Robustness, Evolvability, and Accessibility in the Signal-Integration Space of Gene Regulatory Circuits

Joshua L. Payne[1] and Jason H. Moore[1]

[1]Dartmouth College, Hanover, NH 03755
Joshua.Payne@Dartmouth.edu

Abstract

Gene expression is commonly modulated by a set of regulating gene products, which bind to a gene's *cis*-regulatory region. This region encodes an input-output function, referred to as signal-integration logic, that maps a specific combination of regulatory signals (inputs) to a particular gene expression state (output). The space of all possible signal-integration functions (genotypes) is vast and highly redundant: for the same set of inputs, many functions yield the same expression output (phenotype). Here, we exhaustively characterize signal-integration space within a computational model of genetic regulation. Our goal is to understand how the inherent redundancy of signal-integration space affects the relationship between robustness and evolvability in regulatory circuits. Among a number of results, we show that robust phenotypes are (i) evolvable, (ii) easily identified by random mutation, and (iii) mutationally biased toward other robust phenotypes. We then explore the implications of these results for mutation-based evolution by conducting an ensemble of random walks between randomly chosen source and target phenotypes. We demonstrate that the time required to identify the target phenotype is independent of the properties of the source phenotype.

Introduction

Living organisms exhibit two seemingly paradoxical properties: They are robust to genetic change, yet highly evolvable (Wagner, 2005). These properties appear contradictory because the former requires that genetic alterations leave the phenotype intact, while the latter requires these alterations to be used for the exploration of new phenotypes. Despite this apparent contradiction, several empirical analyses of living systems, particularly at the molecular scale, have revealed that robustness often facilitates evolvability (Bloom et al., 2006; Ferrada and Wagner, 2008; Isalan et al., 2008). In the cytochrome P450 BM3 protein, for example, increased protein stability — defined as the tendency of a protein to adopt its native structure in the face of mutation — increases the probability that mutants can exploit new substrates (Bloom et al., 2006).

To clarify the relationship between robustness and evolvability, several theoretical models have been proposed (*e.g.*, Newman and Engelhardt (1998); Wagner (2008a); Draghi et al. (2010)). A common feature of these models is the concept of a genotype network (a.k.a. neutral network). In such a network, each node represents a genotype and edges connect genotypes that share the same phenotype and can be interconverted via single mutational events. In the case of RNA, for example, nodes represent DNA sequences and two nodes are connected if their corresponding sequences confer the same secondary structure, yet differ by a single nucleotide (Schuster et al., 1994). Large genotype networks thus correspond to robust phenotypes, where most mutations are neutral and therefore leave the phenotype unchanged. Phenotypic robustness confers evolvability because a population can diffuse neutrally throughout the genotype network (Huynen et al., 1996) and build up genetic diversity, which allows access to novel phenotypes through non-neutral point mutations into adjacent genotype networks (Wagner, 2008a).

Genotype networks have been used to explore the relationship between robustness and evolvability in a variety of biological systems, ranging from the molecular (Schuster et al., 1994; Cowperthwaite et al., 2008; Ferrada and Wagner, 2008; Wagner, 2008b) to the cellular level (Aldana et al., 2007; Ciliberti et al., 2007a,b; Mihaljev and Drossel, 2009). In the latter case, the phenotype of interest is typically a gene expression pattern and its corresponding genotype is a gene regulatory network, which consists of a structured set of gene products that activate and inhibit one another's expression. Gene expression is controlled by a gene's *cis*-regulatory region (Fig. 1A), which can be thought to perform a computation (Fig. 1B), using the regulating gene products as inputs. The regulatory program that encodes this computation is referred to as signal-integration logic.

Previous studies of the robustness and evolvability of gene regulatory networks have focused on the specific case where genetic perturbations alter network structure by adding or deleting regulatory interactions (Aldana et al., 2007; Ciliberti et al., 2007a,b; Mihaljev and Drossel, 2009). In this case, two gene regulatory networks are connected in the genotype network if they confer the same gene expression pattern, yet differ in a single regulatory interaction. The correspond-

ing genotype network is therefore a "network of networks" (Ciliberti et al., 2007b). These analyses have revealed several general properties of gene regulatory networks. First, robustness is an evolvable trait (Ciliberti et al., 2007b; Mihaljev and Drossel, 2009). Second, phenotypes are made up of vast genotype networks that span throughout the space of all possible genotypes (Ciliberti et al., 2007a; Mihaljev and Drossel, 2009); and third, highly robust phenotypes are often highly evolvable (Aldana et al., 2007; Ciliberti et al., 2007a).

While these studies have helped to elucidate the relationship between robustness and evolvability in gene regulatory networks, they are limited by their assumption that genetic perturbations primarily affect network structure. It is well known that the presence or absence of regulatory interactions is not the only determining factor of gene expression patterns (Setty et al., 2003; Mayo et al., 2006; Kaplan et al., 2008; Hunziker et al., 2010). By altering the arrangement of promoters and transcription factor binding sites (Fig. 1A, shaded boxes) in a gene's *cis*-regulatory region, the signal-integration logic of gene regulation can be dramatically influenced. For example, by simply rearranging the location of transcription start sites in the promoter region of a reporter gene in the galactose network of *Escherichia Coli*, it is possible to generate 12 out of the 16 possible Boolean outputs (Hunziker et al., 2010). Thus, it is not only the structure of regulatory interactions that affects robustness and evolvability, but also the logic of signal-integration used in the *cis*-regulatory region of each gene. When genetic perturbations correspond to changes in the signal-integration logic, two gene regulatory networks are connected in the genotype network if they are topologically identical and confer the same gene expression pattern, yet differ in a single element of their signal-integration logic. The extent to which genetic perturbations in the signal-integration logic of gene regulatory networks affect robustness and evolvability remains largely unexplored. Further, the ease with which a phenotype is accessed by blind mutation, and how this relates to robustness and evolvability in the signal-integration logic of gene regulation, has not been addressed.

Here, we investigate the relationship between robustness and evolvability in the signal-integration logic of model gene regulatory circuits. These small circuits are ideal for this investigation because their genotype networks are exhaustively enumerable, which allows for a full characterization of the relationship between robustness and evolvability. To understand how robustness and evolvability influence mutation-based evolution, we conduct an ensemble of random walks between randomly chosen source and target phenotypes. We discuss the implications of our results and present directions for future work.

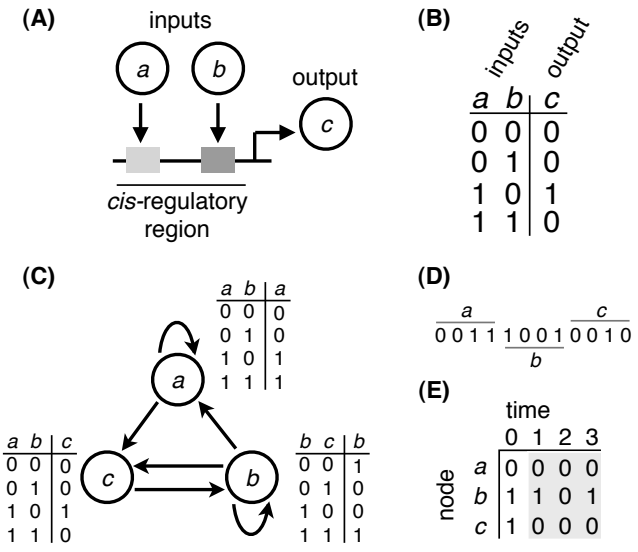

Figure 1: (A) Schematic of genetic regulation, where gene products a and b serve as regulatory inputs, attaching to their respective binding sites (gray shaded boxes) in the *cis*-regulatory region of gene c to influence its expression. The input-output function encoded in this regulatory region is called signal-integration logic and can be modeled as (B) a discrete function that explicitly maps all of the 2^z input-output combinations of a z-input function. Here, $z = 2$. (C) All interactions between gene products a, b, and c can be represented as a Random Boolean Circuit (RBC) with $N = 3$ nodes. Gene product c possesses the same regulatory inputs and signal-integration logic as in (A) to clearly depict how the RBC abstraction captures genetic regulation. (D) The signal-integration logic of every node in the RBC can be simultaneously represented with a single rule vector by concatenating the rightmost columns of each node's look-up table. (E) The dynamics of the RBC begin with an initial state (*e.g.*, $\langle 011 \rangle$) and eventually settle into an attractor (gray shaded region).

Methods

Random Boolean Circuits

We use Random Boolean Circuits (RBCs) to model genetic regulation (Kauffman, 1969). RBCs are composed of nodes and directed edges (Fig. 1C). Nodes represent gene products and edges represent regulatory interactions. Two nodes a and c are connected by a directed edge $a \rightarrow c$ if the expression of gene c is regulated by gene product a. Node states are binary, reflecting the presence (1) or absence (0) of a gene product, and dynamic, such that the state of a node at time $t + 1$ is dependent upon the states of its regulating nodes at time t. This dependence is captured by a look-up table associated with each node, which explicitly maps all possible combinations of regulatory input states to an output expression state. This look-up table is analogous to the

signal-integration logic encoded in *cis*-regulatory regions. The signal-integration logic of all of the nodes in the network can be simultaneously represented using a single *rule vector* (Fig. 1D).

The dynamics of RBCs occur in discrete time with synchronous updating of node states (Fig. 1E). The dynamics begin at a pre-specified initial state, which can be thought to represent regulatory factors upstream of the circuit (Ciliberti et al., 2007a; Martin and Wagner, 2009). The dynamics then unfold according to the circuit's structure and signal-integration logic. Since the system is both finite and deterministic, its dynamics eventually settle into an attractor (Kauffman, 1969), which represents the gene expression pattern, and is referred to as the phenotype. We refer to the combination of circuit structure, rule vector, and initial state as an *instance* of a RBC.

While simple, the Boolean abstraction has proven capable of precisely replicating specific properties of genetic regulation in natural systems. For example, variants of the model have emulated the expression patterns of the fruit fly *Drosophila melanogaster* (Albert and Othmer, 2003), the plant *Arabidopsis thaliana* (Espinosa-Soto et al., 2004), and the yeast *Saccharomyces pombe* (Davidich and Bornholdt, 2008). Due to their accuracy in capturing the dynamics of genetic regulation, and because the signal-integration logic of each gene is explicitly represented, RBCs are ideal synthetic systems for investigating the relationship between robustness and evolvability when genetic perturbations correspond to changes in signal-integration logic.

Dynamical Regimes of RBCs

An important feature of RBCs is that they exhibit three dynamical regimes: ordered, critical, and chaotic (Kauffman, 1969). In the ordered regime, gene expression patterns are relatively insensitive to perturbations, while in the chaotic regime they are highly sensitive. The critical regime delineates these two extremes. For randomly constructed circuits, the transitions between regimes are controlled by two parameters: the average in-degree z and the probability ρ of gene expression (*i.e.*, the probability of observing a 1 in the rule vector). Letting $S = 2\rho(1-\rho)z$, the RBC lies in the ordered regime when $S < 1$, the critical regime when $S = 1$, and the chaotic regime when $S > 1$. When there is an equal probability of observing a 0 or a 1 in the rule vector ($\rho = 0.5$) the dynamical regime is determined solely by the average in-degree, with $z < 2$ yielding the ordered regime, $z = 2$ the critical regime, and $z > 2$ the chaotic regime. In this study, $\rho = 0.5$.

Genotype Networks

We refer to the signal-integration logic of a RBC, as represented by its rule vector (Fig. 1D), as the genotype. There are a total of 2^L unique genotypes for a given combination of circuit structure and initial state, where $L = N2^z$. We refer to this set of genotypes as the genotype space, or equivalently, as the signal-integration space. For the RBCs considered here, the size of the genotype space ranges from 2^6 for the ordered regime to 2^{24} for the chaotic regime.

These genotypes map to a significantly smaller set of phenotypes. This high level of redundancy is a general feature of RBCs, and can be formalized using a genotype network, in which rule vectors are represented as nodes, and edges connect rule vectors that differ by a single bit, yet yield the same gene expression pattern (*i.e.*, phenotype). Thus, we define a neutral point mutation as a single change to an element of the genotype that does not lead to a change in phenotype. Such a mutation is analogous to a change in the position of a transcription factor binding site in the *cis*-regulatory region that leaves the gene expression pattern unchanged. Genotype networks are measured using an exhaustive breadth-first search, thus discovering all genotypes that yield the same phenotype and are accessible via neutral point mutations, starting from the original genotype of the RBC instance.

The quantity v_{ij} captures the number of unique non-neutral point mutations to genotypes in the genotype network of phenotype i that lead to genotypes in the genotype network of phenotype j. We call phenotypes i and j adjacent if $v_{ij} > 0$. By enumerating all of the phenotypes that are adjacent to phenotype i, and their corresponding genotype networks, we capture the mutational biases between adjacent phenotypes.

Robustness, Evolvability, and Accessibility

Several definitions of robustness and evolvability have been proposed, at both the genotypic and phenotypic scales (Aldana et al., 2007; Wagner, 2008b; Mihaljev and Drossel, 2009; Draghi et al., 2010). Here, we focus on these properties at the level of the phenotype. We define robustness R_i as the proportion of signal-integration space occupied by the genotype network of phenotype i. This metric is independent of rule vector length L, and captures the fraction of all genotypes that yield the same phenotype and can be accessed via neutral point mutations.

We define evolvability using two metrics. The first $E_{1,i}$ is simply the number of phenotypes that can be accessed through non-neutral point mutations from the genotype network of phenotype i (Wagner, 2008b). The second $E_{2,i}$ captures the mutational biases that exist between the genotype networks of adjacent phenotypes (Cowperthwaite et al., 2008). Letting

$$f_{ij} = \frac{v_{ij}}{\sum_{k \neq i} v_{ik}} \tag{1}$$

denote the fraction of non-neutral point mutations to genotypes of phenotype i that result in genotypes of phenotype j, we define the evolvability $E_{2,i}$ of phenotype i as

$$E_{2,i} = 1 - \sum_j f_{ij}^2. \tag{2}$$

Since $\sum_j f_{ij}^2$ captures the probability that two randomly chosen non-neutral point mutations to genotypes of phenotype i result in genotypes with identical phenotypes, its complement $E_{2,i}$ captures the probability that these same mutations result in genotypes with distinct phenotypes. This metric takes on high values when a phenotype is adjacent to many other phenotypes and its non-neutral point mutations are uniformly divided amongst these phenotypes. The metric takes on low values when a phenotype is adjacent to only a few other phenotypes and its non-neutral point mutations are biased toward a subset of these phenotypes.

In addition to measuring evolvability, which captures the uniformity of non-neutral mutations from phenotype i into adjacent phenotypes, we also consider accessibility

$$A_i = \sum_j f_{ji}, \tag{3}$$

which captures the propensity to mutate into phenotype i (Cowperthwaite et al., 2008). This metric takes on high values if the phenotypes adjacent to phenotype i are mutationally biased toward i and low values otherwise.

Lastly, we measure the robustness of all phenotypes that are adjacent to phenotype i, in proportion to the probability that these phenotypes are encountered through a randomly chosen, non-neutral point mutation from phenotype i (Cowperthwaite et al., 2008). We refer to this quantity as adjacent robustness,

$$B_i = \sum_j f_{ij} \times R_j. \tag{4}$$

This metric takes on high values when a phenotype is mutationally biased toward robust phenotypes and low values otherwise.

Simulation Details and Data Analysis

For all RBC instances, the rule vector and initial state are generated at random with $\rho = 0.5$. The circuit structure is also generated at random, but subject to the constraint that each node has exactly z inputs. Self-loops are permitted, mimicking autoregulation. We separately consider RBCs in the ordered, critical, and chaotic regimes by setting $z = 1, 2, 3$, respectively. The initial state and circuit structure are held fixed for each RBC instance. To ensure that all of the genotype networks considered in this study are amenable to exhaustive enumeration, we restrict our attention to RBCs with $N = 3$ nodes. While small, sensitivity analysis (Derrida and Pomeau, 1986) confirms that these RBCs exhibit the same dynamical regimes as larger networks, albeit with shorter attractors. To assess the strength and significance of the trends in our data, we employ Pearson's correlation coefficient.

Results

Characteristics of Genotype Networks

To characterize the genotype networks of signal-integration space in RBCs, we randomly generate 2500 RBC instances for each dynamical regime and exhaustively characterize the genotype networks of their corresponding phenotypes, and the genotype networks of all adjacent phenotypes.

The range of phenotypic robustness R varies with dynamical regime, with ordered RBCs spanning the smallest range ($3.12 \times 10^{-2} \leq R \leq 1.25 \times 10^{-1}$), critical RBCs spanning an intermediate range ($4.88 \times 10^{-4} \leq R \leq 1.25 \times 10^{-1}$), and chaotic RBCs spanning the largest range ($1.19 \times 10^{-7} \leq R \leq 1.25 \times 10^{-1}$). The maximum value of phenotypic robustness is independent of dynamical regime, and corresponds to fixed-point attractors. Since these attractors comprise a single state, only N bits of the rule vector are accessed during the RBC's dynamics, leaving $L - N$ bits unused. Thus, the corresponding genotype network is of size 2^{L-N}, with phenotypic robustness $R_{\max} = 2^{-N} = 1.25 \times 10^{-1}$. The average phenotypic robustness decreases from the ordered ($R = 9.44 \times 10^{-2}$) to the critical ($R = 4.12 \times 10^{-2}$) to the chaotic ($R = 3.02 \times 10^{-2}$) regime.

Evolvability E_1 and phenotypic robustness R are positively correlated (Fig. 2A), and the strength of correlation increases from the ordered ($r = 0.75, p \ll 0.01$) to the critical ($r = 0.90, p \ll 0.01$) to the chaotic ($r = 0.98, p \ll 0.01$) regime. This indicates that, in this system, no trade-off exists between robustness and the number of phenotypes accessible via non-neutral point mutations; the more robust the phenotype, the higher its evolvability. Average evolvability E_1 increases faster than linearly with increasing z, indicating a rapid increase in the number of adjacent phenotypes as the dynamical regime shifts from ordered to chaotic (Fig. 2A, inset).

When mutational biases between adjacent phenotypes are taken into account using E_2, a slightly different relationship is observed between evolvability and phenotypic robustness (Fig. 2B). RBCs in the ordered regime exhibit a weak and insignificant correlation between E_2 and R ($r = 0.02, p = 0.41$). In contrast, RBCs in the critical and chaotic regimes exhibit weak, but significant correlations, with the strength of correlation increasing from the critical ($r = 0.10, p \ll 0.01$) to the chaotic regime ($r = 0.42, p \ll 0.01$). The average value of E_2 increases approximately linearly as z increases (Fig. 2B, inset). Thus, the average probability that two randomly chosen, non-neutral point mutations lead to distinct phenotypes is only $\approx 15\%$ higher in chaotic RBCs than in ordered RBCs, despite the four order-of-magnitude difference in the absolute number of adjacent phenotypes (Fig. 2A, inset).

Accessibility A and phenotypic robustness R are positively correlated (Fig. 2C), with the strength of correlation again increasing from the ordered ($r = 0.88, p \ll 0.01$)

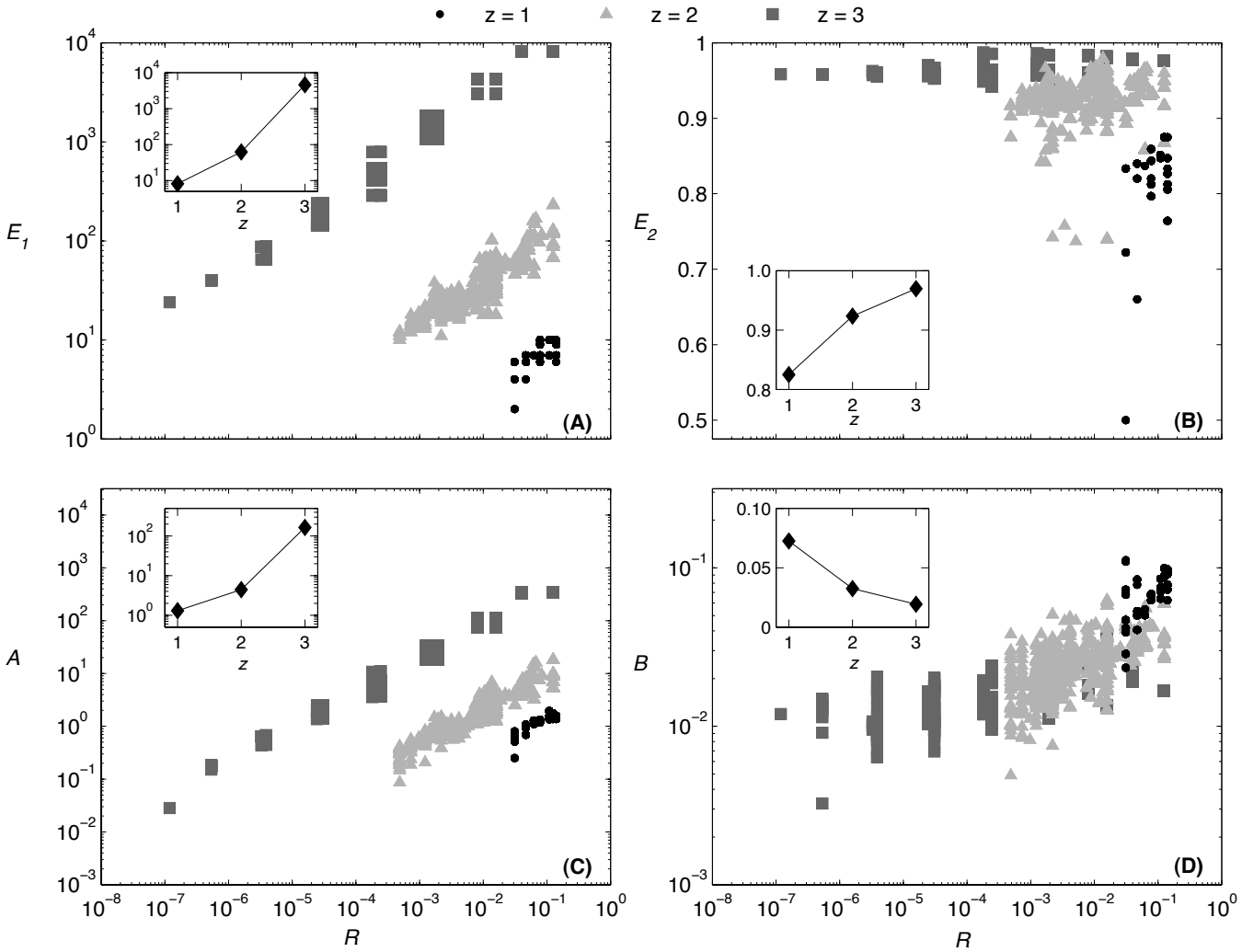

Figure 2: (A,B) Evolvability, (C) accessibility, and (D) adjacent robustness as a function of phenotypic robustness R for each of the three dynamical regimes: ordered ($z = 1$), critical ($z = 2$), and chaotic ($z = 3$). Each data point represents one of 2500 RBC instances for each dynamical regime. The insets depict the corresponding averages, as a function of z. Lines are provided as a guide for the eye.

to the critical ($r = 0.94, p \ll 0.01$) to the chaotic ($r = 0.98, p \ll 0.01$) regimes. This implies that, for all three dynamical regimes, random point mutations are more likely to lead to robust phenotypes than to non-robust phenotypes. Average accessibility increases faster than linearly as z increases (Fig. 2C, inset), indicating a rapid increase in the relative ease with which phenotypes are found by random mutation as the dynamical regime shifts from ordered to chaotic.

Adjacent robustness B and phenotypic robustness R are positively correlated, with the strength of correlation decreasing from the ordered ($r = 0.81, p \ll 0.01$) to the critical ($r = 0.66, p \ll 0.01$) to the chaotic regimes ($r = 0.35, p \ll 0.01$). This implies that non-neutral point mutations to genotypes within robust phenotypes often lead to other robust phenotypes, but that the strength of this tendency weakens as RBCs approach the chaotic regime. The average adjacent robustness B decreases approximately linearly as z increases (Fig. 2D, inset), indicating that the expected robustness of a phenotype encountered via non-neutral point mutation decreases as the dynamical regime shifts from ordered to chaotic.

Taken together, these results suggest that a series of random point mutations will tend toward phenotypes of increased robustness (Fig. 2D) and correspondingly increased evolvability (Fig. 2A,B). Further, the ease with which such a blind evolutionary process identifies an arbitrary phenotype should increase with that phenotype's robustness (Fig. 2C) and as the dynamical regime shifts from ordered to critical to chaotic (Fig. 2C, inset).

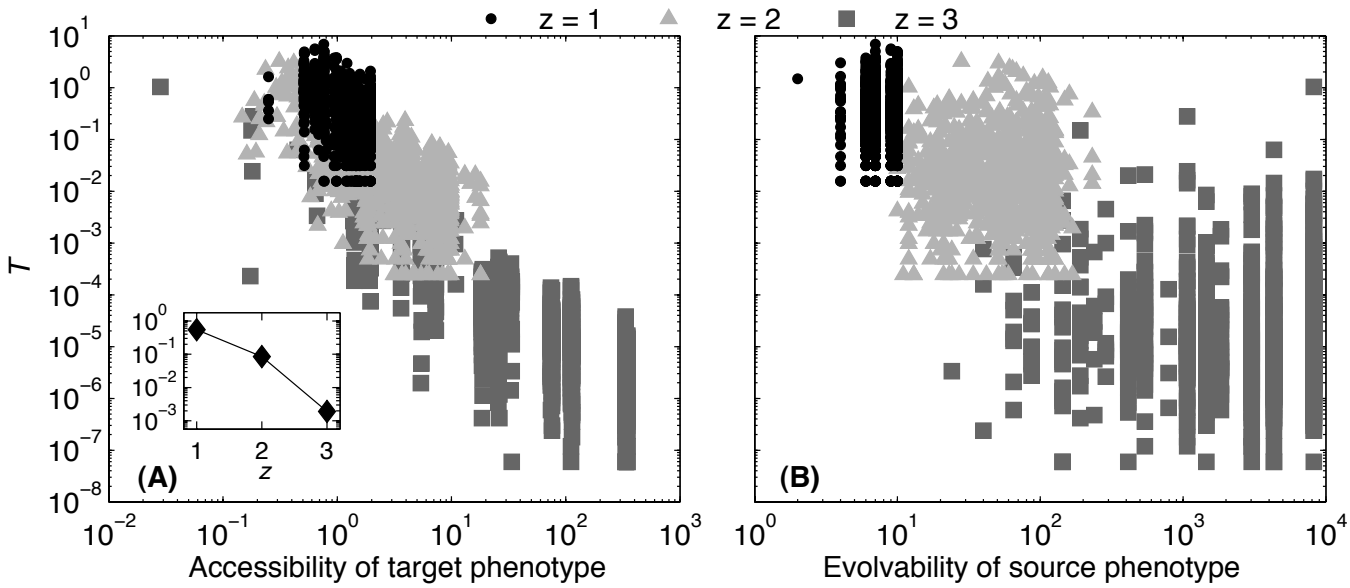

Figure 3: Waiting time of a random walk $T = S/2^L$ as a function of (A) the target phenotype's accessibility A and (B) the source phenotype's evolvability E_1, for each of the three dynamical regimes: ordered ($z = 1$), critical ($z = 2$), and chaotic ($z = 3$). The inset in (A) depicts the average waiting time T as a function of z. Lines are provided as a guide for the eye.

Random Walks Through Signal-Integration Space

To investigate how robustness, evolvability, and accessibility influence blind, mutation-based evolution, we conduct an ensemble of random walks. For each dynamical regime, we randomly generate 1000 RBC instances and identify the phenotype of each instance as a source phenotype. For each instance, we then sample the genotype space at random until we discover a genotype that yields a different phenotype from the source phenotype, and we identify this as the target phenotype. For each pair of source and target phenotypes, we then perform a random walk, starting from the instance's genotype and ending when the random walk reaches any genotype in the target phenotype. Each step in the random walk corresponds to a single point mutation to the genotype. We record the number of steps S required to reach the target phenotype, which we normalize by the size of the signal-integration space 2^L, and refer to as the waiting time $T = S/2^L$.

Waiting time T decreases faster than linearly as z increases (Fig. 3A, inset). For all three dynamical regimes, waiting time is strongly negatively correlated with the accessibility A of the target phenotype (Fig. 3A), and the strength of correlation increases from the ordered ($r = -0.41, p \ll 0.01$) to the critical ($r = -0.67, p \ll 0.01$) to the chaotic ($r = -0.82, p \ll 0.01$) regime. In contrast, the correlation between waiting time T and the evolvability E_1 of the source phenotype is weak and insignificant ($z = 1 : r = -0.03, p = 0.38$; $z = 2 : r = 0.01, p = 0.82$; $z = 3 : r = -0.02, p = 0.56$) (Fig. 3B). Similarly weak and insignificant correlations were observed between waiting time T and other characteristics of the source phenotype, such as E_2, A, and B. These results indicate that the time required for a blind evolutionary search to identify a target phenotype is independent of the phenotypic properties of the starting point and solely dependent upon the phenotypic properties of the target.

Discussion

This study has provided the first characterization of genotype networks in the signal-integration space of Random Boolean Circuits (RBCs), highlighting the relationship between robustness and the evolvability and accessibility of phenotypes. We found a positive correlation between robustness and evolvability, as measured by either the absolute number of adjacent phenotypes E_1 (Fig. 2A) or by the probability that two non-neutral point mutations lead to distinct phenotypes E_2 (Fig. 2B). Our results corroborate the observation made in previous studies that gene regulatory networks can simultaneously exhibit robustness and evolvability (Aldana et al., 2007; Ciliberti et al., 2007a,b). Further, our analyses extend these previous studies by providing an explicit description of this relationship and by considering genetic perturbations that alter the signal-integration logic encoded in *cis*-regulatory regions, instead of genetic perturbations that alter circuit structure.

We also found a positive correlation between robustness and accessibility (Fig. 2C), a measure that captures the relative ease with which a phenotype can be identified by mutation-based evolution. This result supports the intuitive notion that phenotypes comprising many genotypes are eas-

ier for evolution to identify than those comprising few genotypes. In addition, robust phenotypes are mutationally biased toward other robust phenotypes (Fig. 2D), indicating that the robustness of phenotypes encountered by blind mutation-based evolution should, on average, tend to increase.

To understand how phenotypic robustness, evolvability, and accessibility in signal-integration space influence mutation-based evolution, we considered an ensemble of random walks between pairs of source and target phenotypes. We found that the number of random mutations required to reach the target phenotype was entirely dependent upon its accessibility (Fig. 3A) and independent of any properties of the source phenotype (Fig. 3B). This suggests that a random walk through signal-integration space quickly loses any memory of its starting location. Consequently, extant evolvability metrics cannot be expected to predict the duration of a random walk between phenotypes.

The majority of our results are consistent with those made in RNA systems (Cowperthwaite et al., 2008; Wagner, 2008b). However, there is one difference worth emphasizing: the correlation between robustness and evolvability E_2 is negative in RNA (Cowperthwaite et al., 2008). Since the relationship between robustness and adjacent robustness B is positive in RNA systems, Cowperthwaite et al. (2008) concluded that robust phenotypes act as "evolutionary traps." That is, random mutation will tend toward phenotypes of higher robustness, which in turn are less evolvable, and therefore stagnate evolutionary search. Since we observed a positive correlation between (i) robustness and evolvability E_2 and (ii) robustness and adjacent robustness B, we conclude that robust phenotypes in the signal-integration space of RBCs are not evolutionary traps, but instead facilitate the discovery of novel phenotypes. Such contrast between model systems highlights the fact that the relationships between robustness, evolvability, and accessibility are system dependent.

Evolvability increased monotonically as z increased (Fig. 2A,B, insets) and the maximum achievable robustness was independent of z ($R_{\max} = 2^{-N}$). Taken together, these results indicate that robustness and evolvability can be simultaneously maximized in chaotic RBCs. This result contrasts with previous analysis (Aldana et al., 2007), which found robustness and evolvability to be simultaneously maximized in critical RBCs. This discrepancy can be understood by considering the two primary differences between the analyses. First, Aldana et al. (2007) focused on genetic perturbations that altered circuit structure (and consequently, in some cases, signal-integration logic) while we focused solely on genetic perturbations that altered signal-integration logic. Second, and of greater importance, the measures of robustness and evolvability considered by Aldana et al. (2007) were not based on genotype networks. Instead, robustness was defined as the ability of a single mutated genotype to maintain the phenotypic landscape (*i.e.*, the set of all phenotypes observed across all possible initial states), and evolvability was defined as the capacity of the mutated genotype to expand the phenotypic landscape (*i.e.*, add new phenotypes to the set of existing phenotypes). Thus, Aldana et al. (2007) focused on robustness and evolvability at the level of the genotype rather than the phenotype (Wagner, 2008b). While these definitions are reasonable and insightful, our departure from their use precludes any direct comparison between the two studies. That said, our observation that chaotic RBCs simultaneously optimize robustness and evolvability must be interpreted with caution. For all dynamical regimes, robustness is maximal for fixed point attractors, and these occur with decreasing frequency as z increases. Thus, while it is only possible to simultaneously observe maximal robustness and maximal evolvability in chaotic RBCs, this case represents the exception rather than the rule.

Future work will seek to understand how evolution navigates signal-integration space. Is it possible for mutation and selection to identify the high-robustness, high-evolvability phenotypes of chaotic RBCs? If so, can they out-compete critical and ordered RBCs in static (Oikonomou and Cluzel, 2006) or dynamic (Greenbury et al., 2010) environments? How are these evolutionary outcomes affected by mutation rate (Wilke et al., 2001) or recombination (Martin and Wagner, 2009)? Future research will also focus on larger systems, moving from an analysis of circuits to entire networks. To accomplish this, Monte Carlo sampling methods will be required (Jörg et al., 2008), as the increased size of the signal-integration space will prohibit the exhaustive enumeration of genotype networks. In addition, future work will seek to understand both the influence of canalyzing functions (Kauffman et al., 2004) and the probability of gene expression ρ on the size and structure of genotype networks. These directions, among others, will lead to a more thorough understanding of how the genetic flexibility of *cis*-regulatory regions influence evolutionary processes.

Acknowledgements

The authors would like to thank Davnah Urbach and Dov Pechenick for their insightful comments, which greatly improved the clarity and presentation of this manuscript. This work was partially supported by NIH grants R01-LM009012, R01-LM010098, and R01-AI59694. J.L.P. was supported by NIH grant K25-CA134286.

References

Albert, R. and Othmer, H. G. (2003). The topology of the regulatory interactions predicts the expression pattern of the segment polarity genes in *Drosophila melanogaster*. *Journal of Theoretical Biology*, 223:1–18.

Aldana, M., Balleza, E., Kauffman, S., and Resendiz, O. (2007). Robustness and evolvability in genetic regulatory networks. *Journal of Theoretical Biology*, 245:433–448.

Bloom, J. D., Labthavikul, S. T., Otey, C. R., and Arnold, F. H. (2006). Protein stability promotes evolvability. *PNAS*, 103:5869–5874.

Ciliberti, S., Martin, O. C., and Wagner, A. (2007a). Innovation and robustness in complex regulatory gene networks. *PNAS*, 104:13591–13596.

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

Cowperthwaite, M. C., Economo, E. P., Harcombe, W. R., Miller, E. L., and Meyers, L. A. (2008). The ascent of the abundant: how mutational networks constrain evolution. *PLoS Computational Biology*, 4:e10000110.

Davidich, M. I. and Bornholdt, S. (2008). Boolean network model predicts cell cycle sequence of fission yeast. *PLoS ONE*, 3:e1672.

Derrida, B. and Pomeau, Y. (1986). Random networks of automata: a simple annealed approximation. *Europhysics Letters*, 1:45–49.

Draghi, J. A., Parsons, T. L., Wagner, G. P., and Plotkin, J. B. (2010). Mutational robustness can facilitate adaptation. *Nature*, 463:353–355.

Espinosa-Soto, C., Padilla-Longoria, P., and Alvarez-Buylla, E. R. (2004). A gene regulatory network model for cell-fate determination during *Arabidopsis thaliana* flower development that is robust and recovers experimental gene expression profiles. *The Plant Cell*, 16:2923–2939.

Ferrada, E. and Wagner, A. (2008). Protein robustness promotes evolutionary innovations on large evolutionary time-scales. *Proceedings of the Royal Society London B*, 275:1595–1602.

Greenbury, S. F., Johnston, I. G., Smith, M. A., Doye, J. P. K., and Louis, A. A. (2010). The effect of scale-free topology on the robustness and evolvability of genetic regulatory networks. *Journal of Theoretical Biology*, 267:48–61.

Hunziker, A., Tuboly, C., Horváth, P., Krishna, S., and Semsey, S. (2010). Genetic flexibility of regulatory networks. *PNAS*, 107:12998–13003.

Huynen, M. A., Stadler, P. F., and Fontana, W. (1996). Smoothness within ruggedness: The role of neutrality in adaptation. *PNAS*, 93:397–401.

Isalan, M., Lemerle, C., Michalodimitrakis, K., Horn, C., Beltrao, P., Raineri, E., Garriga-Canut, M., and Serrano, L. (2008). Evolvability and hierarchy in rewired bacterial gene networks. *Nature*, 452:840–846.

Jörg, T., Martin, O. C., and Wagner, A. (2008). Neutral network sizes of biological RNA molecules can be computed and are not atypically small. *BMC Bioinformatics*, 9:464.

Kaplan, S., Bren, A., Zaslaver, A., Dekel, E., and Alon, U. (2008). Diverse two-dimensional input functions control bacterial sugar genes. *Molecular Cell*, 29:786–792.

Kauffman, S. A. (1969). Metabolic stability and epigenesis in randomly constructed genetic nets. *Journal of Theoretical Biology*, 22:437–467.

Kauffman, S. A., Peterson, C., Samuelsson, B., and Troein, C. (2004). Genetic networks with canalyzing Boolean rules are always stable. *Proceedings of the National Academy of Sciences*, 101:17102–17107.

Martin, O. C. and Wagner, A. (2009). Effects of recombination on complex regulatory circuits. *Genetics*, 138:673–684.

Mayo, A. E., Setty, Y., Shavit, S., Zaslaver, A., and Alon, U. (2006). Plasticity of the *cis*-regulatory input function of a gene. *PLoS Biology*, 4:e45.

Mihaljev, T. and Drossel, B. (2009). Evolution of a population of random Boolean networks. *European Physics Journal B*, 67:259–267.

Newman, M. and Engelhardt, R. (1998). Effects of selective neutrality on the evolution of molecular species. *Proceedings of the Royal Society London B*, 265:1333–1338.

Oikonomou, P. and Cluzel, P. (2006). Effects of topology on network evolution. *Nature Physics*, 2:532–536.

Schuster, P., Fontana, W., Stadler, P. F., and Hofacker, I. L. (1994). From sequences to shapes and back: a case study in RNA secondary structures. *Proceedings of the Royal Society London B*, 255:279–284.

Setty, Y., Mayo, A., Surette, M., and Alon, U. (2003). Detailed map of a *cis*-regulatory input function. *PNAS*, 100:7702–7707.

Wagner, A. (2005). *Robustness and Evolvability in Living Systems*. Princeton University Press.

Wagner, A. (2008a). Neutralism and selectionism: a network-based reconciliation. *Nature Reviews Genetics*, 9:965–974.

Wagner, A. (2008b). Robustness and evolvability: a paradox resolved. *Proceedings of the Royal Society London B*, 275:91–100.

Wilke, C. O., Wang, J. L., Ofria, C., Lenski, R. E., and Adami, C. (2001). Evolution of digital organisms at high mutation rates leads to survival of the flattest. *Nature*, 412:331–333.