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Methods for and results from the study of design principles in molecular systems

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ABSTRACT

Most aspects of molecular biology can be understood in terms of biological design principles. These principles can be loosely defined as qualitative and quantitative features that emerge in evolution and recur more frequently than one would expect by chance alone in biological systems that perform a given type of process or function. Furthermore, such recurrence can be rationalized in terms of the functional advantage that the design provides to the system when compared with possible alternatives. This paper focuses on those design features that can be related to improved functional effectiveness of molecular and regulatory networks. We begin by reviewing assumptions and methods that underlie the study of such principles in molecular networks. We follow by discussing many of the design principles that have been found in genetic, metabolic, and signal transduction circuits. We concentrate mainly on results in the context of Biochemical Systems Theory, although we also briefly discuss other work. We conclude by discussing the importance of these principles for both, understanding the natural evolution of complex networks at the molecular level and for creating artificial biological systems with specific features.

1. Introduction

One of the most important goals in biology is the understanding of how the molecular features of biological systems have emerged and become fixed. Emergence of these features during evolution is random, due to different mechanisms such as mutation and recombination. Fixation of the different alternative features can be accidental, due to chance. Another possibility is that they become fixed because they generate molecular variants that make fitter organisms that survive and reproduce better. In this process, the predominance of a given molecular feature is a consequence of natural selection acting as a process that increases the frequency of designs with a better functional performance. Differentiating between the two possibilities allows researchers to identify the biological design principles of the molecular systems of interest [1,2].

In this context, biological design principles can be defined as repeated qualitative and quantitative features of biological components and their interactions that are observed in molecular systems at high frequencies and improve the functional performance of a system that executes a specific process. Such principles have been found in many aspects of molecular biology [3].

For example, sequence biases that facilitate the control of gene expression under different conditions, with appropriate timing, are recurrent and can be rationalized as having evolved under a selective pressure to minimize the metabolic cost associated to the process of synthesis [4–6]. As another example, certain types of protein domains that are more abundant in proteomes and are associated to specific functional requirements for protein stability suggest a recurrent evolutionary design associated to that specific function [7,8]. As a final example, an interesting structural design principle is found in glycogen. This molecule has evolved to provide a reservoir of glucose and to make that glucose available quickly and in large amounts when required. Melendez-Hevia and co-workers showed that the branching in the structure of glycogen is an optimal solution to the problem of optimizing storage space and fast glucose mobilization [9,10].

If design principles emerge from the evolution of complex biological systems, one may expect to identify such principles at all organizational levels, from metabolic and gene networks, organs, and physiology, to organisms and their interactions [2]. Here we centre our attention on the evolution of structure and regulation of molecular networks in cells. These include gene networks, metabolic pathways, signal transduction cascades, cell cycle, immune response, and other molecular networks. The study of design principles in the context of regulatory molecular networks started as early as the seventies (see, for example, [11–20]). These early

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studies were performed in the context of *BST* (*Biochemical Systems Theory*) [20,21], a body of work providing a set of tools that facilitate the creation and analysis of mathematical models for biological systems [21]. The current surge in interest towards network motifs and the modular structure of molecular networks is partially a consequence of those early studies. However, it is also a consequence of the amount and complexity of the biological information that continuously accumulates and becomes available to us. This creates a situation where learning how biology works hinges on the possibility of understanding general organizational principles in biological systems, rather than through memorizing massive "grocery lists" of biological facts.

There have been several methods developed within the *BST* framework specifically to studying design principles in molecular networks. One of these methods is that of the *Mathematically Controlled Comparisons* [11,12,22,23]. This technique facilitates the analysis and comparison of the differences in systemic behavior between alternative designs for the same network. The compared behaviors range from steady-state characteristics to dynamic behavior and parameter robustness. The comparisons are mathematically constrained in a way that ensures that any differences in behavior are a consequence of the differences in design and not of other spurious changes between systems. Recently, design space representations that provide a simplified way to analyze the different phenotypic regions of systemic behavior were developed within the same theoretical framework [24–26].

In this paper, we review some of these methods, and results of their application to the study of molecular networks. We focus mainly on studies within the context of Biochemical Systems Theory, although we also briefly discuss other relevant work. We conclude by discussing the importance of biological design principles and how they can be organized in the future.

2. Molecular circuits vs. the molecular network of the cell

This special issue of Mathematical Biosciences focuses mostly on design principles that can be inferred for the structure and regulation of molecular circuits that are responsible for specific biological functions, and on how such principles correlate to those functions.

These biological design principles are a consequence of evolution selecting for particular features that make some circuits more effective in performing their biological function. Given that evolution acts on organisms and populations [27], it is fair to ask two questions about the previous sentence. The first question is how appropriate is it to identify functional effectiveness of a specific circuit or module rather than that of the entire network of circuits. The second question is how can we be sure that a particular design it is a consequence of selection because it is functionally more effective, rather than an accident of evolution.

To answer the first question one must consider two aspects. To begin, one must admit that at the molecular level living organisms seem to evolve in a modular way. Several examples point to this. Proteins evolve mostly through domain recombination, and specific functions are associated to each type of protein domains [28]. The expression of the genes coding for the proteins of many pathways is coordinated in operons and regulons [29,30]. In addition, recent work suggests that, given the parallel and multiple demands that biological systems have to cope with during evolution, it is likely that their functionality has evolved in a modular fashion [31–35]. Considering that such modularity appears to be extended in biology, one must also consider that most mutations in a circuit are likely to cause malfunctioning of that circuit. The malfunction of the circuit contributes to decrease the fitness of the organism (see, for example, [36]). These two considerations suggest that it is indeed appropriate to consider functional effectiveness of circuits, when isolated from the entire molecular network of the cell.

To answer the second question one must consider that alternative designs come about randomly for any given molecular circuit, through the natural forces and events that generate diversity in biology (mutation, cross-over, etc.). If various alternatives are selected during evolution under different conditions, this implies that not all network designs are functionally equivalent and that each design would provide for a better functionality under the conditions in which it was selected.[1]

3. Functional effectiveness of molecular networks

A fundamental aspect in the study of biological design principles is how to define functional effectiveness criteria for a given circuit and how to analyze the effect of changes in the design of the circuit on those criteria. In essence, one should understand the biology that a given type of circuit is involved in, find out what the specific role of that circuit is, and identify physiologically relevant aspects of that role that can be associated to improved or decreased functionality.

It is hard to propose an algorithm to define functional criteria that are applicable to every type of circuit one could be interested in. For example, in signal transduction circuits, one should consider specific criteria related to signal interpretation, such as amplification, delay, frequency response, noise propagation, correlation between input and output [37,38]. In contrast, in some moiety conservation cycles, one would apply considerations that are similar to those engineers apply when designing batteries [24,39]. However, there are some general criteria that are applicable, in a broad sense, to different modules. For example, the ability to maintain performance under small perturbations in parameters values (robustness) seems to be a desirable characteristic for many different systems [40,41].

In essence, several physiologically relevant criteria are simultaneously important for the appropriate function of a circuit. Analyzing the molecular circuits that perform a given biological process provides important insights into what interactions determine that the circuit functions as it should under different conditions [42]. Furthermore, it helps understanding if different design characteristics of those circuits are linked, making it so that if one characteristic is selected for or against, others are automatically implemented or excluded (see, for example, [43,44]). In addition, contradictory functional demands may be placed upon a molecular biological network of interest, constraining its evolution. These considerations imply that it is difficult to intuitively understand how a design may have been selected for or against. Such an understanding requires the use of appropriate analytical tools to evaluate how changes in the design can simultaneously affect all relevant functional aspects of the circuit.

4. Methods to analyze design principles

An accurate analysis of the effect of alternative designs of a molecular circuit on the circuit's function requires that one understands what that function is. This knowledge is essential to identify the relevant performance criteria that need to be analyzed in order to understand the selection of alternative designs for the circuit. As stated above, some of these criteria will be quite general (robustness, stability, etc.), while others will be system specific.

[1] In this discussion we disregard the effect of random drift and population size. We assume that the population is always sufficiently large so that the effect of Muller's ratchet in fixing deleterious mutations is small.

When characterizing the effect of a circuit's design on the performance of that circuit, one is typically interested in understanding either (a) the functional performance limits of a given design or (b) why analogous systems have alternative designs under different conditions or in diverse organisms. The performance limits of the circuit can be analyzed from qualitative (for example, can a given network structure generate oscillatory behavior or multistationarity?) or quantitative (for example, by how much should gene expression change during some adaptive response in order to ensure organism survival?) perspectives. Whatever the perspective, the analysis is typically done by building mathematical models that represent the circuit and analyzing these models using one or two of an array of different methods.

Methods to determine the functional performance limits of a given circuit include, for example, approaches such as *Reaction Network Theory* (*RNT*) [45–55]. *RNT* permit identifying necessary conditions in the structure of mass actions circuits that lead to robustness, oscillations and multistationarity, independent of the parameter values. This is done using a combination of graph theory and differential equations theory in order to analyze the stoichiometric matrix of the circuit [54–64]. RNT calculates (a) the rank of the stoichiometric matrix of the network, (b) the number of different sets of reactants and/or products of individual reactions in the network, and (c) the number of isolated subnetworks in which the circuit can be decomposed. With these three numbers, a deficiency is calculated for the network and, based on this deficiency, the necessary conditions for different types of dynamic behavior are determined (Fig. 1).

Other qualitative methods, such as the pentose phosphate pathway (PPP) game [56,57], have been used to understand what is being optimized during the evolution of a particular solution for the structure of a circuit or network. The PPP game considers all possible reaction paths that a set of biological enzymes can generate between different metabolites. Then, it compares these alternative paths to the ones that naturally evolved in organisms (Fig. 2). These comparisons have led to the inference that minimization of the number of steps is a significant driving force in the evolution of metabolic circuits [57,58].

Limits of functional performance of circuits can also be characterized through the use of numerical methods. For example, the physiological constraints that may shape the evolution of changes in gene expression during heat shock response of the yeast *Saccharomyces cerevisiae* have been systematically studied [59–61]. An initial approach to the problem led to the creation of a mathematical model representing the main metabolic pathways involved in this response. Then, the numerical criteria that represented minimal requirements for survival were identified. Finally, a large scale Monte Carlo (MC) sampling of the parameters of the system was performed, eliminating all parameter combinations that generate systems that did not meet the minimal criteria. Once this was done, an analysis of the parameter sets generating systems that were feasible led to the identification of numerical design principles for these parameters that can be justified by the functionality of the system. This approach can be applied to similar problems, although it can become computationally demanding for systems wide increasing dimensions. Recently, a more efficient approach to the problem was developed and applied. Instead of using large scale MC sampling, global optimization methods are used to map the parameter space in such a way that all regions of this space that meet minimal functionality criteria can be identified [59,62].

The concept of *design spaces* has been recently systematized and proposed by Savageau and co-workers as an alternative to fully characterize the different phenotypical regimes of a molecular circuit [24–26]. These different regimes are identified with regions in the parameter space in which different elementary processes dominate the dynamic change in the level of each variable of the circuit. In short, one creates a model for the circuit of interest and then performs dimensional reduction on the model in such a way that the number of parameters is minimized. Then, the parameter space of the reduced model is divided into regions where different dominant elementary processes regulate the production and consumption of each of the variables in the system. The borders between regions identify approximate boundaries for the different phenotypes of the model in the parameter space (Fig. 3).

There are also methods that are specifically tailored to address questions about why alternative designs exist for analogous circuits performing the same function. The first method specifically developed to address these questions was published in the early 1970s by Michael Savageau [14–16,18–20]. In this pioneering work, he developed the first version of what is now known as

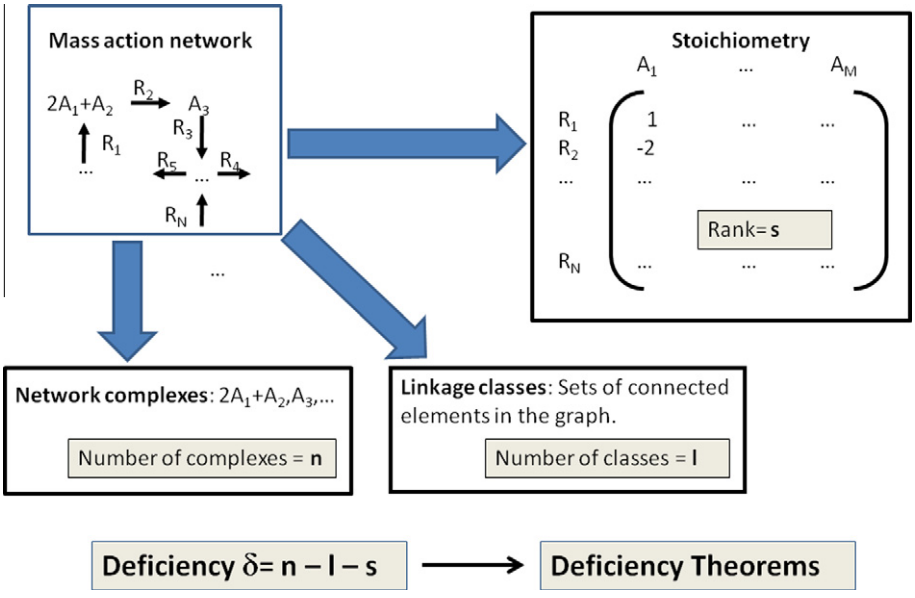

Fig. 1. Reaction Network Theory (RNT). By analyzing the structure of (usually mass action) reaction networks, RNT derives deficiency-related theorems that, depending on that deficiency d, certify existence of single or multiple steady state and/or limit cycles. The theory, in its basic forms, requires knowing the rank of the stoichiometic matrix of the network, as well as the number of complexes in the reactions and the number of linkage classes.

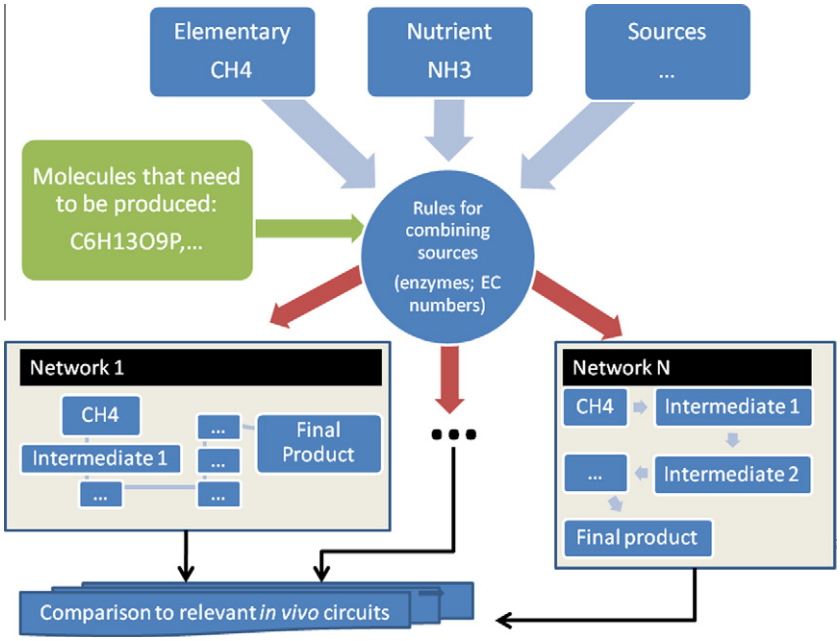

Fig. 2. Pentose-Phosphate-Pathway (PPP) like games. By starting from elementary nutritional sources and using all enzymatic activities that are known, these games determine all possible reaction pathways that lead from the elementary carbon sources to the desired biological molecules. A comparison of these pathways to those occuring in living beings supports the notion that nature selects for the shortest paths between elementary nutritional sources and biological building blocks (see text for details).

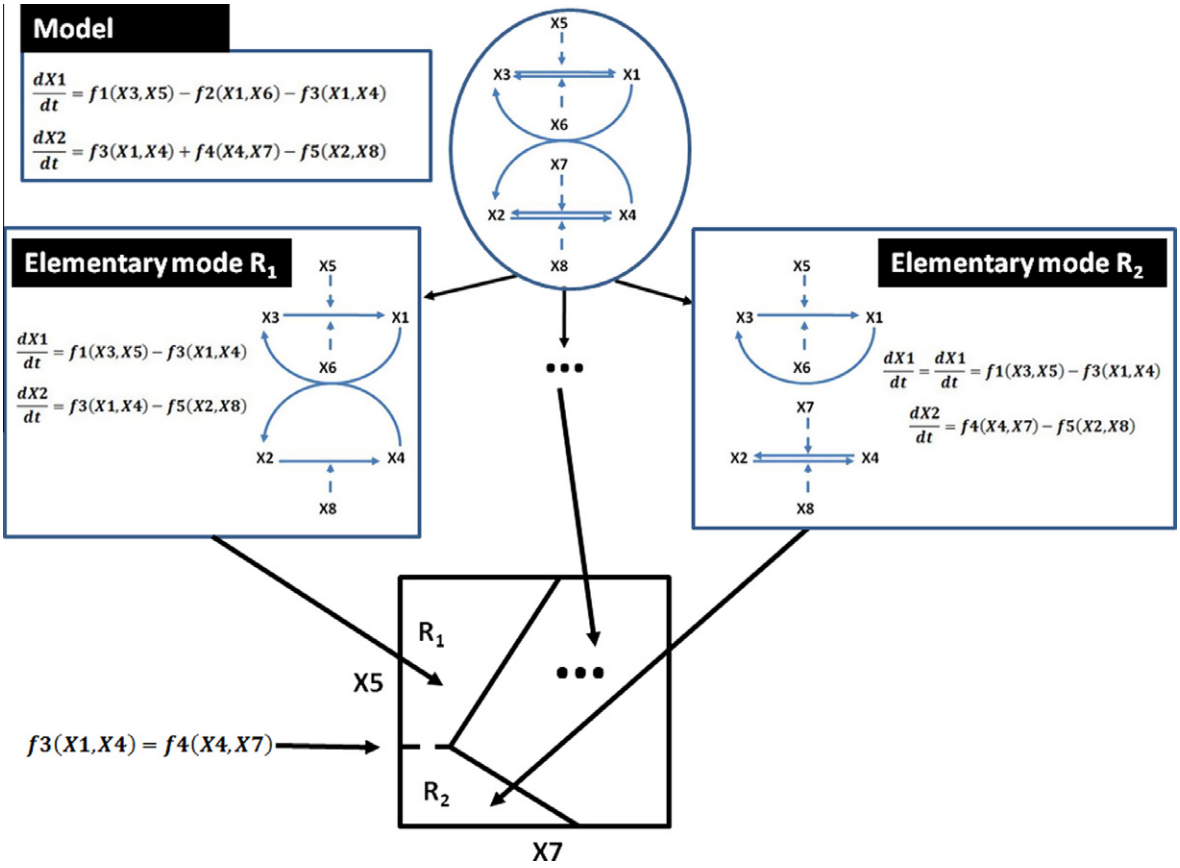

Fig. 3. Design spaces. This method decomposes the mathematical model of the system into the elementary modes of consumption and production of each of the variables. Each combination of these elementary modes is a region Ri in the design space. By analyzing the simpler models in the space of variables and/or parameters one is interested in, one can identify "pure" possible phenotypes for the circuits. The borders between the regions correspond to zone where two production (or consumption) terms for a given variable have the same numerical value (see text for details).

Mathematically Controlled Comparisons. Later, this method was further developed and applied to various biological problems [11,12,22,23]. These comparisons can be done in fully analytical form or numerically [22,23,63], depending on the models being compared and on the questions one is asking [46,49,72]. Mathematical details can be found in the literature [11,12,64–67]. Briefly, the use of this method requires (see also Fig. 4):

(i) Defining the functional requirements for the biological process or network under analysis.
(ii) Defining alternative designs for the system.
(iii) Defining basic criteria of *internal equivalency* between alternative designs. In general, all processes that are identical in the alternative networks are considered to have exactly the same parameter values in the two systems. This is equivalent to making control experiments in a wet lab.
(iv) For each pair of comparisons, the system in which the process that has alternative designs is characterized by the largest number of parameters and is usually taken as the reference, while the other system is taken as the alternative. Then, one defines *external equivalency* conditions. The reasoning underlying such conditions is as follows. There are certain behaviors of the system that are important for its function. If the reference process had mutated in such a way that it became the alternative process, then, in the best of all possible worlds nature could mutate the parameters of this alternative such that it would make both systems equivalent with respect to at least some of those behaviors. Therefore, if one takes each of the parameters of the alternative system and imposes that a specific behavioral trait is the same in the alternative and in the reference, one can fix the value for each of the alternative parameters. This comparison process assumes that evolution has an infinite amount of tries and time to make alternative designs as equivalent as possible when a specific functionality is required. Although this may not be the case in biological evolution, the results obtained by using the method, so far, indicate that these assumptions are reasonable for successfully identifying design principles in many cases.
(v) When maximal external equivalency is achieved, any remaining differences in the behavior of the systems are exclusively attributable to the differences in design. Then, advantages in the functional performance of the system can be highlighted and related to the emergence of a particular design under specific conditions.

Other approaches to identify and study biological design principles are also available. For example, one can study a catalog of network designs to identify functional alternatives that have been implemented by nature during evolution [68]. For example, this approach was used to identify design principles for biochemical oscillators [69]. The analysis and classification of network motifs according to their dynamical behavior also follows this strategy [70,71].

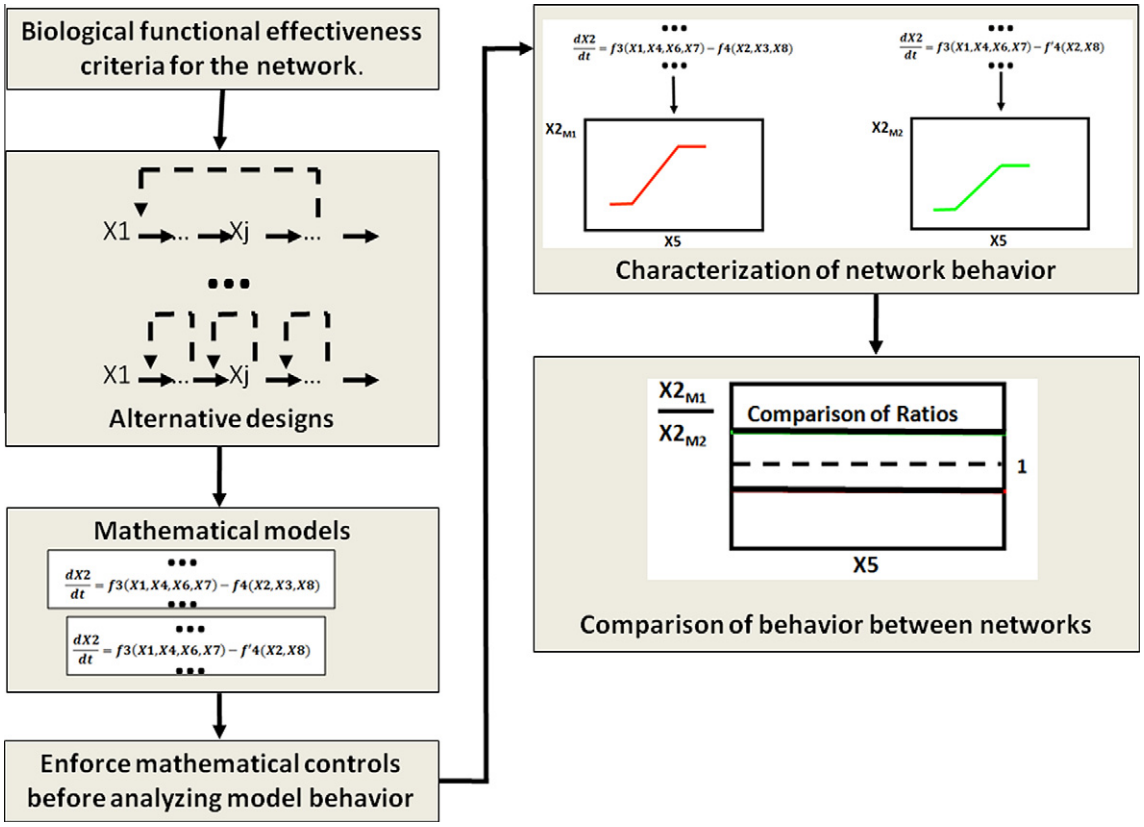

Fig. 4. Mathematically controlled comparisons. This method permits comparing the functional effectiveness of alternative circuits for biological networks that perform the same function. This is done through the creation of mathematical models for the alternative designs. Then, one implements a set of controls to ensure that any differences between the behavior of the two models is only due to the differences in network structure. Typically, the comparison is done by taking the ratio of the property of interest in the reference system [M1] to the corresponding property in the alternative system (s) [M2]. If the property is always larger in the reference system, the ratio will always be larger than one [upper line in the last panel of the figure]. If the property is always smaller in the reference system, the ratio will always be smaller than one [lower line in the last panel of the figure] (see text for details).

5. Design principles in molecular systems

5.1. Design principles in gene circuits

Gene regulation networks show a number of recurrent motifs that could represent a fundamental topology of regulatory circuits that is independent of the specific genes involved in the circuit. One of the most prevalent motifs is a feed forward loop in which a transcription factor X regulates another transcription factor Y and both regulate a given gene Z. This motif can generate eight different basic designs. In four of these designs the direct effect of X on the gene expression of Z is similar to the indirect of X on the gene expression of Y compounded with the effect of Y on the gene expression of Z. These are called coherent designs. Four other designs are incoherent (Fig. 5). An initial theoretical analysis of the different designs shows that coherent loops are advantageous for delaying response to a signal, while incoherent loops work more effectively as accelerators of response to a signal [72]. This lead to the suggestion that incoherent feed-forward loops should be selected in environments where the distribution of the input pulse duration is sufficiently broad [73]. More recent work shows that both types of loop can accelerate or delay response to a signal, depending on parameter values [74]. Incoherent loops have also been proposed as a functionally more effective mechanism for detecting fold-change in gene regulation [14].

One of the earliest case studies where design principles have been identified in molecular circuits regards the relationship between mode of regulation for gene expression and the demand for the gene product, leading to the proposal of the *demand theory for gene expression* [14,66,67,75]. The theoretical results correctly predict that positive regulation is preferentially selected for genes whose product is required over a large fraction of the life cycle of the individual (high demand genes), while negative regulation is preferentially selected for genes whose product is required for a small fraction of that life cycle (low demand genes) [64,65,76–78]. The biological explanation for the prediction boils down to a "use it or lose it" principle. The effect of losing the binding site for the regulation is proportional to the fraction of time that it is under use. For example, if a positively regulated gene is under low demand, there is a much smaller fraction of the life cycle of the individual when losing this regulation will affect the individual. Conversely, if a negatively regulated gene is under high demand, there is a much smaller fraction of the life cycle of the individual when losing this regulation will also affect the individual. In other words, this theory proposes that rate at which Muller's ratchet will turn for deleterious mutations in the binding sites is proportional to the fraction of time that those sites are active (Fig. 6).

Recently, however, some doubts have been presented with respect to this interpretation, and similar predictions were shown to arise if one considers how the different modes of regulation minimize errors during transcription. Systems in which free sites are more error-prone (exposed to binding by non-specific factors) than sites bound to their cognate partner, will tend to evolve mechanisms that keep the sites bound most of the time, thus minimizing errors [79]. Noise filtering was also put forward as a possible explanation for the different modes of gene regulation [37,80,81]. Approaching the problem from an alternative perspective showed that gene circuits with negative regulation are better at filtering noise out of signals with high intensity, while positively regulated circuits are more efficient in filtering noise out of low intensity signals.

These explanations for selection between alternative modes of gene regulation may not be mutually exclusive. Classical demand theory [66] predicts that loss of binding sites has a smaller effect on fitness if those binding sites are rarely used. Therefore, to keep regulation, it should be implemented using the type of binding site that is used most often for the gene in question in the organism of interest. The noise-related variation of the theory states that fitness is affected mostly because of inappropriate binding in the absence of the cognate regulator, resulting in deleterious gene expression [79]. However, these two aspects are complementary. Under low demand, with a positive regulator, the binding site would be available for binding. If this binding leads to expression of the gene when it is not needed, there would be a deleterious effect that would select for sites where such binding would not occur. This could cause loss of the negative regulatory effect through selection, while classical demand theory argues that such loss could come about even by drift. A similar argument can be made for positive regulation in a low demand environment. It is conceivable that both evolutionary effects could contribute for the observed regulatory pattern under different conditions. There are studies that hint at such complementarity. Effective population size and the typical time scale of environmental variations appear to be key parameters in determining the fitness advantage of the different modes of regulation [82]. The "use-it-or lose-it" principle

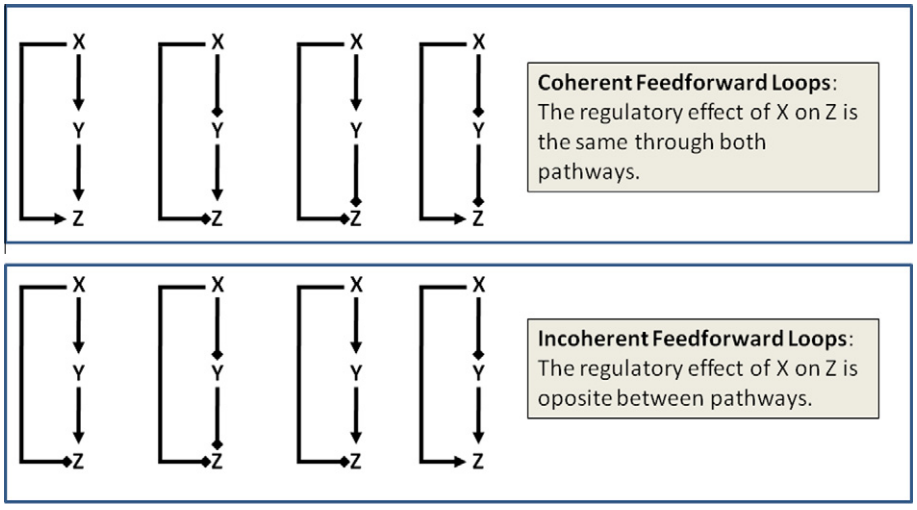

Fig. 5. All possible types of feedforward loops in three-species genetic circuits. Arrows with triangular heads indicate activation, while heads with square heads indicate inhibition.

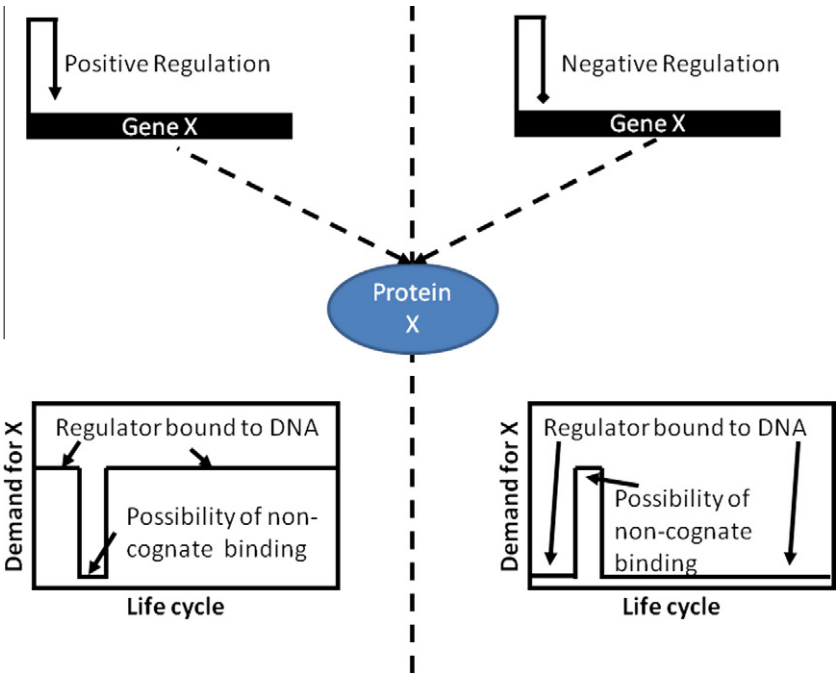

Fig. 6. Demand Theory: Arrows with triangular heads indicate activation, while heads with square heads indicate inhibition. Originally, demand theory predicted that negative regulation of gene expression is observed under low demand, while positive regulation is observed under high demand. This was explained by higher probability of loosing negative regulation sites under high demand and positive regulation sites under low demand. More recently, it was suggested that the correlation between mode of regulation and demand this is a consequence of the fraction of the life cycle in which binding of non-cognate regulators could lead to noise in gene expression (see text for details).

that underlies classical demand theory is valid for small populations with long time scales of environmental variations. Conversely, a complementary principle will be valid for populations with large effective sizes in rapidly changing environments [82]. Under these conditions, one would expect that both, positive and negative regulation, be stable.

Design principles have also been identified for other aspects of how gene circuits function and for the interplay between genotype and phenotype. One example of this are the design principles described for the organization of the gene networks that are responsible for regulating the development of sea urchin embryos, suggesting a number of strategies that may play similar roles in different organisms [83,84].

The quantitative design aspects of the regulation of gene expression have also been analyzed. One example of this is the study that shows that the minimal requirement for network dosage compensation to exist in genetic circuits is that the circuit is regulated by both, a positive and a negative regulator [85]. Another example has to do with regulation of changes in gene expression during stress response. Such changes enable organisms to regulate pathway fluxes and metabolite concentrations in ways that permit an appropriate adaptive response to changing environmental conditions. Adaptive responses are fundamental for survival and can be achieved following different strategies that change gene expression from a given reference initial state to the adapted state. Analyzing these strategies reveals that, in *Escherichia coli* amino acid biosynthetic pathways, genes from the same transcript are translated into proteins in such a way that each subsequent enzyme in a pathway becomes available when enough of its substrate is produced by the previous enzyme of the pathway (Fig. 7) [86].

Operative changes in gene expression that are required to attain a given adaptive response while maintaining a set of basic physiological requirements have been investigated by Sorribas and co-workers [59,60,62]. Based on previous work by Voit and Radivoyevitch [61], they have identified the physiological requirements that constraint the quantitative changes in gene expression during the adaptive response of yeast to heat shock, using a Monte-Carlo based approach [60]. More recently a global optimization method that exactly maps the operating regions of gene expression space that meet the physiological requirements for cell survival has been developed [59,62]. The results of applying this method to the analysis of changes in gene expression during yeast stress response are consistent with those from the Monte-Carlo approach (Fig. 8).

This new technique allows for identifying feasibility regions in the enzyme activities so that a number of physiological constraints required for cell survival are met. These feasibility regions contain many admissible expression values for the genes that are compatible with a given set physiological requirements. As such, one expects that evolution selects gene expression patterns that fall within these regions. The available experimental data is consistent with the computational predictions, suggesting that the physiological constraints that were used to identify the feasibility regions are close to those that are active *in vivo*.

5.2. Design principles in RNA circuits

In the 1970s, Savageau and co-workers found evidence for parallel processing as a design principle in RNA splicing. Such processing decreases the losses of immature intermediates, has shorter processing times, and is more amenable to evolutionary refinements [87]. The current surge of interest in RNA circuits has led to the identification of additional design principles in new types of RNA circuits [88–91]. For example, consider the following three regulatory mechanisms for riboswitch action: transcriptional termination, translational repression and mRNA destabilization. The ratio between reversible and irreversible rate constants is shown to have a critical impact on the performance of the circuit, establishing three operating regimes with distinct tuning properties. Regulation of gene expression by small RNAs has also been analyzed [92,93]. It was found that such regulation has features that

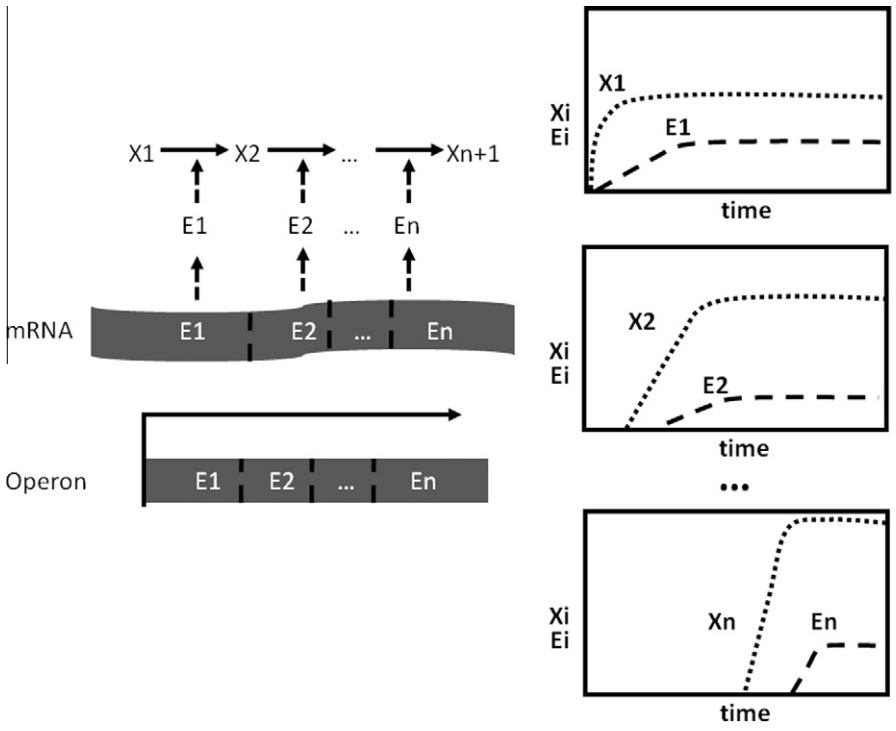

Fig. 7. Design principles in translation of multicystronic mRNAs. In biosynthetic pathways, it appears that the accumulation of enzymes after translation lags behind the accumulation of the substrate for that enzyme. This makes biological sense, as the cell would not spend resources building enzymes before it needs then at sufficiently high concentrations (see text for details).

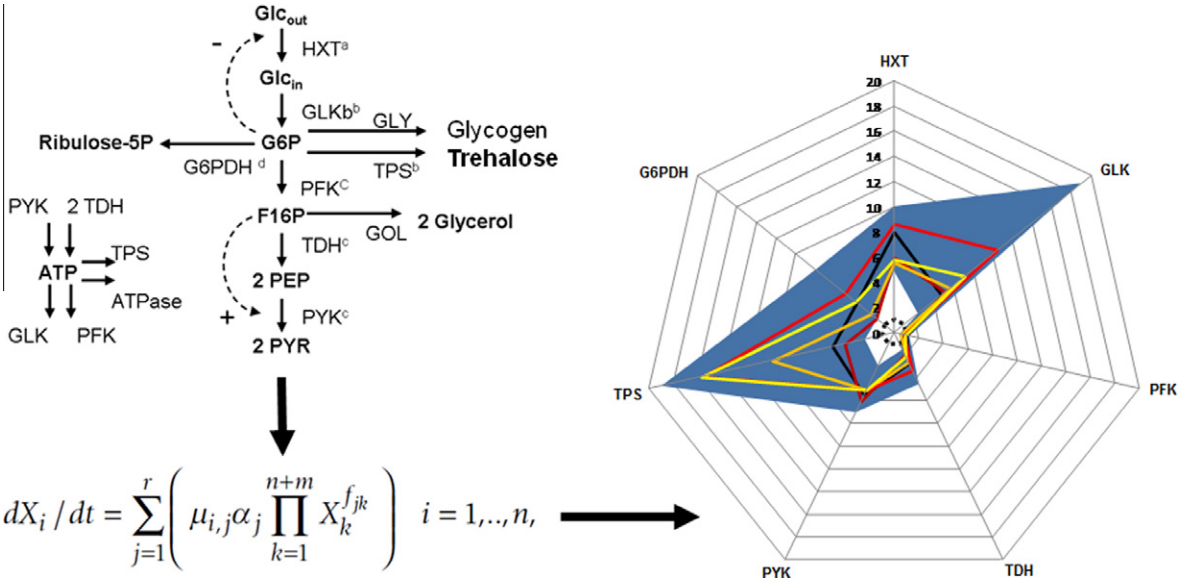

Fig. 8. Design principles for changes in gene expression during stress response. A minimal model of metabolism that still accounts for important changes was built. Subsequently, this model was cast into non-linear form. Finally, global optimization methods were used to determine the ranges of changes in gene expression with respect to the basal level that would allow the cell to survive. These ranges are represented in blue in the spider plot on the right of the figure. Each axis of the graph represents one of the different genes in the model. Full lines indicate experimentally measure microarray profiles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are distinct from protein-mediated gene regulation. The strength of repression is set by the ratio between transcription rates of sRNA and the target gene: at target's high expression, sRNA may have no effect. The threshold value is tunable through controlling the rate of sRNA transcription. The model predicts reduced variance in protein level for sRNA-mediated regulation (attenuation of noise), and high sensitivity to changes in sRNA near the threshold. Different mRNA species are expected to compete for binding with the same pool of sRNA in a hierarchical cross-talk where targets of a given binding strength affect (but are not affected by) targets of lower binding strength. This form of regulation also provides a very fast temporal responsiveness, making sRNA mediated repression a good system when levels of mRNA need to shift reversibly and quickly in response to signals [94].

5.3. Design principles in metabolic networks

One of the first problems to be analyzed by means of Mathematical Controlled Comparisons was the regulation of a biosynthetic pathway by overall negative feedback of the end product to the first reaction of the pathway (Fig. 9). By comparing this design to other possible modes of feedback inhibition, it is seen that the overall negative feedback from the final product of an unbranched pathway to the first reaction of the pathway had several physiological advantages [15,19,95,96]. These advantages include a production of the pathways' end product that is better regulated by cellular demand and less sensitive to spurious interactions with the environment. Later on, it was shown that overall feedback was the most functionally advantageous regulatory loop by inhibitory feedback that such pathways can have [97].

It was also found that a feedforward inhibition of the Aminoacyl-tRNA synthase by an intermediate of the amino acid biosynthesis pathway stabilizes that biosynthesis [95,96]. Additionally, it was found that when reversible reactions are at the beginning of these pathways, regulation by demand is more effective, as is speed of adaptation to cellular demand signal [98].

Recently, it was found that the robustness of the activity of one of the enzyme isocitrate dehydrogenase in the glyoxylate bypass regulation relies, in addition to other known features of the system, on the existence of a ternary protein complex where the kinase activity is higher than the phosphatase activity. This model is quite general: it may apply to other systems with a bifunctional enzyme that catalyzes antagonistic reactions [99].

Other metabolic modules that have been analyzed in search for design principles are moiety conservation cycles. An analysis of the glucose 6-phosphate dehydrogenase (G6PD)–glutathione reductase (GSR) pathway, which catalyzes the reversible redox cycle of NADPH/NADP, found that each enzyme is designed with different functional demands. The activity of the NADP-reductive G6PD far exceeds the capacity of human erythrocytes for a steady NADPH supply, which is limited upstream of G6PD. The analysis indicates that maintaining such a surplus of G6PD activity ensures sufficient robustness of the NADPH concentration and responsiveness of the NADPH supply. These results suggest that large excess capacities found in some biochemical and physiological systems, rather than representing large safety factors, may reflect a close match of system design to unscrutinized performance requirements [44]. These results are complemented by the analysis of the kinetic activity of the GSR enzyme. The normal activity of GSR is under selective pressure by virtue of its ability to minimize the accumulation of oxidized glutathione. Contrary to the assumption of a single functional requirement, natural selection for the normal activities of the distinct enzymes in the pathway is mediated by different requirements. Much, if not most, of the enzymes may thus be fulfilling functional demands other than flux [100].

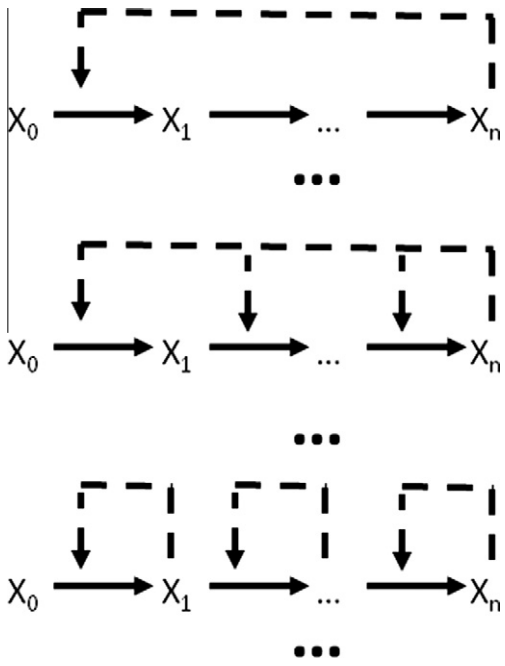

Fig. 9. Design principles for negative feedback in unbranched biosynthetic pathways. All possible alternatives were considered. Even in the presence of additional feedback, overall feedback (top-most reaction scheme) increases the functional effectiveness of the circuit (see text for detail).

It was also found that even though negative feedback is often used in biochemical networks to achieve homeostasis, under certain conditions this feedback can cause the steady state to lose stability and be replaced by spontaneous oscillations of metabolites. The conditions for oscillation are: sufficient "memory" (or time delay) in the negative feedback loop, sufficient nonlinearity in the reaction kinetics, and proper balancing of the timescales of components in the loop [69]. Another interesting and well known result is that the coupling of positive feedback loops and the decrease of negative feedback loops in a network increase the stability of its steady state. A recent report that support this design principle analyzes both random networks and models of specific biological networks to conclude that concatenate negative feedback loops decrease the stability of steady states while concatenated positive feedback loops increase that stability [101].

Melendez-Hevia and co-workers used the pentose phosphate pathway game (Fig. 2; see above) to understand how some of the more central metabolic pathways have evolved. These researchers developed and used the pentose-phosphate pathway game (Fig. 2) to build alternative pathways to get from one metabolite to another in a metabolic network. By combining constraints about the minimal number of carbon atoms that could exchanged between metabolites with optimality principles favoring a minimal number of pathway steps between metabolites, they concluded that the principle of the minimal number of steps is consistent with pathway evolution in general [56–58]. Latter, this method was combined with thermodynamic constraints and used to argue that glycolysis is quantitatively designed in an optimal way with respect to flux optimization, ATP production and ATP usage [102–104]. However, it should be stressed that different *a priori* thermodynamic constraints could change the results of this analysis. Sometime latter Mittenthal and co-workers developed a more complex version of the game [105–107]. They generated alternative networks relaxing the number of carbons that could be exchanged between metabolites, include a larger fraction of irreversible reactions in the networks and considering additional types of reactions and inputs. Pathway evolution was shown to be consistent with the rules of the modified game, because the predicted pathway was the same as those observed in real organisms. Recently, an evolution of this method was applied to study if central metabolism in *E. coli* follows a similar optimality principle. The new rules consider that exchange of chemical groups between metabolites is limited by the functionality of enzymes described in the EC classification. With these rules, it was found that central metabolism is structured in a way that uses the minimal number of steps to connect the key precursor metabolites essential for biomass and energy production. Paths between consecutive precursors cannot be made shorter. The non-precursor compounds in the network form the shortest possible bridges between the precursors. Thus, central metabolism appears to be a minimal walk in chemical space between precursors [108]. This minimization of the number of steps between precursors could be driven by

constraints imposed to the growth of *E. coli* by protein synthesis [109]. This biosynthetic process is often growth limiting, which would imply that cells with shorter pathways may have a competitive advantage due to their economy in proteins. Furthermore, short pathways have fewer intermediate and generate higher flux than long pathways of equally effective enzymes [110,111]. This optimality principle allows making predictions: in organisms where a precursor is no longer essential, a shortcut would evolve that bypasses that precursor compound; and if a longer-than-minimal path is found between two compounds, an essential metabolite lies on that path. One question is that most pairs of precursors separated by more than one step could have been connected by several other alternative paths of the same length (but not shorter). Why the particular minimal path that occurs in the cell was selected out of these alternatives? Possibilities to explore include effects that can differentiate between paths of equal length, such as energy and reduction potential, toxicity effects of intermediate compounds and differential enzyme efficiency in each possible path.

Design principles at the molecular level have also started to be linked to macroscopic organism fitness. For example, ammonia was used to analyze a fitness tradeoff between resource abundant and resource limited environments for *S. cerevisiae*. This was done by analyzing the level of noise in Gdh1p expression and correlating it to the relative balance between resistance to toxic levels of ammonia and fitness in lower levels. It was found that as the noise in Gdh1p expression increased, this conferred enhanced resistance to ammonia toxicity. On the other hand, lower variation (noise) in Gdh1p levels exhibits greater fitness in physiological concentrations of ammonia [36].

Global metabolic responses have also been analyzed in search for design principles. For example, analyzing yeast data, it was found that the metabolic pathway map and the protein–protein interaction network (PIN) have significant positive correlation between the shortest paths across both network types. The sub-systems of the entire PIN appears to follow specific organizing principles: while physical interactions between proteins are generally dissortative (proteins of high degree interact with proteins of low degree), interactions between metabolic enzymes were observed to be assortative (enzymes frequently interact with other enzymes of similar degree or number of links associated with a node) [112].

5.4. Design principles in cellular rhythms

The presence of a negative feedback loop in a network is a necessary condition for that network to be able to generate oscillations. Thus, different topological circuits can be associated to this dynamical behavior [69]. Oscillatory phenomena are the basis of cellular rhythms and may be found in different contexts, from metabolism [113] to development [114] and circadian rhythms [115]. Understanding the fundamental biological design principles underlying the networks generating such cyclic behaviors is an important question.

One of the most well studied cellular oscillators is cell cycle. Basic design principles have been identified for the networks regulating the cellular process. Models created by using molecular information suggests that the molecular mechanism regulating the eukaryotic cell cycle is composed of two bistable switches (governing G1-S and G2-M transitions) and an oscillator (controlling mitotic exit) [116]. The bistable switches are controlled by a molecular antagonism between CDKs and their antagonists. This switch has two alternative states: G1 (low CDK activity) and S-G2-M (high CDK activity) [117–119]. "Starter Kinases" (SKs) and "Exit Proteins" (EPs) flip the switch back and forth. Transitions between these states are controlled by two negative-feedback loops. The Start transition (G1-S) is triggered by a class of SKs that are downregulated by the very species they are aiding. The Exit transition (M to G1) is promoted by a class of EPs that kill the very species they depend on. This topology creates a dynamic of irreversible transitions. Start and Exit checkpoints block progression through the cycle if any serious problems are encountered (DNA damage blocks Start, incorrect chromosome alignment block Exit). A size checkpoint at the Start transition ensures balanced growth and division. This control system of cell cycle regulation has four fundamental properties: alternation of S and M; checkpoints; irreversibility; balanced growth and division. Variations of this model also account for alternative modes of cell division, such as oogenesis (cell growth without division), fertilized egg division (rapid mitotic cycle without growth), endoreplication (repeated rounds of DNA synthesis without mitosis) and meiosis. Recent work by the groups of Nurse and Cross suggests that the different cell cycles have evolved from duplication and divergence from a primordial cell cycle with a single cyclin. The accumulation of this cyclin throughout the cell cycle allowed for the progression of the cycle. Cell division led to an abrupt decrease in that concentration, restarting the cycle [118,120–123].

Other important biological oscillators are the networks responsible for regulating the circadian rhythm of organisms. These biological processes appear to have evolved independently for different groups of organisms [124]. For example, the proteins that regulate the circadian clocks of cyanobacteria and those of multicellular organisms evolved from different ancestors and generated networks that have diverse regulatory loops. On top of a stable oscillation, the networks of genes and proteins responsible for the circadian clock need also appropriate mechanisms for input signals that are required to reset and entrain the clock when conditions change. Inputs that are known to entrain the clock include light, temperature, and food. All known circadian clock networks use a multi-loop structure to obtain circadian oscillations that can, in principle be obtained with a single negative feedback loop. The presence of these multifeedback loops appear to provide the clocks with higher flexibility that allows these clocks to be entrained and have their phase more easily reset by the input signals, while remaining fairly insensitive to noise and having a robust period [125,126]. This makes evolutionary and biological sense, because organisms on earth have a constant circadian period that often requires phase resets either due to changes in the day–night cycle or to moves between different time zones. A linear analysis of a non-mechanistic model for the circadian clock of *Arabidopsis* further suggests that the circadian clock of this plant requires a mechanism for rapid light inputs if the clock is to adjust to photoperiod-dependent changes [127]. More complex instances of circadian clocks have also been analyzed. For example, in mammals, several thousand neurons of the suprachiasmic nucleus generate rhythms of approximately 24 h [128]. A mathematical model of the systems suggests that the neurotransmitter feedback loop plays an important role in the appropriate synchronization of the ligth/dark cycles, allowing the network to resynchronizing the clock after a perturbation that simulates a 'jet-lag' of several hours. Other design principles have been proposed for networks to achieve phase-splitting behavior [129].

Another important issue about the networks that regulate biological rhythms is to understand in which situations one can expect the networks that regulate each autonomous rhythm to interact. Furthermore, how does that interaction benefit the fitness of the organism? Finally, are there specific modes of interaction (design principles) that improve the functional effectiveness of the interactions under different conditions?

The answer to the first question is positive [130]. Cell cycle is also regulated by the circadian clock in *Synechococcus elongatus* [131] and in mice [130]. This regulation is consistent with a model

where cell cycle rate decreases during the night [132]. The structure of the network that integrates both oscillators is still unclear. Thus, the answers to the second and third question are still missing. Nevertheless, in *S. elongatus,* a phosphorylation cascade of circadian clock proteins that signal to the putative transcription factor RpaA is involved in linking the two processes. It is tempting to speculate that in a photosynthetic organism such as *S. elongatus* it would make physiological sense to decrease the rate of cell cycle during the night, as the main source of energy for the cell is shut-off. If availability of resources is an important selective pressure in the coupling of the circadian and cell cycle oscillators, one might expect that cells from diurnal animals will go through cell cycle faster during the day, while cells from nocturnal animals will have a faster cell cycle during the night. An analysis of available data for nocturnal rodents is consistent with this prediction (see figures in [133,134]).

5.5. Design principles in signal transduction networks

Signal transduction is another area where design principles have been studied, both in prokaryotes and eukaryotes. The identification of many types of design principles for these networks has been reported. Here, we will discuss only a few of these reports, focusing mostly on phosphorylation cascades, both in prokaryotes and in eukaryotes.

In prokaryotes, signal transduction through phosphorylation events is mediated by Two Component Systems (TCS) or Phosphorelays (PR). In these systems, a sensor protein modifies its own phosphorylation state in response to some signal from the environment. The phosphate is then transferred to a response regulator protein that either modulates physiological response (in TCS) or transfers it again to a second histidine kinase that will subsequently transfer the same phosphate to a second response regulator (in PR; see Fig. 10). TCS are ubiquitous in bacteria, and homologous pathways have been identified in several eukaryotic organisms as well, including *S. cerevisiae, Arabidopsis thaliana, Neurospora crassa* and *Dictyostelium discoideum*.

The modular aspect of TCS and PR circuits has facilitated the evolution of a variety of signal transduction modules. One circuitry motif that exemplifies this versatility is the four-step His-Asp-His-Asp PR. Different PR show the same alternating pattern of histidine and aspartate phosphorylation sites, but can utilize a different pattern of covalent linkage between individual protein domains: the four phosphorylation sites of the Kin-Spo0 pathway (in *Bacillus subtilis*) are found in independent proteins, whereas one protein can join the first two or three members of the PR (Sln1p-Ypd1p-Ssk1p and BvgS-BvgA pathways, in *S. cerevisiae* and *Bordetella pertussis*, respectively). The discovery that the yeast Sln1 pathway employs a PR mechanism with the same his-asp-his-asp configuration reported for the Kin-SpoO and BvgS-BvgA systems suggests that this signaling strategy may be widely utilized by eukaryotes as well as prokaryotes. However, it appear to be absent in mammals [135–138].

Several aspects of the physiological regulation by TCS have been analyzed. One of these is the apparent insensitivity of the input–output relationship of TCS modules to changes in the concentrations of the system's components [54]. It was found that this insensitivity can justify a design of the TCS that require three biochemical features: (i) ATP dependence of dephosphorylation; (ii) sensor kinase bifunctionality (the sensor catalyzes the phosphorylation of the response-regulator but also the dephosphorylation of the phosphorylated RR); and finally, (iii) the two-step nature of the sensor-kinase (autophosphorylation and phosphotransfer) [139]. In contrast, it was found that TCS mediating responses that require hysteresis should have a channel for response regulator (RR) dephosphorylation that is independent from the sensor protein. In addition it is also required that the dephosphorylated forms of sensor and RR form a reversible dead-end complex [38,140]. It has also been shown that TCS modules where the sensor kinase is bifunctional should be preferentially selected in physiological responses that need to be buffered against cross-talk, while TCS with monofunctional sensors should be selected in situations where the physiological response requires the integration of signals [140]. However, the use of signaling pathways with multiple inputs and a single output entails a loss of information about input signals. How cells integrate information from multiple inputs to modulate their gene expression states is poorly understood. Information theory can be adapted to study a biological circuit performing information processing and signal integration. The analysis of quorum sensing in *Vibrio harveyi* revealed that information transmission is primarily limited by interference from other signals, not by noise. Cells must tune the kinase

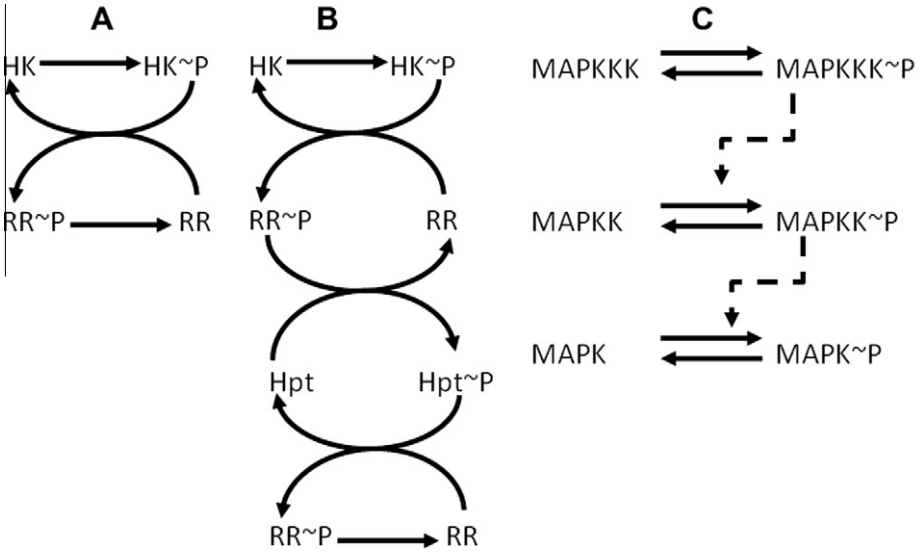

Fig. 10. Prototypical Two Component Systems (A), Phosphorelays (B), and MAP kinase cascades (C). HK – histine kinases; RR – response regulators; Hpt – intermediate phosphotransfer protein, accepting phosphate on a histidine. MAPK – MAP kinase; MAPKK – MAPK kinase; MAPKKK – MAPKK kinase; ~P – phosphorylated form of the proteins. See text for mechanistic details.

activity of each signaling branch of the quorum sensing circuit to simultaneously learn about individual inputs. Cells can increase how much they learn about individual signals by manipulating the different autoinducer production rates. Bacteria can learn preferentially about a particular input in a particular environment by using simple feedback loops to control receptor numbers. This analysis suggests that the need to minimize interference between signals probably imposes strong constraints on the design of signal-integration networks [141].

Some TCSs are positively autoregulated: the regulon controlled by active RR often includes the TCS operon, leading to a feedback loop. Positive autoregulation does not necessarily give rise to overall positive feedback. Mathematical model analysis shows that the effective sign of this feedback is determined by the values of the kinetic parameters of the system, making TCSs capable of tuning feedback sign, switching between positive and negative feedback to achieve appropriate outputs in different circumstances. Attainment of negative feedback depends on sensor bifunctionality (so that the sensor protein of the TCS can both increase and decrease the fraction of active RR) and RR activation independent of its cognate sensor. The feedback sign is physiologically relevant, since negative feedback reduces noise and gives rise to fast overshooting responses and positive loops lead to bistability, phenotypic heterogeneity and a stronger learning effect [142].

How does feedback lead to bistability [143]? The effect of the interplay of two positive feedbacks on the network bistability has been studied theoretically and experimentally. One example is the mycobacterial stress-response network which consists of the MprA/MprB TCS along with the σ^E -RseA sigma/anti-sigma factor system, involved in persistence in mycobacteria. This network contains two positive feedback loops. Positive autoregulation of the *mprAB* operon by MprA-P gives rise to a positive feedback. A second positive feedback arises from the transcriptional activation of σ^E by MprA-P and subsequent upregulation of *mprAB* operon by σ^E. The analysis of reduced versions of the network, to understand the role of each component, shows that the second feedback involving σ^E makes the network bistable, but only due to the post-translational regulation of σ^E by its anti-sigma factor RseA, which increases effective cooperativity and leads to bistability [144]. Bifunctionality of the sensor kinase avoids bistability in the positively regulated TCS.

Recently, the effect of the number of steps in the signaling of PR cascades was analyzed [145]. Under simplifying mechanistic assumptions, models for cascades with less than four steps are not capable of ultrasensitivity responses to signals. Thus, the authors suggest that 4-step PR cascades are the simplest evolutionary solution to the problem of high signal amplification in bacterial signal transduction.

The eukaryotic equivalent of TCS and PR are MAP cascades (Fig. 10). These cascades are composed of three proteins. The first step in the cascade is the MAPKKK protein. It becomes phosphorylated in response to some signal and it in turn phosphorylate the second proteins of the cascade, the MAPKK. MAPKKs in turn phosphorylate MAPK, which then regulate the physiological response. Unlike TCS and phosphorelays, ATP is consumed in each phosphorylation event in MAPKs. It was shown that this type of signal transmission could account for high signal amplification [146,147], and that the most energy efficient way to regulate this signal transduction is by signaling both the phosphorylating and dephosphorylating enzymes that control the cascade [148]. Such amplification depended on the existence of a highly cooperative mechanism in the phosphorylation of the proteins in the cascade and on an increase in the concentration of protein in each subsequent step of the cascade. Nevertheless, several questions about the design of these cascades remain unanswered.

For example, why do MAPK cascade use three kinases instead of one? (other membrane-to-nucleus signaling pathways, such as the cAMP/protein kinase A and the Jak/Stat pathways, employ a single kinase). A numerical analysis of a MAPK cascade model shows that, with typical parameter values, the three step cascade behaves like a highly cooperative enzyme, even if none of the individual enzymes is regulated cooperatively. The degree of ultrasensitivity increases as the cascade is descended and depends critically on the assumption that the dual phosphorylation of MAPKK and MAPK occurs through a two-collision mechanisms [149]. Thus, MAP cascades can convert graded inputs into switch-like outputs, filter out noise and flip from off to on over a narrow range of input stimuli. This sort of behavior would be appropriate for a signaling system that mediates processes where cells switch rapidly between discrete states without assuming intermediate positions, like in mitogenesis, cell-fate induction, and oocyte maturation.

Other questions that regard the design of MAP cascades concern the relationship between the concentrations of the enzymes in the three steps of the cascade [150–152]. Computational analysis provides rationale for why the MAPK and MAPKK concentrations are similar. The response time of the cascade is critically dependent on specific combinations of ranges of cellular MAPK and MAPKK concentrations. Concentrations of these signaling components fall within a region where the cascade seems to achieve optimal efficiency and rapid activation. When the MAPKK concentration becomes very different from the concentration of MAPK an undesirable delay is predicted in the response. Both increases and decreases in the MAPK and MAPKK concentrations result in a reduction in the efficiency of this initial response [151]. The way that MAPK cascades interact has also been analyzed. Interacting MAPK cascades are capable of implementing useful logic and amplitude-dependent signal processing functions (“exclusive-or” function and an in-band detector or two-sided threshold) and their implementation requires only limited crosstalk. This behavior cannot be achieved with a single cascade or with non-interacting cascades. A significant challenge still remaining is to determine if this potential is actually realized in the cell and if the computationally evolved solution resembles the solution chosen in the evolution of life. We also have yet to consider the cascade in a larger context, embedded in feedback loops, engaged in crosstalk with other signaling networks or protected from crosstalk by scaffolds [152].

As mentioned above, signal transduction networks regulate their response using (typically negative) feedback loops. Such down-regulation of the response to signals can increase the correlation between the input and the output of the network [153,154]. Recent work suggests that evolution of feedback as mechanism to regulate the response in signal transduction networks must optimize opposing goals. On one hand this mechanism should increase the correlation between signal and output. On the other it should be able to decrease the transmission of noise through the network. A network that maximizes the correlation signal-output also increases the effect of noise on that output [155]. This is easy to understand because by perfectly correlating input and output, a network will also perfectly correlate noise in the input to noise in the output. Thus, depending on the particular system one might expect feedback loops that preferably buffer the response of the network against noise, while in other the feedback loops will preferably maximize the correlation between input and output.

6. Final remarks

To be able to write this paper we struggled with the question of what is a biological design principle. The definition we gravitated towards is by no means the only one available. However, once we accepted it as a working definition, we could review some of

the work that has improved our understanding of such principles in molecular circuits. The importance of that work is justified because it improves our understanding of how biology works. The appropriateness of considering functional effectiveness of molecular circuits rather than fitness of the whole organism in the analysis is also discussed in this review. After establishing a framework for thinking about design principles, we discuss the different theoretical and mathematical methods that are usually applied to study them. We finish by presenting examples of those principles in different types of molecular circuits. We restricted the discussion mostly to intracellular networks, with some exceptions [84]. This means that most of the work that deals with design principles in molecular networks that regulate development is not included (for example, see [71,156–158]). Nevertheless, the examples given here present a general view of the research in this field.

Considering the work reviewed and presented here, one could feel that many of the design principles are somewhat *ad hoc* and too system specific. This view raises the important question of whether, over time, something like a ''periodic table'' of universal design principles that are valid for all types of biological circuits can be built. In other words, can we identify network elements, either qualitative or quantitative, that are almost always associated to specific types of behavior?

There appear to be cases where the answer is positive. For example, it is well known that the existence of a positive feedback loop is a necessary condition for multistability in molecular networks [143]. Also, a sort of ''uncertainty principle'' was proposed for feedback in biological systems [155]. This principle roughly states that feedback can be used to maximize correlation between input and output of a biological system at the cost of increasing noise amplification or used to decrease noise amplification at the cost of decreasing correlation between input and output. This imposes fundamental limits to how much evolution can optimize response to noise in molecular systems through the evolution of feedback interactions. Results of Reaction Network Theory that relate the structure of the network with the possibility of different types of dynamical behaviors may also fit into this category of basic design principles [48,51,54,55]. The common link between all these principles is the fact that they are independent of the specific function of the circuit being analyzed and represent hard constraints to dynamical behavior imposed by network structure.

As opposed to these ''elementary'' design principles, most of the principles discussed in this review hinge heavily on understanding the function of the circuit under analysis. Showing that a given feature improves the function of the circuit is crucial to explain why that feature is fixed during evolution. Such features are specific elements in the network (for example, bifunctionality in bacterial two component systems [140]), particular ranges of parameter values that enable a given dynamic response (for example, survival during heat shock adaptation in yeast [60]), or both (for example, only specific network designs with a given range of parameter values permit creating a developmental system with one stripe [71]). Take the analogy of a ''periodic table of design principles'' a bit further, many of the principles discussed in this special issue may be more like ''molecules'', for which no periodic table exists, rather than like ''atoms'', for which it does.

This does not in any way demeans the usefulness of these principles for understanding the way biological systems work and how they came to be as they are. If fact, an engineer might argue that proof of understanding of a system comes from building instances of the system that work under different regimes and demand specifications. From this perspective, creating more restricted catalogues that associate a specific functional behavior in a given type of system to a specific design element for that system may be more useful that a general periodic table. Such catalogues could become extremely useful for Synthetic Biology, enabling the construction of artificial biological circuits of a certain type with specific properties and behavior.

Synthetic Biology is the major body of work that is absent from this review. This choice was made because many good and extensive reviews on the subject have been published recently. We refer the readers to some of those reviews for more details [159–175]. Researchers are using decades of accumulated molecular knowledge to engineer new circuits within organisms that either implement new functionality or test some of the predictions made in the past through the analysis of design principles (see, for example, [16,176]). Synthetic biologists design and implement non-naturally occurring biological networks that perform a given function. Identification of design principles, on the other hand, focuses on understanding the emergence of these designs from evolution. Both activities are complementary and design principles can greatly assist and guide the development of Synthetic Biology applications (see, for example, [177] for a more detailed discussion on this subject). The merging of Design Principle analysis to Synthetic Biology creates a field of opportunities that may immensely potentiate our understanding of how organisms work at the molecular level and why they came to work like they do [178].

Biomedical research is another area that may in the future benefit from the study of biological design principles. If principles that guide shifts between pathogenic and healthy states can be identified, these can be used to devise strategies for better treatments. Furthermore, host-pathogen interactions might also have evolved in such a way that these interactions and their regulation can be classified into a small set of principles that can be used to facilitate host survival.

In summary, it seems to us that there may come a time when a hierarchy of design principles will need to be established and accepted for molecular networks. It is hard to imagine what such a hierarchy will look like. One possibility is that it becomes organized along the lines discussed above. It could be that there will be a set of design principles that are universal and constrained by network structure. Then, on top of these, and specific to the networks that regulate the biological processes of interest, one will identify principles that explain if and why such networks have been selected to perform the process. If this is the case, then we believe that the work reviewed here constitutes a very encouraging head start towards the goal of such a classification.

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