Emergence of cooperative bistability and robustness of gene regulatory networks

Shintaro Nagata\(^1\,\,^2\) and Macoto Kikuchi\(^2\,\,^1\,\,^3\)

\(^1\)Department of Physics, Osaka University, Toyonaka 560-0043, Japan
\(^2\)Cybermedia Center, Osaka University, Toyonaka 560-0043, Japan
\(^3\)Graduate School of Frontier Bioscience, Osaka University, Suita 565-0871, Japan

Gene regulatory networks (GRNs) are complex systems in which many genes mutually regulate their expressions for changing the cell state adaptively to the environmental conditions. Besides the functions, the GRNs utilized by living systems possess several kinds of robustness. Here, the robustness means that the GRNs do not lose their functions when exposed to mutation or noises. Both the adaptive response and the robustness have been acquired through the evolution. In this respect, real GRNs are rare among "all the possible GRNs". In this study, we explore the fitness landscape of GRNs and investigate how the robustness emerge in the "well-fitted" GRNs. For that purpose, we employ the Multi-Canonical Monte Carlo method, which can sample GRNs randomly in wide range of fitness. We consider a toy model of GRNs having one input gene and one output gene. The difference in the expression levels between the input states "on" and "off" is taken as the fitness. Thus the more sensitively a GRN responds to the input, the fitter it is. We show the following properties for the GRNs in the "fittest ensemble": (1) They distinguish two different states of the input by switching the fixed points. Thus they exhibit bistability, which necessarily emerges as the fitness becomes high. (2) They are robust against noises thanks to the bistability. (3) Many GRNs in the fittest ensemble are robust against mutation. These properties are universal irrespective of the evolutionary pathway, because we did not perform evolutionary simulations.

INTRODUCTION

Living systems have been formed through a long history of Darwinian evolution. And thus they have acquired totally distinct properties from other physical systems. First of all, they have developed biological functions. For example, in the molecular level, many different proteins have evolved so that they work under the physiological conditions. In a larger scale, the cells have developed metabolism and proliferation.

Another significant property of living systems is a several kinds of robustness\(^{1-3}\). Here, the robustness means that the system does not lose its functions when disturbed by mutation or noises. Among them the mutational robustness is of particular importance. Since the living systems are constantly exposed by mutation of genes, the systems that are not robust against mutation cannot survive in the course of evolution. Thus, it is widely considered that the mutational robustness has been acquired through the evolution. On the other hand, if we regard the process of developing functions as an optimization process, the highly optimized systems are intuitively considered as fragile against disturbances and thus it is natural to consider that they readily lose their functions by mutation. In this prospect, the process of evolution should be something different from the simple optimization process. The mutational robustness have long been discussed from many different point of views. Robustness against several types of noises, such as disturbance from the environment and the noises occur within the cell, in particular the fluctuation due to the finiteness of the number of molecules, are also important, because the living systems work stochastically in the noisy world where we are living.

As Wagner pointed out\(^2\), it is difficult to investigate the mutational robustness experimentally. Thus, numerical simulations based on mathematical models provide important information. In this paper, we investigate the relationship between the development of functions and robustness using a toy model of gene regulatory networks (GRNs). GRNs are complex networks of genes which mutually regulate their expressions for changing the cell state according to the environmental conditions. There have been a number of theoretical researches on the evolution of GRNs, most of which used evolutionary simulations\(^4-11\). But the knowledges drawn from this type of studies strongly depend on evolutionary pathways.

In this paper, departing from the evolutionary process, we discuss universal aspects of function and the robustness irrespective of the evolutionary pathway. Namely, we investigate the abovementioned relationship for randomly generated GRNs and try to obtain insights on evolution from them. In other words, we explore the landscape picture of evolution. Landscape pictures have been discussed on living systems in many different levels. Examples are the energy landscape for protein folding\(^{12}\) and the epigenetic landscape for development\(^{13}\). Fitness landscape (or alternatively, adaptiveness landscape), in which the fitness distribution in the multidimensional genotypic space is considered, is an old concept for discussing the evolutionary processes\(^{14}\). But it has long been left just as an abstract concept except for a very simple models until recently. For example, ruggedness of the fitness landscape has been discussed using the NK model\(^{15-17}\). Quite recently, the empirical fitness landscapes are becoming available\(^{18}\).
We explore the different type of the fitness landscape in which the fitness is taken as a parameter instead of the genotype. Namely, we investigate what properties emerge as the fitness becomes higher. Research in this direction was made by Ciliberti et al.\cite{19, 20}. They sampled GRNs randomly and found that a majority of functional GRNs are connected with each other by successive mutations like the neutral network found for RNA sequence space \cite{21}. In other words, they form a large cluster in the neutral space, which means the genotypic populations that share the same fitness \cite{2}. The genotypes belong to such cluster are consider to robust against mutations. Namely, for such genotypes the possibility that they stays in the same neutral space is high even when some of the genes are mutated. The concept of the neutral space is also a landscape-type point of view. It should be noted, however, that the fitness of the model used by Ref. \cite{19} was two valued: whether viable or not. This simplification made it possible to investigate the neutral space by the random sampling of the GRNs.

We consider a model of GRNs that has a continuous fitness. We construct ensembles of GRNs in which possible networks are classified according to the fitness, and investigate how characteristic properties of GRNs change with the fitness. The constant-fitness ensemble introduced in this study is a very close in concept to the neutral space. But we do not discuss how the functional GRNs connect to each other through mutation in this paper. If the evolution is the process that the fitness is gradually optimized, it should be a successive transition between the constant-fitness ensembles to the direction of higher fitness. If there are some universal properties emerging in that process, they should be observed irrespective of the evolutionary pathway.

Since GRNs having high fitness are rare, the simple random sampling method is not appropriate for obtaining sufficient number of samples of such GRNs. Thus we employ a rare event sampling method, which has been developed in the field of the equilibrium statistical mechanics.

**MODEL**

We consider random GRNs which has one input gene and one output gene. It can be regarded either as a GRN directly respond to the environmental change or as a part of a larger GRN. We ignore detailed mechanism of expression of transcription factors, and deals only the regulatory relations between the genes. Namely, we consider a connectionism-type model \cite{22}. A GRN is represented by a directed random graph in which nodes correspond to the genes and edges correspond to the regulatory interactions. In order to exclude genes not participating in the function, we require that paths from the input gene to all the other genes exist and also paths to the output gene from all the other genes exist. For simplicity, both the mutual regulation of two genes and the self-regulating loop are prohibited. Although they are frequently observed in real GRNs, we think these restrictions do not cause any problem for the purpose of this study. Regulations on the input gene from other genes and regulations from the output gene on other genes are permitted.

The GRNs have $N$ nodes and $K$ edges. The average number of edges connected to a node is represented as $C \equiv 2K/N$. The node number one is assigned to the input node. How to determine the output edge will be described later. Fig.1 shows an illustrative example of a randomly-generated GRN of $N = 6$ and $K = 15$.

Each node is assigned a variable called "expression" $x_i$, where $i$ is the node number and $x_i$ takes a continuous value in $[0, 1]$. The expression obeys the following discrete time dynamics, which is similar to that of a neural network model:

$$x_i(t + 1) = R \left( I(t) \delta _{i,1} + \sum _{j \neq i} J_{ij} x_j(t) \right),$$  \hspace{1cm} (1)$$

$$R(x) = \frac{\tanh x + 1}{2},$$  \hspace{1cm} (2)$$

where $I(t)$ is the input signal at time $t$, which is applied only to the input node. $J_{ij}$ expresses the regulating interaction from $j$-th node to $i$-th node. For simplicity, all the amplitudes of the regulation are taken as $|J_{ij}| = 1$, and 0 or $\pm 1$ are assigned randomly to $J_{ij}$: $J_{ij} = 0$ means that there is no regulating interaction from $j$-th gene to $i$-th gene, and $J_{ij} = 1$ and $-1$ represent the activation and the repression, respectively. Since the self-regulation

![FIG. 1. An example of the random GRN having one input node and one output node for $N = 6$ and $K = 15$. I and O indicate the input node and the output node, respectively. The blue lines are activating regulations and red ones are repressing regulations.](image-url)
is not permitted, \( J_{ii} = 0 \). \( J_{ji} = 0 \) when \( J_{ij} \neq 0 \), because the mutual regulation is absent.

The response function \( R(x) \) is a gradually-varying sigmoidal function, which reflects the fact that the responses in the living systems are imprecise and stochastic (or "sloppy" according to Ref. [11]). Each node exhibits the spontaneous expression \( x_i = 0.5 \) even when no input is provided. Although the Hill function is frequently used to express the response of GRN, we consider there is no essential problem in using this tanh function \([8]\).

This model is basically the same as the one proposed by Wagner \([4, 5]\). But there are two different point. First, only the first node receive the input signal from outside. Second, the response function is a sigmoidal function instead of the sign function, and thus the expression takes continuous value. In this respect, the model is close to the one used in Ref\([6]\). Thanks to the second point, we can define a continuous fitness, which will be explained below. This model can also be regarded as a discrete-time version of the one used by Inoue and Kaneko \([10]\), apart from the normalization and the degradation effect. Since the expression decays as transmitted from one gene to the next in the sequential circuit, the feed-forward type regulations are mandatory in order that a large expression to occur at the output gene.

Assuming that the environment takes two different states, we require GRN to respond to the difference between \( I = 0 \) and 1 as sensitively as possible. Namely, we make a discriminating circuit for 0 and 1. For that purpose, we define the response of \( i \)-th gene to the input as the temporal average of the expression at the steady state:

\[
\bar{x}_i(t) = \frac{1}{T} \sum_{t=t_0}^{t_0+T} x_i(t),
\]

where \( t_0 \) is the time required for the initial relaxation, and \( T \) is a long enough time for taking the average. If the dynamical system reaches the fixed point, taking the average is not necessary. In fact, as we will see later, the system reaches the fixed point in most cases. If the system exhibits a limit cycle or chaos, although very rare, the response takes comparatively small value because of the temporal average. As the initial state, we assume that all the genes exhibit the spontaneous expression and set \( x_i = 0.5 \). The initial state dependence will be discussed later.

The sensitivity of \( i \)-th gene is defined as the difference of the response of the gene for \( I = 0 \) and 1 as follows:

\[
s_i = |\bar{x}_i(1) - \bar{x}_i(0)|.
\]

It should be noted that we only require the absolute value of the difference between \( \bar{x}_i(1) \) and \( \bar{x}_i(0) \) to become large in this definition and which one is larger is not relevant. The node that exhibits the largest sensitivity except the input node is selected as the output node. Thus no other node has larger sensitivity than the output node: this definition is just for convenience. We regard the sensitivity \( s_{out} \) of the output node as the sensitivity of the whole network and call it as the "fitness" \( f \). Since we will not perform the evolutionary simulation, the term "fitness" simply means the degree of functionality.

**METHOD**

Our purpose is to classify the randomly generated GRNs according to the fitness and investigate their universal properties. To this end, we would like to sample a large number of GRNs having a variety of values of the fitness. But since GRNs with high fitness are expected to be rare, simple random sampling is considered to be useless. Then we employ the rare-event sampling based on the multicanonical Monte Carlo (McMC) method\([23, 24]\). McMC belongs to the Markov chain Monte Carlo methods and has originally been developed in the field of the equilibrium statistical mechanics. In terms of the statistical mechanics, it realizes the uniform sampling in energy. For that to be possible, the appearance probability of the microscopic states of the energy \( E \) should be made inversely proportional to the number of states \( \Omega(E) \) having the same energy. In the Metropolis method, the following detailed balance is assumed between the transition probability \( W_{ij} \) from \( j \)-the state to \( i \)-th state and its inverse process:

\[
W_{ij}P_{eq}(E_j) = W_{ji}P_{eq}(E_i),
\]

where \( P_{eq}(E) \) represents the appearance probability of the microscopic state of the energy \( E \) in thermal equilibrium. While the Gibbs distribution is used as \( P_{eq}(E) \) in ordinary Metropolis method, the flat energy distribution is obtains if \( P_{eq}(E) \propto 1/\Omega(E) \).

Although the number of states \( \Omega(E) \) is not known beforehand, only a rough estimation is sufficient for the sampling purpose. Thus we perform the computation in two steps: The first is the learning process to determine the weight, namely the approximate value of the appearance probability, for each energy. The second step is the measurement process by the Metropolis method using those weights. In case that the energy takes continuous values, the whole range of the energy is divided into bins and \( \Omega(E) \) is approximated by a piecewise linear function in the original McMC. Then the probability distribution of the energy in each bin is regarded as the canonical distribution of constant temperature which differs from bin to bin. But we assign a constant weight within each bin. In this case, the energy distribution in each bin is the microcanonical distribution. This latter method is called the "entropic sampling" \([25]\), which is one of variants of McMC. By using this method, we can sample the microscopic states having wide range of energy.
randomly in principle. We can estimate the appearance probability of the energy corresponding to each bin from the histogram and the weight. The energy distribution in a bin obtained by the entropic sampling is not uniform but proportional to the number of states having the same energy. Thus for the thermodynamic systems the number of states that appear in the simulation within each bin decays exponentially as the energy increases. It should be noted that since this method is based on the Markov chain Monte Carlo, the successive samples resemble each other and thus samples should be taken at interval to reduce the correlations.

By regarding quantities other than the physical energy as the energy, this method can be applied to a variety of problems. It is particularly suitable for counting the rare state or for estimating the appearance probability of rare events[26–31].

In this study, we perform the entropic sampling by regarding the fitness as the energy. We estimate the appearance probability of each fitness and sample GRNs having wide range of the fitness. As the elementary process of the Monte Carlo method, we replace a randomly chosen edge to another place and select the input and output nodes as the abovementioned conditions are satisfied. Thus K is kept constant. This elementary process is only for sampling GRNs in McMC and do not relate to any evolutionary process.

For the networks having K edges, we define K trials of elementary processes as one Monte Carlo step (MCS). We obtain the fitness at every MCS; on the other hand, we sample GRNs at every 10 MCS to reduce the correlations. We employ the Wang-Landau method [32, 33] to determine the weights. Since the fitness f takes continuous values within [0, 1], we divide this range into 100 bins. From now on, we call the set of GRNs having the maximum fitness, that is, the set of GRNs in the bin \( f \in [0.99, 1] \) as "the fittest ensemble". In order to check the consistency of the result, we perform 5 independent runs of the entropic sampling. Thus 5000 GRNs on average are obtained as a total for each bin.

**RESULTS**

We performed computations on the networks of \( N = 16 \sim 32 \) and \( C = 5 \) and 6. In the following, we show mainly the results for \( C = 5 \), otherwise stated.

**Fitness Landscape**

Figure 2 shows the relative number of states \( \Omega(f) \) of GRNs in each bin \([f, f + 0.01]\). The sum of \( \Omega(f) \) is normalized to unity. Although this figure does not represent a conventional fitness landscape in which the fitness are drawn in the genotopic space, we may call it as "fitness landscape" in the same sense as the energy landscape in the protein folding problem. In fact, since the vertical axis is in the logarithmic scale, it can be interpreted as the entropy of each bin of the fitness. A majority of GRNs have small \( f \); for example, in case of \( N = 32 \), more than 97% of GRNs are within the range \( f < 0.2 \). The fitness landscape bends at \( f \approx 0.2 \) and the GRNs become exponentially rare as \( f \) increases. The slopes are different for different \( N \). For \( f > 0.8 \), the GRNs become rarer with \( f \) more than exponentially. The probability of the GRNs participating in the fittest ensemble is as small as about \( 3 \times 10^{-19} \) for \( N = 32 \).

**Emergence of cooperative bistability**

We investigated how the steady-state response changes with the input. Fig.3 show the response \( x_{out} \) against \( I \), with the initial state being set as \( x_0(0) = 0.5 \), namely all the nodes exhibit the spontaneous expression initially. The fixed-point values of randomly chosen 20 GRNs for (a) the bin \( f \in [0.7, 0.71] \) and (b) the fittest ensemble are plotted. Since the fitness expresses the difference of the response for \( I = 0 \) and 1, the response can either be an increasing function or decreasing function of \( I \). In the cases of \( f \in [0.7, 0.71] \), most of the GRNs respond smoothly to the change of input; some of them are ultrasensitive[34, 35] and a few among the ultrasensitive GRNs exhibit discontinuous response. On the other hand, all the GRNs exhibit discontinuous step-like response in the cases of the fittest ensemble. This type of responses have been observed for real GRNs and sometimes called the "genetic toggle switch"[36].

This toggle-switch like responses realize by two successive saddle-node bifurcation with \( I \) as the bifurcation parameter[37]. Namely, there are two stable fixed point and one unstable fixed point in some range of \( I \). Although it is difficult to identify the unstable fixed point,
FIG. 3. Examples of steady-state response of GRNs with the input $I$ as a parameter for (a) $f \in [0.7, 0.71]$ (b) the fittest ensemble. The results for randomly selected 20 samples for $N = 32$ and $C = 5$ are shown for both cases. For each value of $I$, all the expressions are set as $x_i = 0.5$ in the initial state. The temporal averages of the expression of the output gene are plotted. But GRNs reach the fixed point in all the cases shown. Thus we in fact plotted the fixed-point values.

There should be a bistable region between two bifurcation points, and the hysteresis is expected to be observed between increasing and decreasing of $I$. We examined the hysteresis of a GRN by the following way: First, taking the steady-state at $I = 0$ as the initial state, we increased $I$ by 0.001 and again ran the dynamics until the steady-state is reached. This procedure was repeated up to $I = 1$. Next, we performed the inverse process, that is, starting from the steady-state at $I = 1$ and decreased $I$ to $I = 0$. Fig. 4a shows an example of a GRN belonging to the fittest ensemble, which exhibits a clear hysteresis. We also checked GRNs that do not show the bifurcation in the ensemble for $f \in [0.7, 0.71]$ and confirmed that the response follow the same trajectory both in the increasing process and the decreasing process of $I$.

Then, we study the fitness dependence of the proportion $P_2$ of GRNs exhibiting the bifurcation. Since it is difficult to examine the bifurcation of dynamical systems with a large degrees of freedom rigorously, we employed a heuristic method: Changing $I$ at the interval 0.001 and when difference larger than 0.01 was observed in steady-state value of some $I$ between increasing process and decreasing process, we regarded that the bifurcation took place. Since a very weak bifurcation may be missed by this criterion, obtained $P_2$ is the lower limit. The cases that the bistable region includes $I = 0$ or 1 (sometimes called “one-way switch”[37]) or both are classified into the bifurcating cases. Fig 5 shows $P_2$ against $f$. $P_2$ exhibits a sigmoidal increase and for the fittest ensemble for $N = 32$ it reaches 99.9% (4524 among 4528 for $C = 5$ and 4785 among 4787 for $C = 6$). Since we do not observe a significant size dependence, it is not considered as a phase transition. But there is a characteristic value of $f$ that the bistable GRNs starts to appear. From the tendency of increase, we expect that $P_2$ approaches 1 as $f \to 1$. This means that the GRNs necessarily become bistable as the fitness becomes high, irrespective of the evolutionary pathway.

Of course, in case that the bistable range includes $I = 0$ or 1 or both, the GRN may not be able to follow the dy-
namical change of input. Fig.6 shows an example of dynamics of a GRN in the fittest ensemble, which can follow the change of input; the instantaneous response $x_{out}(t; I)$ is plotted in case that $I = 0$ for the first 1000 steps, 1 for the next 1000 steps and again 0 for the last 1000 steps. But there are GRNs that cannot follow the same change of $I$ because being trapped by the wrong fixed point, as will be shown later. For $N = 32$, we found that 28% of GRNs (1263 out of 4528) in the fittest ensemble can follow the input. Although we did not require the ability to follow the input in defining the fitness, we may add it to this requirement afterwards. For example, the fitness for GRNs which does not follow the input change may be defined as 0. Then the fittest ensemble will be reduced in size to 28%. We can add or modify fitness in this way after the computation is done in our method. From now on, we consider the fittest ensemble that includes GRNs which lack an ability to follow the change of input.

Robustness against input noise

In what follows we discuss several kind of robustness for all the 4528 GRNs in the fittest ensemble. First, the robustness against the input noise is considered. We suppose the number fluctuation of the input molecules as the source of the noise. We observed the instantaneous response for the change of input $I = 0 \rightarrow 1 \rightarrow 0$ as before. But this time the uniform random number in the range $[-0.3, 0.3]$ was added as the noise. Although $I$ become negative from time to time by this procedure, we think it does not cause problem for investigating the effect of the noise qualitatively.

Fig.6a is the dynamics of the same GRN as used in Fig.5 with the noise added. Both the input and the response are plotted. We see that the response is stable despite the noisy input. This is clearly the consequence of the bistability and the GRN works as a low-pass filter. We confirmed that the GRNs that can follow the input properly in the absence of the noise can follow also the noisy input. Moreover, the number of the GRNs that can follow the input increased to be 1506 under this condition. An example is shown in Fig.6b. This implies that the lock to the wrong fixed point comes off by the effect of the noise. This effect may be called as “the noise-induced response” (NIR). We will discuss NIR in detail in the discussion on the internal noise.

Robustness against internal noise

Next, we discuss the robustness against the internal noise. The number fluctuation of, this time, the transcription factors are supposed as the noise source. The dynamics is modified as

$$x_i(t + 1) = R \left( I(t) \delta_{i,1} + \sum_{j \neq i} J_{ij}(x_j(t)) + \xi_{ij}(t) \right), \quad (6)$$
where $\xi_{ij}(t)$ is the internal noise added to the expression of $j$-th gene when regulating $i$-th gene. The uniform random numbers in the range $[-0.1, 0.1]$ were used as $\xi_{ij}(t)$.

The temporal responses to the changing input both the cases with and without the internal noise are plotted in Fig.7. Figure 7a is for the same GRN as Fig.5, which can follow the change of input. On the other hand, Fig.7b is for a GRN which cannot follow the input without noise because of being trapped by the wrong fixed point. This GRN successfully follow the input when the internal noise is applied, although a bit noisy. Namely, NIR by the internal noise is observed in this case. We found that the number of GRNs having the ability to follow the input increases to 46% (2086 out of 4528) for this condition. The ratio depends on the amplitude of the noise. When a larger noise $\xi_{ij} \in [-0.2, 0.2]$ is applied, while the ratio of NIR increases, some GRNs which can follow the input without noise loose that ability.

The robustnesses against both the input noise and the internal noise are the consequences of the bistability. In spite of the fact that we have not required these robustnesses as the fitness, the bistable GRNs appear as the fitness grows and as a byproduct they acquire the robustness against noises automatically. That is, the robustness against the noises is an accompanying property of the high fitness.

**Robustness against mutation**

Finally, we discuss the robustness against mutation. We investigated the effect of the simplest mutation, that is, the deletion of one of the edges. This mutation is regarded to represent the situation that the affinity between a gene and a TF is lowered by a slight mutation occurring at the TF or at the TF binding site.

For investigating the robustness, we computed the fitness $f^\prime$s after all the possible mutation for all the GRNs in the fittest ensemble. For each GRN, the input gene and the output gene were kept same as those before the mutation. The color map in Fig.8a shows the logarithm fo the probability distribution of $f^\prime$, $\log_{10} P(f^\prime)$, against $f$. The sum of $P(f^\prime)$ in each bin of $f$ is normalized as unity. The bins that $P(f^\prime)$s are less than $1/1000$ are not indicated.

An inverse-$\lambda$ distribution is clear seen. First, most of $f^\prime$s do not differ largely from the original $f$s. That is, the fitness does not change much by the single-edge deletion. But the edges that the fitness drops significantly when deleted start to appear near $f \approx 0.6$. As $f$ increases, a peak of $f^\prime$ becomes visible near $f^\prime \approx 0$ and the proportion of the edges having intermediate $f^\prime$ decreases. Figure8b shows the distribution of all $f^\prime$ for the fittest ensemble. The edges are divided into two: ones that $f^\prime$ stays high and ones that $f^\prime$ become almost zero. Although there are a few intermediate edges, they are scarce and not visible in the figure. Namely, as the fitness becomes high, the lethal edges start to appear and edges are divided into neutral ones and lethal ones. A majority of the edges are neutral against mutation and the lethal edges are less even for the fittest ensemble.

We counted the number of the lethal edges $n_L$ for each GRN in the fittest ensemble. Figure 8c shows the probability distribution $P(n_L)$. Here we take a large threshold and regarded $f^\prime < 0.9$ as the criterion for the lethal edges. But since most of the edges are divided into those with $f^\prime \approx 1$ and $f^\prime \approx 0$, this choice of the threshold affect the result only a little. Although there is a size effect in $P(n_L)$, interestingly the peak position does not depend on size significantly. The typical numbers of the lethal edges are about 6 and 7 for $C = 5$ and 6, respectively. This implies that large GRNs readily become relatively more robust than smaller ones. There are completely robust GRNs without lethal edge, although scarce. For $N = 32$, the number of such GRNs is 17 out of 4528, which is a small number but they are not extremely rare
FIG. 8. (a) Probability distribution $P(f')$ of the fitness $f'$ after the single-edge deletion against $f$ for $N = 32$ and $C = 5$. The values of $f'$ are divided into 100 bins as $f$. Sum of $P(f')$ for each bin of $f$ is normalized to unity. The bins for $P(f') < 0.001$ are not shown. (b) Probability distribution $P(f')$ for the fittest ensemble for $N = 32$ and $C = 5$. (c) Probability distribution $P(n_L)$ of the number of the lethal edges $n_L$ for $C = 5$.

As a stronger mutation, we tried the single-node deletion, that is, a knock out of a single gene. Again, the genes are divided into neutral ones and lethal ones for the fittest ensemble. The probability distribution of the number of the lethal nodes are plotted in Fig.10. Because the effect of the mutation is strong, we did not find GRNs without lethal nodes. But relatively robust GRNs considering the rareness of the fittest ensemble. An example of such GRN is given in Fig.9.

FIG. 9. An example of the GRN without a lethal edge in the fittest ensemble for $N = 32$ and $C = 5$. I and O indicate the input gene and output one, respectively. The blue lines and the red lines are the activation and the repression, respectively.

FIG. 10. Probability distribution $P(n_{LN})$ of the number of the lethal nodes $n_{LN}$ for $C = 5$ that have only a few lethal nodes are not extremely rare.
Discussions

We have performed a multicanonical Monte Carlo computation to sample from a random gene regulatory network (GRN). By classifying them according to the fitness, which is high when the sensitive response to the input is realized, we have investigated fitness-dependent properties of these GRNs. The result strongly suggests that all the GRNs having the maximum fitness exhibit bistability (saddle-node bifurcation).

The bistability and the hysteresis are observed widely in the living systems such as lac operon, the family of MAPK cascades, and the bacteriophage λ, and have been extensively studied both experimentally and theoretically [36–59]. Thus the bistability certainly plays important roles in cells. However, most of the bistable systems so far studied consist of a relatively small number of components. In contrast, the bistable GRNs we found in this study consist of larger number of genes, and the bistability is realized as a cooperative phenomenon of them. The present result indicates the possibility that large bistable GRNs are generated by evolution. The positive feedback in the network is considered as a key ingredient for realizing the bistability[40, 58]. But the bistable GRNs we obtained are very much complex so that the analysis of the network topology will be left for future studies.

Inoue and Kaneko[11] argued that a large number of genes are needed to work cooperatively for reliable response in case that the genes are "sloppy". Although they did not observe bistability, our result is consistent with their observation because the cooperative bistability in the present case is a cause of the reliable response.

Importance of the noise originated from the finiteness of the number of molecules such as the transcription factors in the gene regulation systems have been emphasized by a number of studies[8, 11, 29, 53, 55, 56]. In spite of the fact that we have not required the robustness against noise as the fitness, both the robustness against the input noise and the internal noise was acquired automatically as a byproduct of the bistability. The cause and the result can be reversed. If we require robustness against noise as fitness, bistable GRNs are expected to evolve because the bistable GRNs are highly robust. We also found the noise-induced response (NIR) to a change of the input. There have been some studies that the origin of bimodal distribution of the cell state is attributed to the switching of the bistable systems induced by several kinds of noise[36, 41, 55, 56]. Our NIR systems should also show the bimodal distribution after the environmental state changes if a number of the identical cells are considered.

The emergence of the new fixed points can be considered as an "innovation"[60] or "a big evolutionary jump". Then, what can we infer about the evolution based on them? The cooperative bistability and the robustness against noises are the consequence of the high fitness. Thus, we can say that this "evolutional jump" occurs inevitably as the fitness increases irrespective of the evolutionary pathway. We may call this as "the universality" of evolution. In other words, the possible phenotypes are restricted by the fitness function and the GRNs having two stable fixed points necessarily appear in the course of evolution. Consider that the evolution is rewinded and rebooted. Then the evolving genotype will become different but the phenotype will share the above properties in common as long as the same fitness function is used. This can also be interpreted as a possible mechanism of the convergent evolution.

As for the robustness against mutations, we found that the regulating interactions split into two categories: neutral and lethal. The lethal interactions are rather scarce. Similar result was also obtained for genes. Although direct comparison is difficult because the context is different, there is an evidence from a comprehensive single gene knockout experiment that the lethal genes are in fact scarce[61]. These lethal genes are considered to be essential for function of GRNs. Interestingly, we found a few GRNs that have no lethal interactions. In such cases, the function is realized cooperatively by all the interactions. We also found that larger GRNs become relatively more robust than small ones.

What we can say about the mutational robustness from this study is that the robust GRNs are not rare among the well-fitted GRNs. In other words, the high fitness is not necessarily accompanied by fragility. Even if the evolution was a simple optimization process, there would be a great chance for robust GRNs to evolve. Of course, the evolutionary process is far from the random sampling process. Rather, it is considered that the robustness is enhanced during the evolution. But since the destination of the evolution can be chosen only from the repertoires available in the set of possible GRNs, the fact that the robust GRNs are not rare is important because it implies that the robust GRNs are readily reached by the evolution.

There have been some numerical evidences that the mutational robustness and the noise robustness are correlated[8, 19, 29]. But in the present study, while the robustness against the noise is clearly a direct consequence of the bistability, the relation between the bistability and the mutational robustness is not clear. This point should be explored further and will be left for a future study.

The rare event sampling method we used can be readily extended to investigate multidimensional landscape in which the evolutionary pathway will pass through, and will be useful to explore the structure of the neutral space. Comparative study with the evolutionary simulation is currently ongoing.

The authors thank Koich Fujimoto, Masayo Inoue, Kunihiko Kaneko, Katsuyoshi Matsushita, Tomoyuki Obuchi, Ken Suito, Hiroki Sayama, Kei Tokita, and Ha-
jime Yoshino for fruitful discussions and comments. This work was supported by JPSJ KAKENHI Grant Number 15K05246.