

Contents lists available at SciVerse ScienceDirect

Journal of Theoretical Biology



journal homepage: www.elsevier.com/locate/yjtbi

The influence of assortativity on the robustness of signal-integration logic in gene regulatory networks

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ARTICLE INFO

Article history: Received 5 August 2011 Received in revised form 23 November 2011 Accepted 30 November 2011 Available online 8 December 2011

Keywords: Boolean networks Regulatory regions In-components Genetic regulation

ABSTRACT

Gene regulatory networks (GRNs) drive the cellular processes that sustain life. To do so reliably, GRNs must be robust to perturbations, such as gene deletion and the addition or removal of regulatory interactions, GRNs must also be robust to genetic changes in regulatory regions that define the logic of signal-integration, as these changes can affect how specific combinations of regulatory signals are mapped to particular gene expression states. Previous theoretical analyses have demonstrated that the robustness of a GRN is influenced by its underlying topological properties, such as degree distribution and modularity. Another important topological property is assortativity, which measures the propensity with which nodes of similar connectivity are connected to one another. How assortativity influences the robustness of the signal-integration logic of GRNs remains an open question. Here, we use computational models of GRNs to investigate this relationship. We separately consider each of the three dynamical regimes of this model for a variety of degree distributions. We find that in the chaotic regime, robustness exhibits a pronounced increase as assortativity becomes more positive, while in the critical and ordered regimes, robustness is generally less sensitive to changes in assortativity. We attribute the increased robustness to a decrease in the duration of the gene expression pattern, which is caused by a reduction in the average size of a GRN's in-components. This study provides the first direct evidence that assortativity influences the robustness of the signal-integration logic of computational models of GRNs, illuminates a mechanistic explanation for this influence, and furthers our understanding of the relationship between topology and robustness in complex biological systems.

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1. Introduction

Living organisms are constantly beset by environmental and genetic perturbations, which threaten to compromise the intricate processes that sustain life. Despite the harmful potential of such perturbations, organisms show remarkable robustness at many levels of biological organization (Wagner, 2005). For example, the three-dimensional structure and function of biological macromolecules, such as RNA and proteins, are robust to point mutations in primary sequence (Schuster et al., 1994; Bloom et al., 2005). Similarly, the biological networks that drive cellular processes are robust to topological reorganization, as exemplified by the removal of nodes in the protein–protein interaction network of the yeast *Saccharomyces cerevisiae* (Jeong et al., 2001) and

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the rewiring of the gene network in the bacterium *Escherichia coli* (Isalan et al., 2008), both of which regularly fail to elicit a change in growth rate.

There are several sources of robustness in biological systems (Kitano, 2004; Wagner, 2005). Of fundamental importance is the many-to-one mapping of genotype to phenotype. In RNA, for example, myriad primary sequences (genotypes) may result in the same secondary structure (phenotype) upon folding in three dimensions. These distinct sequences are often connected by neutral point mutations, and form a vast web of primary sequences that all yield identical secondary structure (Schuster et al., 1994). Such webs are referred to as genotype networks, wherein vertices represent genotypes and edges connect two genotypes that can be interconverted via neutral point mutations (Wagner, 2008a). Within this context, a genotype network, and a phenotype is said to be robust if its underlying genotype network is made up of many robust genotypes (Wagner, 2008b).

The conceptual framework of a genotype network (Van Nimwegen et al., 1999) has been used to study the robustness of numerous biological systems, particularly at the molecular scale (Schuster et al., 1994; Huynen et al., 1996; Ferrada and

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^{0022-5193/\$ -} see front matter \circledcirc 2011 Elsevier Ltd. All rights reserved. doi:10.1016/j.jtbi.2011.11.029

Wagner, 2008; Cowperthwaite et al., 2008; Wagner, 2008b; Rodrigues and Wagner, 2011). However, this framework has been less frequently applied to the study of robustness at higher levels of biological organization, such as gene regulatory networks (GRNs). GRNs consist of collections of genes and gene products that interact to produce the gene expression patterns that drive cellular processes. The genotype of a GRN typically represents the topology of regulatory interactions (Ciliberti et al., 2007a,b) and the phenotype represents some property of the GRN's expression pattern (Mihaljev and Drossel, 2009; Szejka and Drossel, 2007, 2010). The corresponding genotype network is therefore a "network of networks" (Ciliberti et al., 2007b), wherein two GRNs are directly connected in the genotype network if they yield the same phenotype, yet differ in a single regulatory interaction (Ciliberti et al., 2007a,b). Using these definitions, recent analyses have revealed three general properties of GRNs. First, the topology of a GRN has a governing influence on phenotypic robustness (Aldana and Cluzel, 2003; Greenbury et al., 2010). Second, robust phenotypes are comprised of vast genotype networks that span the space of all possible genotypes (Mihaljev and Drossel, 2009; Szejka and Drossel, 2007, 2010). Third, these genotype networks harbor a reservoir of genetic diversity (Bergman and Siegal, 2003; Ciliberti et al., 2007a) that promotes adaptation to novel environments (Espinosa-Soto et al., 2011) and allows for the evolution of genotypic robustness without phenotypic alteration (Ciliberti et al., 2007b).

While these analyses have improved our understanding of robustness in GRNs, they are limited by the assumption that genetic perturbations primarily correspond to topological alterations. 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). Gene expression may also be influenced by the arrangement of transcription factor binding sites in a gene's regulatory region (Hunziker et al., 2010). This region encodes an input-output function, referred to as signal-integration logic, that maps a specific combination of regulatory signals to a particular gene expression state. Such regulatory programs offer an additional source of robustness, as many rearrangements of a gene's regulatory region yield the same expression output (Little et al., 1999; Mayo et al., 2006). Thus, not only can the topology of a GRN confer robustness (Aldana and Cluzel, 2003; Greenbury et al., 2010), so too can the signal-integration logic encoded in regulatory regions.

Despite the known importance of signal-integration logic as a source of robustness in GRNs, its theoretical analysis has received little attention. In a recent study, Payne and Moore (2011) explored the genotype networks of three-node GRNs under the assumption that genetic perturbations correspond solely to alterations in regulatory regions. In this case, the genotype represents the signal-integration logic and two GRNs are directly connected in a genotype network if they are topologically identical and yield the same phenotype, yet differ in a single element of their signal-integration logic. Under these assumptions, their analysis revealed that robust phenotypes only occupy a small fraction of the space of possible regulatory programs and that these phenotypes are often mutationally biased toward other robust phenotypes.

The results of Payne and Moore (2011) pertain to topologically random GRNs. However, recent characterizations of the GRNs of biological organisms have uncovered several nonrandom topological properties, including heavy-tailed degree distributions, hierarchical organization, and modularity (Ravasz et al., 2002), many of which are known to affect the robustness of GRNs (Aldana and Cluzel, 2003; Variano et al., 2004; Aldana et al., 2007; Poblanno-Balp and Gershenson, 2011). Another important topological property is degree-degree assortativity (Newman, 2002), which captures the propensity with which nodes of similar degree are connected to one another. Assortativity has been shown to vary in biological systems (Newman, 2002; Foster et al., 2010) and its functional significance has been demonstrated in a number of dynamical network processes (Newman, 2002; Rong et al., 2007; Payne et al., 2009; Payne and Eppstein, 2009), including the stability of GRNs (Pomerance et al., 2009). However, it is not yet known how assortativity affects the robustness of the signal-integration logic encoded in the regulatory regions of GRNs.

Here, we use computational models of genetic regulation to address this open question. We separately consider three degree distributions, two of theoretical interest and one that resembles the topological properties of biological GRNs (Aldana and Cluzel, 2003; Aldana et al., 2007), and use a simple edge-swapping algorithm to tune the assortativity of the GRNs while holding the degree distribution constant. To quantify robustness, we use ensembles of random walks, which explore the genotype networks of possible signal-integration functions. We find that assortativity influences the robustness of signal-integration logic in a computational model of GRNs, and we provide a mechanistic explanation for the relationship between these two quantities. We close with a discussion of our results and present several directions for future research.

2. Methods

2.1. Boolean networks: genotype and phenotype

We used Boolean networks to model GRNs (Kauffman, 1969). Boolean networks are made up of nodes, which represent genes, and directed edges, which represent regulatory interactions between genes. Each node *i* has a binary state $\sigma_i(t) \in \{0, 1\}$, which represents whether or not the gene is being expressed at time *t*. Therefore the number of possible expression states for a Boolean network with *N* nodes is 2^N . The dynamics of the system occur in discrete time with synchronous updating of node states. Specifically, the state of each node σ_i is updated at time *t*+1 according to a Boolean function f_i , such that

$$\sigma_i(t+1) = f_i(\sigma_{i_1}(t), \dots, \sigma_{i_{k_{i_n}}}(t)),$$
(1)

where $\sigma_{i_1}, \ldots, \sigma_{i_{k_{in,i}}}$ are the states of the $k_{in,i}$ inputs to node *i*. The function f_i is defined by a look-up table containing a binary entry for each of the $2^{k_{in,i}}$ possible combinations of states for the $k_{in,i}$ input nodes (Fig. 1A). The binary string generated by concatenating the rightmost output columns of these look-up tables is referred to as the rule vector (Fig. 1B). We consider the rule vector to be the GRN's genotype, because it represents the signal-integration logic that defines the regulatory program for the entire GRN (Payne and Moore, 2011).

For deterministic Boolean functions, a unique combination of the states of the $k_{in,i}$ input nodes realized at any time *t* always results in the same state for node *i* at time t+1. Since there are a finite number of possible expression states for the GRN (2^N), some expression state must eventually repeat. This results in a sequence of unique states that is repeated, which is referred to as an attractor (Fig. 1C); its length corresponds to the duration of the gene expression pattern. We consider the attractor to be the GRN's phenotype, as it represents the gene expression pattern that defines cell type (Huang et al., 2005).

While abstract, the Boolean model has proven quite accurate in predicting the dynamics of GRNs in certain biological systems. For example, the model has recapitulated precise expression dynamics for the gap gene system of the fruit fly *Drosophila melanogaster* (Albert and Othmer, 2003) and for the primordial



Fig. 1. A Boolean network example. (A) This Boolean network is composed of three nodes and four directed edges. Each node possesses a look-up table that determines the dynamics of the Boolean network by defining the expression state of the node at time t+1 as a function of the states of its inputs at time t. For example, the look-up table for node b shows how each possible combination of expression states $\sigma_a(t)$ and $\sigma_c(t)$ of the inputs at time t dictate the expression state $\sigma_b(t+1)$. (B) The rule vector, which captures the signal-integration logic for the entire GRN, is obtained by concatenating the rightmost output columns of the look-up tables for all nodes. (C) Starting with initial states at t=0, the states are updated according to the look-up tables until they repeat, forming an attractor (shaded region). In this example, the attractor length is two.

floral organ cells of the plant *Arabidopsis thaliana* (Espinosa-Soto et al., 2004). The model has also accurately reproduced the avalanche distributions that result from gene knockout in *S. cerevisiae* (Serra et al., 2004). Due to its accuracy in emulating gene expression dynamics and its explicit representation of signal-integration logic, the Boolean model provides a compelling synthetic system with which to carry out this study.

2.2. Degree distributions

Three distinct combinations of input and output degree distributions were considered. The first, referred to as fixed-input/ Poisson-output (FIPO), was constructed by fixing the number of inputs to a constant, $k_{in} = c$, and randomly selecting *c* inputs for each node. The second, Poisson-input/Poisson-output (PIPO), was constructed by drawing k_{in} for each node from a Poisson distribution,

$$p(k_{\rm in}) = \frac{\lambda^{k_{\rm in}} e^{-\lambda}}{k_{\rm in}!}.$$
(2)

The third, Poisson-input/Power-law-output (PIPLO), was constructed by drawing k_{out} for each node from a power-law distribution (Darabos et al., 2009),

$$p(k_{\text{out}}) = \frac{1}{Z(\gamma)} k_{\text{out}}^{-\gamma}$$
(3)

with the normalization constant $Z(\gamma) = \sum_{j=1}^{N} j^{-\gamma}$, where *N* is the number of nodes in the GRN. In the first two cases, we specified the input degree distribution and in the last case, we specified the output degree distribution. Once the specified distribution was established, edges were laid down at random to satisfy the distribution (Newman, 2003). This naturally resulted in a Poisson output distribution in the first two cases and a Poisson input distribution in the last case (Aldana et al., 2007).

2.3. Dynamical regimes

Boolean networks exist in one of three dynamical regimes: ordered, critical, and chaotic. The behavior of Boolean networks in the different regimes is characterized by the rates of divergence between similar initial states, where similarity between states is characterized by Hamming distance (Derrida and Pomeau, 1986). In the chaotic regime, the distance between two such states increases exponentially as the states get updated. In the ordered regime, the distance decreases, because on average such a perturbation affects fewer than one node. The critical regime occupies the boundary between ordered and chaotic regimes, and this along with the ordered regime are thought to be relevant to biological systems (Shmulevich et al., 2005; Nykter et al., 2008).

The dynamical regime of a Boolean network is a function of the properties of its degree distribution, such as average degree (Kauffman, 1993; Aldana and Cluzel, 2003). For each of the three degree distributions considered, we specified the average degree necessary to yield the three dynamical regimes (see Section 2.7).

2.4. Assortativity

Degree–degree assortativity, $r \in [-1, 1]$, is a global network property that measures the propensity for nodes of similar degree to be connected. In assortative networks, r > 0, edges often exist between nodes with similar degree, whereas in disassortative networks, r < 0, edges often exist between nodes with dissimilar degree. In directed networks, nodes possess both an in- and an out-degree, and for the purposes of this study we looked at the assortativity between the out-degrees of connected nodes (Foster et al., 2010). This out–out degree assortativity, referred to henceforward simply as assortativity, was calculated as a Pearson's correlation coefficient (Newman, 2002):

$$r = \frac{M^{-1} \sum_{i} j_{i} k_{i} - [M^{-1} \sum_{i} \frac{1}{2} (j_{i} + k_{i})]^{2}}{M^{-1} \sum_{i} \frac{1}{2} (j_{i}^{2} + k_{i}^{2}) - [M^{-1} \sum_{i} \frac{1}{2} (j_{i} + k_{i})]^{2}},$$
(4)

where j_i and k_i are the out-degrees of the nodes at the ends of the *i*th edge, and i=1,...,M, where *M* is the number of edges in the network.

A standard edge-swapping method was employed for tuning the assortativity of a network to a desired value (Milo et al., 2003; Payne and Eppstein, 2009). In each iteration of this method two edges $i \rightarrow j$ and $x \rightarrow y$ were selected at random. Edges were then swapped, resulting in two new edges $i \rightarrow y$ and $x \rightarrow j$. These new edges replaced $i \rightarrow j$ and $x \rightarrow y$ if they changed assortativity in the desired direction. Otherwise, the new edges were discarded and the old edges were kept. Such edge swaps preserved the in- and out-degrees of all nodes involved, thereby keeping the degree distribution intact.

2.5. Robustness

Several definitions of robustness exist at both the genotypic and phenotypic scales (Aldana et al., 2007; Wagner, 2008b; Mihaljev and Drossel, 2009; Draghi et al., 2010). Genotypic robustness is commonly measured as the connectivity of a genotype in a genotype network, capturing the total number of neutral mutations available to the genotype (Wagner, 2008b). Phenotypic robustness is often measured as the average genotypic robustness of a phenotype's underlying genotype network (Wagner, 2008b). Here, we are primarily concerned with phenotypic robustness, and we will use the term "robustness" as shorthand for "phenotypic robustness" unless stated otherwise.

The size of the GRNs considered in this study (see Section 2.7) prohibited the exhaustive enumeration of all genotype networks, so we approximated robustness using random walks on genotype networks. A potential step in the random walk was assessed by flipping the bit of a single randomly chosen entry in the rule vector, and then recording the corresponding attractor generated from the same initial state. If the attractor remained unchanged, the step was neutral and the new rule vector was kept (fulfilling the requirement that the random walk remain on the genotype network). Otherwise, the flipped bit was restored to what it was immediately before that step. Thus, we quantified robustness as

the proportion of total attempted steps in a random walk that were neutral. This proportion serves as a proxy for measuring the average genotypic robustness of the connected set of genotypes that comprise a phenotype.

2.6. In-components

An in-component (IC) is a group of weakly connected nodes in a directed network that receives no input connections from other nodes in the network (Fig. 2). Therefore, the IC of a node *i* is the set of all nodes that directly or indirectly has an influence on *i*. The IC was determined by identifying all nodes that provide input to *i*, all nodes that provide input to those nodes, and so on until there were no nodes outside the IC that provide input to nodes in the IC. Mean IC size (\overline{S}) for a GRN was calculated as follows:

$$\overline{S} = \frac{\sum_{i=1}^{N} S_i}{N},\tag{5}$$

where S_i is the number of nodes in the IC of node *i*.

2.7. Simulation details

Weakly connected FIPO, PIPO, and PIPLO GRNs of size N=30 were generated without self-loops in the ordered, critical, and chaotic dynamical regimes, resulting in a total of nine combinations of degree distribution and dynamical regime. To generate ordered, critical, and chaotic FIPO GRNs, fixed k_{in} was set to c=1, 2, 3, respectively. To generate ordered, critical, and chaotic PIPO GRNs, the average in-degree, \overline{k}_{in} , was set to $\lambda = 1.3$, 2, 3, respectively (the average of 1.3 was chosen because it is difficult to ensure weak-connectivity with $\lambda = 1$). To generate ordered, critical, and chaotic PIPLO GRNs, $\gamma = 3.00$, 2.25, 1.81 were chosen to yield power-law distributions with $\overline{k}_{in} = 1.3$, 2, 3, respectively. These \overline{k}_{in} were calculated for the specific size of the GRNs considered in this study (Aldana et al., 2007)

$$\overline{k}_{in} = \frac{\sum_{j=1}^{N} j^{1-\gamma}}{\sum_{i=1}^{N} j^{-\gamma}}$$
(6)

 $(\overline{k}_{in} = 1.3 \text{ was chosen because } \overline{k}_{in} = 1 \text{ only exists in the limit of } \gamma \rightarrow \infty$). Visual inspection of Derrida plots (Derrida and Pomeau, 1986) confirmed that the selected parameters for each degree distribution produced networks in the three respective regimes. After the topology of a GRN was created, its rule vector was generated at random, such that the probability of choosing a 0 or

Fig. 2. In-components of GRNs. An in-component (IC) can be drawn around each node in the GRN. The IC of node *j* consists of five nodes (lightly shaded region), of which the IC of node *i* is a subset (darkly shaded region).

1 was equal. Each GRN was also paired with its own randomly generated initial state, which was held constant throughout the random walk.

For each of the nine combinations of degree distribution and dynamical regime, 75,000 GRNs were generated with assortativity values that lie within the bounds shown in Table 1. While the theoretical bounds of assortativity are $r \in [-1, 1]$, in practice they are constrained by degree distribution (Dodds and Payne, 2009). Approximate bounds were experimentally determined for each of the nine combinations by performing 2000 edge-swaps toward an assortativity of -1 or 1 (see Section 2.4) on 100 GRNs. This number of edge-swaps was chosen as a balance between computational efficiency and achieving an assortativity value approaching the absolute bound (Fig. 3A). From each resulting distribution of assortativity values, a representative value was chosen as a bound. Within each set of bounds, 15 linearly spaced values were chosen as the assortativity targets, and 5000 networks were tuned to within 0.01 of each target, preserving weak-connectivity and prohibiting self-loops.

As with assortativity bounds, the range of assortativity values expected at random for a particular GRN depends on the degree distribution and dynamical regime (Foster et al., 2010; Johnson et al., 2010). To determine this null distribution, 1000 GRNs were generated for each combination of degree distribution and dynamical regime. Subsequently, $10 \times M$ random edge-swaps were performed, taking care to preserve the degree distribution, weak connectivity, and lack of self-loops (Maslov and Sneppen, 2002). These random edge swaps ensured the removal of any structural bias introduced during the construction of the GRNs. After the edges were thoroughly randomized, assortativity and mean IC size were measured, providing a null distribution of these quantities for each combination of degree distribution and dynamical regime. These null distributions were used to verify that the bounds of assortativity considered in this study (Table 1) lie outside what is expected at random.

To calculate robustness, the length of the random walks was set at 500 attempted steps, which again was chosen to balance computational efficiency with accuracy (Fig. 3B). Average robustness for each GRN was calculated from random walks for 100 random initial states and rule vector pairings, and mean attractor length was calculated from the resulting 100 attractors. Our study was limited to networks of size of N=30 because the attractor lengths of chaotic networks grow exponentially with N (Aldana, 2003), placing computational constraints on the feasible length of such random walks. The choice of N=30 therefore reflects a balance between network size and the accuracy of the robustness estimate.

Statistical significance of all reported trends was determined using Pearson's correlation, and the direction and strength of the trends are approximated by the slope of the best linear fit to the data.

Table 1				
Lower an	d upper	bounds	on ass	ortativity.

Degree distribution	Regime	Assortativity (r) bounds
Fixed-input/Poisson-output (FIPO)	Ordered Critical Chaotic	$\begin{array}{l} -0.82 \leq r \leq 0.47 \\ -0.86 \leq r \leq 0.64 \\ -0.82 \leq r \leq 0.67 \end{array}$
Poisson-input/Poisson-output (PIPO)	Ordered Critical Chaotic	$\begin{array}{l} -0.83 \leq r \leq 0.51 \\ -0.84 \leq r \leq 0.61 \\ -0.82 \leq r \leq 0.66 \end{array}$
Poisson-input/Power-law-output (PIPLO)	Ordered Critical Chaotic	$-0.31 \le r \le 0.51 \\ -0.46 \le r \le 0.21 \\ -0.56 \le r \le 0.05$





Fig. 3. Parameters for tuning assortativity and estimating robustness. (A) Assortativity (*r*) is shown after each of 5000 edge-swaps for 100 critical FIPO GRNs, as *r* is tuned toward 1. The grey lines represent individual GRNs, and the solid black line depicts the corresponding average. The vertical dashed line shows our choice for the maximum number of edge-swaps allowed to generate each GRN. The horizontal dashed line shows the upper assortativity bound selected for critical FIPO GRNs (Table 1). (B) Residual robustness is shown after each of 2000 steps in a random walk on the genotype networks of 100 critical FIPO GRNs. Residual robustness is calculated as the difference between the robustness measured at each step and the robustness measured at step 2000. The vertical dashed line shows our choice for the maximum number of steps allowed to estimate robustness, and the horizontal dashed line provides a reference for convergence.

3. Results

3.1. The influence of assortativity on robustness

The sensitivity of robustness to changes in assortativity varied between dynamical regimes (Fig. 4). Ordered and critical GRNs displayed slopes close to zero, indicating a general insensitivity to changes in assortativity. In contrast, the slopes for chaotic GRNs were steeper. Chaotic GRNs are therefore more sensitive to changes in assortativity, with robustness increasing as the GRN becomes more positively assortative. These trends were consistent across the three degree distributions tested.

3.2. The influence of assortativity on attractor length

The sensitivity of attractor length to changes in assortativity also varied between dynamical regimes (Fig. 5). Except for the ordered PIPO GRNs (Fig. 5D), the directions of the trends were the opposite of those observed for robustness (Fig. 4), indicating that shorter attractors result in higher robustness. Structurally altering the topology of GRNs in order to achieve different assortativity values therefore influences their dynamical behavior.

3.3. Assortativity, in-components, and attractor length

For each combination of degree distribution and dynamical regime, mean IC size was negatively correlated with assortativity (Fig. 6). An increase in assortativity was therefore marked by a corresponding decrease in mean IC size, which had a direct influence on attractor length (Fig. 7). In all but a single case (see Section 3.5), attractor length increased as mean IC size increased. Thus, an increase in assortativity leads to a decrease in mean IC size, which generally leads to a reduction in attractor length.

3.4. Relating in-components and attractor length to robustness

To provide a mechanistic explanation for the relationship between assortativity and robustness, we draw the following connection between mean IC size and attractor length.

High assortativity can be achieved by increasing the number of edges that exist between highly connected nodes, while simultaneously reducing the number of edges that exist between these nodes and the rest of the GRN. For the degree distributions considered in this study, there are few highly connected nodes. Thus, placing edges between them and encouraging their isolation from the other nodes in the GRN results in the presence of small ICs. As such, increasing assortativity leads to the formation of smaller ICs (Fig. 6).

Since they receive no inputs from the rest of the GRN, ICs behave autonomously. Therefore, an IC operates like a small Boolean network that is independent of, yet nested within, a larger Boolean network (Fig. 8). Smaller Boolean networks generate shorter attractors (Kauffman, 1993), so a small IC will provide a simple regulatory signal (in the form of a short attractor) to other parts of the GRN, reducing the attractor length of the GRN as a whole. Decreasing the mean IC size thus leads to a decrease in attractor length (Fig. 7).

Attractor length is directly related to robustness. Attractors are determined by accessing entries in the rule vector until an expression state repeats, and longer attractors access more entries than shorter attractors. Robustness is higher when there are more unaccessed entries in the rule vector, as perturbing these entries does not affect the attractor. Thus, reducing attractor length increases robustness (Figs. 4 and 5).

Hence, the mechanism by which assortativity influences robustness: increased assortativity reduces mean IC size, which reduces the length of attractors, which increases robustness. This effect is most apparent in chaotic GRNs (Figs. 4–7). An intriguing counterexample is provided by the ordered FIPO GRNs.

3.5. The counterexample: in-components as forcing structures

In almost all cases, there is a positive correlation between mean IC size and attractor length (Fig. 7). However, ordered FIPO GRNs display a negative trend, where larger mean IC size is associated with shorter attractors (Fig. 7A). This phenomenon can be understood as follows.

FIPO GRNs have connectivity restrictions that limit each node to exactly one input. This causes the smallest IC in a GRN to form a cycle (Fig. 8A and B). Such a cycle acts as a "forcing structure" (Kauffman, 1990), where the state of a single node in the cycle decides the states of all other nodes in the cycle. When the smallest IC is a large forcing structure (Fig. 8B), the propagation of such "frozen nodes" (Kauffman, 1990) reduces the attractor



Fig. 4. Robustness as a function of assortativity. Observed robustness is shown for three different degree distributions: (A–C) Fixed-in/Poisson-out (FIPO), (D–F) Poisson-in/Poisson-out (PIPO), and (G–I) Poisson-in/Power-law-out (PIPLO), and three different dynamical regimes: (A,D,G) ordered, (B,E,H) critical, and (C,F,I) chaotic. In each panel we provide data for 75,000 GRNs: 5000 at each of 15 different linearly spaced assortativity values that lie between the assortativity bounds for that combination of degree distribution and dynamical regime (Table 1). We place observations in two-dimensional hexagonal bins and shade these bins by their counts (darker colors indicate higher counts) to show relative densities. Note that the gray scale in each panel is normalized to the data in that panel, and therefore bin shade combination of degree distribution and dynamical regime (see Section 2.7). Solid best-fit lines are provided as visual guides, where *m* is slope and *R* is Pearson's coefficient (all $p \leq 0.001$).

length. Thus, in FIPO GRNs, the reduction of mean IC size that accompanies increased assortativity results in an increase in mean attractor length (Fig. 5A) and a corresponding reduction in robustness (Fig. 4A).

3.6. Sensitivity to changes in assortativity

Assortativity affects robustness more strongly in the chaotic regime than in the critical and ordered regimes (Fig. 4). One possible explanation for this is that chaotic GRNs are more sensitive to change in general, either in topology, rule vector, or initial state (Kauffman, 1993). An alternative explanation is that the sensitivity of a GRN to factors that influence its robustness is a function of its variance in attractor length. We therefore plot the strengths of the trends observed in Fig. 4 against the standard deviations of attractor lengths for each combination of degree distribution and dynamical regime (Fig. 9). The positive

correlation suggests that if a GRN can access attractors whose lengths deviate widely from the mean, changing its assortativity will have a greater impact on robustness.

4. Discussion

This study provides the first direct evidence that degreedegree assortativity influences the robustness of the signalintegration logic of computational models of gene regulatory networks (GRNs). This occurs via the modification of in-component (IC) sizes, which has a direct effect on attractor length, and thus robustness. To the best of our knowledge, this is the first study to show a direct relationship between IC sizes and the dynamics of computational models of GRNs.

We used linear regression to approximate the sensitivity of robustness to assortativity. However, some of the observed trends



Fig. 5. Mean attractor length as a function of assortativity. Observed mean attractor length is shown for the same GRNs as in Fig. 4. Hexagonal binning and dashed lines are as in Fig. 4. The GRNs with the largest 1% mean attractor lengths have been excluded for ease of visualization. Best-fit lines of displayed data are provided as visual guides, where *m* is slope and *R* is Pearson's coefficient (all $p \ll 0.001$). Note the logarithmic scale on the *y*-axis.

were nonlinear, exhibiting insensitivity below a critical assortativity value and sensitivity above that value. This may reflect a phase transition in the structure of ICs as assortativity increases, similar to the percolation of the giant component in undirected networks (Newman, 2002).

To clarify the relationship between the dynamics of GRNs and robustness, we intuitively linked increasing attractor length to decreasing robustness. Additional supporting evidence for this relationship can be found in studies of evolving populations of GRNs (Bornholdt and Sneppen, 2000; Mihaljev and Drossel, 2009), where selection for increased robustness leads to a decrease in attractor lengths, relative to random GRNs. Further support can be found in related models of GRNs (Wagner, 1996), where robustness scales inversely with attractor length (Luo and Turner, 2011).

The relationship between assortativity and robustness was consistent across the three degree distributions tested, contrasting with the dynamics of GRNs reported in some related studies, which vary between scale-free and Poisson degree distributions (Aldana and Cluzel, 2003; Aldana et al., 2007; Oikonomou and Cluzel, 2006). This general insensitivity to degree distribution results from the fact that changing assortativity leads to the rewiring of nodes in a fashion that is independent of degree distribution, and attractor lengths were therefore similarly affected across the different degree distributions tested.

In contrast, the relationship between assortativity and robustness varied between dynamical regimes. Specifically, ordered and critical GRNs were relatively insensitive to changes in assortativity, whereas chaotic GRNs exhibited a greater sensitivity. Empirical evidence indicates that biological GRNs may operate in the critical or ordered regimes (Shmulevich et al., 2005; Nykter et al., 2008). For example, studies have been conducted that measured the information theoretic content of global gene expression patterns produced by the underlying GRNs of HeLa cells (Shmulevich et al., 2005) and murine bone marrow-derived macrophages (Nykter et al., 2008). These results were then compared to the information contained in the attractors of Boolean models operating in the ordered, critical, or chaotic regimes, and it was shown that critical models produced dynamics with information complexity most similar to that of the biological datasets. While such results suggest that biological GRNs may be insensitive to changes in assortativity, our results further indicate that the relationship between assortativity and



Fig. 6. Mean in-component size as a function of assortativity. Mean in-component (IC) size is shown for the same GRNs as those in Fig. 4. Hexagonal binning and dashed lines are as in Fig. 4. Best-fit lines are provided as visual guides, where *m* is slope and *R* is Pearson's coefficient (all $p \ll 0.001$).

robustness may also be a function of the diversity of attractor lengths that a particular GRN can access. Since attractor length increases with the number of nodes in a GRN (Kauffman, 1993), the relatively larger GRNs of biological organisms may be more sensitive to changes in assortativity than the smaller GRNs tested herein, even if they operate in the critical regime.

Evidence that assortativity may influence the dynamics of GRNs was initially set forth by Pomerance et al. (2009), who introduced a framework for predicting the stability of GRNs with varying assortativity. Their measure of stability averaged the Hamming distance between expression states over time as the signal-integration logic was continuously perturbed. This "semiannealed" procedure found disassortative GRNs to be the least stable, which agrees with our observation that robustness is positively correlated with assortativity in critical and chaotic GRNs.

4.1. Future directions

Robustness is defined as a function of the specific type of perturbation a system may suffer, and in this study we examined the effects of point mutations to the signal-integration logic of GRNs. Another source of genetic variation to consider is recombination. Using a variation of the model considered here (Wagner, 1996), Martin and Wagner (2009) investigated the effects of both point mutations and recombination on the phenotypic robustness of GRNs, and found that GRNs are more robust to recombination than to mutation. Understanding how assortativity influences a GRN's robustness to recombination is another exciting direction for future research.

In addition to robustness, evolvability is also an important property of biological systems (Wagner, 2005). While robustness is characterized by a system's insensitivity to perturbations, evolvability is characterized by a system's capacity to utilize these perturbations for the exploration of novel phenotypes. Theoretical studies of the relationship between these two properties have shown that evolving systems often tend toward robust phenotypes (Cowperthwaite et al., 2008; Payne and Moore, 2011), allowing the population to diffuse neutrally throughout the underlying genotype network. This permits the accumulation of genetic diversity (Huynen et al., 1996), which increases evolvability by facilitating access to novel phenotypes (Wagner, 2008b; Draghi et al., 2010). Since robustness is influenced by assortativity, evolvability may be as well, and future work will examine how assortativity impacts the relationship between robustness and evolvability.



Fig. 7. Mean attractor length as a function of mean in-component size. Mean attractor length is shown for the same GRNs as those in Fig. 4. Hexagonal binning is as in Fig. 4. The GRNs with the largest 1% mean attractor lengths have been excluded for ease of visualization. Vertical dashed lines show minimum and maximum mean IC size for the middle 95% of the null distribution for each combination of degree distribution and dynamical regime (see Section 2.7). Best-fit lines of displayed data are provided as visual guides, where *m* is slope and *R* is Pearson's coefficient (all $p \ll 0.001$). Note the different scales on the *y*-axes.

This relationship has been investigated at the genotypic level in uncorrelated Boolean networks subject to random node duplication (Aldana et al., 2007). Critical GRNs were found to simultaneously maximize the conservation of existing attractors (robustness) and the generation of new attractors (evolvability), which was cited as evidence supporting the hypothesis that realworld biological GRNs would benefit most from being critical, as opposed to chaotic. However, the influence of nonrandom assortativity was not considered. Our results show that chaotic GRNs exhibit increasingly robust phenotypes with increasing assortativity, whereas critical GRNs are relatively insensitive to such changes. Therefore, an examination of how the robustness and evolvability of assortative chaotic GRNs compares to uncorrelated critical GRNs is warranted.

As a proxy for measuring evolvability, previous computational studies have investigated the ability of GRNs to match prespecified target expression patterns (Oikonomou and Cluzel, 2006; Greenbury et al., 2010), which represent adaptation to some environment. While several topological properties, including degree distribution, have been found to impact the target-matching abilities of GRNs (Oikonomou and Cluzel, 2006; Greenbury et al., 2010), the influence of assortativity is not yet known, and requires further investigation.

The results presented in this study link assortativity to robustness in a computational model of GRNs, which leaves open the question of whether assortativity might influence real biological GRNs. Over the last decade a wealth of data on biological GRNs has been collected, which promises to vield new insights into how GRNs ensure robust gene expression (Macneil and Walhout, 2011). Methods such as chromatin immunoprecipitation (ChIP) have been used to experimentally identify transcription factor (TF) binding sites in the fly, D. melanogaster, during specific stages of development, which was shown to be predictive of gene expression patterns across both spatial and temporal dimensions (Zinzen et al., 2009). ChIP data is also being generated and analyzed by the ENCODE (ENCODE Project Consortium et al., 2011) and modENCODE (Celniker et al., 2009) projects, one of whose ambitious goals is to catalogue the genome-wide binding events of hundreds of TFs across multiple independent systems that span human, mouse (Mus musculus), fly (D. melanogaster), and nematode (Caenorhabditis elegans). In a separate approach, yeast one-hybrid (Y1H) assays have been used to experimentally



Fig. 8. The smallest in-components in examples of FIPO GRNs. Highlighted in grey is the smallest in-component (IC) that exists in each GRN for the ordered (A and B), critical (C and D), and chaotic (E and F) regimes. GRNs are assortative (r > 0.3) in the top row (A,C,E) and disassortative (r < -0.3) in the bottom row (B,D,F). The smallest ICs in disassortative GRNs are considerably larger than those found in assortative GRNs. Although we report that mean IC size influences GRN dynamics, we here show the smallest ICs for ease of visual display. Mean IC size and the size of the smallest IC are correlated in FIPO GRNs.



Fig. 9. The slope of robustness versus assortativity is correlated with the standard deviation of the mean attractor lengths. The letters associated with each symbol correspond to the letters shown in the panels of Figs. 4–7. Symbol types denote dynamical regime (squares: ordered, triangles: critical, circles: chaotic). Y-axis slope values are the same as those reported in Fig. 4.

map a post-developmental GRN of metabolic genes in the nematode, *C. elegans* (Arda et al., 2010), and a root stele tissue-specific GRN with both TFs and miRNAs in the plant, *A. thaliana* (Brady et al., 2011). Efforts have also been aimed at reconstructing GRNs from gene expression datasets, for example inferring and systematically testing a GRN controlling the murine dendritic cell response to pathogens (Amit et al., 2009), and reverse-engineering a glioma-specific GRN to identify how mesenchymal genes are turned on during malignancy (Carro et al., 2010). Another recent enterprise was the inference of the whole-genome set of micro-RNA regulatory interactions implicated in glioblastoma (Sumazin et al., 2011), which provided a layer of regulatory information complementary to that of genome-wide TF binding.

The comparison and integration of data from these different approaches will produce a more complete picture of biologically relevant GRNs (Walhout, 2011), the prospects of which will be the ability to examine these networks for topological features that explain their functional properties. Therefore, an important next step is to investigate whether robustness might come about through changes in assortativity in these biological networks. Foster et al. (2010) analyzed a variety of directed real-world networks to examine whether the assortativity values that characterize such networks are any different from those expected at random. In a separate study, Johnson et al. (2010) established that highly heterogeneous degree distributions are expected to produce disassortative networks for statistical reasons, and provided examples of disassortative biological and technological networks that fail to differ from the neutral expectation. Such analyses have yet to be applied to biological GRNs, and are among the means that are necessary to determine (1) if nonrandom assortativity exists, and if so, (2) how and why assortativity evolved in these biological networks. These extensions, among others, will further our understanding of the relationship between assortativity, IC size, and robustness in biological GRNs.

Acknowledgements

D.A.P. was supported by NIH Grant Nos. LM010098, LM009012, AI59694, and Award Number T32GM008704 from the National Institute of General Medical Sciences. J.L.P. was supported by NIH Grant No. K25-CA134286. The authors would

like to thank Davnah Urbach for her discerning comments on an earlier version of this manuscript, which greatly improved both its clarity and overall quality.

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