting) transcription factors and their (*cis*-acting) DNA-binding sites, that is, in the individual components of gene regulatory circuits. We then review the more global mechanisms of robustness and evolvability that emerge at the level of whole gene regulatory circuits.

2 Robustness and Evolvability of Gene Regulatory Circuit Components

2.1 Robustness and Evolvability of Transcription Factors

Genotype of a TF: Amino acid sequence of the protein
Phenotype of a TF: Ability to bind DNA specifically and regulate gene transcription

One of the most useful and productive distinctions in biology is that between genotype and phenotype, which can be defined at different levels of biological organization, ranging from biological macromolecules to whole organisms (Wagner, 2011). In this section, we define the genotype of a TF as its amino acid sequence. A TF can have multiple phenotypes. For example, its ability to bind a specific short DNA sequence with a given binding affinity or its ability to recruit RNA polymerases at a given rate.

TFs may have several functional domains—conserved protein segments that can function independently, each with a different function (Bornberg-Bauer & Albà, 2013; Stormo, 2013; Toll-Riera & Albà, 2013). TFs typically have just one DNA-binding domain, which can function autonomously (Stormo, 2013). Other TF domains are responsible for dimerization, with many TFs functioning as homodimers or heterodimers. Finally, some TF domains mediate interactions with other proteins to form large molecular complexes that regulate the rate of transcription. For instance, many TFs have an activation domain that interacts with the basal transcriptional machinery and coactivator complexes to initiate transcription (Latchman, 2008). TFs can be classified into families based on the structures and sequence similarity of their DNA-binding domains (Weirauch & Hughes, 2011; Stormo, 2013). TFs from the same family have similar structures, and thus bind DNA with the same overall geometry of interaction (Stormo, 2013). TFs from the same family usually also have a common ancestry, and have diverged through evolutionary processes such as gene duplication and species diversification.

2.1.1 The Robustness of the Protein Structure of Transcription Factors

While most protein mutations tend to be deleterious (Eyre-Walker & Keightley, 2007), the structure and biological activity of proteins are to some extent robust to mutations. However, different proteins, and even different domains within a single

protein, can vary widely in their level of mutational robustness. The robustness of TFs against mutations has important implications for human disease, because numerous Mendelian diseases are caused by mutations in TFs, especially in homeodomain TFs (Veraksa et al., 2000). Similarly, somatic mutations in Cys_2-His_2 zinc finger (C2H2-ZF) domains are commonly mutated in cancer cells, thus likely contributing to the transcriptional dysregulation that is characteristic of this disease (Munro et al., 2018). Even among putatively healthy individuals, there are more than 50,000 polymorphisms segregating in the human population that are found in the DNA-binding domains of sequence-specific TFs (Barrera et al., 2016). Many of these variants are likely to affect binding activity, and thus contribute to phenotypic heterogeneity and disease. However, of 177 nonsynonymous polymorphisms chosen because they are predicted to have an effect on TF activity, 40 were not found to affect DNA binding and/or specificity in vitro (Barrera et al., 2016). This shows how regulatory proteins can be robust to mutational change, including mutations in their DNA-binding domains. This is further exemplified by the human basic helix-loop-helix (bHLH) TF Max, which interfaces DNA with five amino acid residues. Three of these positions can be mutated into any other amino acid without altering binding specificity, although some amino acid substitutions in two of these three positions modulate binding affinity (Maerkl & Quake, 2009). However, the general rule is that mutations in the DNA-binding domains of TFs cause changes in binding specificity (Cook et al., 1994; Mathias et al., 2001; Noyes et al., 2008; Aggarwal et al., 2010; De Masi et al., 2011).

The activation domains of TFs are less conserved across species and less structured than DNA-binding domains (Latchman, 2008). They are intrinsically disordered domains. Activation domains are more robust against amino acid replacements than DNA-binding domains (Majithia et al., 2016; Staller et al., 2018). For example, the function of the human nuclear receptor PPARγ is less affected by amino acid substitutions in its activation domain AF-1 than its DNA-binding domain (Majithia et al., 2016). Strikingly, the activation domain is even less sensitive to mutations than the "hinge" region connecting the DNA-binding domain to the ligand-binding domain of this TF.

Many transcription factors cooperatively bind DNA with other protein factors. Such interactions may alleviate the consequences of mutations in a TF's DNA-binding domain or mutations in its binding sites. Protein–protein interactions with a different TF may stabilize the binding of a TF to particular genomic locations while its protein–DNA interactions evolve gradually. For example, the conserved interaction of Matα1 with Mcm1 may explain the dramatic changes in the binding specificity of this regulator of mating type in ascomycete fungi over relatively short evolutionary time scales (Baker et al., 2011). Similarly, the cooperativity between Mcm1 and Rap1 can stabilize the binding of Mcm1 to weak binding sites (Sorrells et al., 2018). Thus, interactions with protein partners can provide a source of mutational robustness for both TFs and their binding sites.

2.1.2 Robustness in Duplicated Transcription Factors

Gene duplication is one of the main forces shaping eukaryotic genomes (Zhang, 2003). Because gene duplicates (paralogs) are initially redundant, one of the copies can act as a backup, compensating deleterious mutations in the other copy (Keane et al., 2014). Therefore, gene duplication is an important mechanism of mutational robustness (Gu et al., 2003; Conant & Wagner, 2004; Fares, 2015), and plays an important role in the evolution of TFs (Babu & Teichmann, 2003; Teichmann & Babu, 2004). Many paralogous TFs recognize the same or very similar sets of binding sites in vitro (Weirauch et al., 2014), and they also bind many of the same genomic regions in vivo (Hollenhorst et al., 2007), indicating that they may be fully or partially redundant. Even distant TF paralogs can partially compensate one another against loss-of-function mutations (Kafri et al., 2005; He & Zhang, 2006; Tischler et al., 2006). Additionally, TFs with similar binding specificities tend to regulate sets of genes with similar biological functions. This may minimize the negative consequences of the "cross-talk" that occurs when different TFs bind similar sets of sites (Itzkovitz et al., 2006), or the negative consequence of specificity-changing mutations in a TFs DNA-binding domain.

2.1.3 Many Transcription Factors Are Clients of the Molecular Chaperone HSP90

A protein is classified as a client of HSP90 if it interacts physically with the chaperone and if the inhibition of HSP90 function reduces protein client activity. The most common protein clients of the eukaryotic chaperone HSP90 are TFs, including nuclear steroid receptors, but also PAS family TFs, p53, STAT3, and chromatin proteins such as trithorax (Taipale et al., 2010). BES1, a TF in the steroid hormone pathway in *Arabidopsis thaliana*, is a client of HSP90, but its closest paralog, BZR1, is not an HSP90 client. This difference in the client status of two highly similar proteins facilitates a test of whether HSP90 can enhance the mutational robustness of a TF. *BES1* shows relaxed selection compared to *BZR1* as expected if HSP90 allows BES1 to explore a greater fraction of genotype space without losing function (Lachowiec et al., 2013). Similarly, HSP90 clients in yeast, including many TFs, evolve faster than their nonclient paralogs, suggesting that HSP90 can increase the mutational tolerance of its client TFs (Lachowiec et al., 2013; Alvarez-Ponce et al., 2019).

2.1.4 The Evolvability of Transcription Factors

New gene expression patterns can evolve by changes in TFs and their binding sites that lead to the rewiring of a gene regulatory circuit. However, the adaptive evolution of a TF can be heavily constrained by both epistasis and pleiotropy. Epistasis between different residues can severely restrict the evolutionary trajectories of any

evolving protein (Starr & Thornton, 2016), including TFs, and mutations in a TF can have strong pleiotropic effects on a regulatory circuit because all its gene targets can be affected by changes in its binding specificity (Britten & Davidson, 1969; Stern, 2000; Carroll, 2005; Wray, 2007).

For these reasons, while there are clear cases of mutations in TFs contributing to the adaptive evolution of gene regulation (Galant & Carroll, 2002; Ronshaugen et al., 2002; Lynch et al., 2008, 2011), most studies of regulatory evolution have focused on the evolution of *cis*-regulatory elements, such as promoters and enhancers (Prud'homme et al., 2007; Carroll, 2008; Stern & Orgogozo, 2008). However, some properties of a TF can promote its evolvability. For example, the organization of TFs in functionally autonomous protein domains that can evolve independently allows for the evolutionary emergence of new TFs by domain rearrangement, including the gain or loss of protein domains, the shuffling of already existing domains, and expansions or contractions in the number of a given protein domain (Bornberg-Bauer & Albà, 2013). Such domain rearrangements in TF families can lead to a major functional shift and the subsequent expansion of new sub-families of TFs (Schmitz et al., 2016). Changes in TF function can be mediated by either a change in DNA-binding specificity or changes in protein–protein interactions with other TFs or signaling proteins. Together with gene duplication, domain rearrangement was a potent force in the evolution of major TF families (Schmitz et al., 2016), including bHLH TFs (Amoutzias et al., 2004; Morgenstern & Atchley, 2018).

The robustness of a protein can facilitate the acquisition of novel functions (Bloom et al., 2006). In other words, genetic robustness and evolvability can be synergistic (Wagner, 2008). For example, the robustness of TFs due to the existence of paralogous TFs can promote the evolution of novel adaptive regulatory roles. Gene duplication can facilitate the evolution of TFs with divergent binding specificities that control different sets of genes and facilitate adaptation to new niches (Perez et al., 2014). A duplication of a Hox3 TF in two paralogs deep in the lineage of Cyclorrhaphan flies allowed one of the two paralogs (Bicoid, Bcd) to gain the important developmental role of controlling anterior-posterior patterning in fly embryos (Stauber et al., 1999). After the emergence by duplication of Bcd, this TF acquired at least two large-effect mutations that changed its DNA specificity and played a major role in the evolution of this TFs controlling role during early fly development (Liu et al., 2018). In combination with protein domain rearrangements, and given enough evolutionary time, the diversification and expansion of TF families can have deep evolutionary consequences, and it tends to be associated with increases on organismic complexity, both morphological and in terms of the number of cell types (Carroll et al., 2001; De Bodt et al., 2003; Irish, 2003; Levine & Tjian, 2003; Degnan et al., 2009; Vaquerizas et al., 2009; Ruiz-Trillo et al., 2013; Albertin et al., 2015; Schmitz et al., 2016).

C2H2-ZF TF are the most common class of TFs in metazoans (Vaquerizas et al., 2009). The binding specificity of a C2H2-ZF domain is mainly conferred by four DNA-contacting residues within the domain's α-helix (Pabo et al., 2001). A C2H2-ZF domain can bind a wide range of three or four base pairs. C2H2-ZF

TFs typically contain tandem arrays of these domains that bind contiguous DNA sites, which allows this type of TFs the ability to recognize an incredibly large diversity of DNA sequences of variable length (Basciotta et al., 2013). Domain rearrangement and gene duplication have played an important role in the expansion and diversification of C2H2-ZF TFs in animals (Schmitz et al., 2016). Even without gene duplication, the binding specificities of C2H2-ZFs can change over short evolutionary timescales. For example, one-to-one orthologous C2H2-ZF TFs typically show divergence in their DNA-contacting residues across closely related *Drosophila* species (Nadimpalli et al., 2015). The predicted DNA-binding specificities of these domains gradually change as a function of phylogenetic distance, suggesting that single-copy TFs can diverge in their DNA-binding specificities via small evolutionarily viable steps. Robustness may be behind this process of binding specificity modifications in single-copy TFs. While the binding energy of other eukaryotic C2H2-ZF TFs largely depends on base-contacting amino acids, C2H2-ZF TFs from metazoans use non-base contacting amino acids to establish hydrogen bonds with the phosphate backbone of DNA that increase their overall binding energy (Najafabadi et al., 2017). These non-base contacting amino acids provide robustness to mutations in base-contacting amino acids, which may have led to the ability of single-copy C2H2-ZF TFs to rapidly diverge in binding preference. This robustness of the C2H2-ZF domain may have played an important role in the regulatory evolution not only of *Drosophila* but also of other metazoans, including humans, where there is evidence of adaptive evolution in C2H2-ZF domains (Emerson & Thomas, 2009). Similarly, robustness played a role in the evolution of binding specificity in steroid receptors (Starr et al., 2017; Payne & Wagner, 2019).

Besides specificity-altering mutations in DNA-binding domains, gene regulation can also evolve via mutations that change how TFs respond to upstream signaling pathways. For example, a TF that plays an essential role during pregnancy, CEBPB, changed its response to cAMP/PKA signaling from repression to activation due to three amino acid replacements that affected phosphorylation sites in an internal regulatory domain (Lynch et al., 2011). This novel function evolved coincident with the evolution of pregnancy in placental mammals. The alteration of post-translational modification sites is therefore an additional mechanism by which TFs evolve. Such changes facilitate gene regulatory innovations in signaling-dependent transcriptional circuits by altering the function of TFs in specific cell types, while avoiding or minimizing deleterious pleiotropic effects on other cellular functions.

In sum, the regulatory proteins involved in transcriptional regulation often exhibit robustness to mutation, which can facilitate evolvability.

2.2 Robustness and Evolvability of Transcription Factor Binding Sites

Genotype of a *cis*-regulatory element: short DNA sequence
Phenotype of a *cis* element: molecular ability to bind a regulatory protein

2.2.1 The Robustness of Regulatory Sequences

In the previous section, we reviewed how regulatory proteins can be robust to mutational change, and how such robustness can enhance evolvability. The regulatory regions that these proteins bind are also robust to genetic change, and similarly their robustness can synergize with their evolvability. The very short length of TF binding sites confers an additional source of robustness against mutations. Shorter binding sites are less easily disrupted by mutations because they offer a smaller mutational target, a benefit that comes at the expense of reduced specificity (Stewart et al., 2012). However, TF binding sites can also be intrinsically robust to mutations. TFs can specifically bind to dozens or hundreds of different short DNA sequences (Sengupta et al., 2002; Berger et al., 2006; Badis et al., 2009; Wong et al., 2013; Weirauch et al., 2014), and these sequences tend to be mutationally interconnected to one another forming genotype networks of TF binding sites (Payne & Wagner, 2014; Khalid et al., 2016; Aguilar-Rodríguez et al., 2018). In such a genotype network, vertices represent DNA sequences with the ability to specifically bind a particular TF, and two vertices are connected by a link if their associated sequences differ by just a single small mutation, such as a point mutation. The existence of genotype networks of TF binding sites implies that mutations to a binding site will often create mutant sequences that are still able to bind the same TF, thus conferring mutational robustness. Additionally, mutational neighbors in a genotype network tend to have similar binding affinity for a given TF, indicating that binding affinity is also robust to mutation (Payne & Wagner, 2014; Aguilar-Rodríguez et al., 2017). This is important because changes in binding affinity can lead to changes in gene expression (Kasowski et al., 2010; Shultzaberger et al., 2010; Sharon et al., 2012), and both large (Giaever et al., 2002; Gerdes et al., 2003; Dietzl et al., 2007; Hillenmeyer et al., 2008; Ramani et al., 2012; Hart et al., 2015) and small (Dykhuizen et al., 1987; Dekel & Alon, 2005; Rest et al., 2013; Keren et al., 2016) deviations from an optimal mean level of expression can be detrimental to organismal fitness. However, some genes have a nonlinear fitness-expression function with a plateau of maximal fitness for a wide range of expression levels (Rest et al., 2013; Bergen et al., 2016; Keren et al., 2016; Duveau et al., 2017). Therefore, the promoters of these genes are robust to many *cis*-regulatory mutations. For example, nearly all mutations and polymorphisms in the promoter of the yeast gene *TDH3* have no significant effect on fitness in a rich medium containing glucose (Duveau et al., 2017).

The robustness of TF binding sites allows the accumulation of genetic diversity in binding sites, both within species (Aguilar-Rodríguez et al., 2017, 2018), and between species (Weirauch & Hughes, 2010). Intra-specific variation in TF sites is pervasive (Zheng et al., 2011; Garfield et al., 2012; Spivakov et al., 2012; Khurana et al., 2013; Arbiza et al., 2013), and such differences often do not impact the expression level of target genes (Kasowski et al., 2010; Zheng et al., 2010). Similarly, over longer evolutionary time scales, regulatory sequences can diverge considerably at the sequence level without a corresponding divergence at

the phenotypic level of the gene expression patterns they control (Ludwig et al., 2000; Odom et al., 2007).

Another important source for robustness of TF binding sites is the presence of multiple binding sites for the same TF in close proximity to one another (Johnson et al., 1979; Giniger & Ptashne, 1988; Carey et al., 1990; Thanos & Maniatis, 1995; Wasserman & Fickett, 1998; Krivan & Wasserman, 2001; Pfeiffer et al., 2002; Ezer et al., 2015). These homotypic clusters of binding sites are common across all domains of life (Lifanov et al., 2003; Gotea et al., 2010; Gama-Castro et al., 2011). Mutations in a TF binding site can be compensated by the presence of nearby non-mutated TF binding sites (Somma et al., 1991; Spivakov et al., 2012; Kilpinen et al., 2013). This mechanism of mutational robustness has already been reviewed more extensively elsewhere (Payne & Wagner, 2015).

However, TF binding sites are not robust to all mutations, and mutations in these regulatory sequences can often be deleterious and cause disease (Musunuru et al., 2010; Harismendy et al., 2011). Indeed, the majority of single-nucleotide variants in DNA regulatory regions associated with different human diseases tend to alter TF binding sites (Maurano et al., 2012). For example, de novo mutations in DNA regulatory elements active in the human brain are associated with different neurodevelopmental disorders and predicted to increase the binding affinity of the binding sites in which they fall (Short et al., 2018). Mutations that fall in TF binding sites can increase cancer risk (Pomerantz et al., 2009; Khurana et al., 2013; Weinhold et al., 2014; Katainen et al., 2015; Melton et al., 2015). For example, noncoding single-nucleotide variants associated to breast cancer can modulate TF binding affinity resulting in transcriptional misregulation (Liu et al., 2017), which is a hallmark of many cancer types (Lee & Young, 2013; Bhagwat & Vakoc, 2015).

2.2.2 The Evolvability of Regulatory Sequences

Substitutions in *cis*-regulatory sequences may produce novel gene expression patterns associated to evolutionary innovations and adaptations (Wray, 2007; Prud'homme et al., 2007). Single-base pair substitutions in a TF binding site can change the regulatory control of a target gene from one TF to another (Payne & Wagner, 2014; Aguilar-Rodríguez et al., 2018), and this may lead to profound changes in development, physiology or behavior. For example, in rice, a single mutation in the promotor of a C2H2-ZF TF gene reduces its expression by creating a binding site for the transcriptional repressor MYB, and this change in expression increases resistance against rice blast—a fungal disease that can cause significant crop loss (Li et al., 2017). Standing genetic variation within TF binding sites can also contribute to evolutionary adaptation. For example, a recent high-throughput precise genome editing screen found that among 16,006 natural genetic variants in yeast, 572 variants with a significant fitness effect in glucose media were highly enriched in promoters, particularly in TF binding sites (Sharon et al., 2018). The genetic diversity accumulated within binding sites as a consequence of their robustness to mutational change provides an ideal "testing ground" for new mutations by

allowing the exploration of many different genetic backgrounds (Aguilar-Rodríguez et al., 2017, 2018). Robustness is a way to explore new mutational neighborhoods while preserving a phenotype, because it is almost always possible to transform one site into another via a series of mutations that preserve TF binding. Some of these new mutations may create binding sites for a different TF, which may lead to adaptive changes in gene expression (Payne & Wagner, 2014). Therefore, the genetic robustness of TF binding sites can synergize with their evolvability. For example, a comparative analysis of two well-studied transcriptional repressors from phages, showed that it was easier to evolve a cognate site from a non-cognate site for the repressor whose cognate sites are more robust to mutations (Igler et al., 2018). The structure of the genotype networks of TF binding sites furthers our understanding of the "robust-yet-evolvable" nature of these DNA sequences. They tend to be "small-world" (Watts & Strogatz, 1998; Aguilar-Rodríguez et al., 2018), which indicates that binding sites tend to be highly clustered in genotype space (robustness), but also that it is possible to traverse the network with just a few mutations, thus providing efficient access to adjacent genotype networks of other TFs (evolvability).

TF binding sites are short enough that it is possible to study their evolvability comprehensively (Rowe et al., 2010; Jimenez et al., 2013). For example, one can easily measure how strongly a TF binds tens of thousands of different DNA sequences (Berger et al., 2006), and this information is available for thousands of TFs from hundreds of species comprising multiple TF families (Weirauch et al., 2014). Binding affinity is an important molecular phenotype because it is an important contributor to a TFs ability to activate or repress a target gene, and the gene expression patterns that emerge from such TF-DNA interactions embody fundamental biological processes. The regulatory effect on gene expression of a TF can be either fine-tuned or even radically transformed by affinity-altering mutations in TF binding sites (Shultzaberger et al., 2010; Sharon et al., 2012). The mapping of a DNA sequence to binding affinity can be described as an adaptive landscape where one can study how mutation and natural selection can change the capacity of a DNA sequence to bind a particular TF (Berg et al., 2004). A recent study of more than a thousand such landscapes characterized their ruggedness using a variety of measures and found that they are highly navigable via a Darwinian process of mutation and selection, indicating that binding affinity—and thereby gene expression—is readily fine-tuned via mutations in TF binding sites (Aguilar-Rodríguez et al., 2017). These landscapes typically have just a single peak, and these peaks tend to be accessible from any location in the landscape via mutational pathways that increase monotonically in binding affinity. This type of smooth landscape promotes the evolvability of TF binding sites because mutation can bring forth beneficial phenotypic variation from any location on the landscape (Payne & Wagner, 2019). Therefore, the navigability of these TF binding affinity landscapes may have contributed to the enormous success of altering transcriptional regulation as a way to generate variation and innovation throughout evolution.

3 Robustness and Evolvability of Whole Gene Regulatory Circuits

Genotype of a gene circuit: genes for TFs and *cis*-regulatory sequences
Phenotype of a gene circuit: spatiotemporal gene expression pattern

The genotype of a gene regulatory circuit comprises the DNA sequences that encode the circuit's constituent transcription factors, as well as the binding sites for these factors in the promoters and enhancers of the circuit's genes. The phenotype of a gene regulatory circuit is its spatiotemporal gene expression pattern. Most of what we know about the robustness and evolvability of regulatory circuits comes from abstract computational models, such as Boolean circuits (Kauffman, 1969; Wagner, 1996). These studies have shown that there are many genotypes that have the same phenotype, meaning that a large number of circuit configurations are capable of driving the same gene expression pattern. Additionally, these genotypes tend to be arranged as a genotype network, such that it is usually possible to mutate any one regulatory circuit with a given phenotype into any other via a series of intermediates that also produce the phenotype (Ciliberti et al., 2007a, b). Such genotype networks confer robustness and evolvability to regulatory circuits, as they do at the level of the circuit components.

Our understanding of the robustness and evolvability of regulatory circuits continues to advance, with recent studies uncovering how circuit architecture influences the robustness of gene expression patterns to variation in morphogen production rates (Raspopovic et al., 2014; Li et al., 2018), how the robustness of regulatory circuits makes their evolution contingent upon chance mutational events (Starr et al., 2017), and how the evolvability of a regulatory circuit can transcend that of its constituent components (Lagator et al., 2017). In addition, a series of recent studies have shown how robustness and evolvability depend upon the dynamical mechanism a circuit uses to generate its phenotype (Jiménez et al., 2015; Schaerli et al., 2018), and how genotype networks facilitate "system drift," which enhances evolvability (Nocedal et al., 2017; Jaeger, 2018). These recent insights, realized using a combination of increasingly sophisticated computational models and experiments, are the focus of this section.

Gene regulatory circuits can produce the same gene expression pattern using distinct dynamical mechanisms. For example, Cotterell and Sharpe (2010) produced an atlas of regulatory circuits that interpret a morphogen gradient to produce a single stripe of gene expression, using a model based on the gap gene circuit, which drives segmental patterning in dipteran insects. This atlas includes circuits that produce stripes using six distinct dynamical mechanisms—unique spatiotemporal patterns of expression that all converge on a stripe. Analysis of the circuits employing each dynamical mechanism revealed variation in mutational robustness, measured either as the number of distinct circuit topologies, or as the volume of parameter space for a specific topology, that produce a stripe. Thus, identical gene expression phenotypes

can vary in their mutational robustness, depending upon the dynamical mechanism used to generate the phenotype.

The underlying explanation for this phenomenon is that distinct genotype networks are formed by the different sets of stripe-forming circuits with each dynamical mechanism. This means that it is generally not possible to smoothly transition via mutation from one dynamical mechanism to another. Jiménez et al. (2015) explored the consequences of this genotype network fragmentation for evolvability, defined as the ability of mutation to bring forth novel expression phenotypes, such as spatial gradients, inverse stripes, or multiple stripes. They found that evolvability is mechanism-dependent. Circuits using distinct mechanisms differ not only in which phenotypes mutation can bring forth, but also in the relative likelihood of mutations bringing forth such phenotypes. These findings were recently validated with experiments using synthetic stripe-forming regulatory circuits, in which random mutations to two circuits with distinct dynamical mechanisms produced different distributions of novel phenotypes (Schaerli et al., 2018). Such mechanism-dependent evolvability is an example of evolutionary constraint, in which circuits with the same phenotype, but different dynamical mechanisms, differ in their ability to generate phenotypic variation via mutation.

A population of circuits with any one phenotype can accrue mutations that alter the quantitative and qualitative features of the circuit, without affecting the circuit's gene expression phenotype. This phenomenon is referred to as system drift (Weiss & Fullerton, 2000). For example, the final patterning output of the gap gene circuit is conserved among *Drosophila melanogaster* and the scuttle fly *Megaselia abdita*, two species that last shared a common ancestor approximately 180 million years ago. In contrast, the dynamical mechanisms used to generate this phenotype show significant quantitative differences (Wotton et al., 2015). Specifically, gap domains appear more posteriorly and retract from the pole later in *M. adbita* than in *D. melanogaster*. Analysis of a data driven mathematical model of gap gene expression demonstrates that such system drift occurs because the set of gap gene circuits that drive segmental patterning—even those employing distinct dynamical mechanisms—are arranged as a genotype network (Crombach et al., 2016). This permits the accumulation of mutations that affect the strength and identity of a circuit's regulatory interactions, without affecting the final patterning phenotype. This also influences evolvability, because the potential for mutation to cause phenotypic variation varies across a genotype network (Wagner, 2011).

Phenotype-preserving mutations can thus serve as stepping-stones for evolutionary innovations. Another example of this phenomenon is the fungal transcriptional regulator Ndt80, a DNA-binding protein that is conserved across a large group of fungal species that last shared a common ancestor approximately 300 million years ago (Nocedal et al., 2017). Ndt80 is part of a regulatory circuit controlling meiosis and sporulation in most of these species, but it controls the formation of biofilms in *Candida albicans*. This shift in function resulted from system drift. This is evidenced by a comparative analysis of six fungal lineages, which uncovered extensive rewiring in the regulon of Ndt80, even in those lineages where the function of Ndt80 had not changed (Nocedal et al., 2017). This rewiring facilitated

the exploration of new regulatory circuits, potentiating the evolution of the novel phenotype of biofilm formation.

Such rewiring can be extreme. A comparative analysis of the regulatory circuits controlling the conversion of galactose to glucose-1-phosphate in *S. cerevisiae* and *C. albicans* provides an illustrative example (Dalal et al., 2016). In both species, three *GAL* genes are needed for this conversion. In *S. cerevisiae*, these genes are activated by the TF Gal4, whereas in *C. albicans*, they are activated by the TFs Rtg1 and Rtg3. This rewiring primarily occurred via changes in the *cis*-regulatory sequences of the *GAL* genes, which not only resulted in a qualitative change to the structure of the circuit, but also to quantitative changes in the induction ratios of the *GAL* genes and in their response to non-galactose signals. Some of these quantitative changes may have been adaptive for *S. cerevisiae*, because they contribute to the rapid fermentation of different sugars.

In sum, the robustness of gene regulatory circuits facilitates system drift, which enhances evolvability. The extent to which system drift occurs can depend upon the dynamical mechanism a circuit uses to generate its phenotype, which may constrain evolvability. However, system drift need not preserve dynamical mechanism, and some quantitative changes to dynamical mechanism may themselves be adaptive.

4 Concluding Remarks

While the idea behind genotype-phenotype maps can be traced back to the work of Sewall Wright (1932) and John Maynard Smith (1970), the term genotype-phenotype map ("genotype-phenotype mapping") itself was only coined in 1970 by Jim Burns (1970), who outlined the research goals of evolutionary systems biology before the development of systems biology made it feasible. He recognized early on the importance of integrating the mechanistic perspective of biochemistry, cell and molecular biology within the unifying framework of evolutionary biology:

> *It is the quantitative phenotype, arising from the genotypic prescriptions and the environment, which is of critical importance for the cell's survival and which therefore features in population genetic theory. A study of this synthetic problem would thus, by providing genotype-phenotype mappings for simple synthetic systems, help to connect two major areas of biological theory: the biochemical and the population genetic.*

The term "genotype-phenotype map" was re-introduced in 1991 by the developmental biologist Pere Alberch as a useful concept for the integration of genetics into the study of the complex developmental processes that generate morphological phenotypes such as the vertebrate limb (Alberch, 1991). Gene regulatory circuits occupy a central position in the map that goes from a genome to the high-level morphological phenotypes that interested Alberch.

In this chapter, we have reviewed the robustness of such circuits against genetic change, and how such robustness may have contributed to the enormous success of transcriptional regulation as a source of evolutionary novelty. We have

explored some of the mechanistic causes for the robustness and evolvability of transcription factors, the regulatory DNA sequences that they bind, and the gene circuits that emerge from the complex interactions between transcription factors and their binding sites. Space constraints do not allow us to review other molecular mechanism of evolvability in transcriptional regulation, such as stochastic gene expression that can promote evolvability by generating phenotypic heterogeneity in isogenic populations (Payne & Wagner, 2019). Also, we have focused on transcriptional regulation, although there are important levels of gene expression regulation, such as regulatory noncoding RNA, alternative splicing, epigenetic gene regulation, and protein posttranslational modifications. However, less is known about the mechanisms of robustness and evolvability at these levels, or about how they interact with the mechanisms reviewed here, although there is progress being made in this area (Payne et al., 2018). In the foreseeable future, the concepts and tools of evolutionary systems biology, aided by new technological advances, will further our understanding of the mechanisms by which gene regulation is robust to genetic change, yet capable of bringing forth evolutionary adaptations and innovations.

Acknowledgments We apologize to our peers whose important works in this rapidly advancing field were not cited due to space constraints. J.A.-R. acknowledges support by Swiss National Science Foundation grant P2ZHP3_174735 and European Molecular Biology Organization grant ALTF 724-2018. J.L.P. acknowledges support by Swiss National Science Foundation grant PP00P3_170604.

References

Aggarwal, P., Das Gupta, M., Joseph, A. P., et al. (2010). Identification of specific DNA binding residues in the TCP family of transcription factors in *Arabidopsis*. *Plant Cell, 22*, 1174–1189. https://doi.org/10.1105/tpc.109.066647

Aguilar-Rodríguez, J., Payne, J. L., & Wagner, A. (2017). A thousand empirical adaptive landscapes and their navigability. *Nature Ecology and Evolution, 1*, 45. https://doi.org/10.1038/s41559-016-0045

Aguilar-Rodríguez, J., Peel, L., Stella, M., et al. (2018). The architecture of an empirical genotype-phenotype map. *Evolution, 72*, 1242. https://doi.org/10.1111/evo.13487

Alberch, P. (1991). From genes to phenotype: Dynamical systems and evolvability. *Genetica, 84*, 5–11.

Albertin, C. B., Simakov, O., Mitros, T., et al. (2015). The octopus genome and the evolution of cephalopod neural and morphological novelties. *Nature, 524*, 220–224. https://doi.org/10.1038/nature14668

Alonso, C. R., & Wilkins, A. S. (2005). The molecular elements that underlie developmental evolution. *Nature Reviews. Genetics, 6*, 709–715. https://doi.org/10.1038/nrg1676

Alvarez-Ponce, D., Aguilar-Rodríguez, J., & Fares, M. A. (2019). Molecular chaperones accelerate the evolution of their protein clients in yeast. *Genome Biology and Evolution, 11*(8), 2360–2375.

Amoutzias, G. D., Robertson, D. L., Oliver, S. G., & Bornberg-Bauer, E. (2004). Convergent evolution of gene networks by single-gene duplications in higher eukaryotes. *EMBO Reports, 5*, 274–279. https://doi.org/10.1038/sj.embor.7400096

Arbiza, L., Gronau, I., Aksoy, B. A., et al. (2013). Genome-wide inference of natural selection on human transcription factor binding sites. *Nature Genetics, 45*, 723–729. https://doi.org/10.1038/ng.2658

Babu, M. M., & Teichmann, S. A. (2003). Evolution of transcription factors and the gene regulatory network in Escherichia coli. *Nucleic Acids Research, 31*, 1234–1244. https://doi.org/10.1093/nar/gkg210

Badis, G., Berger, M. F., Philippakis, A. A., et al. (2009). Diversity and complexity in DNA recognition by transcription factors. *Science, 324*, 1720–1723. https://doi.org/10.1126/science.1162327

Baker, C. R., Tuch, B. B., & Johnson, A. D. (2011). Extensive DNA-binding specificity divergence of a conserved transcription regulator. *Proceedings of the National Academy of Sciences, 108*, 7493–7498. https://doi.org/10.1073/pnas.1019177108

Barrera, L. A., Vedenko, A., Kurland, J. V., et al. (2016). Survey of variation in human transcription factors reveals prevalent DNA binding changes. *Science, 351*, 1450–1454. https://doi.org/10.1126/science.aad2257

Basciotta, M. D., Christensen, R. G., Celniker, S. E., et al. (2013). Global analysis of *Drosophila* Cys_2-His_2 zinc finger proteins reveals a multitude of novel recognition motifs and binding determinants. *Genome Research, 23*, 928–940. https://doi.org/10.1101/gr.151472.112

Berg, J., Willmann, S., & Lässig, M. (2004). Adaptive evolution of transcription factor binding sites. *BMC Evolutionary Biology, 4*, 42. https://doi.org/10.1186/1471-2148-4-42

Bergen, A. C., Olsen, G. M., & Fay, J. C. (2016). Divergent MLS1 promoters lie on a fitness plateau for gene expression. *Molecular Biology and Evolution, 33*, 1270–1279. https://doi.org/10.1093/molbev/msw010

Berger, M. F., Philippakis, A. A., Qureshi, A. M., et al. (2006). Compact, universal DNA microarrays to comprehensively determine transcription-factor binding site specificities. *Nature Biotechnology, 24*, 1429–1435. https://doi.org/10.1038/nbt1246

Bergman, A., & Siegal, M. L. (2003). Evolutionary capacitance as a general feature of complex gene networks. *Nature, 424*, 549–552. https://doi.org/10.1038/nature01765

Bhagwat, A. S., & Vakoc, C. R. (2015). Targeting transcription factors in cancer. *Trends in Cancer, 1*, 53–65. https://doi.org/10.1016/j.trecan.2015.07.001

Bloom, J. D., Labthavikul, S. T., Otey, C. R., & Arnold, F. H. (2006). Protein stability promotes evolvability. *Proceedings of the National Academy of Sciences, 103*, 5869–5874. https://doi.org/10.1073/pnas.0510098103

Bornberg-Bauer, E., & Albà, M. M. (2013). Dynamics and adaptive benefits of modular protein evolution. *Current Opinion in Structural Biology, 23*, 459–466.

Britten, R. J., & Davidson, E. H. (1969). Gene regulation for higher cells: A theory new facts regarding the organization of the genome. *Science, 25*, 349–357. https://doi.org/10.1016/j.ympev.2006.05.020

Burns, J. (1970). The synthetic problem and the genotype-phenotype relation in cellular metabolism. In C. H. Waddington (Ed.), *Towards a theoretical biology. Volume 3: Drafts. An IUBS Symposium* (pp. 47–51). Aldine Publishing Company.

Carey, M., Lin, Y. S., Green, M. R., & Ptashne, M. (1990). A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives. *Nature, 345*, 361–364. https://doi.org/10.1038/345361a0

Carroll, S. B. (2005). Evolution at two levels: On genes and form. *PLoS Biology, 3*, e245. https://doi.org/10.1371/journal.pbio.0030245

Carroll, S. B. (2008). Evo-devo and an expanding evolutionary synthesis: A genetic theory of morphological evolution. *Cell, 134*, 25–36. https://doi.org/10.1016/j.cell.2008.06.030

Carroll, S. B., Grenier, J. K., & Weatherbee, S. D. (2001). *From DNA to diversity. Molecular genetics and the evolution of animal design*. Blackwell.

Ciliberti, S., Martin, O. C., & Wagner, A. (2007a). Innovation and robustness in complex regulatory gene networks. *Proceedings of the National Academy of Sciences, 104*, 13591–13596. https://doi.org/10.1073/pnas.0705396104

Ciliberti, S., Martin, O. C., & Wagner, A. (2007b). Robustness can evolve gradually in complex regulatory gene networks with varying topology. *PLoS Computational Biology, 3*, 0164–0173. https://doi.org/10.1371/journal.pcbi.0030015

Conant, G. C., & Wagner, A. (2004). Duplicate genes and robustness to transient gene knock-downs in Caenorhabditis elegans. *Proceedings of the Royal Society B: Biological Sciences, 271*, 89–96. https://doi.org/10.1098/rspb.2003.2560

Cook, W. J., Mosley, S. P., Audino, D. C., et al. (1994). Mutations in the zinc-finger region of the yeast regulatory protein ADR1 affect both DNA binding and transcriptional activation. *The Journal of Biological Chemistry, 269*, 9374–9379.

Cotterell, J., & Sharpe, J. (2010). An atlas of gene regulatory networks reveals multiple three-gene mechanisms for interpreting morphogen gradients. *Molecular Systems Biology, 6*, 425. https://doi.org/10.1038/msb.2010.74

Crombach, A., Wotton, K. R., Jiménez-Guri, E., & Jaeger, J. (2016). Gap gene regulatory dynamics evolve along a genotype network. *Molecular Biology and Evolution, 33*, 1293–1307. https://doi.org/10.1093/molbev/msw013

Dalal, C. K., Zuleta, I. A., Mitchell, K. F., et al. (2016). Transcriptional rewiring over evolutionary timescales changes quantitative and qualitative properties of gene expression. *eLife, 5*, 1–24. https://doi.org/10.7554/eLife.18981

De Bodt, S., Raes, J., Van De Peer, Y., & Theißen, G. (2003). And then there were many: MADS goes genomic. *Trends in Plant Science, 8*, 475–483. https://doi.org/10.1016/j.tplants.2003.09.006

De Masi, F., Grove, C. A., Vedenko, A., et al. (2011). Using a structural and logics systems approach to infer bHLH-DNA binding specificity determinants. *Nucleic Acids Research, 39*, 4553–4563. https://doi.org/10.1093/nar/gkr070

de Visser, J. A. G. M., Hermisson, J., Wagner, G. P., et al. (2003). Perspective: Evolution and detection of genetic robustness. *Evolution (N Y), 57*, 1959–1972. https://doi.org/10.1554/02-750R

Degnan, B. M., Vervoort, M., Larroux, C., & Richards, G. S. (2009). Early evolution of metazoan transcription factors. *Current Opinion in Genetics & Development, 19*, 591–599. https://doi.org/10.1016/j.gde.2009.09.008

Dekel, E., & Alon, U. (2005). Optimality and evolutionary tuning of the expression level of a protein. *Nature, 436*, 588–592. https://doi.org/10.1038/nature03842

Dietzl, G., Chen, D., Schnorrer, F., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature, 448*, 151–156. https://doi.org/10.1038/nature05954

Duveau, F., Toubiana, W., & Wittkopp, P. J. (2017). Fitness effects of cis-regulatory variants in the *Saccharomyces cerevisiae* TDH3 promoter. *Molecular Biology and Evolution, 34*, 2908–2912. https://doi.org/10.1093/molbev/msx224

Dykhuizen, D. E., Dean, A. M., & Hartl, D. L. (1987). Metabolic flux and fitness. *Genetics, 115*, 25–31.

Emerson, R. O., & Thomas, J. H. (2009). Adaptive evolution in zinc finger transcription factors. *PLoS Genetics, 5*, e1000325. https://doi.org/10.1371/journal.pgen.1000325

Eyre-Walker, A., & Keightley, P. D. (2007). The distribution of fitness effects of new mutations. *Nature Reviews. Genetics, 8*, 610–618. https://doi.org/10.1038/nrg2146

Ezer, D., Zabet, N. R., & Adryan, B. (2015). Homotypic clusters of transcription factor binding sites: A model system for understanding the physical mechanics of gene expression. *Computational and Structural Biotechnology Journal, 10*, 63–69. https://doi.org/10.1016/j.csbj.2014.07.005

Fares, M. A. (2015). The origins of mutational robustness. *Trends in Genetics, 31*, 373–381. https://doi.org/10.1016/j.tig.2015.04.008

Galant, R., & Carroll, S. B. (2002). Evolution of a transcriptional repression domain in an insect Hox protein. *Nature, 415*, 910–913. https://doi.org/10.1038/nature717

Gama-Castro, S., Salgado, H., Peralta-Gil, M., et al. (2011). RegulonDB version 7.0: Transcriptional regulation of *Escherichia coli* K-12 integrated within genetic sensory response units (Gensor Units). *Nucleic Acids Research, 39*, D98–D105. https://doi.org/10.1093/nar/gkq1110

Garfield, D., Haygood, R., Nielsen, W. J., & Wray, G. A. (2012). Population genetics of cis-regulatory sequences that operate during embryonic development in the sea urchin *Strongylocentrotus purpuratus*. *Evolution & Development, 14*, 152–167. https://doi.org/10.1111/j.1525-142X.2012.00532.x

Gerdes, S. Y., Scholle, M. D., Campbell, J. W., et al. (2003). Experimental determination and system level analysis of essential genes in *Escherichia coli* MG1655. *Journal of Bacteriology, 185*, 5673–5684. https://doi.org/10.1128/JB.185.19.5673

Giaever, G., Chu, A. M., Ni, L., et al. (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature, 418*, 387–391. https://doi.org/10.1038/nature00935

Giniger, E., & Ptashne, M. (1988). Cooperative DNA binding of the yeast transcriptional activator GAL4. *Proceedings of the National Academy of Sciences of the United States of America, 85*, 382–386.

Gotea, V., Visel, A., Westlund, J. M., et al. (2010). Homotypic clusters of transcription factor binding sites are a key component of human promoters and enhancers. *Genome Research, 20*, 565–577. https://doi.org/10.1101/gr.104471.109

Gu, Z., Steinmetz, L. M., Gu, X., et al. (2003). Role of duplicate genes in genetic robustness against null mutations. *Nature, 421*, 63–66. https://doi.org/10.1038/nature01198

Guttman, M., & Rinn, J. L. (2012). Modular regulatory principles of large non-coding RNAs. *Nature, 482*, 339–346. https://doi.org/10.1038/nature10887

Harismendy, O., Notani, D., Song, X., et al. (2011). 9p21 DNA variants associated with coronary artery disease impair interferon-γ 3 signalling response. *Nature, 470*, 264–270. https://doi.org/10.1038/nature09753

Hart, T., Chandrashekhar, M., Aregger, M., et al. (2015). High-resolution CRISPR screens reveal fitness genes and genotype-specific cancer liabilities. *Cell, 163*, 1515–1526. https://doi.org/10.1016/j.cell.2015.11.015

He, X., & Zhang, J. (2006). Transcriptional reprogramming and backup between duplicate genes: Is it a genomewide phenomenon? *Genetics, 172*, 1363–1367. https://doi.org/10.1534/genetics.105.049890

Hillenmeyer, M. E., Fung, E., Wildenhain, J., et al. (2008). The chemical genomic portrait of yeast: Uncovering a phenotype for all genes. *Science, 320*, 362–365. https://doi.org/10.1126/science.1150021

Hollenhorst, P. C., Shah, A. A., Hopkins, C., & Graves, B. J. (2007). Genome-wide analyses reveal properties of redundant and specific promoter occupancy within the ETS gene family. *Genes & Development, 21*, 1882–1894. https://doi.org/10.1101/gad.1561707

Igler, C., Lagator, M., Tkačik, G., et al. (2018). Evolutionary potential of transcription factors for gene regulatory rewiring. *Nature Ecology and Evolution, 2*, 1633–1643. https://doi.org/10.1038/s41559-018-0651-y

Irish, V. F. (2003). The evolution of floral homeotic gene function. *BioEssays, 25*, 637–646. https://doi.org/10.1002/bies.10292

Itzkovitz, S., Tlusty, T., & Alon, U. (2006). Coding limits on the number of transcription factors. *BMC Genomics, 7*, 1–15. https://doi.org/10.1186/1471-2164-7-239

Jaeger, J. (2018). Shift happens: The developmental and evolutionary dynamics of the gap gene system. *Current Opinion in Systems Biology, 11*, 65–73. https://doi.org/10.1016/j.coisb.2018.08.004

Jiménez, A., Munteanu, A., Sharpe, J., et al. (2015). Dynamics of gene circuits shapes evolvability. *Proceedings of the National Academy of Sciences of the United States of America, 112*, 2103–2108. https://doi.org/10.1073/pnas.1411065112

Jimenez, J. I., Xulvi-Brunet, R., Campbell, G. W., et al. (2013). Comprehensive experimental fitness landscape and evolutionary network for small RNA. *Proceedings of the National Academy of Sciences of the United States of America, 110*, 14984–14989. https://doi.org/10.1073/pnas.1307604110

Johnson, A. D., Meyer, B. J., & Ptashne, M. (1979). Interactions between DNA-bound repressors govern regulation by the lambda phage repressor. *Proceedings of the National Academy of Sciences of the United States of America, 76*, 5061–5065.

Kafri, R., Bar-Even, A., & Pilpel, Y. (2005). Transcription control reprogramming in genetic backup circuits. *Nature Genetics, 37*, 295–299. https://doi.org/10.1038/ng1523

Kasowski, M., Grubert, F., Heffelfinger, C., et al. (2010). Variation in transcription factor binding among humans. *Science, 328*, 232–235. https://doi.org/10.1126/science.1183621

Katainen, R., Dave, K., Pitkänen, E., et al. (2015). CTCF/cohesin-binding sites are frequently mutated in cancer. *Nature Genetics, 47*, 818–821. https://doi.org/10.1038/ng.3335

Kauffman, S. A. (1969). Metabolic stability and epigenesis in randomly constructed genetic nets. *Journal of Theoretical Biology, 22*, 437–467. https://doi.org/10.1354/vp.45-1-111

Keane, O. M., Toft, C., Carretero-Paulet, L., et al. (2014). Preservation of genetic and regulatory robustness in ancient gene duplicates of *Saccharomyces cerevisiae*. *Genome Research, 24*, 1830–1841. https://doi.org/10.1101/gr.176792.114

Keren, H., Lev-Maor, G., & Ast, G. (2010). Alternative splicing and evolution: Diversification, exon definition and function. *Nature Reviews. Genetics, 11*, 345–355. https://doi.org/10.1038/nrg2776

Keren, L., Hausser, J., Lotan-Pompan, M., et al. (2016). Massively parallel interrogation of the effects of gene expression levels on fitness. *Cell, 166*, 1282–1294.e18. https://doi.org/10.1016/j.cell.2016.07.024

Khalid, F., Aguilar-Rodríguez, J., Wagner, A., & Payne, J. L. (2016). Genonets server–a web server for the construction, analysis and visualization of genotype networks. *Nucleic Acids Research, 44*, W70–W76.

Khurana, E., Fu, Y., Colonna, V., et al. (2013). Integrative annotation of variants from 1092 humans: Application to cancer genomics. *Science, 342*, 1235587. https://doi.org/10.1126/science.1235587

Kilpinen, H., Waszak, S. M., Gschwind, A. R., et al. (2013). Coordinated effects of sequence variation on DNA binding, chromatin structure, and transcription. *Science, 342*, 744–747. https://doi.org/10.1126/science.1242463

Krivan, W., & Wasserman, W. W. (2001). A predictive model for regulatory sequences directing liver-specific transcription. *Genome Research, 11*, 1559–1566. https://doi.org/10.1101/gr.180601

Lachowiec, J., Lemus, T., Thomas, J. H., et al. (2013). The protein chaperone HSP90 can facilitate the divergence of gene duplicates. *Genetics, 193*, 1269–1277. https://doi.org/10.1534/genetics.112.148098

Lagator, M., Sarikas, S., Acar, H., et al. (2017). Regulatory network structure determines patterns of intermolecular epistasis. *eLife, 6*, 1–22. https://doi.org/10.7554/eLife.28921

Latchman, D. S. (2008). *Eukaryotic transcription factors*. Elsevier Science.

Lawrence, P. A. (1992). *The making of a fly: The genetics of animal design*. Wiley-Blackwell.

Lee, T. I., & Young, R. A. (2013). Transcriptional regulation and its misregulation in disease. *Cell, 152*, 1237–1251. https://doi.org/10.1016/j.cell.2013.02.014

Lehner, B. (2010). Genes confer similar robustness to environmental, stochastic, and genetic perturbations in yeast. *PLoS One, 5*, e9035. https://doi.org/10.1371/journal.pone.0009035

Levine, M., & Tjian, R. (2003). Transcription regulation and animal diversity. *Nature, 424*, 147–151. https://doi.org/10.1038/nature01763

Li, P., Markson, J. S., Wang, S., et al. (2018). Morphogen gradient reconstitution reveals Hedgehog pathway design principles. *Science, 360*, 543–548. https://doi.org/10.1126/science.aao0645

Li, W., Zhu, Z., Chern, M., et al. (2017). A natural allele of a transcription factor in rice confers broad-spectrum blast resistance. *Cell, 170*, 114–126.e15. https://doi.org/10.1016/j.cell.2017.06.008

Lifanov, A. P., Makeev, V. J., Nazina, A. G., & Papatsenko, D. A. (2003). Homotypic regulatory clusters in Drosophila. *Genome Research, 13*, 579–588. https://doi.org/10.1101/gr.668403

Liu, Q., Onal, P., Datta, R. R., et al. (2018). Ancient mechanisms for the evolution of the bicoid homeodomain's function in fly development. *eLife, 7*, 1–28. https://doi.org/10.7554/eLife.34594

Liu, Y., Walavalkar, N. M., Dozmorov, M. G., et al. (2017). Identification of breast cancer associated variants that modulate transcription factor binding. *PLoS Genetics, 13*, 1–21. https://doi.org/10.1371/journal.pgen.1006761

Ludwig, M. Z., Bergman, C., Patel, N. H., & Kreitman, M. (2000). Evidence for stabilizing selection in a eukaryotic enhancer element. *Nature, 403*, 564–567. https://doi.org/10.1038/35000615

Lynch, V. J., May, G., & Wagner, G. P. (2011). Regulatory evolution through divergence of a phosphoswitch in the transcription factor CEBPB. *Nature, 480*, 383–386. https://doi.org/10.1038/nature10595

Lynch, V. J., Tanzer, A., Wang, Y., et al. (2008). Adaptive changes in the transcription factor HoxA-11 are essential for the evolution of pregnancy in mammals. *Proceedings of the National Academy of Sciences, 105*, 14928–14933. https://doi.org/10.1073/pnas.0802355105

Madan Babu, M., Teichmann, S. A., & Aravind, L. (2006). Evolutionary dynamics of prokaryotic transcriptional regulatory networks. *Journal of Molecular Biology, 358*, 614–633. https://doi.org/10.1016/j.jmb.2006.02.019

Maerkl, S. J., & Quake, S. R. (2009). Experimental determination of the evolvability of a transcription factor. *Proceedings of the National Academy of Sciences of the United States of America, 106*, 18650–18655. https://doi.org/10.1073/pnas.0907688106

Majithia, A. R., Tsuda, B., Agostini, M., et al. (2016). Prospective functional classification of all possible missense variants in PPARG. *Nature Genetics, 48*, 1570–1575. https://doi.org/10.1038/ng.3700

Masel, J., & Trotter, M. V. (2010). Robustness and evolvability. *Trends in Genetics, 26*, 406–414. https://doi.org/10.1016/j.tig.2010.06.002

Mathias, J. R., Zhong, H., Jin, Y., & Vershon, A. K. (2001). Altering the DNA-binding specificity of the yeast Matalpha 2 homeodomain protein. *The Journal of Biological Chemistry, 276*, 32696–32703. https://doi.org/10.1074/jbc.M103097200

Maurano, M. T., Humbert, R., Rynes, E., et al. (2012). Systematic localization of common disease-associated variation in regulatory DNA. *Science, 337*, 1190–1195. https://doi.org/10.1126/science.1222794

Maynard Smith, J. (1970). Natural selection and the concept of a protein space. *Nature, 225*, 563–564. https://doi.org/10.1038/225563a0

Melton, C., Reuter, J. A., Spacek, D. V., & Snyder, M. (2015). Recurrent somatic mutations in regulatory regions of human cancer genomes. *Nature Genetics, 47*, 710–716. https://doi.org/10.1038/ng.3332

Morgenstern, B., & Atchley, W. R. (2018). Evolution of bHLH transcription factors: Modular evolution by domain shuffling? *Molecular Biology and Evolution, 16*, 1654–1663.

Munro, D., Ghersi, D., & Singh, M. (2018). Two critical positions in zinc finger domains are heavily mutated in three human cancer types. *PLoS Computational Biology, 14*, 1–17. https://doi.org/10.1371/journal.pcbi.1006290

Musunuru, K., Strong, A., Frank-Kamenetsky, M., et al. (2010). From noncoding variant to phenotype via SORT1 at the 1p13 cholesterol locus. *Nature, 466*, 714–719. https://doi.org/10.1038/nature09266

Nadimpalli, S., Persikov, A. V., & Singh, M. (2015). Pervasive variation of transcription factor orthologs contributes to regulatory network evolution. *PLoS Genetics, 11*, e1005011. https://doi.org/10.1371/journal.pgen.1005011

Najafabadi, H. S., Garton, M., Weirauch, M. T., et al. (2017). Non-base-contacting residues enable kaleidoscopic evolution of metazoan C2H2 zinc finger DNA binding. *Genome Biology, 18*, 1–15. https://doi.org/10.1186/s13059-017-1287-y

Nocedal, I., Mancera, E., & Johnson, A. D. (2017). Gene regulatory network plasticity predates a switch in function of a conserved transcription regulator. *eLife, 6*, 1–20. https://doi.org/10.7554/elife.23250

Noyes, M. B., Christensen, R. G., Wakabayashi, A., et al. (2008). Analysis of homeodomain specificities allows the family-wide prediction of preferred recognition sites. *Cell, 133*, 1277–1289. https://doi.org/10.1016/j.cell.2008.05.023

Odom, D. T., Dowell, R. D., Jacobsen, E. S., et al. (2007). Tissue-specific transcriptional regulation has diverged significantly between human and mouse. *Nature Genetics, 39*, 730–732. https://doi.org/10.1038/ng2047

Pabo, C. O., Peisach, E., & Grant, R. A. (2001). Design and selection of novel Cys2His2 zinc finger proteins. *Annual Review of Biochemistry, 70*, 313–340. https://doi.org/10.1146/annurev.biochem.70.1.313

Pauli, A., Rinn, J. L., & Schier, A. F. (2011). Non-coding RNAs as regulators of embryogenesis. *Nature Reviews. Genetics, 12*, 136–149. https://doi.org/10.1038/nrg2904

Payne, J. L., Khalid, F., & Wagner, A. (2018). RNA-mediated gene regulation is less evolvable than transcriptional regulation. *Proceedings of the National Academy of Sciences of the United States of America, 115*, E3481–E3490. https://doi.org/10.1073/pnas.1719138115

Payne, J. L., & Wagner, A. (2014). The robustness and evolvability of transcription factor binding sites. *Science, 343*, 875–877. https://doi.org/10.1126/science.1249046

Payne, J. L., & Wagner, A. (2015). Mechanisms of mutational robustness in transcriptional regulation. *Frontiers in Genetics, 6*, 322. https://doi.org/10.3389/fgene.2015.00322

Payne, J. L., & Wagner, A. (2019). The causes of evolvability and their evolution. *Nature Reviews. Genetics, 20*, 24–38. https://doi.org/10.1038/s41576-018-0069-z

Pelechano, V., & Steinmetz, L. M. (2013). Gene regulation by antisense transcription. *Nature Reviews. Genetics, 14*, 880–893. https://doi.org/10.1038/nrg3594

Perez, J. C., Fordyce, P. M., Lohse, M. B., et al. (2014). How duplicated transcription regulators can diversify to govern the expression of nonoverlapping sets of genes. *Genes & Development, 28*, 1272–1277. https://doi.org/10.1101/gad.242271.114

Pfeiffer, B. D., Berman, B. P., Levine, M., et al. (2002). Exploiting transcription factor binding site clustering to identify cis-regulatory modules involved in pattern formation in the *Drosophila* genome. *Proceedings of the National Academy of Sciences, 99*, 757–762. https://doi.org/10.1073/pnas.231608898

Pomerantz, M. M., Ahmadiyeh, N., Jia, L., et al. (2009). The 8q24 cancer risk variant rs6983267 shows long-range interaction with MYC in colorectal cancer. *Nature Genetics, 41*, 882–884. https://doi.org/10.1038/ng.403

Prud'homme, B., Gompel, N., & Carroll, S. B. (2007). Emerging principles of regulatory evolution. *Proceedings of the National Academy of Sciences, 104*, 8605–8612. https://doi.org/10.1073/pnas.0700488104

Ptashne, M., & Gann, A. (2002). *Genes & signals*. Cold Spring Harbor Laboratory Press.

Ramani, A. K., Chuluunbaatar, T., Verster, A. J., et al. (2012). The majority of animal genes are required for wild-type fitness. *Cell, 148*, 792–802. https://doi.org/10.1016/j.cell.2012.01.019

Raspopovic, J., Marcon, L., Russo, L., & Sharpe, J. (2014). Modeling digits. Digit patterning is controlled by a Bmp-Sox9-Wnt Turing network modulated by morphogen gradients. *Science, 345*, 566–570. https://doi.org/10.1126/science.1252960

Rest, J. S., Morales, C. M., Waldron, J. B., et al. (2013). Nonlinear fitness consequences of variation in expression level of a eukaryotic gene. *Molecular Biology and Evolution, 30*, 448–456. https://doi.org/10.1093/molbev/mss248

Ronshaugen, M., McGinnis, N., & McGinnis, W. (2002). Hox protein mutation and macroevolution of the insect body plan. *Nature, 415*, 914–917. https://doi.org/10.1038/nature716

Rowe, W., Platt, M., Wedge, D. C., et al. (2010). Analysis of a complete DNA-protein affinity landscape. *Journal of the Royal Society Interface, 7*, 397–408. https://doi.org/10.1098/rsif.2009.0193

Ruiz-Trillo, I., de Mendoza, A., Torruella, G., et al. (2013). Transcription factor evolution in eukaryotes and the assembly of the regulatory toolkit in multicellular lineages. *Proceedings of the National Academy of Sciences, 110*, E4858–E4866. https://doi.org/10.1073/pnas.1311818110

Schaerli, Y., Jiménez, A., Duarte, J. M., et al. (2018). Synthetic circuits reveal how mechanisms of gene regulatory networks constrain evolution. *Molecular Systems Biology, 14*, e8102. https://doi.org/10.15252/msb.20178102

Schmitz, J. F., Zimmer, F., & Bornberg-Bauer, E. (2016). Mechanisms of transcription factor evolution in Metazoa. *Nucleic Acids Research, 44*, 6287–6297. https://doi.org/10.1093/nar/gkw492

Sengupta, A. M., Djordjevic, M., & Shraiman, B. I. (2002). Specificity and robustness in transcription control networks. *Proceedings of the National Academy of Sciences, 99*, 2072–2077. https://doi.org/10.1073/pnas.022388499

Sharon, E., Chen, S. A., Khosla, N. M., et al. (2018). Functional genetic variants revealed by massively parallel precise genome editing. *Cell, 175*, 544–557.e16. https://doi.org/10.1016/j.cell.2018.08.057

Sharon, E., Kalma, Y., Sharp, A., et al. (2012). Inferring gene regulatory logic from high-throughput measurements of thousands of systematically designed promoters. *Nature Biotechnology, 30*, 521–530. https://doi.org/10.1038/nbt.2205

Short, P. J., McRae, J. F., Gallone, G., et al. (2018). De novo mutations in regulatory elements in neurodevelopmental disorders. *Nature, 555*, 611–616. https://doi.org/10.1038/nature25983

Shultzaberger, R. K., Malashock, D. S., Kirsch, J. F., & Eisen, M. B. (2010). The fitness landscapes of cis-acting binding sites in different promoter and environmental contexts. *PLoS Genetics, 6*, e1001042. https://doi.org/10.1371/journal.pgen.1001042

Smith, Z. D., & Meissner, A. (2013). DNA methylation: Roles in mammalian development. *Nature Reviews. Genetics, 14*, 204–220. https://doi.org/10.1038/nrg3354

Somma, M. P., Pisano, C., & Lavia, P. (1991). The housekeeping promoter from the mouse CpG island HTF9 contains multiple protein-binding elements that are functionally redundant. *Nucleic Acids Research, 19*, 2817–2824. https://doi.org/10.1093/nar/19.11.2817

Sorrells, T. R., Johnson, A. N., Howard, C. J., et al. (2018). Intrinsic cooperativity potentiates parallel cis-regulatory evolution. *eLife, 7*, 1–29. https://doi.org/10.7554/eLife.37563

Spivakov, M., Akhtar, J., Kheradpour, P., et al. (2012). Analysis of variation at transcription factor binding sites in *Drosophila* and humans. *Genome Biology, 13*, R49. https://doi.org/10.1186/gb-2012-13-9-r49

Staller, M. V., Holehouse, A. S., Swain-Lenz, D., et al. (2018). A high-throughput mutational scan of an intrinsically disordered acidic transcriptional activation domain. *Cell Systems, 6*, 444–455. https://doi.org/10.1016/j.cels.2018.01.015

Starr, T. N., Picton, L. K., & Thornton, J. W. (2017). Alternative evolutionary histories in the sequence space of an ancient protein. *Nature, 549*, 409–413. https://doi.org/10.1038/nature23902

Starr, T. N., & Thornton, J. W. (2016). Epistasis in protein evolution. *Protein Science, 25*, 1204–1218. https://doi.org/10.1002/pro.2897

Stauber, M., Jäckle, H., & Schmidt-Ott, U. (1999). The anterior determinant bicoid of *Drosophila* is a derived Hox class 3 gene. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 3786–3789. https://doi.org/10.1073/pnas.96.7.3786

Stern, D. L. (2000). Evolutionary developmental biology and the problem of variation. *Evolution, 54*, 1079–1091. https://doi.org/10.1111/j.0014-3820.2000.tb00544.x

Stern, D. L., & Orgogozo, V. (2008). The loci of evolution: How predictable is genetic evolution? *Evolution (N Y), 62*, 2155–2177. https://doi.org/10.1111/j.1558-5646.2008.00450.x

Stewart, A. J., Hannenhalli, S., & Plotkin, J. B. (2012). Why transcription factor binding sites are ten nucleotides long. *Genetics, 192*, 973–985. https://doi.org/10.1534/genetics.112.143370

Stormo, G. D. (2013). *Introduction to protein-DNA interactions: Structure, thermodynamics, and bioinformatics*. Cold Spring Harbor Laboratory Press.

Stormo, G. D., & Zhao, Y. (2010). Determining the specificity of protein-DNA interactions. *Nature Reviews. Genetics, 11*, 751–760. https://doi.org/10.1038/nrg2845

Taipale, M., Jarosz, D. F., & Lindquist, S. (2010). HSP90 at the hub of protein homeostasis: Emerging mechanistic insights. *Nature Reviews. Molecular Cell Biology, 11*, 515–528. https://doi.org/10.1038/nrm2918

Teichmann, S. A., & Babu, M. M. (2004). Gene regulatory network growth by duplication. *Nature Genetics, 36*, 492–496. https://doi.org/10.1038/ng1340

Thanos, D., & Maniatis, T. (1995). Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. *Cell, 83*, 1091–1100. https://doi.org/10.1016/0092-8674(95)90136-1

Tian, B., & Manley, J. L. (2016). Alternative polyadenylation of mRNA precursors. *Nature Reviews. Molecular Cell Biology, 18*, 18–30. https://doi.org/10.1038/nrm.2016.116

Tischler, J., Lehner, B., Chen, N., & Fraser, A. G. (2006). Combinatorial RNA interference in *Caenorhabditis elegans* reveals that redundancy between gene duplicates can be maintained for more than 80 million years of evolution. *Genome Biology, 7*, 1–13. https://doi.org/10.1186/gb-2006-7-8-r69

Toll-Riera, M., & Albà, M. M. (2013). Emergence of novel domains in proteins. *BMC Evolutionary Biology, 13*, 47. https://doi.org/10.1186/1471-2148-13-47

Tsong, A. E., Tuch, B. B., Li, H., & Johnson, A. D. (2006). Evolution of alternative transcriptional circuits with identical logic. *Nature, 443*, 415–420. https://doi.org/10.1038/nature05099

Vaquerizas, J. M., Kummerfeld, S. K., Teichmann, S. A., & Luscombe, N. M. (2009). A census of human transcription factors: Function, expression and evolution. *Nature Reviews. Genetics, 10*, 252–263. https://doi.org/10.1038/nrg2538

Veraksa, A., Del Campo, M., & McGinnis, W. (2000). Developmental patterning genes and their conserved functions: From model organisms to humans. *Molecular Genetics and Metabolism, 69*, 85–100. https://doi.org/10.1006/mgme.2000.2963

Wagner, A. (1996). Does evolutionary plasticity evolve? *Evolution (N Y), 50*, 1008. https://doi.org/10.2307/2410642

Wagner, A. (2005). *Robustness and evolvability in living systems*. Princeton University Press.

Wagner, A. (2008). Robustness and evolvability: A paradox resolved. *Proceedings of the Royal Society B, 275*, 91–100. https://doi.org/10.1098/rspb.2007.1137

Wagner, A. (2011). *The origins of evolutionary innovations: A theory of transformative change in living systems*. Oxford University Press.

Wasserman, W. W., & Fickett, J. W. (1998). Identification of regulatory regions which confer muscle-specific gene expression. *Journal of Molecular Biology, 278*, 167–181. https://doi.org/10.1006/jmbi.1998.1700

Watts, D. J., & Strogatz, S. H. (1998). Collective dynamics of 'small-world' networks. *Nature, 393*, 440–442. https://doi.org/10.1038/30918

Weinhold, N., Jacobsen, A., Schultz, N., et al. (2014). Genome-wide analysis of noncoding regulatory mutations in cancer. *Nature Genetics, 46*, 1160–1165. https://doi.org/10.1038/ng.3101

Weirauch, M. T., & Hughes, T. R. (2010). Conserved expression without conserved regulatory sequence: The more things change, the more they stay the same. *Trends in Genetics, 26*, 66–74. https://doi.org/10.1016/j.tig.2009.12.002

Weirauch, M. T., & Hughes, T. R. (2011). A catalogue of eukaryotic transcription factor types, their evolutionary origin, and species distribution. In T. R. Hughes (Ed.), *A Handbook of transcription factors* (pp. 25–73). Springer.

Weirauch, M. T., Yang, A., Albu, M., et al. (2014). Determination and inference of eukaryotic transcription factor sequence specificity. *Cell, 158*, 1431–1443. https://doi.org/10.1016/j.cell.2014.08.009

Weiss, K. M., & Fullerton, S. M. (2000). Minireview: Phenogenetic drift and the evolution of the genotype–phenotype relationships. *Theoretical Population Biology, 57*, 187–195.

Wong, K. C., Chan, T. M., Peng, C., et al. (2013). DNA motif elucidation using belief propagation. *Nucleic Acids Research, 41*, 1–12. https://doi.org/10.1093/nar/gkt574

Wotton, K. R., Jiménez-Guri, E., Crombach, A., et al. (2015). Quantitative system drift compensates for altered maternal inputs to the gap gene network of the scuttle fly *Megaselia abdita*. *eLife, 4*, 1–28. https://doi.org/10.7554/eLife.04785

Wray, G. A. (2007). The evolutionary significance of cis-regulatory mutations. *Nature Reviews. Genetics, 8*, 206–216. https://doi.org/10.1038/nrg2063

Wright, S. (1932). The roles of mutation, inbreeding, crossbreeding and selection in evolution. In D. F. Jones (Ed.), *Proceedings of the Sixth International Congress on Genetics* (pp. 356–366). The Genetics Society of America.

Zhang, J. (2003). Evolution by gene duplication: An update. *Trends in Ecology & Evolution, 18*, 292–298. https://doi.org/10.1016/S0169-5347(03)00033-8

Zhao, B. S., Roundtree, I. A., & He, C. (2016). Post-transcriptional gene regulation by mRNA modifications. *Nature Reviews. Molecular Cell Biology, 18*, 31–42. https://doi.org/10.1038/nrm.2016.132

Zheng, W., Gianoulis, T. A., Karczewski, K. J., et al. (2011). Regulatory variation within and between species. *Annual Review of Genomics and Human Genetics, 12*, 327–346. https://doi.org/10.1146/annurev-genom-082908-150139

Zheng, W., Zhao, H., Mancera, E., et al. (2010). Genetic analysis of variation in transcription factor binding in yeast. *Nature, 464*, 1187–1191. https://doi.org/10.1038/nature08934

Understanding the Genotype-Phenotype Map: Contrasting Mathematical Models

Isaac Salazar-Ciudad, Miquel Marín-Riera, and Miguel Brun-Usan

Abstract In this chapter, we review and compare existing theoretical models of the relationship between genetic and phenotypic variation or genotype-phenotype map (GPM). By doing that, we introduce the reader to concepts and assumptions of evolutionary genetics and contrast them with concepts and models coming from developmental biology. Although these two approaches can be regarded as complementary to study the same underlying problem, phenotypic variation and evolution, they contradict each other in a number of ways.

The evolutionary genetics models on the GPM consider genetic interactions but not epigenetic interactions. This simplicity has been used to argue that they are the most general (Wagner, Trends Ecol Evol 26: 577–584, 2011). We argue, in contrast, that epigenetic factors are crucial to understand the GPM. We understand epigenetic factors as nongenetic factors that are instrumental in building the phenotype during development (Waddington, Beyond reductionism, 1968). We argue that models including epigenetic factors exhibit features found in real GPMs that are not found in purely genetic models. Since these features are widely found in real GPMs,

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_10

no model can be considered general without those and, then, models including epigenetic factors are more general than purely genetic models, even when the details of the former are specific of certain types of phenotypes.

1 Introduction

The twentieth century can be seen as the century of genetics. We have learned that phenotypes and most of their variation have, ultimately, a specific genetic basis. We know what is at the bottom, the genome, and what is at the top, the phenome, but we do not understand well enough the processes in between to explain which changes at the genetic level lead to which specific changes at the phenotypic level (and why to those changes and not to others). It is our perception, and that of many others (e.g., Houle et al., 2010), that early twenty-first century biology would be, largely, about understanding the genotype-phenotype map.

The genotype-phenotype map, or GPM, is simply the pattern of association between each specific phenotypic variant and its underlying genetic variant. Its domain of application is not fixed and, thus, one can talk of the GPM of an organism, a part of an organism or even of a gene network affecting some aspect of the phenotype.

A central tenet of developmental evolutionary biology is that the GPM is, at least for the case of morphology, determined by embryonic development (Alberch, 1991). By morphology, we mean the distribution of cells and extracellular matrix, ECM, in space. During the process of embryonic development, a single zygotic cell gives rise to a functional organism characterized by a complex distribution of cell types in space. If one defines morphology as the distribution of cells and extracellular matrix in space, it follows that cells have to do things to change their position from the early embryo to the adult. These things are the cell behaviors (cell division, cell adhesion, cell contraction, etc.) and interactions, either mechanical or chemical through cell–cell signaling. In other words, specific morphologies arise in development because individual cells do specific things (e.g., divide, die, secrete ECM) in specific places and times along development. Genes and gene networks have an effect on morphology because they affect the spatiotemporal regulation of these cell behaviors and interactions (Forgacs & Newman, 2005).

There is a complex interdependence between gene networks, cell behaviors, and cell interactions (Salazar-Ciudad et al., 2003). On one hand, some of the gene products in networks are extracellular diffusible signals. These can alter the behavior and network dynamics of the neighboring cells that receive them. On the other hand, changes in cell behaviors and cell mechanical properties lead to changes in mechanical interactions between cells. These lead to changes in the shape of the space in which these signals are diffusing (i.e., the embryo morphology). By affecting this shape, biomechanics indirectly affect the spatial distribution of signals and then which cells are receiving which extracellular signals. This, in turn, affects which genes are expressed and where. In that sense, gene networks, signaling, and biomechanics reciprocally affect each other.

As a result of these interdependent interactions between gene networks and cellular processes, gene expression, cell location, cell shape and ECM change over time and space to produce the complex morphology of the adult.

Since morphology arises through complex networks of gene, cell, and tissue-level interactions, it follows that single genes do not have intrinsic morphological effects. In other words, no individual gene codes, or has the information, for a specific phenotype or phenotypic variant. On the contrary, the morphological effect of a gene, and by extension of genetic variation in it, is totally dependent on the gene networks in which it is embedded and on the effect of these networks on the cell behaviors and interactions by which morphology is built (Oster & Alberch, 1981; Alberch, 1991). We call a gene network regulating cell behaviors and mechanical properties a *developmental mechanism* (Salazar-Ciudad et al., 2003). The cell behaviors and properties are also part of the developmental mechanism.

Natural selection is a crucial factor determining the direction of evolutionary change. Natural selection, however, can only act by eliminating phenotypic variation in each generation. Within an individual, the ultimate cause of phenotypic variation is genetic and environmental variation (Griffiths, 2002). Which phenotypic variation will arise from such genetic and environmental variation, however, is determined by development since, as we come to discuss, development has a crucial role in determining the GPM. It follows then that development, in addition to natural selection, is crucial to understand the direction of phenotypic evolution (de Beer, 1930; Goldschmidt, 1940; Waddington, 1957; Alberch, 1982; Arthur, 2001; Salazar-Ciudad, 2006a), at least for the case of morphological phenotypes.

In this chapter, we review existing models of the GPM. We aim to show that many prevailing genetic models, although claiming to be general, fail to recreate some fundamental properties of real GPMs. We will argue that this is largely because they do not consider epigenetic factors (e.g., cell behaviors). Instead, phenotypes are often conceptualized as arising purely from gene product interactions or even from genes themselves. We will argue that it is only because of epigenetic factors that complex multicellular phenotypes, or even just phenotypes, are possible at all. Based on these premises, we will also argue that models including epigenetic factors are not only more realistic, they also uncover general characteristics of real GPMs that are simply invisible to purely genetic models.

In this article, we will focus on the GPMs for morphological phenotypes but we will also discuss similarities between these GPMs and those studied at other phenotypic levels. The article is arranged in sections: a section describing what we mean by epigenetic factors, one section per each kind of GPM model and sections describing each main difference between the models that include epigenetic factors and the models that do not.

2 Epigenesis and Epigenetic Factors

The concept of epigenetic factors has two meanings. An epigenetic factor can be anything that is not in the DNA sequence (such as the patterns of methylation on the DNA) but that is heritable and has a causal role in development, physiology or any other biological process. Epigenetic is also the adjective of epigenesis (Haig, 2004) and, thus, epigenetic factor would be any "factor" related to epigenesis. Epigenesis is one of the two alternative views Aristotle proposed about embryonic development. Epigenesis is the view that during development new organization arises from previously existing organization that was not equal or trivially similar to it (Jablonka & Lamb, 2005; Müller, 2007). The other view is preformationism: the view that nothing really new arises in development and that most body parts and organization are already present, at a smaller scale, within the gametes.

At the most general conceptual level, development can be described as the process by which specific arrangements of cell types, what we call *developmental patterns* (Salazar-Ciudad et al., 2003), transform into other developmental patterns. Over developmental time, these pattern transformations occur constantly and lead from a simple developmental pattern, the zygote, to a quite complex one, the adult organism. In that sense, embryonic development is in concordance with Aristotle's epigenesis, except that there is something that remains constant during development and that is faithfully copied between generations: the genotype.

An example of an epigenetic factor, in the epigenesis sense, is the asymmetric spatial distribution of many proteins and RNAs in the oocytes of many species (Newman, 2011a). These distributions are relatively simple but most animals have an asymmetry along the animal-vegetal pole and many of them have also asymmetries along other axes (Gilbert & Raunio, 1997; Gilbert & Barresi, 2016). These asymmetries are absolutely required for development, if they are experimentally disturbed, embryos become spherically symmetric and their development gets arrested very early (Kandler-Singer & Kalthoff, 1976; Gilbert & Barresi, 2016). These spatial asymmetries arise from spatial asymmetries present in the gonads of the parents, typically their cell-level apical-basal asymmetries (Bastock & St Johnston, 2008), or are transferred to the oocyte from an apical-basally polarized epithelium through short-range signaling (Neuman-Silberberg & Schupbach, 1993; Roth & Lynch, 2009).

One may argue that the asymmetries in the mother's gonads are due to gene product interactions in the earlier development of the mother. This is indeed the case, but these asymmetries in the mother's gonad required also that the oocyte that gave rise to the mother had the same spatial asymmetries, otherwise, the mother's development would have arrested early on. The spatial asymmetries in the oocyte are, thus, not reducible to gene product interactions. This interdependence between genetic and spatial epigenetic factors is not exclusive of multicellular organisms, but applies to all organisms (Jablonka & Lamb, 2005).

Another example of epigenetic factors is the *developmental patterns* themselves (see Figs. 1 and 2). Gene product interactions are crucial in determining which

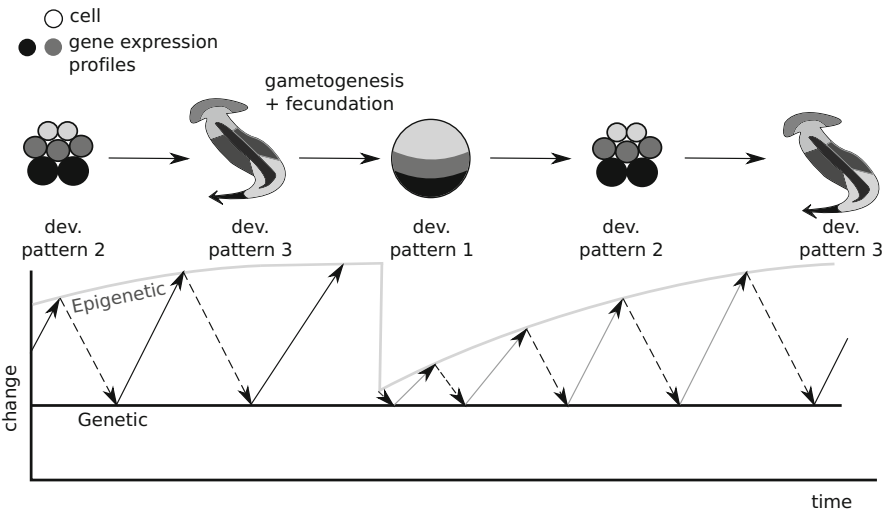

Fig. 1 Schema of the interplay between genetic and epigenetic factors along successive stages of organismal development. The top row shows different developmental patterns, different distributions of cell types in space, from the zygote to the adult of one organism and its parent. In the case of the zygote, the developmental pattern comes from the asymmetric distribution of gene products mediated by the mother during gametogenesis. The plot below shows the amount of genetic and epigenetic accumulated change within an individual and between generations in respect to the zygote. The genotype does not change per se during development, it is the interplay between it and the epigenetic factors (solid and dashed arrows) that builds the developing organism. This interaction (the arrows) should be understood, as discussed in the text, as different epigenetic factors affecting where and when different genes get expressed, while at the same time, which epigenetic factors are encountered in each time and place in the embryo depends on previous genetic and epigenetic interactions (as the arrows abstractly depict)

developmental patterns arise from which previous developmental patterns, but so are these previous developmental patterns themselves. The same developmental mechanism (i.e., gene network plus cell behaviors and mechanical properties) can lead to different final developmental patterns depending on which developmental pattern it acts on (Salazar-Ciudad et al., 2000, 2003). In each developmental stage, existing developmental patterns depend on previous gene product interactions acting on previous developmental patterns. Thus, these patterns, starting from the asymmetries in the oocyte, are both a consequence and a cause of developmental dynamics. In this process, genes and epigenetic factors are intricately interdependent, but not reducible to each other.

Other epigenetic factors relevant for embryonic development include the mechanical properties of cell collectives (Newman & Comper, 1990; Newman & Müller, 2000) and basic cell behaviors such as cell division, cell adhesion, apoptosis, extracellular signal secretion, etc. (Salazar-Ciudad et al., 2003). All these factors are often regulated by gene products, but their existence is not due to, or merely reducible to, genes or genetic interactions. In fact, many cells and tissue

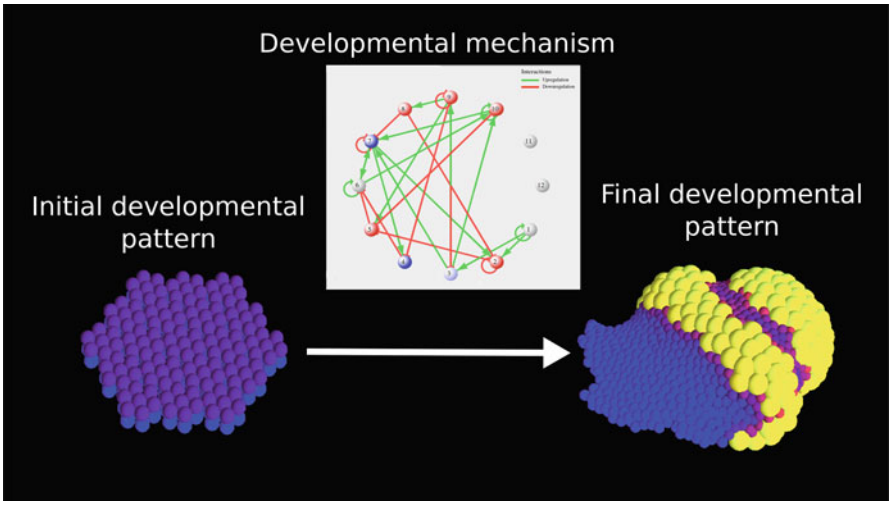

Fig. 2 Example of a genetic-epigenetic model. The figure shows, in the left, an initial developmental pattern the model takes as input and the developmental mechanism it implements, top. In the right, the resulting developmental pattern is shown (the output of the model). In the left, each purple sphere represents the apical side of an epithelial cell and each sphere in blue the basal side. In the developmental mechanism, positive gene regulation is shown by green arrows and negative gene regulation by red arrows. Each sphere is a different gene product, gene product in red can diffuse between cells. In the final developmental pattern, the colors show the expression of gene 2 in the developmental mechanism (yellow for the maximal expression and blue for the minimal expression). The model is implemented in EmbryoMaker (Marin-Riera et al., 2016)

mechanical properties that are relevant to understand morphogenesis are also found, in some rudimentary form, in other systems such as in liposomes devoid of proteins (Newman & Comper, 1990). Gene interactions can then be seen as just a way to more precisely regulate properties and behaviors that are intrinsic to cell clusters (Newman & Müller, 2000). Understanding those cell properties and behaviors is fundamental to understand development, as has been widely discussed (Newman & Comper, 1990; Beloussov, 1998; Oyama, 2000; Newman, 2011b; Guillot & Lecuit, 2013) under different names and slightly different concepts: epigenetic mechanisms (Newman & Müller, 2000), soft-matter properties (Newman & Comper, 1990), developmental resources (Oyama, 2000), phenogenesis (Weiss & Fullerton, 2000), and epigenotype (Waddington, 1942).

Epigenetic factors are heritable but their variation is usually not (Jablonka & Lamb, 2005; Salazar-Ciudad, 2008). One could then argue that the epigenetic factors are not necessary to understand the GPM. After all, the GPM is defined as the association between genetic variation and phenotypic variation. However, as we come to explain, genetic and epigenetic factors need to interact for complex phenotypes and their variation to be possible at all. In other words, genetic variation does not have phenotypic effects unless it affects some epigenetic factors. Thus, to understand the phenotypic consequences of a genetic change, and then the GPM,

the interaction between genetic and epigenetic factors needs to be considered too (Waddington, 1968; Oster & Alberch, 1981).

3 GPM Models

Models about the GPM coming from an evolutionary genetics tradition do not consider epigenetic factors (Wagner, 1994; Hansen & Wagner, 2001). In fact, it has been claimed that these purely genetic models (Wagner, 2011) are the most general because, allegedly, they make fewer assumptions and do not include epigenetic factors that are always specific of certain phenotypic levels. In this chapter, we will argue that purely genetic models are not more general, but more particular because they do not exhibit some general features of real phenotypic variation and GPMs. In addition, they make a number of simple but strong assumptions. When these assumptions are considered explicitly, as we will do in this article, it becomes clear that the purely genetic models are not more general than the models that consider epigenetic factors.

4 Mendelian and Quantitative Genetics

The study of the GPM has not been central to evolutionary genetics and, by extension, to the modern evolutionary synthesis, also called neo-Darwinism (Mayr & Provine, 1980). Although it was very early recognized (Wright, 1932) that such a map was likely to be complex, most models in evolutionary genetics assume or require a simple GPM. Such an assumption may be justified depending on the questions being addressed. Thus, for example, if the aim is to show that natural selection can lead to the fixation of some alleles (i.e., some variants of a gene), then one may ignore the GPM and consider only the average phenotypic effect of an allele on the phenotype (Fisher, 1930). For more advanced questions, as we will discuss, this assumption is not tenable.

Evolutionary genetics is based in population genetics. The way the relationship between genetic and phenotypic variation is understood in population genetics, is based on Mendelian genetics (Griffiths, 2002). Specific discrete phenotypic variation was conceived to be associated with some particles in the nucleus, the genes. Later on, these particles were found to be made of DNA (Griffiths, 2002). Finding this association is a major achievement of twentieth century biology. However, it is rarely the case that a discrete phenotypic character can be simply associated with a gene as in Mendelian genetics. Even in the best cases, there is substantial penetrance and expressivity: different proportions of the individuals bearing a gene exhibit the corresponding phenotype and when they do it, they do it to different degrees.

Most characters do not depend on one but several genes, i.e., they are polygenic (Griffiths, 2002). In that case, Mendelian genetics considers that each different combination of alleles could be associated with a different state in that character, a different variant phenotype. Mendelian genetics, however, has no way to tell which these character states would be. In other words, Mendelian genetics can tell us about the frequency of certain phenotypic states (e.g., a pea being green) given that those states are statistically associated with specific combinations of alleles, but it cannot tell us why some alleles are associated with some specific character states, and it cannot tell us which character states, or phenotypic variation, are possible (Alberch, 1982). This relevant information has to be found by other means, i.e., observation or other theories. In essence, genes in Mendelian genetics are defined based on their statistical association with phenotypic variants. However, in some of the applications of Mendelian genetics that we will discuss, genes become understood as actual bearers of the information necessary to build specific phenotypes and phenotypic variants.

Quantitative genetics is a theory that aims at providing such information. Quantitative genetics is concerned with the inheritance of quantitative characters or traits. These are characters that can be described by continuous, rather than discrete, values (e.g., weight, limb length). Quantitative genetics was originally conceived as an extension of multilocus (i.e., polygenic) population genetics following the so-called infinitesimal model (Rice, 2004), that in its turn, was based in Mendelian genetics. According to this model, each phenotypic trait is determined by the sum of the phenotypic effect of a large number of Mendelian loci (i.e., genes) of small quantitative effect on the phenotype. In addition, loci's phenotypic effects are supposed to be additive. This means that the phenotypic effect of a locus is independent from the effect of other loci. Thus, each different allele in a locus (i.e., each variant of a gene) adds a value to the phenotype independently from the other alleles in the other loci in the genome. Contrary to what we proposed in the introduction, then, genes are conceived to have intrinsic phenotypic effects. Note also that the assumption that the effects of alleles are small and independent from each other is also a mechanistic assumption on how genes interact in order to construct the phenotype.

The infinitesimal model leads to a clear view on which phenotypic variation should be possible by genetic variation. This model is, thus, implicitly a model about the GPM. Mutations would affect genes and lead to new alleles. These would have slightly larger or smaller intrinsic phenotypic effects than their non-mutated version. It is then assumed that the values of phenotypic traits are able to increase or decrease by the replacement of old alleles by specific new alleles over time. This simple model implies that, in principle, all quantitative traits should be able to vary gradually and increase or decrease forever as long as there is selection acting on them. Since the phenotypic effect of alleles is small and additive (independent) one is assuming a linear GPM: small genetic changes lead to small phenotypic changes.

In practice, quantitative genetics uses information about the genetic relatedness between individuals to estimate which proportion of the phenotypic variation between these individuals is due to shared genetics. This information is based

on genealogies or on direct information of the genotypes of individuals (such as in GWAS studies) (Uricchio, 2020). The GPM is conceptualized through the G matrix, the matrix of covariances between traits due to shared genetics (Lande & Arnold, 1983). Describing the GPM through covariances is convenient if the GPM is assumed to be linear. In such an approach, all traits can change indefinitely, but not all of them are necessarily equally variable. Since some genes may be affecting several traits at the same time these traits may not be able to vary independently from each other.

It is important to note that although the quantitative genetics approach is eminently statistical and claimed to be applicable to any phenotype (Roff, 2007), the assumption of linearity implies that it should only be accurate when the GPM is linear. When the GPM is nonlinear, quantitative genetics will still provide predictions but these would often be inaccurate (Pigliucci, 2006; Milocco & Salazar-Ciudad, 2020).

There is the general perception that the predictions of quantitative genetics are experimentally accurate (Roff, 2007). Artificial selection experiments are usually seen as supporting this perception (Roff, 2007). This evidence, however, exists only for selection on single traits. Some authors claim that most studies only show that the quantitative genetics approach works better than nothing since no alternative approaches are used (Pigliucci, 2006). In addition, more general experiments selecting for a trait and looking at the response in others or experiments selecting for multiple traits at the same time are rare. The few studies that attempt that are sometimes compatible with the expectations of multivariate quantitative genetics, but some other times they are not (Roff, 2007).

5 Evolutionary Genetics Models on Epistasis

There are several theoretical models that are conceptually related to quantitative genetics but that explicitly consider that the phenotypic effects of loci depend on each other, although linearly (Barton & Turelli, 1987; Jones et al., 2004). As an example, we will discuss one such model in some more detail: Hansen's and Wagner's multilinear model (Hansen & Wagner, 2001). The multilinear model was developed with the explicit intention of (quoting this original work; Hansen & Wagner, 2001, p. 76):

> *The current body of theory in quantitative genetics lacks an operational theory of gene interaction [. . .] The multilinear theory is [. . .] the only current suggestion that allows for a systematic non-statistical way of incorporating gene interactions in quantitative genetic theory.*

The multilinear model bears an assumption about the GPM: The interactions between gene effects are linear; changing the genetic background for a locus causes a linear transformation of the effects of all substitutions at this locus. Thus, the value of each trait is determined by a fixed phenotypic (^{i}y) effect from each locus

i, defined as the phenotypic change of a substitution in that locus in respect to an arbitrary reference genotype, and a term multiplying all combinations of phenotypic effects by an epistatic coefficient ε. This coefficient can be different for each gene interaction ($^{ij}\varepsilon$ for two loci interactions, $^{ijk}\varepsilon$ for three loci interactions, etc.).

$$x = x_0 + \sum_i {}^i y + \sum_i \sum_{j>i} {}^{ij}\varepsilon^i y^j y + \sum_i \sum_{j>i} \sum_{k>j} {}^{ijk}\varepsilon^i y^j y^k y + \ldots \quad (1)$$

For several traits we have

$$x_a = x_{a0} + \sum_i {}^i y_a + \sum_i \sum_{j>i} \sum_b \sum_c {}^{ij}\varepsilon_{abc}{}^i y_b{}^j y_c + \sum_i \sum_{j>i} \sum_{k>j} \sum_b \sum_c \sum_d {}^{ijk}\varepsilon_{abcd}{}^i y_b{}^j y_c{}^k y_d + \ldots \quad (2)$$

Where a, b, c ... are the successive traits conforming the phenotype. There are a number of models that make similar linear assumptions (Zhivotovsky & Feldman, 1992; Gavrilets & Dejong, 1993; Turelli & Barton, 1994; Nowak et al., 1997; Wagner et al., 1997). Other than linearity, this model does not propose much about how the GPM is (i.e., nothing is said about the distribution of the epistatic coefficients or about whether epistasis happens between two, three or more loci at the same time). However, the actual applications of the model do take more detailed assumptions about the GPM: they assume that (Hansen et al., 2006; Fierst & Hansen, 2010) all epistatic coefficients are equally likely to change by mutation. In other words, since the coefficients in Eqs. (1) and (2) can acquire any arbitrary value through mutations, the GPM is totally plastic to change in any direction over generations. This is a common feature of the models based on quantitative genetics (Barton & Turelli, 1987; Jones et al., 2004). These models consider that loci's phenotypic effects depend on each other but they assume that these dependencies are free to change, over time, by mutation in any way. This is a sort of second-order neo-Darwinism: Traits are not assumed to all be equally likely to change as in the neo-Darwinian approach. There are genetic covariances between traits but these covariances are free to change in any way by the accumulation of small adaptive mutations. This is like applying the infinitesimal model to the evolution of the GPM rather than directly to the evolution of the phenotype (Roff, 2000; Wagner et al., 2007; Cheverud, 2007; Crow, 2010).

6 Wagner's Model

Wagner's purely genetic model (Wagner, 1994) has also been used to study the evolution of the GPM and its effect on phenotypic evolution (Le Cunff & Pakdaman, 2012; Fierst, 2011; Pinho et al., 2012; Draghi & Whitlock, 2012). In contrast to

Hansen's model, this is a nonlinear model in which gene products regulate each other expression. The phenotype is the level of expression of the genes in the network.

The level of expression of a gene i in a given time $t + 1$, $S_i(t + 1)$, is a nonlinear function, σ, of the sum of the regulatory inputs from each other gene j, w_{ij}, each multiplied by the expression of those genes $S_j(t)$.

$$S_i\ (t + 1) = \sigma \left[\sum_{i=1}^{N} w_{ij} S_j(t) \right] \tag{3}$$

The σ function is a sigmoidal function that it effectively acts as a threshold: it gives -1 if its argument is smaller than 0 and 1 if its argument is larger than 0 (and 0 if its argument is zero). The matrix of all w_{ij} values effectively determines gene network topology and the interaction strength between genes.

In Wagner's model, the number of traits is the same as the number of genes. For that model to be generally applicable to the GPM, it is required that the phenotype can be explained, at least in its main features, solely on the bases of gene expression levels. To explain the phenotype, thus, Wagner's model still takes the idea that genes have intrinsic phenotypic effects. In this case, simply, it is not the genes but their expression that have intrinsic phenotypic effects. Thus, there are no epigenetic factors nor space.

The main improvement of this model in respect to previous models is that there are gene networks and that the gene interactions in this network are nonlinear. Although this approach is an improvement over other purely genetic approaches, it still fails to capture some important features of GPMs, as we later discuss.

7 Genetic-Epigenetic Models

In this section, we include those models in which the phenotype is understood to arise from genetic factors, epigenetic factors and their interactions. The models we will discuss in most detail are those of embryonic development. Most models of embryonic development (e.g., Odell et al., 1981; Meinhardt, 1982; Jaeger et al., 2004; Honda et al., 2008; Salazar-Ciudad & Jernvall, 2010; Zhu et al., 2010; Osterfield et al., 2013) have some elements in common: gene networks, cells, and some epigenetic factors in an explicit spatial context. These models always include some initial developmental pattern (i.e., an initial condition), and often, some cell behaviors (e.g., cell division, cell contraction, cell adhesion, etc.). There is often the diffusion of extracellular signals in space. Similar models can be applied to other phenotypic levels such as cell biology (Vik et al., 2011; Karr et al., 2012), organ physiology (Noble, 2002; Gjuvsland et al., 2013) and neurophysiology (Skinner, 2012; Goldberg & Bergman, 2011), RNA secondary structure (Schuster et al., 1994; Schuster et al., 1994; Cowperthwaite & Meyers, 2007) and protein

conformation (Silva et al., 2009; Ferrada & Wagner, 2012). Models at each phenotypic level include epigenetic factors that are level-specific (e.g., cell signaling and other cell behaviors, the rules of nucleotide base pairing in RNA models, or the stereochemistry of amino acids in proteins).

In the case of development, most models focus on a specific organ and incorporate experimental information on the genetic, cellular, and other epigenetic factors known to be important for that organ development, just to cite a few: epithelial buckling (Odell et al., 1981), skin coat patterns (Meinhardt, 1982), early fly segmentation (Jaeger et al., 2004), mammal blastulation (Honda et al., 2008), tooth morphogenesis (Salazar-Ciudad & Jernvall, 2010), limb morphogenesis (Zhu et al., 2010), and egg shape (Osterfield et al., 2013).

There are genetic-epigenetic models that are not specific to any organ but that intend to apply to the whole of animal development (Hogeweg, 2000; Hagolani et al., 2019). They implement basic equations for gene network dynamics, cell behaviors, extracellular signal diffusion, and cell mechanical interactions. One can then explore the different GPMs arising from the theoretically possible developmental mechanisms (Hogeweg, 2000; Marin-Riera et al., 2016; Hagolani et al., 2019). With these models, it has been recently suggested that the development of complex morphologies can be achieved if most cells in an embryo activate cell behaviors (mostly cell division and contraction). For these complex morphologies to be also stable (i.e., that they develop in the same way in spite of noise), it is required that the embryo is partitioned in relatively small areas of gene expression where cell behaviors are regulated differently (Hagolani et al., 2019). These models, however, have not yet been applied to better understand the GPM directly.

One limitation of the genetic-epigenetic models is that there is no genotype as such. There are parameter's that specify the strength of some genetic interactions in the model. Changes in the values of these parameters are usually used as proxies for genetic variation but, in fact, the relationship between the values of these parameters and genetic variation is likely to be quite complex since it may depend in process such as protein folding, RNA folding, enzymatic catalysis, etc.

8 The Lattice Pattern Formation Model

The simplest genetic-epigenetic models, are those that include gene networks and two epigenetic factors: the cell behavior of cell signaling, and the physical process of signal diffusion in the extracellular space. These models are framed in a spatial cellular context, e.g., a lattice of cells, and the phenotype of interest is the spatial distribution of gene expression. There are several of those models (Mjolsness et al., 1991; Jaeger et al., 2004; Cotterell & Sharpe, 2010; Jiménez et al., 2015; Rothschild et al., 2016) but only one is being discussed here, the Salazar-Ciudad's lattice model (from here on just the lattice model), because it intends to be general (i.e., applying to all animal development not to a specific organ) and it explicitly focuses in the

GPM and in its evolution (Salazar-Ciudad et al., 2000, 2001a). In essence, this and other models can be seen as adding epigenetic factors to Wagner's model.

In this model, a number of nonmotile cells, each one including an identical gene network, occupy different positions in a 2D regular lattice. Some of the gene products are extracellular diffusible signals that affect gene expression in neighboring cells. In the lattice model the (continuous) change in concentration of a given gene product "*i*" in a given cell "*j*," that is g_{ij}, does not only depend on the gene network dynamics of that cell, but also on the contribution of diffusible gene product coming from other cells:

$$\frac{\partial g_{ii}}{\partial t} = \frac{\phi\left[\sum_{k=1}^{N_g} w_{ij} g_{ik}\right]}{\phi\left[\sum_{k=1}^{N_g} w_{ij} g_{ik}\right] + K_{\mathrm{m}}} - \mu g_{ij} + D_i \nabla^2 g_{ij} \tag{4}$$

As in Wagner's model, w_{jk} values determine the gene network topology and the interaction strength between genes. The heaviside function ϕ prevents negative concentrations in the gene products ($\phi(x)$ is such that if $x > 0$ then $\phi(x) = x$ and if $x \leq 0$ then $\phi(x) = 0$). The first term is a Michaelis–Menten saturating function with a K_{m} coefficient. The degradation rate of the gene products is specified by the μ parameter and D_l is the specific diffusion rate of the gene product l.

The model starts from a very simple initial developmental pattern in which only one gene is expressed and only in a cell in the middle of the lattice (Salazar-Ciudad et al., 2000). As a result of genetic interactions in the network, cell signaling and diffusion, a new developmental pattern arises (i.e., new distributions of gene expression in space). This developmental pattern is the phenotype in this model. There is, thus, some basic morphology in the sense of the distribution of cell types in space where cell types are described by their gene expression. Despite its relative simplicity, lattice models have been used to study the segmentation mechanisms in the early embryos of *Drosophila* and other insects (Salazar-Ciudad et al., 2001b; Jaeger et al., 2004).

The main result of the analysis of the Salazar-Ciudad lattice model is that there are a limited number of gene network topologies that can lead to pattern formation (Salazar-Ciudad et al., 2000). In other words, most gene networks one could build (for example by wiring genes at random as in Salazar-Ciudad et al., 2000) simply do not lead to any changes in gene expression over space, no pattern, even if they promote cell–cell signaling. Somehow there are mathematical constraints or rules on which gene networks can lead to pattern formation. These rules exist, however, because genes affect some epigenetic factors (e.g., the physical process of extracellular signal diffusion). These epigenetic factors allow for phenotypes (i.e., patterns) to arise rather than just being assumed as in the purely genetic models. These constraints simplify the study of the GPM, since one only needs to worry about the GPM in this subset of topologies.

9 Models Including Morphogenesis: The Tooth Model

The Salazar-Ciudad lattice and similar models including cell signaling are still rather unrealistic models of the GPM for morphological phenotypes. First, because morphological variation does not involve just differences in the location of gene expression but also differences in the location of cells themselves and the former cannot be defined without the latter unless one leaves morphology as such as unexplained. Second, during development, cells not only signal to each other, they also activate cell behaviors. In fact, cells only change their position, and thus morphology, as a consequence of these other cell behaviors. There are a number of specific models doing that (Odell et al., 1981; Honda et al., 2008; Salazar-Ciudad & Jernvall, 2010; Osterfield et al., 2013) but most of them are only concerned with wild-type phenotypes or with mutations of large phenotypic effect that are unlikely to be found in natural populations due to their likely highly deleterious effects.

One exception is the tooth model (Salazar-Ciudad & Jernvall, 2010; Salazar-Ciudad & Marín-Riera, 2013). This model integrates experimental knowledge on how genes and cells interact in a specific system, the mammalian teeth, to reproduce their development, their adult morphologies, and their variation. In addition, the model is used to reproduce the multivariate and three-dimensional morphological variation in natural populations. This allows to suggest which changes in development, and possibly in which genetic pathways, may be responsible for the observed patterns of micro-evolutionary phenotypic variation. This model also allows to obtain some mechanistic understanding on why some morphological variation arises and some other does not arise from mutations affecting development.

General models of development including many, or even most, cell behaviors do exist (Hogeweg, 2000; Marin-Riera et al., 2016; Delile et al., 2017) but so far their potential to address general questions about the GPM itself has not been fully explored (except for two attempts, Hogeweg, 2000; Hagolani et al., 2019).

10 The Differences Between Purely Genetic Models and Genetic-Epigenetic Models

Overall, genetic-epigenetic models provide a view on the GPM and the phenotypic variation that differs from that of the purely genetic models in a number of fundamental ways:

10.1 Genetic-Epigenetic Models Reproduce Individual Phenotypes and the GPM

The first difference is that in genetic-epigenetic models the phenotypes arise from the modeling of specific epigenetic factors or even physical interactions (e.g., between gene products, between cells, between nucleotides; see Fig. 1 for an example) while in the purely genetic models the phenotypes arise from the assumed intrinsic phenotypic effect of genes and the distribution of genes among individuals in a population. Wagner's model is somehow in between because it simulates gene product interactions but in order to be general it assumes that gene expression has an intrinsic effect on the phenotype (i.e., it does not depend on epigenetic factors) and that these can arise without considering the interactions between cells (i.e., Wagner's model is a unicellular model).

In the genetic-epigenetic models, the GPM is studied by exploring which phenotypes arise from which changes in its parameters. These models make assumptions at the level of which genetic interactions and epigenetic factors are relevant to explain the phenotype and its variation. Given these assumptions, the GPM arises from the model, i.e., no assumptions are made directly on the nature of the phenotype or the GPM. In the purely genetic models, in contrast, there are features of the GPM that do not arise from the model, they are simply assumed, most notably linearity and that genes have intrinsic phenotypic effects. This inevitably makes the genetic-epigenetic models more general since they make fewer assumptions on the nature of the GPM.

Genetic-epigenetic models often have enough realism as to be quantitatively comparable with a real organ's morphology (Odell et al., 1981; Meinhardt, 1982; Jaeger et al., 2004; Harris et al., 2005; Honda et al., 2008; Newman et al., 2008; Salazar-Ciudad & Jernvall, 2010; Osterfield et al., 2013; Moustakas-Verho et al., 2014; Ray et al., 2015; Onimaru et al., 2016; Brun-Usan et al., 2017; Marin-Riera et al., 2018).

It is relevant to note that genetic-epigenetic models reproducing population-level phenotypic variation can also reproduce the statistical properties of the population that purely genetic models aim at. All models have parameters, e.g., the diffusivity of an extracellular signal in the lattice models that can be varied. This variation leads, through the model, to phenotypic variation. Statistics can then be applied to this variation, as it is done in population and quantitative genetics, and these can be compared to the ones observed in natural populations (as for example in Salazar-Ciudad & Jernvall, 2010; Milocco & Salazar-Ciudad, 2020).

10.2 Epigenetic Factors Inform About the Space of Possible Networks

Epigenetic factors provide informative rules on which networks are biologically plausible. In the lattice model, for example, we have found that cell signaling and signal diffusion allows for pattern formation but only for specific types, or families, of gene network topologies (Salazar-Ciudad et al., 2000). In other words, by including the epigenetic factor of signaling, we learn that not all gene network topologies can be found in nature to perform a given function (e.g., lead to pattern formation in lattices of cells). This is not the case in purely genetic models, not even in Wagner's model. Since these include no epigenetic factors, the space of gene network topologies that can lead to plausible phenotypes is much larger. This is relevant because different conclusions would be reached when studying one space of gene networks or the other. For example, some studies (Wagner, 1996; Draghi & Whitlock, 2012) claim that phenotypes become more robust to environmental changes as a result of conservative natural selection (i.e., selection for a phenotype to remain unchanged over generations). For that to occur there has to be many gene networks that can lead to the same specific phenotype and they have to differ in their environmental robustness. This is the case for Wagner's model. This is not necessarily the case in genetic-epigenetic models. In the genetic-epigenetic models where this has been studied (Salazar-Ciudad & Jernvall, 2005; Hagolani et al., 2019), many phenotypes are only possible for specific genetic-epigenetic networks and for very specific values in their parameters. Conservative natural selection is, thus, unlikely to increase environmental robustness by choosing among the networks that can produce the same morphology. This is because there is not many of them and they are not connected to each other by a small number of mutations. Thus, the result that conservative selection increases robustness depends on a specific unconstrained space of gene networks that does not seem to be realistic.

10.3 In Genetic-Epigenetic Models Not All Aspects of the Phenotype Can Change

The third main difference between genetic-epigenetic models and purely genetic models is that, in the former, not all aspects of the phenotype are equally likely to change by mutation, and in fact, some aspects may not be changeable at all, at least in the short term. This is a general property of genetic-epigenetic models (Oster & Alberch, 1981), although it has been explicitly studied only in a subset of these models (Hogeweg, 2000; Salazar-Ciudad & Marín-Riera, 2013; Crombach et al., 2016; Verd et al., 2019). This coincides with the evo-devo view that, because of how genes and epigenetic factors interact during development, some aspects of morphology are more variable than others (Alberch, 1982; Horder, 1989). Similar results are obtained in the GPM models of RNA and protein folding (Schuster et al.,

1994). In this case, there are also many studies measuring how different phenotypes are more or less likely to arise by mutations and how the same phenotype can arise from different genotypes (Ahnert, 2017; Ferrada & Wagner, 2012).

As explained in the previous sections, the purely genetic models, except for Wagner's model, either assume that all aspects of the phenotype are equally likely to change or have no way to explain why some aspects of the phenotype are more variable than others, other than natural selection-based arguments. In addition, they assume that all traits can change unlimitedly by mutation.

This difference implies that the genetic-epigenetic models provide a more general depiction of the GPM and of the variability of the phenotype. In other words, the purely genetic models only consider the possibility that all traits are equally likely to vary while the genetic-epigenetic models can explain, even within a single model (Salazar-Ciudad & Jernvall, 2005; Cotterell & Sharpe, 2010), many other possibilities. Again, the purely genetic models do in fact make an assumption, that all traits are free to vary indefinitely, that the genetic-epigenetic models do not usually make.

10.4 Genetic-Epigenetic Models Can Explain Changes in Phenotypic Dimensionality and Novelty

The fourth main difference between genetic-epigenetic models and purely genetic models is related to the dimensionality of the phenotypic changes they can consider. Quantitative and evolutionary genetics conceptualize the phenotype as a set of quantitative traits. Although these approaches consider that the value of each trait can vary without limit, they do not consider that the nature and number of these traits can itself evolve (Müller & Wagner, 1991). In Hansen's model, for example, one has to specify the number of traits from the beginning and this number will not change over time. In Wagner's model, since each phenotypic trait is the expression of a gene, the number of genes defines the dimensionality of the phenotype.

Evolution, however, cannot be reduced to quantitative changes in previously existing traits. As an extreme example, it is clear that one cannot derive a human from gradually changing the traits one could measure in a distant bacteria-like ancestor: the nature and number of traits has dramatically changed in evolution. This kind of changes are sometimes called, or related to the concept of, novelty (Müller & Wagner, 1991).

In many genetic-epigenetic models, some novelty can arise. This is specially evident in models that include cell division or growth since there are then new traits being created over the time in the model. In the tooth model, for example, mutations from one individual can lead to individuals with novel cusps. These are due to simple changes on how strongly genes interact with each other or on how strongly they affect cell division (Salazar-Ciudad & Jernvall, 2004, 2010). These new cusps cannot be defined as arising from other cusps or as arising from some

traits measurable in its ancestors: they arise where there was a feature-less flat area on the tooth and the show no relationship to previously existing cusps.

Again the genetic-epigenetic models turn out to be more general since they do not necessarily assume that variation occurs only as quantitative variation in existing traits but can consider a much larger class of phenotypic changes.

10.5 Genetic-Epigenetic Models Can Explain How the GPM Evolves

Ideally, any model on the GPM should give some hints about how the GPM itself evolves, otherwise its utility is restricted to the short time scale in which the GPM itself is not expected to evolve much. Here again, the differences between the two approaches are fundamental. The purely genetic models have no way to address this question other that assuming, as in the second-order neo-Darwinian view described above, that all aspects of the GPM are equally likely to change by mutation and that then, the GPM smoothly changes in the direction imposed by natural selection. From this perspective past natural selection would be the only factor determining the evolution of the GPM.

In the case of the genetic-epigenetic models, as explained, the nature of the GPM arises from the genetic and epigenetic interactions included in the model. The evolution of the GPM can then be studied by making changes in the genetic interactions, i.e., the gene network. This approach has been taken in a number of articles simulating evolution and development (Hogeweg, 2000; Salazar-Ciudad, 2001a; Salazar-Ciudad & Marín-Riera, 2013; Crombach et al., 2016; Hagolani et al., 2019). Some of these articles show, for example, that the most parsimonious way to evolve a complex phenotypes leads to a highly complex, non-linear GPM where parts of the phenotype cannot vary independently (Salazar-Ciudad et al., 2001a, b).

Many other rules or trends of change in development and the GPM have been hypothesized over the years, either based on models or not: constructional constraints on how developmental stages can be put onto each other over developmental time (Alberch & Blanco, 1996), rules arising from the intrinsic material properties of cells and tissues (Newman & Müller, 2000), from the structure of morphogenetic fields (Webster & Goodwin, 1996) from the logic of gene networks (Kauffman, 1993) or from the limited number of ways in which genes and cells can be wired to lead to pattern formation and morphogenesis (Salazar-Ciudad et al., 2000; Von Dassow et al., 2000).

11 Conclusions

From the previous discussion, it should become apparent that the genetic-epigenetic models describe some important and general properties of GPMs that cannot be described by models that do not include epigenetic factors. First, they explain phenotypes and the GPM based on genetic interactions and epigenetic factors without making assumptions on the nature of the GPM itself. Second, they restrict the space of possible gene network topologies to the ones capable of performing biological functions. Third, they can help to explain which directions of phenotypic variation are more likely by genetic mutation and why. Fourth, they can explain some novelty, and, thus, changes in the dimension of phenotypic variation. Fifth, they can explain how the GPM itself evolves and, thus, they have a stronger explanatory power in evolution.

We think that the generality of models should be measured based on the number of features of reality they can reproduce. Purely genetic models cannot reproduce many of the features of GPMs and, thus, should be considered non-general. Genetic-epigenetic models are specific of a specific phenotypic level. In spite of that, however, many of them reproduce more features of real GPMs than purely genetic models. Thus, even if the details may differ between genetic-epigenetic models, they should be regarded, overall, as a more general description of the GPM than purely genetic models. This view has already been put in practice in some research. Models of specific phenotypes, the tooth model, have been used as a general models of evolution under realistically complex GPMs (Salazar-Ciudad & Marín-Riera, 2013; Milocco & Salazar-Ciudad, 2020). Other authors have gone even further and suggested that secondary RNA structure models can be used to model evolution at other phenotypic levels such as morphology because they capture crucial features of the GPM that are not captured by purely genetic models (Fontana, 2002).

Acknowledgments We thank J. Jernvall, I. Salvador-Martinez, P. Hagolani, T. Välikangas, K. Ruiz-Mirazo, A. Moreno, S. A. Newman and R. Zimm for comments. This research was funded by the Finnish Academy (WBS 1250271) and the Spanish Ministry of Science and Innovation (BFU2010-17044) to I.S.C., the Spanish Ministry of Science and Innovation (BES2011-046641) to M.B.U., and the Generalitat de Catalunya (2013FI_B00439) to M.M.R.

References

Ahnert, S. E. (2017). Structural properties of genotype-phenotype maps. *Journal of the Royal Society Interface, 14*(132), 132–141.

Alberch, P. (1982). Developmental constraints in evolutionary processes. In J. T. Bonner (Ed.), *Evolution and development. Dahlem Konferenzen* (pp. 313–332). Springer.

Alberch, P. (1991). From genes to phenotype: Dynamical systems and evolvability. *Genetica, 84*, 5–11.

Alberch, P., & Blanco, M. J. (1996). Evolutionary patterns in ontogenetic transformation: From laws to regularities. *The International Journal of Developmental Biology, 40*(4), 845–858.

Arthur, W. (2001). Developmental drive: An important determinant of the direction of phenotypic evolution. *Evolution & Development, 3*, 271–278.

Barton, N. H., & Turelli, M. (1987). Adaptive landscapes, genetic distance and the evolution of quantitative characters. *Genetical Research, 49*(2), 157–173.

Bastock, R., & St Johnston, D. (2008). *Drosophila oogenesis*. *Current Biology, 18*, R1082–R1087.

Beloussov, L. V. (1998). *The dynamic architecture of a developing organism: An interdisciplinary approach to the development of organisms*. Kluwer Academic Publishers.

Brun-Usan, M., Marin-Riera, M., Grande, C., Truchado-Garcia, M., & Salazar-Ciudad, I. (2017). A set of simple cell processes is sufficient to model spiral cleavage. *Development, 144*, 54–56.

Cheverud, J. M. (2007). The relationship between development and evolution through heritable variation. In G. Bock & J. Goode (Eds.), *Tinkering: The microevolution of development*. John Wiley & Sons, Ltd.

Crombach, A., Wotton, K. R., Jiménez-Guri, E., & Jaeger, J. (2016). Gap gene regulatory dynamics evolve along a genotype network. *Molecular Biology and Evolution, 33*, 1293–1307.

Cotterell, J., & Sharpe, J. (2010). An atlas of gene regulatory networks reveals multiple three-gene mechanisms for interpreting morphogen gradients. *Molecular Systems Biology, 6*, 425.

Cowperthwaite, M. C., & Meyers, L. A. (2007). How mutational networks shape evolution: Lessons from RNA models. *Annual Review of Ecology, Evolution, and Systematics, 38*, 203–230.

Crow, J. F. (2010). On epistasis: Why it is unimportant in polygenic directional selection. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 365*, 1241–1244.

de Beer, G. R. (1930). *Embryology and evolution*. Claredon Press.

Delile, J., Herrmann, M., Peyriéras, N., & Doursat, R. (2017). A cell-based computational model of early embryogenesis coupling mechanical behaviour and gene regulation. *Nature Communications, 8*, 13929.

Draghi, J. A., & Whitlock, M. C. (2012). Phenotypic plasticity facilitates mutational variance, genetic variance, and evolvability along the major axis of environmental variation. *Evolution, 66*(9), 2891–2902.

Ferrada, E., & Wagner, A. (2012). A comparison of genotype-phenotype maps for RNA and proteins. *Biophysical Journal, 102*, 1916–1925.

Fierst, J. L. (2011). A history of phenotypic plasticity accelerates adaptation to a new environment. *Journal of Evolutionary Biology, 24*, 1992–2001.

Fierst, J. L., & Hansen, T. F. (2010). Genetic architecture and postzygotic reproductive isolation: Evolution of Bateson-Dobzhansky-Muller incompatibilities in a polygenic model. *Evolution, 64*, 675–693.

Fisher, R. A. (1930). *The genetical theory of natural selection*. Oxford University Press.

Fontana, W. (2002). Modelling 'evo-devo' with RNA. *BioEssays, 24*(12), 1164–1177.

Forgacs, G., & Newman, S. A. (2005). *Biological physics of the developing embryo*. Cambridge University Press.

Gavrilets, S., & Dejong, G. (1993). Pleiotropic models of polygenic variation, stabilizing selection, and epistasis. *Genetics, 134*, 609–625.

Gilbert, S. F., & Barresi, M. J. F. (2016). *Developmental biology*. Oxford University Press.

Gilbert, S. F., & Raunio, A. M. (1997). *Embryology: Constructing the organism*. Sinauer Associates.

Gjuvsland, A. B., Vik, J. O., Beard, D. A., Hunter, P. J., & Omholt, S. W. (2013). Bridging the genotype-phenotype gap: What does it take? *The Journal of Physiology, 591*, 2055–2066.

Goldberg, J. A., & Bergman, H. (2011). Computational physiology of the neural networks of the primate globus pallidus: Function and dysfunction. *Neuroscience, 198*, 171–192.

Goldschmidt, R. (1940). *The material basis of evolution*. Yale University Press.

Griffiths, A. J. (2002). *Modern genetic analysis: Integrating genes and genomes* (Vol. 2). Macmillan.

Guillot, C., & Lecuit, T. (2013). Mechanics of epithelial tissue homeostasis and morphogenesis. *Science, 340*, 1185–1189.

Hagolani, P. F., Zimm, R., Marin-Riera, M., & Salazar-Ciudad, I. (2019). Cell signaling stabilizes morphogenesis against noise. *Development, 146*(20), 18.

Haig, D. (2004). The (dual) origin of epigenetics. *Cold Spring Harbor Symposia on Quantitative Biology, 69*, 67–70.

Hansen, T. F., Alvarez-Castro, J. M., Carter, A. J., Hermisson, J., & Wagner, G. P. (2006). Evolution of genetic architecture under directional selection. *Evolution, 60*, 1523–1536.

Hansen, T. F., & Wagner, G. P. (2001). Modeling genetic architecture: A multilinear theory of gene interaction. *Theoretical Population Biology, 59*, 61–86.

Harris, M. P., Williamson, S., Fallon, J. F., Meinhardt, H., & Prum, R. O. (2005). Molecular evidence for an activator-inhibitor mechanism in development of embryonic feather branching. *Proceedings of the National Academic Science U S A, 102*, 11734–11739.

Hogeweg, P. (2000). Evolving mechanisms of morphogenesis: On the interplay between differential adhesion and cell differentiation. *Journal of Theoretical Biology, 203*, 317–333.

Honda, H., Motosugi, N., Nagai, T., Tanemura, M., & Hiiragi, T. (2008). Computer simulation of emerging asymmetry in the mouse blastocyst. *Development, 135*, 1407–1414.

Horder, T. J. (1989). Syllabus for an embryological synthesis. In D. B. In Wake & G. Roth (Eds.), *Complex organismal functions: Integration and evolution* (pp. 315–348). John Wiley.

Houle, D., Govindaraju, D. R., & Omholt, S. (2010). Phenomics: The next challenge. *Nature Reviews. Genetics, 11*, 855–866.

Jablonka, E., & Lamb, M. J. (2005). *Evolution in four dimensions: Genetic, epigenetic, behavioral, and symbolic variation in the history of life*. MIT Press.

Jaeger, J., Blagov, M., Kosman, D., Kozlov, K. N., Myasnikova, E., Surkova, S., Vanario-Alonso, C. E., Samsonova, M., Sharp, D. H., & Reinitz, J. (2004). Dynamical analysis of regulatory interactions in the gap gene system of *Drosophila melanogaster*. *Genetics, 167*, 1721–1737.

Jiménez, A., Cotterell, J., Munteanu, A., & Sharpe, J. (2015). Dynamics of gene circuits shapes evolvability. *Proceedings of the National Academy of Sciences of the United States of America, 112*(7), 2103–2108.

Jones, A. G., Arnold, S. J., & Bürger, R. (2004). Evolution and stability of the G-matrix on a landscape with a moving optimum. *Evolution, 58*(8), 1639–1654.

Kandler-Singer, I., & Kalthoff, K. (1976). RNase sensitivity of an anterior morphogenetic determinant in an insect egg (*Smittia* sp, Chironomidae, Diptera). *Proceedings of the National Academic Science U S A, 73*, 3739–3743.

Karr, J. R., Sanghvi, J. C., Macklin, D. N., Gutschow, M. V., Jacobs, J. M., Bolival, B., Jr., Assad-Garcia, N., Glass, J. I., & Covert, M. W. (2012). A whole-cell computational model predicts phenotype from genotype. *Cell, 150*, 389–401.

Kauffman, S. A. (1993). *The origins of order*. Oxford University Press.

Lande, R., & Arnold, S. J. (1983). The measurement of selection on correlated characters. *Evolution, 37*, 1210–1226.

Le Cunff, Y., & Pakdaman, K. (2012). Phenotype-genotype relation in Wagner's canalization model. *Journal of Theoretical Biology, 314*, 69–83.

Marin-Riera, M., Brun-Usan, M., Zimm, R., Välikangas, T., & Salazar-Ciudad, I. (2016). Computational modeling of development by epithelia, mesenchyme and their interactions: A unified model. *Bioinformatics, 32*(2), 219–225.

Marin-Riera, M., Moustakas-Verho, J., Savriama, Y., Jernvall, J., & Salazar-Ciudad, I. (2018). Differential tissue growth and cell adhesion alone drive early tooth morphogenesis: An ex vivo and in silico study. *PLoS Computational Biology, 14*(2), e1005981.

Mayr, E., & Provine, W. (Eds.). (1980). *The evolutionary synthesis: Perspectives on the unification of biology*. Harvard University Press.

Meinhardt, H. (1982). *Models of biological pattern formation*. Academic Press.

Milocco, L., & Salazar-Ciudad, I. (2020). Is evolution predictable? Quantitative genetics under complex genotype-phenotype maps. *Evolution, 74*(2), 230–244.

Mjolsness, E., Sharp, D. H., & Reinitz, J. (1991). A connectionist model of development. *Journal of Theoretical Biology, 152*, 429–453.

Moustakas-Verho, J. E., Zimm, R., Cebra-Thomas, J., Lempiäinen, N. K., Kallonen, A., Mitchell, K. L., Hämäläinen, K., Jernvall, J., & Gilbert, S. F. (2014). The origin and loss of periodic patterning in the turtle shell. *Development, 141*(15), 3033–3039.

Müller, G. B. (2007). Evo-devo: Extending the evolutionary synthesis. *Nature Reviews. Genetics, 8*, 943–949.

Müller, G. B., & Wagner, G. P. (1991). Novelty in evolution: Restructuring the concept. *Annual Review of Ecology and Systematics, 22*, 229–256.

Neuman-Silberberg, F. S., & Schupbach, T. (1993). The Drosophila dorsoventral patterning gene *gurken* produces a dorsally localized RNA and encodes a TGF-alfa like protein. *Cell, 75*, 165–174.

Newman, S. A. (2011a). Animal egg as evolutionary innovation: A solution to the "embryonic hourglass" puzzle. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution, 316*, 467–483.

Newman, S. A. (2011b). The developmental specificity of physical mechanisms. *Ludus Vitalis, 36*, 343–351.

Newman, S. A., Christley, S., Glimm, T., Hentschel, H. G., Kazmierczak, B., Zhang, Y. T., Zhu, J., & Alber, M. (2008). Multiscale models for vertebrate limb development. *Current Topics in Developmental Biology, 81*, 311–340.

Newman, S. A., & Comper, W. D. (1990). 'Generic' physical mechanisms of morphogenesis and pattern formation. *Development, 110*, 1–18.

Newman, S. A., & Müller, G. B. (2000). Epigenetic mechanisms of character origination. *The Journal of Experimental Zoology, 288*, 304–317.

Noble, D. (2002). Modeling the heart – From genes to cells to the whole organ. *Science, 295*, 1678–1682.

Nowak, M. A., Boerlijst, M. C., Cooke, J., & Smith, J. M. (1997). Evolution of genetic redundancy. *Nature, 388*, 167–171.

Odell, G. M., Oster, G., Alberch, P., & Burnside, B. (1981). The mechanical basis of morphogenesis: I Epithelial folding and invagination. *Developmental Biology, 85*, 446–462.

Onimaru, K., Marcon, L., Musy, M., Tanaka, M., & Sharpe, J. (2016). The fin-to-limb transition as the re-organization of a Turing pattern. *Nature Communications, 7*, 11582.

Oster, G. F., & Alberch, P. (1981). Evolution and bifurcation of developmental programs. *Evolution, 36*, 444–459.

Osterfield, M., Du, X., Schüpbach, T., Wieschaus, E., & Shvartsman, S. Y. (2013). Three-dimensional epithelial morphogenesis in the developing *Drosophila* egg. *Developmental Cell, 24*, 400–410.

Oyama, S. (2000). *The ontogeny of information: Developmental systems and evolution* (2nd ed.). Duke University Press.

Pigliucci, M. (2006). Genetic variance–covariance matrices: A critique of the evolutionary quantitative genetics research program. *Biology and Philosophy, 21*(1), 1–23.

Pinho, R., Borenstein, E., & Feldman, M. W. (2012). Most networks in Wagner's model are cycling. *PLoS One, 7*, e34285.

Ray, R. P., Matamoro-Vidal, A., Ribeiro, P. S., Tapon, N., Houle, D., Salazar-Ciudad, I., & Thompson, B. J. (2015). Patterned anchorage to the apical extracellular matrix defines tissue shape in the developing appendages of Drosophila. *Developmental Cell, 34*(3), 310–322.

Rice, S. H. (2004). Developmental associations between traits: Covariance and beyond. *Genetics, 166*(1), 513–526.

Roff, D. A. (2000). The evolution of G-Matrix: Selection or drift? *Heredity, 84*, 135–142.

Roff, D. A. (2007). A centennial celebration for quantitative genetics. *Evolution, 61*, 1017–1032.

Roth, S., & Lynch, J. A. (2009). Symmetry breaking during *Drosophila* oogenesis. *Cold Spring Harbor Perspectives in Biology, 1*, a001891.

Rothschild, J. B., Tsimiklis, P., Siggia, E. D., & François, P. (2016). Predicting ancestral segmentation phenotypes from *Drosophila* to *Anopheles* using *in silico* evolution. *PLoS Genetics, 12*(5), e1006052.

Salazar-Ciudad, I. (2006a). Developmental constraints vs variational properties: How pattern formation can help to understand evolution and development. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution, 306*, 107–125.

Salazar-Ciudad, I. (2008). Evolution in biological and nonbiological systems under different mechanisms of generation and inheritance. *Theory in Biosciences, 127*(4), 343–358.

Salazar-Ciudad, I., Garcia-Fernandez, J., & Solé, R. V. (2000). Gene networks capable of pattern formation: From induction to reaction-diffusion. *Journal of Theoretical Biology, 205*, 587–603.

Salazar-Ciudad, I., & Jernvall, J. (2004). How different types of pattern formation mechanisms affect the evolution of form and development. *Evolution & Development, 6*, 6–16.

Salazar-Ciudad, I., & Jernvall, J. (2005). Graduality and innovation in the evolution of complex phenotypes: Insights from development. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution, 304*, 619–631.

Salazar-Ciudad, I., & Jernvall, J. (2010). A computational model of teeth and the developmental origins of morphological variation. *Nature, 464*, 583–586.

Salazar-Ciudad, I., Jernvall, J., & Newman, S. A. (2003). Mechanisms of pattern formation in development and evolution. *Development, 130*, 2027–2037.

Salazar-Ciudad, I., & Marín-Riera, M. (2013). Adaptive dynamics under development-based genotype-phenotype maps. *Nature, 497*, 361–364.

Salazar-Ciudad, I., Newman, S. A., & Solé, R. V. (2001a). Phenotypic and dynamical transitions in model genetic networks. I Emergence of patterns and genotype-phenotype relationships. *Evolution & Development, 3*, 84–94.

Salazar-Ciudad, I., Solé, R. V., & Newman, S. A. (2001b). Phenotypic and dynamical transitions in model genetic networks. II Application to the evolution of segmentation mechanisms. *Evolution & Development, 3*(2), 95–103.

Schuster, P., Fontana, W., Stadler, P. F., & Hofacker, I. L. (1994). From sequences to shapes and back: A case study in RNA secondary structures. *Proceedings of the Royal Society of London B, 255*, 279–284.

Silva, J. R., Pan, H., Wu, D., Nekouzadeh, A., Decker, K. F., Cui, J., Baker, N. A., Sept, D., & Rudy, Y. (2009). A multiscale model linking ion-channel molecular dynamics and electrostatics to the cardiac action potential. *Proceedings of the National Academic Science U S A, 106*, 11102–11106.

Turelli, M., & Barton, N. H. (1994). Genetic and statistical analyses of strong selection on polygenic traits: What, me normal? *Genetics, 138*, 913–941.

Uricchio, L. H. (2020). Evolutionary perspectives on polygenic selection, missing heritability, and GWAS. *Human Genetics, 139*(1), 5–21.

Verd, B., Monk, N. A. M., & Jaeger, J. (2019). Modularity, criticality, and evolvability of a developmental gene regulatory network. *eLife, 8*, e42832.

Vik, J. O., Gjuvsland, A. B., Li, L., Tøndel, K., Niederer, S., Smith, N. P., Hunter, P. J., & Omholt, S. W. (2011). Genotype-phenotype map characteristics of an in silico heart cell. *Frontiers in Physiology, 2*, 106.

Von Dassow, G., Meir, E., Munro, E. M., & Odell, G. M. (2000). The segment polarity network is a robust developmental module. *Nature, 406*, 188–192.

Waddington, C. H. (1942). The epigenotype. *Endeavour, 1*, 18–20.

Waddington, C. H. (1957). *The strategy of the genes* (Vol. 63, pp. 375–384). Allen and Unwin.

Waddington, C. H. (1968). The theory of evolution today. In A. Koestler & J. R. Smythies (Eds.), *Beyond reductionism*. Columbia Univ. Press.

Wagner, A. (1994). Evolution of gene networks by gene duplications: A mathematical model and its implications on genome organization. *Proceedings of the National Academic Science U S A, 91*, 4387–4391.

Wagner, A. (1996). Does evolutionary plasticity evolve? *Evolution, 50*, 1008–1023.

Wagner, A. (2011). Genotype networks shed light on evolutionary constraints. *Trends in Ecology & Evolution, 26*, 577–584.

Wagner, G. P., Booth, G., & Bagheri, H. C. (1997). A population genetic theory of canalization. *Evolution, 51*, 329–347.

Wagner, G. P., Pavlicev, M., & Cheverud, J. M. (2007). The road to modularity. *Nature Reviews. Genetics, 8*, 921–931.

Webster, G., & Goodwin, B. (1996). Form and transformation. In *Generative and relational principles in biology*. Cambridge University Press.

Weiss, K. M., & Fullerton, S. M. (2000). Phenogenetic drift and the evolution of genotype-phenotype relationships. *Theoretical Population Biology, 57*, 187–195.

Wright, S. (1932). The roles of mutation, inbreeding, crossbreeding and selection in evolution. In D. F. Jones (Ed.), *Proceedings of the Sixth International Congress of Genetics* (Vol. 1, pp. 356–366).

Zhivotovsky, L. A., & Feldman, M. W. (1992). On models of quantitative genetic variability: A stabilizing selection-balance models. *Genetics, 130*, 947–955.

Zhu, J., Zhang, Y. T., Alber, M. S., & Newman, S. A. (2010). Bare bones pattern formation: A core regulatory network in varying geometries reproduces major features of vertebrate limb development and evolution. *PLoS One, 5*, e10892.

Dynamical Modularity of the Genotype-Phenotype Map

Johannes Jaeger and Nick Monk

Abstract An organism's phenotype can be thought of as consisting of a set of discrete traits, able to evolve relatively independently of each other. This implies that the developmental processes generating these traits—the underlying genotype-phenotype map—must also be functionally organised in a modular manner. The genotype-phenotype map lies at the heart of evolutionary systems biology. Recently, it has become popular to define developmental modules in terms of the structure of gene regulatory networks. This approach is inherently limited: gene networks often do not have structural modularity. More generally, the connection between structure and function is quite loose. In this chapter, we discuss an alternative approach based on the concept of dynamical modularity, which overcomes many of the limitations of structural modules. A dynamical module consists of the activities of a set of genes and their interactions that generate a specific dynamic behaviour. These modules can be identified and characterised by phase space analysis of data-driven models. We showcase the power and the promise of this new approach using several case studies. Dynamical modularity forms an important component of a general theory of the evolution of regulatory systems and the genotype-phenotype map they define.

1 Introduction: Modular Traits and the Genotype-Phenotype Map

The question why organisms are able to evolve is one of the most fundamental and unresolved questions in biology today. What kind of systems architecture permits and facilitates the generation of adaptive variation? What are the indispensable

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7_11

characteristics of such an architecture? Modularity is one of the main candidates for such a fundamental structural property of evolving systems. George Cuvier already argued that the functional integration of parts implies that evolution cannot occur; but since then, we have discovered modular elements at all levels of living systems (Schlosser & Wagner, 2004; Callebaut & Rasskin-Gutman, 2005). At the molecular level, genes and their regulatory sequences are thought to be organised in a modular way. At the phenotypic level, the study of evolution relies on the subdivision of the organism into individual characters or traits. These traits provide the foundation for establishing character homologies and their phylogenetic relationships (Wagner, 2014).

Lewontin (1970, 1974) emphasises the "quasi-independence" of evolutionary characters: they can be individuated through functional or structural criteria (see, for example, Riedl, 1975), or in terms of their variational properties (Wagner & Altenberg, 1996). In this sense, traits are functional, structural, and/or variational *modules* (Fig. 1a). Without modularity limiting pleiotropic effects, selection for specific characters would be impossible, and coherent description of evolutionary processes would be unattainable. It may be that some features of an organism may not be separable in this way,[1] but evolutionary theory at a minimum focuses attention on the set of features that do exhibit a significant degree of quasi-independence. This is why it is widely accepted that modularity is a prerequisite for evolvability, the capacity to produce adaptive change (Dawkins, 1989; Wagner & Altenberg, 1996; Pigliucci, 2008).

Herbert Simon (1962, 1973) argues further that "near-decomposability" is a necessary property of complex adaptive systems that evolve under selective pressure. It is characterised by the scarcity of strong interactions between systems parts. Near-decomposability provides a powerful principle for plausible simplification—and hence understanding—of complex adaptive systems by subdividing our explanatory tasks into manageable chunks (Callebaut, 2005).

Character traits—considered as individuated features of an organism's phenotype—are generated by metabolic, physiological, and developmental processes, which constitute a mapping from genotype to phenotype (Fig. 1b) (Burns, 1970; Alberch, 1991; Pigliucci, 2010).[2] These processes compose the epigenotype of the organism (Waddington, 1942, 1953; Goodwin, 1982; Oster & Alberch, 1982).

[1] Gould and Lewontin (1979) use the human chin as an example to criticise the concept of character traits as natural kinds. The chin is not individuated, since it develops from the interaction of two different generative processes (the alveolar and mandibular growth fields), and thus cannot vary independently from other features of the human skull.

[2] Actually, a more accurate view is to consider the central mapping to occur from phenotype to phenotype over the life cycle of an organism. On this view, the genotype provides the parameters of this dynamic map (rather than a source range as shown in Fig. 1b). Genotype space becomes embedded in phenotype space. Here, we use the metaphor of the genotype-phenotype map as a useful idealisation. It should not be interpreted in the sense that genotype space is physically or logically independent of the phenotype space. Rather, it highlights the fact that we should focus more attention on the processes that connect genotype and phenotype. Metaphors are only useful as conceptual tools when applied within their proper limitations.

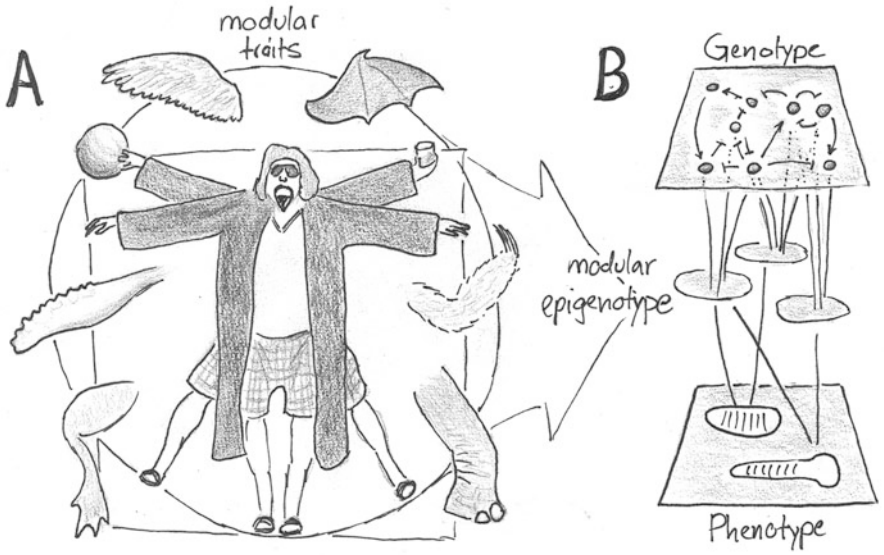

Fig. 1 (**a**) Character traits are functional, structural and/or variational modules, each of which can undergo adaptation quasi-independently of others. (**b**) This implies that the genotype-phenotype map, consisting of the generative processes that produce traits, must also be structured in a modular manner. The argument in this chapter shows that such modularity must occur at the level of the epigenotype, which provides the bridge between genotype and phenotype space. With apologies to the Coen Brothers, Leonardo Da Vinci, and Günter Wagner

Evolutionary change in a phenotypic trait requires corresponding changes in its generative processes (Waddington, 1957; Wagner, 1989, 2014; Amundson, 2005). Since phenotypic variability reflects variability in these processes, the genotype-phenotype map must have a modular architecture as well. Needham (1933) coined the term "dissociability" to describe that generative processes or subsystems are to some degree separable from each other. This suggests that variation in a given individuated trait can be mapped to specific modular subsystems of the epigenotype (Fig. 1). In this chapter, we ask how this is best achieved.[3] After all, understanding the modular architecture of the genotype-phenotype map is the key to reconnecting phylogeny and ontogeny, a central aim of evolutionary developmental biology (Needham, 1933; Waddington, 1957; Goodwin, 1982; Oster & Alberch, 1982; von Dassow & Munro, 1999; Bolker, 2000; Wagner, 2007, 2014). Unfortunately, the nature of modularity remains elusive.

The first difficulty is to precisely define what a module actually is. A general definition should apply across a broad range of specific cases, types of modules,

[3] Another interesting question is how the modular structure of the epigenotype evolved in the first place. This is an important and active field of investigation (reviewed in Wagner, 1996; Wagner et al., 2007; see also Wagner, 2014). We will not pursue this question any further here.

and levels of the organisational hierarchy (Bolker, 2000). In its broadest sense, a module is "a unit that is a component part of a larger system and yet possessed of its own structural and/or functional identity" (Moss, 2001, p. 91). The unit could be any biological entity (a structure, a process, or a pathway) or collective of entities with more internal than external cohesion (Raff, 1996; Hartwell et al., 1999; von Dassow & Munro, 1999; Bolker, 2000; Callebaut, 2005; DiFrisco, 2018; see also Collier, 1988, 2004). In this sense, modules can be considered biological individuals (Hull, 1980). They can be delineated from their surroundings and their behaviour and function emerges as a result of the integrated activity of their parts. Modules require both tight internal cohesion (their "cohesion regime"; Collier, 1988, 2004; DiFrisco, 2018) and relative independence from other modules (cf. Simon, 1962, 1973). They can be defined in terms of their reusability in different contexts (Mireles & Conrad, 2018). Importantly, in the context of our argument, modules must be extended in time: they must persist for long enough to exert their effect (Callebaut, 2005). Finally, as the terms "*near*-decomposability" and "*quasi*-independence" imply, modularity is a matter of degree (Wagner & Altenberg, 1996). All modules interact with their context to some extent, usually in a structured and hierarchical way (Needham, 1933; Riedl, 1975; Raff, 1996; Bolker, 2000).[4]

There are many different ways in which we can distinguish different types of modules. Characters can quite obviously be considered morphological modules (Lewontin, 1970, 1974), and we have already encountered variational modules as a way to individuate traits (Wagner & Altenberg, 1996; Melo et al., 2016). Variational modules are often also functional modules, as shared function implies an increased tendency to co-vary (Wagner et al., 2007). Moreover, they are closely related to the concept of an evolutionary module, which informs the notion of homology and the unit-of-selection debate (e.g. Lewontin, 1970; Raff, 1996; Wagner, 1996, 2014; Brandon, 1999; Callebaut, 2005). Variational/evolutionary modules can be distinguished from developmental modules (Wagner et al., 2007). The latter could provide the basis for a general theory of development, and have received special attention in the field of evolutionary developmental biology (evo-devo) (Raff, 1996; von Dassow & Munro, 1999; Bolker, 2000). Other authors contrast morphological, evolutionary, and developmental modules (Callebaut, 2005). Yet another classification scheme segregates structural from developmental and physiological modules

[4] As a simple example of what is meant here, let us consider the understanding of social behaviour in terms of interactions between individuals. In such a description, each individual is a module—it has tight internal cohesion, with intrinsically determined principles, and relative independence. However, social-level behaviour emerges from the interaction between modules (individuals), so independence is necessarily limited. In this familiar context, there is clear explanatory benefit in representing the population as a collection of quasi-independent individuals, even though one could, in principle, argue that population-level dynamics are "nothing more than" the dynamics of a collective physiology. This provides a simple example of what Simon (1962, 1973) means by near-decomposability, illustrating its evident utility. Note that in this picture, individuals can be thought of as structural, functional and variational modules, depending on the perspective being taken, and the question being addressed.

(Winther, 2001). In this thicket of classifications (Wimsatt, 2007), it is difficult to still see the forest for the trees.

Let us therefore remember that we are interested in the modularity of the genotype-phenotype map and how it relates to phenotypic evolution. Our aim is to explain the modular structure of the genotype-phenotype map in causal-mechanistic terms. Thus, we focus on functional modules, or quasi-independent subsystems of the epigenotype, which are responsible for the generation of specific morphogenetic patterns or phenotypic traits (Fig. 1). We must avoid falling into the trap of a "transcendental argument" by simply postulating such modules (Brandon, 1999). Functional modules have to be identified and characterised empirically. This is not an easy task. It not only requires functional decomposition, but also reconstitution of the integrated activity of modules by systems-level approaches. We review such approaches that use structural and regulatory criteria as proxies for functional ones. We discuss the severe limitations inherent in these criteria. This motivates us to introduce a novel type of module, the dynamical module, which is empirically identifiable in a wide range of developmental processes and intimately related to function. We discuss methods to characterise such modules in formal models and actual developmental systems. We briefly survey known dynamical modules in evolution and development. We conclude that dynamical modules provide a powerful new approach for the causal-mechanistic decomposition of the dynamic and evolvable structure of the genotype-phenotype map, and argue that dynamical modularity can be seen as the conceptual foundation for a new research programme and theoretical perspective on developmental evolution.

2 Functional Modules

Our aim is a functional decomposition of the genotype-phenotype map, because it is the only kind of decomposition that allows us to directly link evolving generative processes to evolving morphological traits (Fig. 1). In this sense, we agree with Hartwell et al. (1999, p. C47), who argue that functional modules are a "critical level of biological organization." These authors define a functional module as "a discrete entity whose function is separable from those of other modules." (ibid., p. C48). Similarly, Mireles and Conrad (2018, p. 1) define a functional module as consisting of "sets of elements that act together in performing some discrete physiological function." These definitions are as concise as they are unworkable since they leave many crucial questions open. How do we define a function? What kind of discrete entity are we looking for? And how do we identify such an entity in the context of a complex regulatory system?[5]

[5]The difficulty of the problem can be illustrated with the following example: it is notoriously hard to formally define the separable function of a "left back" in a football team, even if we have a

For our present purposes, we adopt Cummins' (1975) systems-oriented view of biological functions. For our functional decomposition, we need to know which components of a regulatory system have the capacity to contribute to a given feature of the whole.[6] This contribution is their function.

What kinds of system components are we after? A common reductive approach seeks a decomposition of the generative processes comprising the genotype-phenotype map into a network of interacting elemental regulatory factors. These factors can (but do not have to) be genes and their products. In this context, identifying modular subsystems means identifying subnetworks (also called subcircuits) that contribute to a specific function. This approach goes back to Bonner (1988) who first proposed "gene nets" as discrete units of development.

The methods of molecular biology and genetics provide powerful experimental tools to map individual genes, their products, and their interactions, to specific functions.[7] For instance, Nüsslein-Volhard and Wieschaus (1980) performed a mutagenesis screen that saturated the genome with mutational hits to identify the set of genes involved in the determination of body segments in the early embryo of the vinegar fly *Drosophila melanogaster* (Fig. 2a). Interactions between these genes were then characterised by years of careful and systematic genetic and molecular experimentation (e.g. Akam, 1987; Ingham, 1988). All of these experiments use perturbation assays to determine the contribution of a factor (or an interaction) to segment determination. This research programme led to many revolutionary new insights into the genetic basis of development.[8] But did it also yield a satisfactory characterisation of the segment-determination module?

The answer to this question is a clear and resounding no. Modules are not only dissociable from other modules, but are also characterised by their internal organisation. The components within a module interact, often in complex ways involving regulatory feedback, to generate the activity that defines the module's function. Reductionist experimental approaches for mechanistic decomposition (which attempt to infer pairwise regulatory interactions) are not best suited to the task of reconstructing an integrated module from its genetic or molecular components. They can only establish that a set of regulatory factors is necessary, but not that it is also sufficient, to account for a given function. To make things worse, it is impossible to unambiguously establish the nature and strength of

clear notion of what the role implies in practice. See https://en.wikipedia.org/wiki/Association_football_positions for a good description of a complex network with adaptive functional modules.

[6] Other accounts of function in biology are the etiological account by Wright (1973), which is more suitable to answer the question of why a given function evolved, or the organisational account (e.g., Christensen & Bickhard, 2002; Mossio et al., 2009) which refines Cummins' framework by assessing how a component of a living system contributes to its self-maintenance and self-production.

[7] We will focus on genetic and molecular approaches to functional modularity here. An alternative, more formal and top-down, approach based on "phenotypic building blocks" has been proposed by Mireles and Conrad (2018).

[8] Recognized by Nobel Prizes for Janni Nüsslein-Volhard and Eric Wieschaus in 1995.

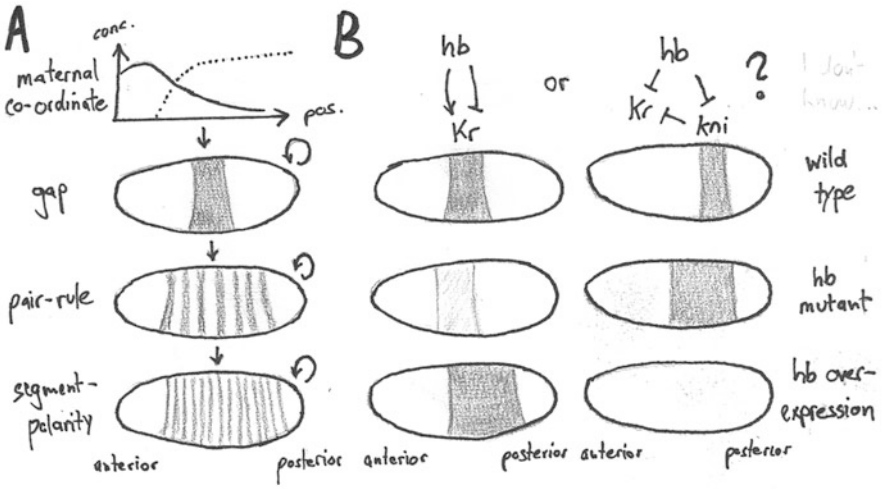

Fig. 2 The tools of genetics and molecular biology establish the necessity, but never the sufficiency, of components and their interactions for a functional trait. (**a**) The functional role of the segmentation gene network in the vinegar fly *Drosophila melanogaster* is to subdivide the body plan into 14 segmental units. The segmentation gene network exhibits a complex hierarchical regulatory structure: protein gradients encoded by maternal-coordinate genes activate gap genes, which, in turn, activate pair-rule and segment-polarity genes. Lower levels in the hierarchy are regulated by, but do not regulate, genes at higher levels. There is extensive cross-regulation within each layer. (**b**) The exact structure of a regulatory network cannot be established by genetic and molecular approaches alone. As an example, we show experiments which aim to establish the regulatory effect of *hunchback* (*hb*) on *Krüppel* (*Kr*). The expression patterns of *Kr* in *hb* loss-of-function mutants and mutants with *hb* overexpression are ambiguous in this regard. Anterior expansion of the *Kr* domain in the mutant, and posterior displacement of its anterior domain boundary upon overexpression indicate that *hb* has a repressive effect on *Kr*. At the same time, *Kr* is weakened in the mutant, and expands posteriorly upon overexpression, which suggests an activating effect. This could either be explained by different regulatory effects at different concentrations of Hb protein, or by an indirect activation via *knirps* (*kni*), which is repressed by *hb* while being a repressor of *Kr*. Which network topology is the correct one cannot be established without additional evidence from quantitative and integrative approaches. See Jaeger et al. (2004a) for discussion; Jaeger (2011) for references to the primary literature

regulatory interactions based on qualitative genetic evidence alone (Fig. 2b) (this issue is discussed, at some length, in the Introduction of Jaeger et al., 2004a). These limitations are fundamental, not merely practical (Isalan & Morrison, 2009; Isalan, 2009), but can be overcome with complementary systems-level and integrative criteria for the definition and identification of functional modules.

We illustrate these difficulties using the example of character identity networks (ChINs) (Wagner, 2007, 2014). ChINs are subnetworks whose function it is to determine the character identity of specific traits. Character identity captures the continuity of a trait in an evolutionary lineage, independent of its precise structure or function. It contrasts with character state, which is defined by the specific size, shape, or colour of a character. For example, “insect forewing” and “hindwing”

are character identities, while "wing blade," "haltere," and "elytra," represent different states of these characters (Fig. 3). Being able to identify functional modules responsible for character identity would yield a mechanistic explanation for homology (Wagner, 2007, 2014). However, the concept of a ChIN is problematic for the general reasons outlined above: the sufficiency of identified components and the precise structure of the subnetwork cannot be established by genetic means alone. In addition, it is rarely easy to pinpoint factors that exclusively contribute to the identity, but not the state, of a trait. The same gene can contribute to both aspects. Moreover, definitions of character identity and state are fluid and context-dependent. Finally, different genes and interactions may be involved in generating homologous trait identities in different lineages due to network drift (Weiss & Fullterton, 2000; True & Haag, 2001; Wagner, 2011). All these issues highlight that there is a fundamental problem of correspondence between this type of genetic network and the functional traits they are supposed to explain, which is "neither trivial nor presently soluble" (von Dassow & Munro, 1999; p. 317). On top of it all, we will encounter an additional issue with explanations in terms of static network structure in the next section. Taken together, this means that ChINs in particular, and networks based on genetic and molecular decomposition in general, only provide a very rough first sketch of a true decomposition of the genotype-phenotype map in terms of functional modularity.[9] In the following sections, we look at different approaches that aim to improve the quality of this sketch.

3 Structural Modules

We have argued in the last section that genetic decomposition needs to be complemented with systems-level criteria to arrive at a satisfactory characterisation of an integrated functional module. The most common approach is to use features of local network structure (or topology) to identify subsystems. The idea is that the internal cohesion of modules is reflected in the type and density of interactions among components within and between subnetworks. We will call modules identified in this way structural modules. This network-based definition is not to be confused with modules that consist of actual physical structures such as protein complexes (Winther, 2001).

We can use informal arguments or formal algorithms to identify structural modules. A prominent example of an informal approach relies on computer metaphors to characterise the developmental and evolutionary roles of subsystems in gene regulatory networks. Through decades of sustained and painstaking genetic

[9] The problems raised here, and their relation to causal-mechanistic explanation in evolution and development, are discussed in detail in DiFrisco and Jaeger (2019). Moreover, DiFrisco et al. (2020) specifically address the problem of correspondence between genetic networks and functional traits by introducing the concept of a character identity mechanism (ChIM), which accommodates nongenetic components and interactions, multi-level causation, and network drift.

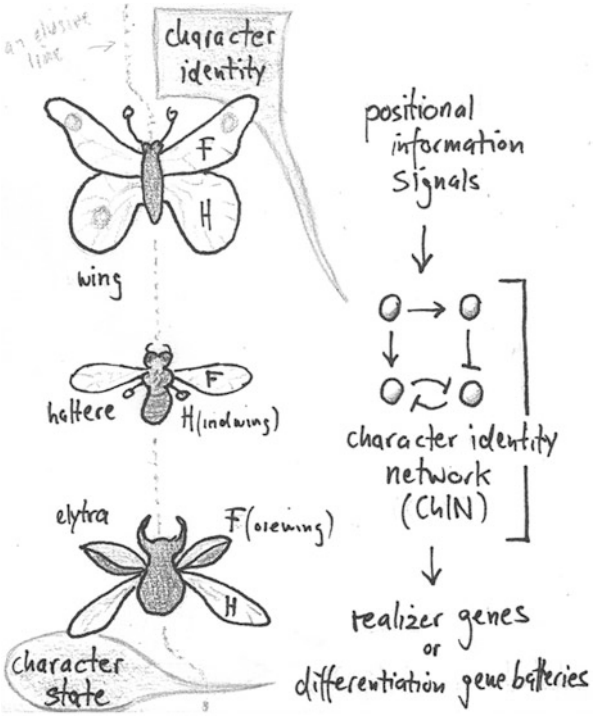

Fig. 3 Character identity networks (ChINs) determine the identity, but not the state, of a character trait. Insect wings are shown as an example. Fore-/hindwing: character identity. Morphology—wing, haltere, elytra: character state. ChINs are activated by general positional information signals, and then activate realiser genes (or differentiation gene batteries) that determine character state. (After Wagner, 2007)

and molecular analysis, Eric Davidson and colleagues have characterised a large regulatory network responsible for the specification of the endomesoderm and its embryonic derivatives during early development in the sea urchin *Stronglyocentrotus purpuratus* (Davidson et al., 2002; Oliveri & Davidson, 2004). This network can be divided into a hierarchy of subcircuits. The authors use an ad hoc combination of functional criteria as a first step to achieve this. Genes are assigned to subcircuits depending on their temporal expression profile (see Sect. 4 below), the specific function they contribute to (e.g. endo- vs. mesoderm specification), and/or the kind of transcription factors that bind to the regulatory sequences that govern their expression (Oliveri & Davidson, 2004; Levine & Davidson, 2005).

The resulting set of subcircuits are then classified into kernels, plug-ins, and differentiation gene batteries connected by input–output switches (Fig. 4a) (Davidson & Erwin, 2006; Erwin & Davidson, 2009). Kernels are highly conserved subcircuits involved in the specification of fundamental features of the body plan. Plug-ins are also conserved to some extent, but contribute to different developmental processes

in different lineages. Gene batteries comprise the downstream effector factors responsible for cell differentiation and morphology. Switches represent connections between modules. Mutational effects depend on where in this modular hierarchy a mutation occurs: micro-evolution is driven by changes in differentiation gene batteries, body plan evolution at higher levels (Davidson, 2011). In particular, plug-ins can be co-opted into new functions by rewiring the switches that connect them (Davidson & Erwin, 2006). Kernels, plug-ins, and gene batteries can be further subdivided into a set of stereotypical minimal regulatory motifs (implementing positive or negative feedback loops, for example), whose functions are supposed to be derivable from their structure (Davidson, 2010, 2011; Peter & Davidson, 2011, 2017). In this analysis, structural arguments supplement functional criteria for the classification of subcircuits and the identification of low-level regulatory motifs.

In addition to such pragmatic approaches, formal methods identify modules based on local features of network structure. We briefly describe two prominent methods that use complementary aspects of the definition of a module. Network motifs are small subcircuits with a specific internal regulatory structure that are detected through their statistical enrichment[10]in large, often genome-wide, regulatory networks (Fig. 4b) (Shen-Orr et al., 2002; Milo et al., 2002; Alon, 2006, 2007). This approach is based on enrichment indicating functional relevance. It assumes that the behaviour of the whole network can be understood as a composite of the well-characterised dynamics of its component motifs. At the very least, we are supposed to learn something about network function from the statistical distribution of motif frequencies (Milo et al., 2004; Shellman et al., 2013). In contrast, algorithms that detect community structure in network graphs rely on differences in the local density of network connections (Girvan & Newman, 2002; Radicchi et al., 2004; Newman, 2006; Newman et al., 2006; Fortunato, 2010). This approach posits that nodes within a module "are joined together in tightly knit groups, between which there are only looser connections" (Fig. 4c) (Girvan & Newman, 2002, p. 7821). On this view, structural modules correspond to "cliques" of densely connected network nodes (Alexander et al., 2009). While network motifs are allowed to overlap, structural modules defined by community structure usually consist of disjoint sets of nodes, although some algorithms yield overlapping subcircuits as well (Palla et al., 2005). The two approaches can be combined to find community structures of clustered network motifs (Benson et al., 2016).

As a means of exploring functional modularity, structural approaches have shortcomings. The most obvious and consequential of these is that network structure constrains, but does not determine function. Even very simple networks, considered in isolation, can exhibit a range of qualitatively different dynamical behaviours depending on the kind and strength of their regulatory interactions and the stability of their components (Mangan & Alon, 2003; Wall et al., 2005; Ingram et al., 2006;

[10]A subnetwork with a given topology is considered as statistically enriched if it occurs more frequently in a given network than in a randomly connected network with equivalent global properties.

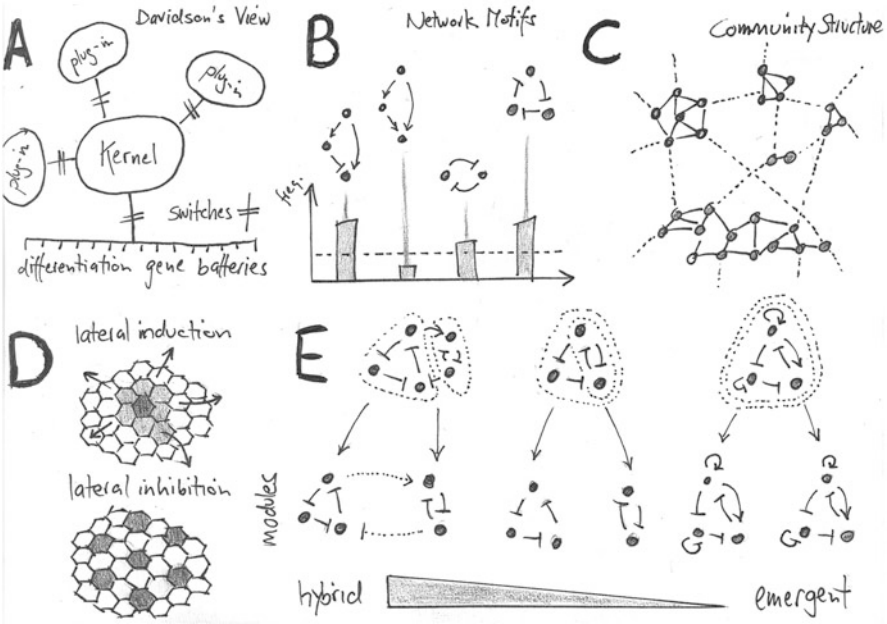

Fig. 4 Structural modularity and its problems. (**a**) Eric Davidson and colleagues divide complex networks into modules that represent conserved kernels, plug-ins (can be co-opted during evolution), and differentiation gene batteries, all connected by switches. Mutational effects depend on where in this hierarchy they occur. (**b**) Network motifs are small networks with a given regulatory structure. They are identified by their enrichment in complex regulatory networks (represented by histogram, expected motif frequency shown as a dashed line). (**c**) Community structure identifies modules by detecting differences in the density of interactions within and between them. (**d**, **e**) Study of multifunctional circuits by Jiménez et al. (2017): (**d**) shows the two functions that each circuit must perform. Lateral induction propagates a signal from its source across a tissue; lateral inhibition leads to a salt-and-pepper pattern. (**e**) Structural modularity in multifunctional circuits occurs along a spectrum: hybrid circuits are composed of completely disjoint modules for each function; emergent circuits show overlap between modules. In extreme cases, the modules performing each function are completely identical to each other. Hybrid circuits are an exception, emergent circuits the norm. See text for details

Siegal et al., 2007; Payne & Wanger, 2015; Ahnert & Fink, 2016; Perez-Carrasco et al., 2018; Page & Perez-Carrasco, 2018). In addition, network motifs can be extremely sensitive to their temporal (Rosenfeld & Alon, 2003; Kim et al., 2011) and network context (Kashtan et al., 2004; Dobrin et al., 2004; Mazurie et al., 2005; Ingram et al., 2006; Solé & Valverde, 2006; Burda et al., 2011; Benson et al., 2016; Gorochowski et al., 2018). The addition of a single regulatory interaction can completely change the range of different dynamical behaviours a network can implement (Perez-Carrasco et al., 2018; Verd et al., 2019). Indeed, such behavioural diversity of regulatory networks is to be expected, and is central in generating adaptive dynamics on developmental, physiological, and evolutionary scales. We therefore cannot assume a network's dynamics to be a straightforward composite of

the behaviour of its modular components. This is illustrated by a series of studies that examined segmentation gene expression in *Drosophila* in terms of network motifs. Despite claims to the contrary, none of these models were able to assign specific patterning functions to motifs, or recover the correct temporal dynamics of the whole system (Zinzen & Papatsenko, 2007; Ishihara & Shibata, 2008; Papatsenko, 2009). Indeed, we are left with a massive explanatory gap between the characterisation of structural modules and their biological function. Any approach that presupposes a close connection between structural and functional modularity in evolved systems is doomed to fail.[11] It is quite telling that even the most stalwart defenders of the idea that structure determines function must rely on dynamical models to understand the behaviour of their network (Peter et al., 2012; Peter & Davidson, 2017).

A second limitation is that many regulatory networks are not structurally modular, even though they are evolvable (e.g. Crombach and Hogeweg, 2008) and exhibit functional modularity (Alexander et al., 2009). This is beautifully illustrated by an in silico screen that searched for small networks with multifunctional behaviour (Jiménez et al., 2017). The screen proceeded in two steps: first, it identified topologies of minimal networks with intercellular signalling that generate both lateral inhibition and lateral induction depending on the strength of regulatory interactions (Fig. 4d). Second, they filtered the selected circuits for networks that can perform both functions in the presence or absence of an external signal. The results indicate two distinct kinds of multifunctional circuits (Fig. 4e). Hybrid circuits, on the one hand, are the sum of their mono-functional structural modules: they use disjoint sets of nodes for each function; emergent circuits, on the other hand, show overlap between the nodes of different modules.[12] Most emergent circuits are only partially modular in the structural sense and, in the most extreme case, the structure of both functional modules completely coincide (Fig. 4e). Many multifunctional circuits are emergent, and therefore only functionally, but not structurally dissociable. Methods based on structural modules will fail for these circuits.

Finally, there is a fundamental conceptual problem with explanations in terms of static network graphs that is essential for our argument. Static graphs cannot, in principle, be causal-mechanistic explanations, because they are not extended in time (Jaeger & Monk, 2015; Nicholson & Dupré, 2018; DiFrisco & Jaeger, 2019). They cannot explain how the network progresses from initial conditions to output. Causality and mechanism must be embedded in time. Modules must persist to exert an effect (Callebaut, 2005). This requires us to search for explanations in terms

[11] This is in contrast to many *engineered* systems, which are designed and constructed specifically to maintain simple and predictable relationships between structural and functional modularity. Such simplicity is appealing, but cannot be expected to hold in naturally evolved networks.

[12] The emergent networks of Jiménez et al. (2017) should not be confused with emergent networks as defined earlier by Salazar-Ciudad et al. (2000, 2001a, b). While "emergent" *sensu* Jiménez indicates heavy overlap between functional modules, "emergent" *sensu* Salazar-Ciudad indicates a flat network structure rich in regulatory feedback, which is contrasted to "hierarchical" networks with a more layered and feed-forward topology.

of the dynamics of modular systems. Dynamic modularity, however, poses its own challenges, since process modules are much more ephemeral than structural ones (Bolker, 2000).

4 Regulatory Modules

Probably the simplest way to identify dynamic modules in a gene regulatory network is to look for factors with correlated or anti-correlated temporal expression profiles (Eisen et al., 1998). Such co-expression clusters or synexpression groups are called regulatory modules[13](Tavazoie et al., 1999; Segal et al., 2003; Bar-Joseph et al., 2003; Babu et al., 2004; Niehrs, 2004). Genes known to be functionally related have products whose expression profiles tend to cluster together (Eisen et al., 1998; Tavazoie et al., 1999). Inversely, evolutionary conservation of regulatory modules can be used as indicator for functional relationships (Teichmann & Babu, 2002; Stuart et al., 2003). Co-expressed genes often share common regulators, which provides an additional criterion for module identification (Segal et al., 2003; Bar-Joseph et al., 2003). This approach scales well to large networks, up to genome scale (Babu et al., 2004; Bolouri, 2014). For our purposes, however, it is limited in two important ways.

One problem is that the resulting network models are probabilistic and not causal-mechanistic (e.g. Bolouri, 2014). This approach cannot move beyond correlational evidence to predict specific mechanistic interactions. Regulatory modules are purely statistical entities. The other problem with regulatory modules is that they are too simplistic. The underlying assumption is that regulation is direct and straightforward: correlated genes are co-activated, anti-correlated factors repress each other. Both cases can be corroborated by finding binding sites for the shared regulator. This should work well for differentiation gene batteries, which are by definition co-regulated and co-expressed (see Sect. 3). It will fail, however, to correctly circumscribe more complex upstream regulatory networks involved in orchestrating cellular and developmental processes. As soon as more than two or three factors are involved in the non-linear regulatory structure of such a network, or as soon as the system is distributed over a spatially differentiated context, indirect effects can create mechanistic interactions resulting in complex patterns beyond simple correlations. Approaches based on correlated co-expression will fail to identify such links.

[13]Segal et al. (2003) also call regulatory modules “module networks.” The terms are equivalent. Since it is easy to confuse module networks with structural networks, we will not use the term here.

5 Dynamical Modules: Definition and Detection

Since our aim is to understand *functional* organisation, we are interested in the full set of dynamics associated with a network architecture. Our starting point is therefore this full set of dynamics, rather than the structure of the network, as discussed above. We describe the dynamics of a network in terms of temporal sequences of states, which define the system's trajectories. The space of all possible trajectories—a network's phase space—has its own characteristic structure, and it is this structure that we seek to interrogate (Strogatz, 2014). A central role is played by attractors: specific state sequences that trajectories approach asymptotically (Jaeger & Monk, 2014). These represent the possible long-term behaviours of the network. Approaches that explore the structure of phase space and its constituent attractors lead to the notion of dynamical modules.

Dynamical modules (also called dynamical subsystems)[14]provide a powerful alternative to structural and regulatory modules. The idea of dynamical modularity first appears implicitly but clearly in Stuart Kauffman's "Origins of Order" (1993). Kauffman's earlier work on ensembles of Boolean network models (Kauffman, 1969, 1974, 1987) had revealed that such systems could be in an ordered or "chaotic" state. "Chaotic" systems, in this context, do not settle into any stable or repeating state within a realistic amount of time, and easily diverge from their original attractor upon perturbation. In contrast, ordered systems exhibit few short attractor cycles and tend to return to their original state after a majority of small state perturbations.

Kauffman (1993) suggests that natural selection drives evolving networks into the border region of the ordered regime (the "edge of chaos"). It does this by altering the topology or density of regulatory interactions or the kind of logical functions that are present in the network. Systems at the edge of chaos contain a large connected component of nodes with ordered dynamics, which percolates around "islands" of chaos. Only such networks exhibit behaviour that is complex, yet controllable, just like biological systems. Perturbations do not travel widely across components, but rather accumulate in the chaotic island they occurred in. Other islands can accumulate their own perturbations relatively independently. Obviously, Kauffman's notion of a "chaotic island" corresponds to some kind of dynamical module of the overall network.

A few years later, von Dassow and Munro (1999) defined a module as a developmental subsystem manifesting quasi-autonomous behaviour. This definition is close to what we mean by a dynamical module here, but is still too vague. A more explicit argument appears in von Dassow and Meir (2004), which states that connectivity is not sufficient: modules are units with their own intrinsic dynamics. An even

[14] Alexander et al. (2009) use the term "activity motifs" for dynamical modules as defined here. We find this potentially confusing due to its similarity with "network motifs" (Alon, 2007). Unlike network motifs, dynamical modules are not defined structurally or in terms of their enrichment within a network.

more specific definition was given by Irons and Monk (2007, pp. 1/2): "Rather than being a protein complex or group of co-expressed genes [a dynamical] module can be viewed as the temporal activity (dynamics) of a group of genes/proteins that controls a specific function in different environmental conditions, cell types and/or tissues. For example, the temporal activity of genes/proteins controlling progression through the cell cycle (in many different environmental conditions and cell types) can be viewed as a module" (see also Irons, 2009). Of course, this definition can be extended to subsystem components that are neither genes nor proteins. Put slightly differently, dynamical modules consist of the activities of a set of network nodes, connected by regulatory interactions, that implement a particular type of behaviour exhibited by the larger network they are embedded in (Verd et al., 2019). In the words of Benítez and Alvarez-Buylla (2010, p. 11), such a module is based on a "set of nodes and interactions that, being part of a larger network, may exhibit a dynamic trait (e.g. a certain number of attractors) in a semi-autonomous way" (see also Benítez et al., 2008, 2011). Dynamical modules can be derived from time-series data (Alexander et al., 2009) or from dynamical computational models (Irons & Monk, 2007; Siebert, 2009, 2011; Verd et al., 2019). They interact in a nested and hierarchical way to generate the overall dynamical repertoire of a complex regulatory network.

Dynamical modules also appear in the study of evolution. Newman and Bhat (2008, p. 2) define a dynamic patterning module as "a set of molecules produced in a cluster of cells, along with one or more physical effects mobilised by these molecules so as to generate an aspect or alteration in the cluster's form or pattern" (see also Newman & Bhat, 2009; Hernández-Hernández et al., 2012). Dynamic patterning modules are used to explain the role of self-organisation in the evolution of early multicellular organisms and more recent developmental processes. The emphasis here is on the interaction of regulatory networks (the "set of molecules") with their cellular and tissue context. Nevertheless, dynamic patterning modules are developmental subsystems that are defined in terms of their dynamic behaviour. In this sense, they are a subtype of dynamical module.

Dynamical modules are not as easy to detect as structural or regulatory modules. To identify them, it is necessary to find patterns in the system dynamics that are present as parts of a wide range of different behaviours of the whole system. They typically do not correspond to clusters in network topology or expression dynamics. The dynamical behaviour they generate can be complex, and assumes no simple correlation between the expression profiles of module components. The fact that dynamical modules are features of the whole dynamical repertoire of a system is what makes their detection challenging. Irons and Monk (2007) introduce an algorithm to identify dynamical modules given a set of discrete-state, discrete-time attractors. These attractor states can be generated by a Boolean dynamical model, or directly identified from experimental data. They capture the non-transient dynamical repertoire of a system. Irons and Monk (2007) use a simple toy model to introduce the method. A brief illustration of the approach will give us a precise understanding of what a dynamical module is.

Boolean networks consist of nodes and their interactions (Fig. 5a). At a given time, each node can either be off (state 0) or on (1). The interactions between nodes are defined by logical tables that connect all possible combinations of inputs to a particular node with specific output states (Fig. 5b). Starting from a given initial state, the system will go through a defined succession of transient states until it reaches a stationary or cyclical attractor. A Boolean network has a finite number of possible states, and so has the advantage that the full repertoire of attractors (and the transient state sequences leading to them) can be enumerated. In this way, one can generate the full set of attractor states, which are the starting point for the identification of dynamical modules. Our toy model has four distinct attractors, three of them cyclical, one stationary (Fig. 5c).

Given the set of attractors, the algorithm identifies subsets of nodes in the network that exhibit a characteristic dynamic (i.e. time-sequence of states) that is conserved in multiple attractors (Irons & Monk, 2007). To achieve this, it examines partial states (states of a subset of nodes in the system) and their state sequences, which are ordered sets of partial states in time. A partial state sequence occurs in an attractor, if its partial states remain constant or continually cycle within an attractor and there is no smaller partial state sequence that does the same. Based on this, the algorithm finds a set of subsystems, which are maximal, nonoverlapping partial state sequences that occur within the given set of attractors (Fig. 5d). Each subsystem generates a typical dynamical behaviour. Put together, they compose the overall behaviour of the whole network. It is important to emphasise that subsystems often overlap in that they share nodes and interactions between each other. Furthermore, subsystems can occur in different combinations in multiple attractors. Therefore, neither their network graphs nor their dynamical functions need be disjoint. Dynamical modules not only can, but are expected to show overlap in both structure and function.

In addition to identifying modular subsystems, the algorithm can be used to examine the hierarchical interactions among subsystems. A subsystem is hierarchically linked to another subsystem if its occurrence entails the occurrence of the other in an attractor. It is said to trigger the other subsystem. By measuring the percentage of perturbations within a subsystem that return to the original attractor, we can assess the robustness of the whole network, as well as the internal and external robustness of each module, that is, its response to internal or external perturbations.

Irons and Monk (2007) illustrate the biological application of their method using a well-established Boolean model of the segment-polarity gene regulatory network of *Drosophila melanogaster* (Albert & Othmer, 2003; Chaves et al., 2005). The model consists of a regulatory network with 13 nodes representing interactions between transcription factors and cell–cell signalling factors, which are distributed over a parasegmental unit represented by a spatial domain of four cells (Fig. 5e, f). This network shows no obvious structural modularity. The system has ten attractors, only three of which correspond to observed gene expression patterns in the embryo (Fig. 5g). Detection of dynamical modules yields a total of 19 subsystems, five of which capture a large proportion of the system's global dynamics (Fig. 5h). These dynamical modules correspond to intercellular variants of core subsystems

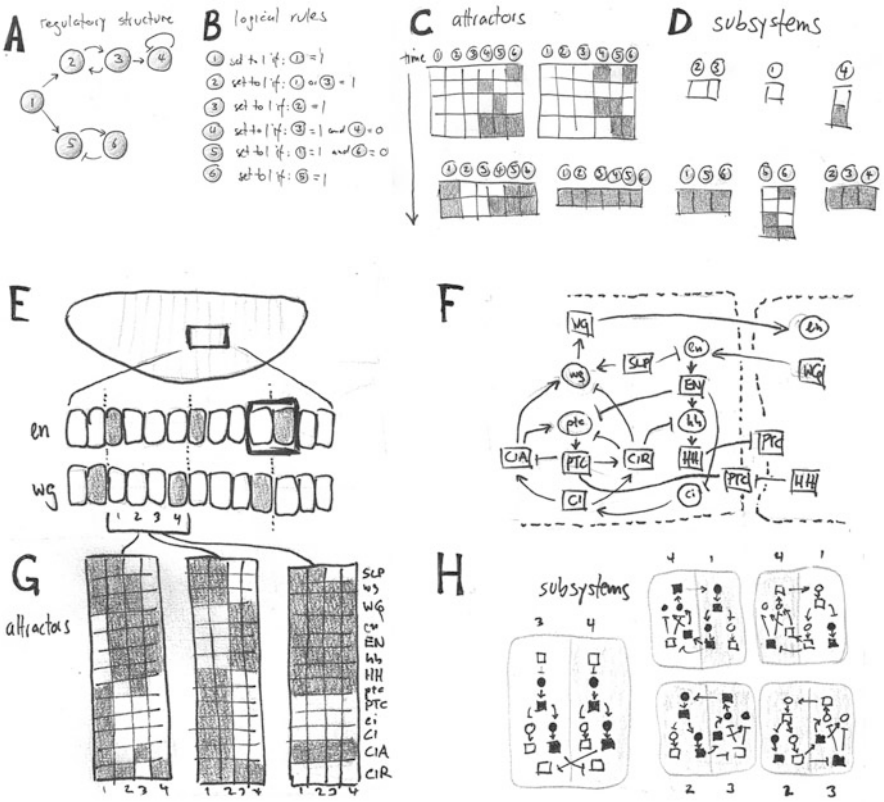

Fig. 5 Dynamical modules in Boolean network models. (**a**–**d**) Toy model, illustrating the algorithmic detection of dynamical modules. Regulatory structure (**a**) is given by logical rules (**b**) that determine the interactions between nodes. This results in multi-stable dynamics with four attractors, shown as temporal sequences of states of the system (**c**). Dynamical modules correspond to a set of subsystems that provide an optimal decomposition of the attractors (**d**). The attractors are simple composites of these subsystems. (**e**–**h**) Application to the segment-polarity system of *Drosophila melanogaster* (see Fig. 2a). (**e**) Segment-polarity genes produce an alternating pattern of *en* and *wg* stripes across four cells within a segmental unit in the embryo. (**f**) Regulatory structure of the segment-polarity network across two cells (indicated by dashed lines). Activating interactions are shown by arrows, repressive interactions by T-bars. (**g**) The system contains three attractors that correspond to empirically observed stable expression patterns (shown across all four cells of a segmental unit). (**h**) Attractors can be decomposed into subsystems as in (**c**) and (**d**). Five main subsystems explain most of the dynamics within the attractors shown in (**g**). These subsystems consist of intercellular feedback circuits that show symmetries across several different cell boundaries. See text for further detail. (After Irons & Monk, 2007)

for segment-polarity patterning that were identified in an earlier in silico screen for robustness in continuous models of three-node networks (Ma et al., 2006). While that screen was based on ad hoc assumptions about the functional core of the network, the algorithm devised by Irons and Monk (2007) provides an unbiased

identification of dynamical modules. Analysis of the hierarchical relationships between subsystems revealed a symmetry in the system centred on the border between the first and the second cell of the parasegment: every subsystem in cells one and four has a counterpart in cells two and three with analogous regulation (Fig. 5h). SLP (Fig. 5f) can be identified as the regulatory factor responsible for setting up this symmetrical pattern. The example highlights how a network that is not structurally modular can be subdivided into dynamical modules, and how the analysis of these modules explains the functionality, that is, the switch-like bistable behaviour of the whole system. The algorithm was also applied to a Boolean model of the yeast cell cycle (Irons, 2009).

6 Dynamical Modules: Gradients, Gap Genes, and the AC/DC Circuit

The algorithm for detection of dynamical modules developed by Irons and Monk applies to networks with discrete-state spaces. While discrete-state models are appropriate for some systems, continuous-state models (e.g. differential equations) are preferable for processes involving gradual changes in regulator concentration. Any generalisation of the algorithm to continuous states must overcome two difficulties. First, information on *multiple* attractors of the system under study is required to define dynamical modules. Second, a suitable measure of "equivalence" of dynamics across attractors replaces the requirement that the dynamics of the module are strictly conserved across sets of attractors. Patterning by morphogen gradients provides many examples of gradual processes that require a continuous approach (see Fig. 2a, top panel) (Wolpert, 1968, 1969; Jaeger et al., 2008; Briscoe & Small, 2015). In these systems, target gene activation depends on the spatiotemporal concentration distribution of the morphogen. Boolean models with discrete on/off states are not suitable in this context. Can the notion of dynamical modularity be extended to such continuous systems?

One particularly well-studied network that drives morphogen-based pattern formation is the gap gene network of *Drosophila melanogaster* (Jaeger, 2011). It constitutes the top-most regulatory layer of the segmentation gene hierarchy we discussed in Sect. 2 (Fig. 2a). It is a relatively simple patterning system that operates in a syncytial embryo without growth or rearrangements of tissue geometry. Gap genes are activated by gradients of maternally expressed transcription factors; in addition, they auto-activate and cross-repress each other, which results in a staggered, overlapping arrangement of their expression domains (Fig. 6a). The study we present results from a research programme to reverse-engineer the gap gene system by fitting dynamic models of the network to quantitative spatiotemporal gene expression data (Jaeger & Crombach, 2012; Chap. 4 by Crombach and Jaeger, this volume). The topology of the gap gene network does not exhibit any structural modularity. And yet, the system implements two clearly distinguishable

dynamical regimes in the anterior versus the posterior of the embryo: while anterior domain boundaries remain at a stationary position and are generated by switch-like multistability, posterior boundaries shift over time and are driven by an underlying damped oscillator (Fig. 6b) (Jaeger et al., 2004a, b; Manu et al., 2009; Ashyraliyev et al., 2009; Crombach et al., 2012; Verd et al., 2017, 2018; Jaeger, 2018). Between the two regimes, a bifurcation occurs (Manu et al., 2009; Gursky et al., 2011; Verd et al., 2017, 2018). In the absence of a suitable algorithm, we took a pragmatic approach to partition a continuous differential-equation model of this system into dynamical modules.

Gap gene patterning does not require diffusion of gap gene products between nuclei (Jaeger et al., 2004b; Manu et al., 2009; Verd et al., 2017). Consequently, the system can be thought of as an ensemble of nucleus-specific gap gene networks, each with a different set of maternal inputs. This provides an ensemble of dynamical repertoires of the network which can be used to detect dynamical modules. Our dynamical decomposition of the gap gene system is based on the observation that patterning never relies on more than three out of four gap genes in any nucleus within the trunk region of the embryo (Verd et al., 2019). This fact can be established rigorously by simulating versions of the network in which one node or gene has been eliminated, and measuring the sensitivity of the system to such in silico knockouts. This approach identifies three distinct subsystems that drive gene expression in the anterior, middle, and posterior of the embryo. These subsystems consist of overlapping sets of genes that all share the same network structure: the AC/DC subcircuit, which was first identified and characterised in the context of morphogen-based patterning in the vertebrate neural tube (Fig. 6c) (Panovska-Griffiths et al., 2013; Perez-Carrasco et al., 2018). AC/DC circuits are simple three-node networks capable of producing oscillations and switch-like behaviour, depending on the strength of their regulatory interactions (Panovska-Griffiths et al., 2013; Perez-Carrasco et al., 2018; Verd et al., 2019).

Next, we examine the dynamical behaviour of each subcircuit with phase space analysis (Strogatz, 2014), and compare it with the dynamical repertoire of the whole gap gene system (Verd et al., 2019). This analysis reveals that each AC/DC circuit, in its own region of influence, is capable of driving the same type of dynamics as observed in the full system (Fig. 6b). AC/DC1, in the anterior, generates expression domain boundaries through multistability: nuclei that express different gap genes find themselves in different basins of attraction due to receiving different maternal inputs. AC/DC3, in the posterior, implements a damped oscillator driving the stereotypical succession of gap gene expression that generates the kinematic shifts of domain boundaries over time. Since they drive a consistent dynamical regime across their regions of influence, these circuits are structurally stable. In the middle, AC/DC2 undergoes a bifurcation within its region of influence, just like the full model. It exhibits switch-like behaviour anterior and damped oscillations posterior of the bifurcation boundary. The bifurcation indicates that this circuit is in a state of criticality, unlike the other two subsystems. The gap gene network is labile to change in some aspects, but robust towards others, which is analogous to Kauffman's notion of the system being at the "edge of chaos" (Kauffman, 1993).

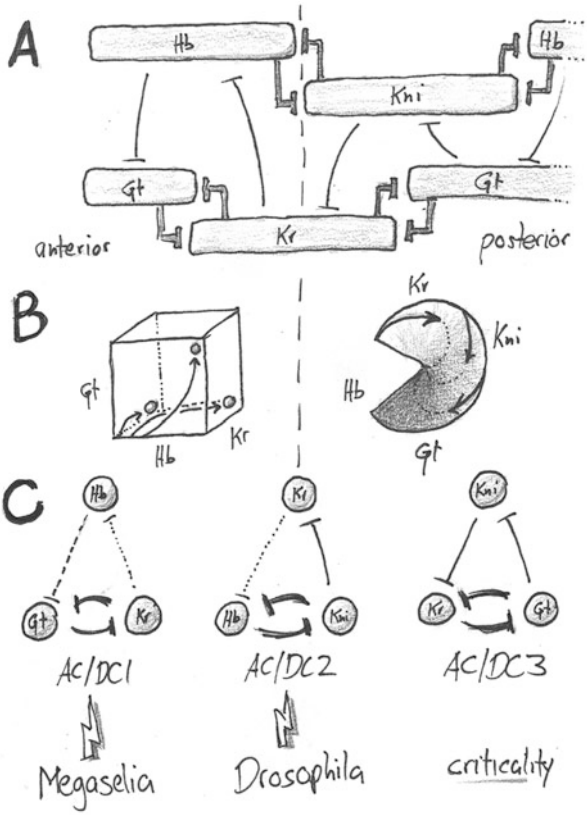

Fig. 6 Modular decomposition of a continuous patterning system, the gap gene network in flies (see Fig. 2a). (**a**) Regulatory structure of the gap gene network. Boxes indicate spatial position of expression domains (anterior is to the left, posterior to the right). Repressive cross-regulation between gap genes is indicated by T-bars; activation by maternal gradients and gap gene auto-activation not shown. Two strong double negative (positive) feedback loops contrast with much weaker repression between overlapping gap gene domains. (**b**) The system implements two distinct dynamical regimes: in the anterior, domain boundaries are set by a multi-stable (switch-like) dynamic, in which the states of nuclei in different domains are converging towards different attractors (circles in box); in the posterior, nuclei cycle through a stereotypical succession of gap gene expression (driven by a damped oscillator) before all converging to the same attractor (indicated by circle with spiralling domains). Each nucleus starts from a different initial condition (dependent on maternal inputs) and thus traverses a different segment of the circle. This results in apparent (kinematic) shifts in the position of gap domains towards the anterior of the embryo, while domains in the anterior remain stationary over time. (**c**) The gap gene network can be decomposed into three AC/DC subsystems in the anterior, middle, and posterior of the embryo. There is extensive overlap in nodes and interactions between these subcircuits. AC/DC circuits reproduce the dynamics of the full system in their respective region of influence. Subcircuits are either structurally stable, or critical (exhibiting a bifurcation within their region of influence). AC/DC2 is critical in *Drosophila*, while AC/DC1 is critical in *Megaselia* (indicated by arrows). See text for details. (After Verd et al., 2019)

A number of points are worth noting about this type of dynamical decomposition. It is a top-down approach, just like the algorithm presented in the last section, using information on a range of different dynamical behaviours to partition the network into interlocking subsystems. Each AC/DC circuit implements a subset of behaviours of the full model. The behaviour of the full system corresponds to a straightforward combination of its modules. In this sense, AC/DC subcircuits fit perfectly into our definition of a dynamical module.[15] Each AC/DC circuit shares two out of three genes with its neighbour (Fig. 6c). This makes the gap gene network heavily emergent (Fig. 4e), which explains why it is not structurally dissociable. Surprisingly, AC/DC modules do not cleanly separate into switch-like or oscillatory behaviour. The bifurcation occurs within the region of influence of AC/DC2. In this regard, dynamical decomposition is not trivial, and requires careful mathematical analysis. In the absence of a formal approach, there are many valid ways to decompose a system dynamically. How we identify subsystems depends, to some extent, on our research question and assumptions. We illustrate this point by comparing our dynamical decomposition with a bottom-up in silico screen that identified core mechanisms for morphogen-based stripe (i.e. domain) formation.

Cotterell and Sharpe (2010) used an approach equivalent to Jiménez et al. (2017) and Ma et al. (2006) (see also Ma et al., 2009) to search for minimal networks that produce a spatial stripe in a non-growing tissue in response to a static morphogen gradient. Their scenario resembles a simplified version of the gap gene system. Their screen yielded six distinct core mechanisms for stripe formation, which can be distinguished by their network topology and dynamical behaviour. Three of the six mechanisms were novel; they have never been observed in an experimental setting. Out of the other three, two can be mapped onto the gap gene network. Surprisingly, the AC/DC circuit is not present, nor do any of the core mechanisms match a subset of interactions within the AC/DC topology. The explanation lies in the assumptions underlying the two different approaches to define subsystems. Dynamical decomposition requires the subsystems to produce well-defined spatial domains, and to reproduce the dynamical regimes observed in the full system. This requirement is much more stringent than the one used by Cotterell et al. and emphasises the importance of studying modules in their native network context.

7 Dynamical Modules Everywhere

We have argued that dynamical modules are more widespread in biological regulatory systems than structural ones. However, the only evidence we have presented so far stems from three isolated case studies: the gap gene network (Verd et al., 2019) and segment-polarity network (Irons & Monk, 2007) of *Drosophila*, as well

[15] Note that for Boolean networks, dynamical modules have dynamics that are *strictly* conserved in multiple attractors; in the continuous-state gap gene system, each AC/DC circuit type corresponds to an equivalence class of dynamics. This provides an illustration of how we can adapt techniques developed in discrete-state systems to continuous-state systems.

as the cell cycle of budding yeast (Irons, 2009). Dynamical modules are detectable in whole-genome data sets (Alexander et al., 2009), but have not yet been formally identified and characterised in developmental systems other than those mentioned above. Plenty of evidence suggests that they will be in the near future. Here we name a few likely candidates. The AC/DC circuit involved in patterning of the vertebrate neural tube exhibits all the criteria to qualify it as a dynamical module: within its region of influence, it is responsible for the main patterning function of the system it is embedded in (Balaskas et al., 2012; Panovska-Griffiths et al., 2013). Turing pattern generators compose another, very broad, class of candidate dynamical modules (Turing, 1952; Meinhardt, 1982; Meinhardt & Gierer, 2010; Kondo & Miura, 2010; Marcon & Sharpe, 2012; Green & Sharpe, 2015). Specifically, the core mechanism underlying proximo-distal and digit patterning in the vertebrate limb, from shark fins to tetrapod appendages, can be seen as a module that dynamically interacts with tissue geometry and morphogens within the growing limb bud (Raspopovic et al., 2014; Onimaru et al., 2016). Various systems driving cellular rhythms should be decomposable in terms of dynamical modularity (Goldbeter, 1997; Novák & Tyson, 2008; Maroto & Monk, 2009). The logical analysis in Irons (2009) could be generalised and extended to continuous models of the cell cycle (see, for example, Chen et al., 2000, 2004; reviewed in Tyson & Novák, 2015). Circadian oscillations provide another interesting case study (Goldbeter, 1995; Asgari-Targhi & Klerman, 2018), for which Boolean models exist (Akman et al., 2012) that are amenable to analysis with the algorithm by Irons and Monk (2007). Dynamical modules have also been described in plants, particularly in patterning the root and leaf epidermis in *Arabidopsis thaliana* (Benítez et al., 2008, 2011; Benítez & Alvarez-Buylla, 2010). This diverse set of examples could be extended as one wishes, but it should be apparent that dynamical modules are omnipresent in cellular and developmental regulatory systems across the kingdoms of life.

Upon closer inspection, a hierarchy of dynamical modules can be discerned. Let us take the segmentation gene network of *Drosophila* as an example. This network in its entirety can be considered a module. It governs a particular aspect of the dynamics of pattern formation that is clearly discernible from other patterning processes. The segmentation gene system can be further subdivided into maternal co-ordinate, gap, pair-rule, and segment-polarity subsystems (Fig. 2a). Again, these subsystems affect separable dynamic aspects of body patterning (Nüsslein-Volhard & Wieschaus, 1980). They interact with other processes, such as those triggered by homeotic genes that specify the identity of body segments (e.g. Akam, 1987; Ingham, 1988). Our work on the gap gene system further divides the network down to the AC/DC subcircuit (Fig. 6d) (Verd et al., 2019). Irons and Monk (2007) partition the segment-polarity network into even smaller subunits of intercellular feedback mechanisms (Fig. 5h). Dynamical decomposition reveals that the dynamic repertoire of systems at higher levels in the hierarchy is dissociable into lower-level dynamical regimes.

This hierarchy of dynamical modules bears striking similarities to the hierarchy of morphogenetic fields from classical embryology (Gilbert et al., 1996). Especially

for higher levels of the hierarchy, we can draw a close parallel between dynamical modules and the field concept. The notion of a morphogenetic field was first introduced by Theodor Boveri in 1910, and was further developed by biologists such as Gurwitsch, Weiss, Harrison, and Needham, who refined its definition and provided experimental support for field phenomena in embryology. In its original meaning, the morphogenetic field is not an incipient undifferentiated tissue, but rather a concept representing the interactions of localised developmental processes to generate a robust pattern (Raff, 1996; see also Goodwin, 1982; Goodwin et al., 1993; Webster & Goodwin, 1996). In this sense, it exhibits obvious similarities to the notion of a developmental module: morphogenetic fields have a cohesive internal regulatory structure and generate dissociable patterns (Needham, 1933). They interact with other fields in a hierarchical and dynamic way: the primary embryonic field, for example, yields to more organ-specific fields over time, which are refined as embryogenesis proceeds (Raff, 1996). In essence, therefore, a dynamical module is a formally defined representation of a morphogenetic field. And, as classical embryology already recognised, morphogenetic fields are the foundational unit of developmental process.

8 Evolutionary Implications

Dynamical modularity has important implications for evolution. We have explained in the "Introduction" why modular traits require functional modularity in the genotype-phenotype map as a prerequisite for evolvability (Wagner & Altenberg, 1996). We have argued in line with Simon (1962, 1973) that near-decomposability (or dissociability, Needham, 1933) is a fundamental property of complex adaptive systems. It enables modules to vary relatively independently, minimising off-target pleiotropic effects, which accounts for the individuality of character traits (Wagner & Altenberg, 1996; Wagner & Zhang, 2011). This kind of functional modularity provides a causal-mechanistic explanation for character identity and homology (Wagner, 2007, 2014). In Sect. 3, we have discussed how modules can be co-opted into new pathways during evolution, generating innovative change (Raff, 1996; von Dassow & Munro, 1999; True & Carroll, 2002; Davidson & Erwin, 2006; Erwin & Davidson, 2009; Monteiro & Podlaha, 2009; Wagner, 2011). How do dynamical modules fit into this picture?

First of all, quasi-independence of character traits implies functional modularity in the generative *processes* or *dynamics* that constitute the underlying genotype-phenotype map (Fig. 1) (e.g. Waddington, 1957; Goodwin, 1982; Wagner, 1989, 2014; Alberch, 1991; Amundson, 2005; Jaeger et al., 2012; Jaeger & Monk, 2014). Different types of generative dynamics must therefore be identifiable and separable in some sense. In engineered systems, dissociability is achieved through modular structure: the components of a laptop screen, for example, map to the trait "display," while the components of the keyboard map to trait "input." These traits can evolve independently by altering the respective structural components. Integration

is achieved by simple input–output maps between the different components. Some aspects of living systems may have evolved to be like this. However, more generally, we expect tighter structural integration, making it difficult to separate modules at the level of network structure (Jiménez et al., 2017). Wimsatt (2007) calls this type of integration "interactional complexity," arguing that it is a natural outcome of selective evolution. Dynamical modules capture the sense in which the genotype-map of such systems can still be dissociated, even if it is not structurally decomposable at all.

A key point is that a given structure (network) in genotype space corresponds not to a single dynamical behaviour, but to a repertoire of different dynamic regimes. This repertoire is captured by the set of attractors and their basins. Note that it is typical, not unusual, that a dynamical system has multiple attractors *for a single set of parameters*: which attractor is manifested depends on the initial and boundary conditions (the context) of the network. In case of the gap gene network, for example, the different attractors are selected by maternal inputs (Manu et al., 2009; Verd et al., 2017, 2018). Dynamical modules give a functional decomposition of the repertoire associated with a given structural network. They reveal the separable aspects of the *dynamics* of the system. Dissociability in the dynamics is not simply related to the decomposability of the structure. This is important in the context of evolution because we can now explore how different dynamical modules respond to changes in the genotype.

Jiménez et al. (2017), and our study of the gap gene system (Verd et al., 2019), illustrate the sometimes extensive structural overlap between dynamical modules (Figs. 4d, e and 6c). At first sight, we must assume that mutations affecting genes or regulatory interactions involved in two or more modules are bound to cause pleiotropic effects. This seems incompatible with limited pleiotropy and the functional dissociability of modules. How can we explain this? The answer lies in the fact that, despite their structural overlap, dynamical modules can show vastly different sensitivities to mutational changes (Verd et al., 2019). In the case of the gap gene network, for example, subcircuits AC/DC1 and AC/DC3 are structurally stable and thus robust. In contrast, AC/DC2 is in a state of criticality, close to a bifurcation boundary, and thus sensitive to even subtle changes in its regulatory interactions. Accordingly, we observe that the overall dynamics and relative arrangement of gap domains is strongly conserved, while the position of the boundary between stable switch-like and shifting oscillatory dynamics differs markedly between *Drosophila* and another dipteran species, the scuttle fly *Megaselia abdita* (Wotton et al., 2015; Crombach et al., 2016). This suggests that AC/DC1 is the critical subcircuit in *Megaselia*, while AC/DC2 is a structurally stable damped oscillator (Fig. 6c) (Verd et al., 2019). This switch in criticality requires surprisingly few and subtle changes in gap gene cross-regulatory interactions.

Such differential sensitivity to change is an important aspect of evolvability that structural or regulatory modules cannot explain, since it requires a causal-mechanistic understanding of the generative process that produces the pattern (Goodwin, 1982; Jaeger et al., 2012; Jaeger & Monk, 2014). The surprising and counterintuitive insight is that, even though genes and interactions are shared

between modules, their effects on the dynamical behaviour of each module are different. This provides an alternative explanation for limited pleiotropy. It corroborates that the structure of networks and regulatory sequences does not necessarily have to be modular to ensure dissociability of functional modules. In fact, structurally more modular (hierarchical) networks can be dynamically less modular, since network structure and the richness of a system's dynamical repertoire are only loosely associated. The prevalence of modular structures in the literature may therefore result from ascertainment bias, rather than a true reflection of the organisation of regulatory systems, because, modular networks and regulatory structure are easier to detect and characterise than the dynamical modularity described here.

The definition of dynamical module in Irons and Monk (2007) requires that a module has a dynamical behaviour that is conserved in multiple different attractors. If the module corresponds to a trait, then evolution can shift the system as a whole to a new attractor, while preserving the trait in question. There is a set of attractors for the whole system, which all incorporate the same subsystem for the trait to be conserved. This subsystem can stay conserved, even if other aspects of the attractors change during evolution. In this way, the decomposition of the dynamical repertoire of a network into dynamical modules gives understanding of the evolutionary potential of the system as a whole. The geometrical arrangement of attractors and their basins in the phase space of the system determines the likelihood of phenotypic transitions (Alberch, 1991; Jaeger & Monk, 2014). Transitions between neighbouring basins are facilitated by large borders between them, while transitions between attractor basins that do not border each other can only be indirect, through intermediate phenotypes. In most cases, phase space geometry will be complex, which means that systems with trivially dissociable genotype-phenotype maps are rare. The norm will be maps that have multiple discernable aspects to their dynamics that are "smeared" across overlapping regions of the network topology. To achieve decomposition in these cases, we need to use techniques that interrogate the dynamics in detail.

Finally, we have seen in Sect. 2 that the identification of subsystems responsible for conferring character identity can provide a powerful conceptual foundation for a mechanistic theory of homology (Wagner, 2007, 2014). We also highlighted why both functional-genetic decomposition and approaches based on structural modularity fall short of reliably identifying and delineating such subsystems. Dynamical modules provide a powerful alternative. The dynamic behaviour of a subnetwork is much more closely related to its function than its regulatory structure. Moreover, dynamical modules provide a causal-mechanistic explanation of the generative process underlying the trait, which accounts for its particular variational properties (Altenberg, 1995; Wagner & Altenberg, 1996; Salazar-Ciudad, 2006). Dynamical modules are morphogenetic fields. Therefore, morphogenetic fields, rather than ChIN networks or structural modules, provide the causal-mechanistic foundation for trait homology. This has always been the case, from classical embryology to the present day (Gilbert et al., 1996). It strongly resonates with the concept of process homology, introduced by Gilbert and Bolker (2001). Homology of process strives

to identify "the ways in which homologous processes are regulated, replicated and changed over time" with the aim "to better understand how changes in development generate changes in morphology" (ibid., p. 10). Process homology is required to make sense of modular functions. Dynamical modules are required to make sense of process homology. The two go hand in hand.

9 Conclusions

Dynamical modules provide a powerful approach to characterise the morphogenetic fields that form the causal-mechanistic basis for the functional modularity of the genotype-phenotype map and the homology of character traits. Dynamical modularity is much more closely related to function than structural or regulatory modules. We demonstrated the practical feasibility of identifying and characterising such modules in models of specific regulatory systems. We outlined promising candidates for the identification of additional dynamical modules. Finally, we discussed the evolutionary implications of our conceptual framework. The identification and characterisation of dynamical modules is an essential component of any theory aiming to mechanistically explain the origin and variability of character traits in terms of the generative processes that produce them (Goodwin, 1982; Wagner & Altenberg, 1996; Wagner et al., 2000).

Characterisation of dynamic modules not only requires decomposition by genetic and molecular approaches, but also the reconstitution of their cohesive internal structure and their coherent integrated activity using dynamical modelling. As von Dassow and Munro (1999, p. 309) stated, mechanism is "an explanatory mode in which we describe what are the parts, how they behave intrinsically, and how those parts are coupled to each other to produce the behaviour of the whole." This last point is emphasised in the framework of dynamic mechanistic explanation (Bechtel & Richardson, 1993; Bechtel & Abrahamsen, 2005, 2010; Bechtel, 2011, 2012) which focuses on the ability of mechanisms to account for patterns of activity and change over time. To understand these dynamic patterns we need models. "[M]odeling provides understanding beyond that which is available from identifying the parts, operations, and organization of the mechanism and mentally rehearsing its functioning" (Bechtel, 2012, p. 244; see also von Dassow & Meir, 2004). This approach is an explicit elaboration and refinement of Simon's theoretical framework of near-decomposability (Bechtel & Richardson, 1993; see also Callebaut, 2005).

As a practical caveat, we point out that it will always be challenging and laborious to rigorously reverse-engineer dynamical modules, since this requires extensive empirical evidence tightly combined with dynamical models of the regulatory process under study. There are good practical reasons why structural approaches have dominated the field: structural modules are relatively easy to identify. It may well be that we have to confine dynamical decomposition to a limited sample of tractable model systems. We are convinced that even such a limited sample would yield interesting and generalisable insights into the causal-mechanistic principles

underlying trait individuality, identity, and homology. These principles can be used for the mechanistic interpretation of results from correlational approaches to variational modules based on quantitative genetics (e.g. Wagner & Zhang, 2011; Nunes et al., 2013). Combined, the two complementary methodologies yield a powerful and general approach to the study of phenotypic evolution.

Another well-known pitfall is to confuse homology at the level of developmental process with homology of characters. The hierarchical organisation and robust behaviour of developmental modules implies that many different molecular mechanisms can lead to the same dynamical behaviour and, therefore, phenotypic outcome (Waddington, 1957; Oster & Alberch, 1982; Goodwin et al., 1993; Wagner, 2011; Jaeger & Monk, 2014). This means that evolution at the genotypic and phenotypic level are at least partially dissociable (von Dassow & Munro, 1999; Bolker, 2000; Gilbert & Bolker, 2001). An important consequence of dissociability is network or developmental system drift[16](Weiss & Fullterton, 2000; True & Haag, 2001; Wagner, 2011; Pavlicev & Wagner, 2012). Regulatory mechanisms can evolve quite freely by rewiring network connections, as long as the phenotypic output of the process remains the same (see Wagner, 2011; Jaeger & Monk, 2014). Process homology of dynamical modules must therefore be established at the level of the epigenotype as the underlying mechanisms can vary considerably even if the dynamics of the process are conserved (Fig. 1b). Even worse, entirely non-homologous generative processes can produce homologous character traits. A good example is insect segmentation: while many insects establish their segments in parallel by partitioning the embryo into territories as in *Drosophila*, this mode of segment determination is derived from an ancestral process that generates segments sequentially through growth and terminal addition (Sander, 1976; Davis & Patel, 2002; Rosenberg et al., 2009; Lynch et al., 2012). The resulting segmented body plan is undoubtedly homologous at the morphological level, yet the underlying processes have diverged radically during evolution.

This provides both a challenge and an opportunity for the study of phenotypic evolution: "[r]ather than view dissociability as a problem for comparative biologists let us recognise it as an architectural feature of evolvable developmental systems, a feature whose origins and consequences deserve attention" (von Dassow & Munro, 1999). We mainly focused on the aspect of identifying and characterising developmental modules, providing the basis for a new research programme for developmental evolution. Our review of the existing literature on modularity indicates this programme is best pursued by a combination of empirical research, ensemble modelling approaches, and data-driven dynamical modelling. Empirical research is necessary for functional decomposition to identify the components of a dynamical modules. Decomposition is and remains an essential precondition for the mechanistic study of phenotypic evolution. Ensemble approaches then provide a map of regulatory possibilities, which helps us to frame our concepts and questions. The study by Jiménez et al. (2017), for example, pointed us to

[16]Also called phenogenetic drift (Weiss & Fullterton, 2000; Weiss, 2005).

the fact that most multifunctional networks may not be structurally modular, and that structural modules are not required for functional modularity. These are two important but highly counterintuitive conclusions. Finally, reverse-engineering specific regulatory systems provides us with the link between functional evidence from decomposition and the possibilities provided by ensemble modelling (Jaeger & Crombach, 2012; Green et al., 2015; Chap. 4 of Crombach and Jaeger, this volume). Together, these complementary approaches will yield a completely new level of mechanistic understanding of the genotype-phenotype map, and its role in character trait evolution.

References

Ahnert, S. E., & Fink, T. (2016). Form and function in gene regulatory networks: the structure of network motifs determines fundamental properties of their dynamical state space. *Journal of the Royal Society Interface, 13*, 20160179.

Akam, M. (1987). The molecular basis for metameric pattern in the *Drosophila* embryo. *Development, 101*, 1–22.

Alberch, P. (1991). From genes to phenotype: dynamical systems and evolvability. *Genetica, 84*, 5–11.

Albert, R., & Othmer, H. (2003). The topology of the regulatory interactions predicts the expression pattern of the segment polarity genes in *Drosophila*. *Journal of Theoretical Biology, 223*, 1–18.

Alexander, R. P., Kim, P. M., Emonet, T., & Gerstein, M. B. (2009). Understanding modularity in molecular networks requires dynamics. *Science Signaling, 2*, pe44.

Alon, U. (2006). *An introduction to systems biology: design principles of biological circuits*. Chapman and Hall/CRC.

Alon, U. (2007). Network motifs: theory and experimental approaches. *Nature Reviews. Genetics, 8*, 450–461.

Altenberg, L. (1995). Genome growth and the evolution of the genotype-phenotype map. In W. Banzhaf & F. H. Eeckman (Eds.), *Evolution and biocomputation: computational models of evolution*. Springer.

Akman, O. E., Watterson, S., Parton, A., Binns, N., Millar, A. J., & Ghazal, P. (2012). Digital clocks: simple Boolean models can quantitatively describe circadian systems. *Journal of The Royal Society Interface, 9*, 2365–2382.

Amundson, R. (2005). *The changing role of the embryo in evolutionary thought*. Cambridge University Press.

Asgari-Targhi, A., & Klerman, E. B. (2018). Mathematical modeling of circadian rhythms. *WIREs Systems Biology and Medicine, 11*, e1439.

Ashyraliyev, M., Siggens, K., Janssens, H., Blom, J., Akam, M., & Jaeger, J. (2009). Gene circuit analysis of the terminal gap gene *huckebein*. *PLoS Computational Biology, 5*, e1000548.

Babu, M. M., Luscombe, N. M., Aravind, L., Gerstein, M., & Teichmann, S. A. (2004). Structure and evolution of transcriptional regulatory networks. *Current Opinion in Structural Biology, 14*, 283–291.

Balaskas, N., Ribeiro, A., Panovska, J., Dessaud, E., Sasai, N., Page, K. M., Briscoe, J., & Ribes, V. (2012). Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. *Cell, 148*, 273–284.

Bar-Joseph, Z., Gerber, G. K., Lee, T. I., Rinaldi, N. J., Yoo, J. Y., Robert, F., Gordon, D. B., Fraenkel, E., Jaakkola, T. S., Young, R. A., & Gifford, D. K. (2003). Computational discovery of gene modules and regulatory networks. *Nature Biotechnology, 21*, 1337–1342.

Bechtel, W. (2011). Mechanism and biological explanation. *Philosophy of Science, 78*, 533–557.

Bechtel, W. (2012). Understanding endogenously active mechanisms: a scientific and philosophical challenge. *European Journal for Philosophy of Science, 2*, 233–248.

Bechtel, W., & Abrahamsen, A. (2005). Explanation: a mechanist alternative. *Studies in History and Philosophy of Biological and Biomedical Sciences, 36*, 421–441.

Bechtel, W., & Abrahamsen, A. (2010). Dynamic mechanistic explanation: computational modeling of circadian rhythms as an exemplar for cognitive science. *Studies in History and Philosophy of Science, 41*, 321–333.

Bechtel, W., & Richardson, R. C. (1993). *Discovering complexity: decomposition and localization as strategies in scientific research*. MIT Press.

Benítez, M., & Alvarez-Buylla, E. R. (2010). Dynamic-module redundancy confers robustness to the gene regulatory network involved in hair patterning of *Arabidopsis* epidermis. *Biosystems, 102*, 11–15.

Benítez, M., Espinosa-Soto, C., Padilla-Longoria, P., & Alvarez-Buylla, E. R. (2008). Interlinked nonlinear subnetworks underlie the formation of robust cellular patterns in *Arabidopsis* epidermis: a dynamical spatial model. *BMC Systems Biology, 2*, 98.

Benítez, M., Monk, N. A., & Alvarez-Buylla, E. R. (2011). Epidermal patterning in *Arabidopsis*: models make a difference. *The Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 316*, 241–253.

Benson, A. R., Gleich, D. F., & Leskovec, J. (2016). Higher-order organization of complex networks. *Science, 353*, 163–166.

Bolker, J. A. (2000). Modularity in development and why it matters to evo-devo. *American Zoologist, 40*, 770–776.

Bolouri, H. (2014). Modeling genomic regulatory networks with big data. *Trends in Genetics, 30*, 182–191.

Bonner, J. T. (1988). *The evolution of complexity by means of natural selection*. Princeton University Press.

Brandon, R. N. (1999). The units of selection revisited: the modules of selection. *Biology and Philosophy, 14*, 167–180.

Briscoe, J., & Small, S. (2015). Morphogen rules: design principles of gradient-mediated embryo patterning. *Development, 142*, 3996–4009.

Burda, Z., Krzywicki, A., Martin, O. C., & Zagorski, M. (2011). Motifs emerge from function in model gene regulatory networks. *Proceedings. National Academy of Sciences. United States of America, 108*, 17263–17268.

Burns, J. (1970). The synthetic problem and the genotype-phenotype relation in cellular metabolism. In C. H. Waddington (Ed.), *Towards a theoretical biology* (Vol. III). Edinburgh University Press.

Callebaut, W. (2005). The ubiquity of modularity. In W. Callebaut & D. Rasskin-Gutman (Eds.), *Modularity–understanding the development and evolution of natural complex systems*. MIT Press.

Callebaut, W., & Rasskin-Gutman, D. (2005). *Modularity–understanding the development and evolution of natural complex systems*. MIT Press.

Chaves, M., Albert, R., & Sontag, E. (2005). Robustness and fragility of Boolean models for genetic regulatory networks. *Journal of Theoretical Biology, 235*, 431–449.

Chen, K. C., Calzone, L., Csikasz-Nagy, A., Cross, F. R., Novak, B., & Tyson, J. J. (2004). Integrative analysis of cell cycle control in budding yeast. *Molecular Biology of the Cell, 15*, 3841–3862.

Chen, K. C., Csikasz-Nagy, A., Gyorffy, B., Val, J., Novak, B., & Tyson, J. J. (2000). Kinetic analysis of a molecular model of the budding yeast cell cycle. *Molecular Biology of the Cell, 11*, 369–391.

Christensen, W. D., & Bickhard, M. H. (2002). The process dynamics of normative function. *The Monist, 85*, 3–28.

Collier, J. (1988). Supervenience and reduction in biological hierarchies. *Canadian Journal of Philosophy, 14*, 209–234.

Collier, J. (2004). Self-organization, individuation and identity. *Revue Internationale de Philosophie, 14*, 209–234.

Cotterell, J., & Sharpe, J. (2010). An atlas of gene regulatory networks reveals multiple three-gene mechanisms for interpreting morphogen gradients. *Molecular Systems Biology, 6*, 425.

Crombach, A., & Hogeweg, P. (2008). Evolution of evolvability in gene regulatory networks. *PLoS Computational Biology, 4*, e1000112.

Crombach, A., Wotton, K. R., Cicin-Sain, D., Ashyraliyev, M., & Jaeger, J. (2012). Efficient reverse-engineering of a developmental gene regulatory network. *PLoS Computational Biology, 8*, e1002589.

Crombach, A., Wotton, K. R., Jiménez-Guri, E., & Jaeger, J. (2016). Gap gene regulatory dynamics evolve along a genotype network. *Molecular Biology and Evolution, 33*, 1293–1307.

Cummins, R. (1975). Functional analysis. *Journal of Philosophy, 72*, 741–765.

Davidson, E. H. (2010). Emerging properties of animal gene regulatory networks. *Nature, 468*, 911–920.

Davidson, E. H. (2011). Evolutionary bioscience as regulatory systems biology. *Developmental Biology, 357*, 35–40.

Davidson, E. H., & Erwin, D. H. (2006). Gene regulatory networks and the evolution of animal body plans. *Science, 311*, 796–800.

Davidson, E. H., Rast, J. P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.-H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otim, O., Brown, C. T., Livi, C. B., Lee, P. Y., Revilla, R., Rust, A. G., Pan, Z. J., Schilstra, M. J., Clarke, P. J. C., … Bolouri, H. (2002). A genomic regulatory network for development. *Science, 295*, 1669–1678.

Davis, G. K., & Patel, N. H. (2002). Short, long, and beyond: molecular and embryological approaches to insect segmentation. *Annual Review of Entomology, 47*, 669–699.

Dawkins, R. (1989). The evolution of evolvability. In C. Langton (Ed.), *Artificial life: the proceedings of an interdisciplinary workshop on the synthesis and simulation of living systems*. Addison-Wesley.

DiFrisco, J. (2018). Biological processes: criteria of identity and persistence. In D. J. Nicholson & J. Dupré (Eds.), *Everything flows: towards a processual philosophy of biology*. Oxford University Press.

DiFrisco, J., & Jaeger, J. (2019). Beyond networks: mechanism and process in Evo-Devo. *Biology and Philosophy, 34*, 54.

DiFrisco, J., Love, A. C., & Wagner, G. P. (2020). Character identity mechanisms: a conceptual model for comparative-mechanistic biology. *Biology and Philosophy, 35*, 44.

Dobrin, R., Beg, Q. K., Barabási, A.-L., & Oltvai, Z. N. (2004). Aggregation of topological motifs in the *Escherichia coli* transcriptional regulatory network. *BMC Bioinformatics, 5*, 10.

Eisen, M. B., Spellman, P. T., Brown, P. O., & Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proceedings. National Academy of Sciences. United States of America, 95*, 14863–14868.

Erwin, D. H., & Davidson, E. H. (2009). The evolution of hierarchical gene regulatory networks. *Nature Reviews. Genetics, 10*, 141–148.

Fortunato, S. (2010). Community detection in graphs. *Physics Reports, 486*, 75–174.

Gilbert, S. F., & Bolker, J. A. (2001). Homologies of process and modular elements of embryonic construction. *The Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 291*, 1–12.

Gilbert, S. F., Opitz, J. M., & Raff, R. A. (1996). Resynthesizing evolutionary and developmental biology. *Developmental Biology, 173*, 357–372.

Girvan, M., & Newman, M. E. (2002). Community structure in social and biological networks. *Proceedings. National Academy of Sciences. United States of America, 99*, 7821–7826.

Goldbeter, A. (1995). A model for circadian oscillations in the *Drosophila* period protein (PER). *Proceedings of the Royal Society of London B, 261*, 319–324.

Goldbeter, A. (1997). *Biochemical oscillations and cellular rhythms: the molecular bases of periodic and chaotic behaviour*. Cambridge University Press.

Goodwin, B. C. (1982). Development and evolution. *Journal of Theoretical Biology, 97*, 43–55.

Goodwin, B. C., Kauffman, S. A., & Murray, J. D. (1993). Is morphogenesis an intrinsically robust process? *Journal of Theoretical Biology, 163*, 135–144.

Gorochowski, T. E., Grierson, C. S., & di Bernardo, M. (2018). Organization of feed-forward loop motifs reveals architectural principles in natural and engineered networks. *Science Advances, 4*, eaap9751.

Green, J. B. A., & Sharpe, J. (2015). Positional information and reaction-diffusion: two big ideas in developmental biology combine. *Development, 142*, 1203–1211.

Gould, S. J., & Lewontin, R. C. (1979). The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. *Proceedings of the Royal Society London B, 205*, 581–598.

Green, S., Fagan, M., & Jaeger, J. (2015). Explanatory integration challenges in evolutionary systems biology. *Biological Theory, 10*, 18–35.

Gursky, V. V., Panok, L., Myasnikova, E. M., Manu, M., Samsonova, M. G., Reinitz, J., & Samsonov, A. M. (2011). Mechanisms of gap gene expression canalization in the *Drosophila* blastoderm. *BMC Systems Biology, 5*, 118.

Hartwell, L. H., Hopfield, J. J., Leibler, S., & Murray, A. W. (1999). From molecular to modular cell biology. *Nature, 402*(Suppl), C47–C52.

Hernández-Hernández, V., Niklas, K. J., Newman, S. A., & Benítez, M. (2012). Dynamical patterning modules in plant development and evolution. *The International Journal of Developmental Biology, 56*, 661–674.

Hull, D. L. (1980). Individuality and selection. *Annual Review of Ecology and Systematics, 11*, 311–332.

Ingham, P. W. (1988). The molecular genetics of embryonic pattern formation in *Drosophila*. *Nature, 335*, 25–34.

Ingram, P. J., Stumpf, M. P., & Stark, J. (2006). Network motifs: structure does not determine function. *BMC Genomics, 7*, 108.

Irons, D. J. (2009). Logical analysis of the budding yeast cell cycle. *Journal of Theoretical Biology, 257*, 543–559.

Irons, D. J., & Monk, N. A. M. (2007). Identifying dynamical modules from genetic regulatory systems: applications to the segment polarity network. *BMC Bioinformatics, 8*, 413.

Isalan, M. (2009). Gene networks and liar paradoxes. *BioEssays, 31*, 1110–1115.

Isalan, M., & Morrison, M. (2009). This title is false. *Nature, 458*, 969.

Ishihara, S., & Shibata, T. (2008). Mutual interaction in network motifs robustly sharpens gene expression in developmental processes. *Journal of Theoretical Biology, 252*, 131–144.

Jaeger, J. (2011). The gap gene network. *Cellular and Molecular Life Sciences, 68*, 243–274.

Jaeger, J. (2018). Shift happens: the developmental and evolutionary dynamics of the gap gene system. *Current Opinion in Systems Biology, 11*, 65–73.

Jaeger, J., Blagov, M., Kosman, D., Kozlov, K. N., Manu, M., Myasnikova, E., Surkova, S., Vanario-Alonso, C. E., Samsonova, M., Sharp, D. H., & Reinitz, J. (2004a). Dynamical analysis of regulatory interactions in the gap gene system of *Drosophila melanogaster*. *Genetics, 167*, 1721–1737.

Jaeger, J., & Crombach, A. (2012). Life's attractors: understanding developmental systems through reverse engineering and *in silico* evolution. In O. Soyer (Ed.), *Evolutionary systems biology*. Springer.

Jaeger, J., Irons, D., & Monk, N. (2008). Regulative feedback in pattern formation: towards a general relativistic theory of positional information. *Development, 135*, 3175–3183.

Jaeger, J., Irons, D., & Monk, N. (2012). The inheritance of process: a dynamical systems approach. *The Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 318B*, 591–612.

Jaeger, J., & Monk, N. (2014). Bioattractors: dynamical systems theory and the evolution of regulatory processes. *The Journal of Physiology, 592*, 2267–2281.

Jaeger, J., & Monk, N. (2015). Everything flows. *EMBO Reports, 16*, 1064–1067.

Jaeger, J., Surkova, S., Blagov, M., Janssens, H., Kosman, D., Kozlov, K. N., Manu, M., Myasnikova, E., Vanario-Alonso, C. E., Samsonova, M., Sharp, D. H., & Reinitz, J. (2004b).

Dynamic control of positional information in the early *Drosophila* embryo. *Nature, 430*, 368–371.

Jiménez, A., Cotterell, J., Munteanu, A., & Sharpe, J. (2017). A spectrum of modularity in multi-functional gene circuits. *Molecular Systems Biology, 13*, 925.

Kashtan, N., Itzkovitz, S., Milo, R., & Alon, U. (2004). Topological generalizations of network motifs. *Physical Review E, 70*, 031909.

Kauffman, S. A. (1969). Metabolic stability and epigenesis in randomly constructed genetic nets. *Journal of Theoretical Biology, 22*, 437–467.

Kauffman, S. A. (1974). The large scale structure and dynamics of gene control circuits. *Journal of Theoretical Biology, 44*, 167–190.

Kauffman, S. A. (1987). Developmental logic and its evolution. *BioEssays, 6*, 82–87.

Kauffman, S. A. (1993). *The origins of order: self organization and selection in evolution*. Oxford University Press.

Kim, M.-S., Kim, J.-R., & Cho, K.-H. (2011). Dynamic network rewiring determines temporal regulatory functions in *Drosophila melanogaster* development processes. *BioEssays, 32*, 505–513.

Kondo, S., & Miura, T. (2010). Reaction-diffusion model as a framework for understanding biological pattern formation. *Science, 329*, 1616–1620.

Levine, M., & Davidson, E. H. (2005). Gene regulatory networks for development. *Proceedings. National Academy of Sciences. United States of America, 102*, 4936–4942.

Lewontin, R. C. (1970). The units of selection. *Annual Review of Ecology and Systematics, 1*, 1–18.

Lewontin, R. C. (1974). *The genetic basis of evolutionary change*. Columbia University Press.

Lynch, J. A., El-Sherif, E., & Brown, S. J. (2012). Comparisons of the embryonic development of *Drosophila*, *Nasonia*, and *Tribolium*. *Wiley Interdisciplinary Reviews: Developmental Biology, 1*, 16–39.

Ma, W., Lai, L., Ouyang, Q., & Tang, C. (2006). Robustness and modular design of the *Drosophila* segment polarity network. *Molecular Systems Biology, 2*, 70.

Ma, W., Trusina, A., El-Samad, H., Lim, W. A., & Tang, C. (2009). Defining network topologies that can achieve biochemical adaptation. *Cell, 138*, 760–773.

Mangan, S., & Alon, U. (2003). Structure and function of the feed-forward loop network motif. *Proceedings. National Academy of Sciences. United States of America, 100*, 11980–11985.

Manu, M., Surkova, S., Spirov, A. V., Gursky, V., Janssens, H., Kim, A. R., Radulescu, O., Vanario-Alonso, C., Sharp, D. H., Samsonova, M., & Reinitz, J. (2009). Canalization of gene expression and domain shifts in the *Drosphila* blastoderm by dynamical attractors. *PLoS Computational Biology, 5*, e1000303.

Marcon, L., & Sharpe, J. (2012). Turing patterns in development: what about the horse part? *Current Opinion in Genetics and Development, 22*, 578–584.

Maroto, M., & Monk, N. A. M. (Eds.). (2009). *Cellular oscillatory mechanisms*. Landes Bioscience/Springer.

Mazurie, A., Bottani, S., & Vergassola, M. (2005). An evolutionary and functional assessment of regulatory network motifs. *Genome Biology, 6*, R35.

Meinhardt, H. (1982). *Models of biological pattern formation*. Academic Press.

Meinhardt, H., & Gierer, A. (2010). Pattern formation by local self-activation and lateral inhibition. *BioEssays, 22*, 753–760.

Melo, D., Porto, A., Cheverud, J. M., & Marroig, G. (2016). Modularity: genes, development, and evolution. *Annual Review of Ecology, Evolution, and Systematics, 47*, 463–486.

Milo, R., Itzkovitz, S., Kashtan, N., Levitt, R., Shen-Orr, S., Ayzenshtat, I., Sheffer, M., & Alon, U. (2004). Superfamilies of evolved and designed networks. *Science, 303*, 1538–1542.

Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D., & Alon, U. (2002). Network motifs: simple building blocks of complex networks. *Science, 298*, 824–827.

Mireles, V., & Conrad, T. O. (2018). Reusable building blocks in biological systems. *Journal of the Royal Society Interface, 15*, 20180595.

Monteiro, A., & Podlaha, O. (2009). Wings, horns, and butterfly eyespots: how do complex traits evolve? *PLoS Biology, 7*, e1000037.

Moss, L. (2001). Deconstructing the gene and reconstructing molecular developmental systems. In S. Oyama, P. E. Griffiths, & R. D. Gray (Eds.), *Cycles of contingency: developmental systems and evolution*. MIT Press.

Mossio, M., Saborido, C., & Moreno, A. (2009). An organizational account of biological functions. *British Journal of the Philosophy of Science, 60*, 813–841.

Needham, J. (1933). On the dissociability of the fundamental processes in ontogenesis. *Biological Reviews, 8*, 180–223.

Newman, M. E. J. (2006). Modularity and community structure in networks. *Proceedings. National Academy of Sciences. United States of America, 103*, 8577–8582.

Newman, M. E. J., Barabási, A.-L., & Watts, D. J. (2006). *The structure and dynamics of networks*. Princeton University Press.

Newman, S. A., & Bhat, R. (2008). Dynamical patterning modules: physico-genetic determinants of morphological development and evolution. *Physical Biology, 5*, 015008.

Newman, S. A., & Bhat, R. (2009). Dynamical patterning modules: a "pattern language" for development and evolution of multicellular form. *The International Journal of Developmental Biology, 53*, 693–705.

Nicholson, D. J., & Dupré, J. (2018). *Everything flows: towards a processual philosophy of biology*. Oxford University Press.

Niehrs, C. (2004). Synexpression groups: genetic modules and embryonic development. In G. Schlosser & G. P. Wagner (Eds.), *Modularity in development and evolution*. University of Chicago Press.

Novák, B., & Tyson, J. (2008). Design principles of biochemical oscillators. *Nature Reviews. Molecular Cell Biology, 9*, 981–991.

Nunes, M. D. S., Arif, S., Schlötterer, C., & McGregor, A. P. (2013). A perspective on micro-evo-devo: progress and potential. *Genetics, 195*, 625–634.

Nüsslein-Volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature, 287*, 795–801.

Oliveri, P., & Davidson, E. H. (2004). Gene regulatory network controlling embryonic specification in the sea urchin. *Current Opinion in Genetics and Development, 14*, 351–360.

Onimaru, K., Marcon, L., Musy, M., Tanaka, M., & Sharpe, J. (2016). The fin-to-limb transition as the re- organization of a Turing pattern. *Nature Communications, 7*, 11582.

Oster, G., & Alberch, P. (1982). Evolution and bifurcation of developmental programs. *Evolution, 36*, 444–459.

Page, K. M., & Perez-Carrasco, R. (2018). Degradation rate uniformity determines success of oscillations in repressive feedback regulatory networks. *Journal of the Royal Society Interface, 15*, 20180157.

Palla, G., Derényi, I., Farkas, I., & Vicsek, T. (2005). Uncovering the overlapping community structure of complex networks in nature and society. *Nature, 435*, 814.

Panovska-Griffiths, J., Page, K. M., & Briscoe, J. (2013). A gene regulatory motif that generates oscillatory or multiway switch outputs. *Journal of the Royal Society Interface, 15*, 20180157.

Papatsenko, D. (2009). Stripe formation in the early fly embryo: principles, models, and networks. *BioEssays, 31*, 1172–1180.

Pavlicev, M., & Wagner, G. P. (2012). A model of developmental evolution: selection, pleiotropy and compensation. *Trends in Ecology & Evolution, 27*, 316–322.

Payne, J. L., & Wanger, A. (2015). Function does not follow form in gene regulatory circuits. *Scientific Reports, 5*, 13015.

Perez-Carrasco, R., Barnes, C. P., Schaerli, Y., Isala, M., Briscoe, J., & Page, K. M. (2018). Combining a toggle switch and a repressilator within the AC-DC circuit generates distinct dynamical behaviors. *Cell Systems, 5*, 521–530.

Peter, I. S., & Davidson, E. H. (2011). Evolution of gene regulatory networks controlling body plan development. *Cell, 144*, 970–985.

Peter, I. S., & Davidson, E. H. (2017). Assessing regulatory information in developmental gene regulatory networks. *Proceedings. National Academy of Sciences. United States of America, 114*, 5862–5869.

Peter, I. S., Faure, E., & Davidson, E. H. (2012). Predictive computation of genomic logic processing functions in embryonic development. *Proceedings. National Academy of Sciences. United States of America, 109*, 16434–16442.

Pigliucci, M. (2008). Is evolvability evolvable? *Nature Reviews. Genetics, 9*, 75–82.

Pigliucci, M. (2010). Genotype-phenotype mapping and the end of the 'genes as a blueprint' metaphor. *Philosophical Transactions of the Royal Society B: Biological Sciences, 365*, 557–566.

Radicchi, F., Castellano, C., Cecconi, F., Loreto, V., & Parisi, D. (2004). Defining and identifying communities in networks. *Proceedings. National Academy of Sciences. United States of America, 101*, 2658–2663.

Raff, R. A. (1996). *The shape of life: genes, development, and the evolution of animal form*. Chicago University Press.

Raspopovic, J., Marcon, L., Russo, L., & Sharpe, J. (2014). Digit patterning is controlled by a Bmp-Sox9-Wnt Turing network modulated by morphogen gradients. *Science, 345*, 566–570.

Riedl, R. (1975). *Die Ordnung des Lebendigen: Systembedingungen der Evolution*. Parey, Hamburg. (English translation: Riedl R (auth.), Jeffries RPS (transl.) (1978) Order in living systems: a systems analysis of evolution. Wiley).

Rosenberg, M. I., Lynch, J. A., & Desplan, C. (2009). Heads and tails: evolution of antero-posterior patterning in insects. *Biochimica et Biophysica Acta, 1789*, 333–342.

Rosenfeld, N., & Alon, U. (2003). Response delays and the structure of transcription networks. *Journal of Molecular Biology, 329*, 645–665.

Salazar-Ciudad, I. (2006). Developmental constraints vs. variational properties: how pattern formation can help to understand evolution and development. *The Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 306B*, 107–125.

Salazar-Ciudad, I., Garcia-Fernández, J., & Solé, R. V. (2000). Gene networks capable of pattern formation: from induction to reaction-diffusion. *Journal of Theoretical Biology, 205*, 587–603.

Salazar-Ciudad, I., Newman, S. A., & Solé, R. V. (2001a). Phenotypic and dynamical transitions in model genetic networks I. Emergence of patterns and genotype-phenotype relationships. *Evolution & Development, 3*, 84–94.

Salazar-Ciudad, I., Solé, R. V., & Newman, S. A. (2001b). Phenotypic and dynamical transitions in model genetic networks II. Application to the evolution of segmentation mechanisms. *Evolution & Development, 3*, 95–103.

Sander, K. (1976). Specification of the basic body pattern in insect embryogenesis. *Advances in Insect Physiology, 12*, 124–238.

Schlosser, G., & Wagner, G. P. (2004). *Modularity in development and evolution*. University of Chicago Press.

Segal, E., Shapira, M., Regev, A., Pe'er, D., Botstein, D., Koller, D., & Friedman, N. (2003). Module networks: identifying regulatory modules and their condition-specific regulators from gene expression data. *Nature Genetics, 34*, 166–176.

Shellman, E. R., Burant, C. F., & Schnell, S. (2013). Network motifs provide signatures that characterize metabolism. *Molecular BioSystems, 9*, 352.

Shen-Orr, S. S., Milo, R., Mangan, S., & Alon, U. (2002). Network motifs in the transcriptional regulation network of *Escherichia coli*. *Nature Genetics, 31*, 64–68.

Siebert, H. (2009). Dynamical and structural modularity of discrete regulatory networks. In R. J. Back, I. Petre, & E. de Vink (Eds.), *Computational Models for Cell Processes (CompMod 2009) EPTCS* (Vol. 6, pp. 109–124).

Siebert, H. (2011). Analysis of discrete bioregulatory networks using symbolic steady states. *Bulletin of Mathematical Biology, 73*, 873–898.

Siegal, M. L., Promislow, D. E. L., & Bergman, A. (2007). Functional and evolutionary inference in gene networks: does topology matter? *Genetica, 129*, 83–103.

Simon, H. A. (1962). The architecture of complexity. *Proceedings. American Philosophical Society, 106*, 467–482.

Simon, H. A. (1973). The organization of complex systems. In H. H. Pattee (Ed.), *Hierarchy theory*. George Braziller.

Solé, R. V., & Valverde, S. (2006). Are network motifs the spandrels of cellular complexity? *Trends in Ecology & Evolution, 21*, 419–422.

Strogatz, S. H. (2014). *Nonlinear dynamics and chaos–with applications to physics, biology, chemistry, and engineering*. Westview Press.

Stuart, J. M., Segal, E., Koller, D., & Kim, S. K. (2003). A gene-coexpression network for global discovery of conserved genetic modules. *Science, 302*, 249–255.

Tavazoie, S., Hughes, J. D., Campbell, M. J., Cho, R. J., & Church, G. M. (1999). Systematic determination of genetic network architecture. *Nature Genetics, 22*, 281–285.

Teichmann, S. A., & Babu, M. (2002). Conservation of gene co-regulation in prokaryotes and eukaryotes. *Trends in Biotechnology, 20*, 407–410.

True, J. R., & Carroll, S. B. (2002). Gene co-option in physiological and morphological evolution. *Annual Review of Cell and Developmental Biology, 18*, 53–80.

True, J. R., & Haag, E. S. (2001). Developmental system drift and flexibility in evolutionary trajectories. *Evolution & Development, 3*, 109–119.

Turing, A. M. (1952). The chemical basis of morphogenesis. *Philosophical Transactions of the Royal Society B: Biological Sciences, 237*, 37–72.

Tyson, J. J., & Novák, B. (2015). Models in biology: lessons from modeling regulation of the eukaryotic cell cycle. *BMC Biology, 13*, 46.

Verd, B., Clark, E., Wotton, K. R., Janssens, H., Jiménez-Guri, E., Crombach, A., & Jaeger, J. (2018). A damped oscillator imposes temporal order on posterior gap gene expression in *Drosophila*. *PLoS Biology, 16*, e2003174.

Verd, B., Crombach, A., & Jaeger, J. (2017). Dynamic maternal gradients control timing and shift-rates for *Drosophila* gap gene expression. *PLoS Computational Biology, 13*, e1005285.

Verd, B., Monk, N. A. M., & Jaeger, J. (2019). Modularity, criticality, and evolvability of a developmental gene regulatory network. *eLife, 8*, e42832.

von Dassow, G., & Meir, E. (2004). Exploring modularity with dynamical models of gene networks. In G. Schlosser & G. P. Wagner (Eds.), *Modularity in development and evolution*. University of Chicago Press.

von Dassow, G., & Munro, E. (1999). Modularity in animal development and evolution: elements of a conceptual framework for EvoDevo. *The Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 285*, 307–325.

Waddington, C. H. (1942). The epigenotype. Endeavour 1: 18–20 (reprinted in 2012). *International Journal of Epidemiology, 41*, 10–13.

Waddington, C. H. (1953). Epigenetics and evolution. In *Symposia of the society for experimental biology VII: evolution*. Cambridge University Press.

Waddington, C. H. (1957). *The strategy of the genes: a discussion of some aspects of theoretical biology*. Allen & Unwin.

Wagner, A. (2011). *The origins of evolutionary innovations: a theory of transformative change in living systems*. Oxford University Press.

Wagner, G. P. (1989). The biological homology concept. *Annual Review of Ecology and Systematics, 20*, 51–69.

Wagner, G. P. (1996). Homologues, natural kinds and the evolution of modularity. *American Zoologist, 36*, 36–43.

Wagner, G. P. (2007). The developmental genetics of homology. *Nature Reviews. Genetics, 8*, 473–479.

Wagner, G. P. (2014). *Homology, genes, and evolutionary innovation*. Princeton University Press.

Wagner, G. P., & Altenberg, L. (1996). Complex adaptations and the evolution of evolvability. *Evolution, 50*, 967–976.

Wagner, G. P., Chiu C.-H., & Laubichler, M. (2000). Developmental evolution as a mechanistic science: the inference from developmental mechanisms to evolutionary processes. *American Zoologist, 40*, 819–831.

Wagner, G. P., Pavlicev, M., & Cheverud, J. M. (2007). The road to modularity. *Nature Reviews. Genetics, 8*, 921–931.

Wagner, G. P., & Zhang, J. (2011). The pleiotropic structure of the genotype-phenotype map: the evolvability of complex organisms. *Nature Reviews. Genetics, 12*, 204–213.

Wall, M. E., Dunlop, M. J., & Hlavacek, S. (2005). Multiple functions of a feed-forward-loop gene circuit. *Journal of Molecular Biology, 349*, 501–514.

Webster, G., & Goodwin, B. C. (1996). *Form and transformation: generative and relational principles in biology*. Cambridge University Press.

Weiss, K. M. (2005). The phenogenetic logic of life. *Nature Reviews. Genetics, 6*, 36–45.

Weiss, K. M., & Fullterton, S. M. (2000). Phenogenetic drift and the evolution of genotype-phenotype relationships. *Theoretical Population Biology, 57*, 187–195.

Wimsatt, W. C. (2007). *Re-engineering philosophy for limited beings: piecewise approximations to reality*. Harvard University Press.

Winther, R. G. (2001). Varieties of modules: kinds, levels, origins, and behaviors. *The Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 291*, 116–129.

Wolpert, L. (1968). The French Flag problem: a contribution to the discussion on pattern development and regulation. In C. H. Waddington (Ed.), *Towards a theoretical biology* (Vol. I). Edinburgh University Press.

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *Journal of Theoretical Biology, 25*, 1–47.

Wotton, K. R., Jiménez-Guri, E., Crombach, A., Janssens, H., Alcaine-Colet, A., Lemke, S., Schmidt-Ott, U., & Jaeger, J. (2015). Quantitative system drift compensates for altered maternal inputs to the gap gene network of the scuttle fly *Megaselia abdita*. *eLife, 4*, e04785.

Wright, L. (1973). Functions. *Philosophical Review, 82*, 139–168.

Zinzen, R. P., & Papatsenko, D. (2007). Enhancer responses to similarlly distributed antagonistic gradients in development. *PLoS Computational Biology, 3*, e84.

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A. Crombach (ed.), *Evolutionary Systems Biology*,
https://doi.org/10.1007/978-3-030-71737-7

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