**ABSTRACT**

**Motivation:** It has been widely reported that biological networks are robust against perturbations such as mutations. On the contrary, it has also been known that biological networks are often fragile against unexpected mutations. There is a growing interest in these intriguing observations and the underlying design principle that causes such robust but fragile characteristics of biological networks. For relatively small networks, a feedback loop has been considered as an important motif for realizing the robustness. It is still, however, not clear how a number of coupled feedback loops actually affect the robustness of large complex biological networks. In particular, the relationship between fragility and feedback loops has not yet been investigated till now.

**Results:** Through extensive computational experiments, we found that networks with a larger number of positive feedback loops and a smaller number of negative feedback loops are likely to be more robust against perturbations. Moreover, we found that the nodes of a robust network subject to perturbations are mostly involved with a smaller number of feedback loops compared with the other nodes not usually subject to perturbations. This topological characteristic eventually makes the robust network fragile against unexpected mutations at the nodes not previously exposed to perturbations.

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**Supplementary information:** Supplementary data are available at Bioinformatics online.

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**1 INTRODUCTION**

Robustness is a key property of biological networks that enables to maintain their functioning against external and internal perturbations. This feature has been ubiquitously observed in various biological examples. For instance, the fate decision of a bacteriophage life cycle is robust against small perturbations at its promoter region (Little *et al*., 1999). *Escherichia coli* is capable of chemotaxis over a wide range of chemo-attractant concentrations (Yi *et al*., 2000). *Drosophila* establishes segmental polarity against perturbations in its initial values and rate constants of molecular interactions (Ingolia, 2004). On the other hand, it has also been reported that biological networks are often fragile against unexpected mutations. For example, the energy control system of our body ensures robustness against common perturbations such as unstable food supply or infections, but the system is fragile against unusual mutations such as high-energy content foods or low-energy utilization lifestyle (Kitano, 2004a). The immune system provides robustness against pathogen threats, but it is fragile against unexpected failures such as dysfunction of MyD88 which is a nonredundant core element (Kitano and Oda, 2006). The segment polarity gene network of *Drosophila* shows robustness against perturbations in its initial condition but shows fragility against a large temporal variability (Chaves *et al*., 2005).

Recent studies showed that robustness and fragility of biological networks are correlated with each other. In particular, Carlson and Doyle (2002) described that complex systems evolved to be robust against general perturbations can extremely be fragile against certain types of rare perturbations. Kitano (2004c) also suggested the trade-offs between robustness and fragility of biological networks with the example of a cancer cell which is robust against a wide range of chemical agents but can be extremely fragile against certain perturbations. Although they brought up an interesting insight into the relationship between robustness and fragility of biological networks, the underlying design principle for such a phenomenon is still largely unknown. Some previous studies have shown that network motifs such as a feed-forward loop and a feedback loop are frequently observed in various biological networks and they could affect dynamical properties (Alon, 2007; Kim *et al*., 2008; Prill *et al*., 2005). In this article, we consider a feedback loop, which is a circular chain of interactions, as an important design principle. The feedback loop has already been proposed as a motif for realizing the robustness of biological networks (Kitano, 2004b, c). For example, the negative feedback loop between MDM2 and p53 maintains an optimal level of p53 and creates certain dynamics of p53 expression levels for a given DNA damage (Lev Bar-Or *et al*., 2000). The *Xenopus* cell cycle is also known robustly controlled against a certain level of perturbations with the help
of several feedback loops engaged (Morohashi et al., 2002). However, the role of feedback loops in realizing robustness is not yet fully investigated, especially for large complex networks containing a number of coupled feedback loops. Moreover, the effect of feedback loops on the fragility of biological networks has not yet been addressed.

In this article, we investigate the robustness and fragility of biological networks through computational experiments on network models with a particular focus on the role of feedback loops. The robustness of biological networks can be interpreted in various ways according to their cellular contexts and functions. Here we consider the robustness of a network defined as the capability of maintaining the stable equilibrium state against perturbations of an initial state. A biological network functions by starting from its initial state and converging to an equilibrium state. Hence, robust biological networks should be able to induce a stable equilibrium state in spite of perturbations in its initial state to a certain extent. In this regard, the robustness can be measured by a probability with which the equilibrium state is maintained against perturbations in its initial state. On the other hand, we note that the robustness of biological networks can be lost by unexpected or unusual mutations. We consider two types of mutations in particular: point mutation and knockout mutation. A point mutation means an inversion of the initial state value of a network node and a knockout mutation implies the deletion of a network node and its related links. The knockout mutation is often called as a loss-of-function mutation. We can measure the fragility by a probability with which the network subject to perturbations are mostly involved with a feedback loops. Moreover, we found that the nodes of a robust network of Figure 1b is robust since it converges to the same state irrespective of the perturbation. We note that both the networks in Figure 1a and 1b have the same number of links. This raises a question about the relationship between the network topology and its robustness. It is also interesting that such robustness can be lost by unexpected mutations. Figure 1c and 1d show examples illustrating the effects of a point mutation at \( v_4 \) and a knockout mutation at \( v_6 \), respectively. As shown in Figure 1c, when the initial value of \( v_4 \) is point mutated from \(-1\) to \(+1\), the robustness of the network in Figure 1b gets lost. On the other hand, the robustness was preserved against the knockout mutation at \( v_6 \) as shown in Figure 1d. Therefore, it turns out that the robust network of Figure 1b is fragile against a point mutation at \( v_4 \) while it is still robust against a knockout mutation at \( v_6 \).

3 MATERIALS AND METHODS

3.1 The number of feedback loops

A feedback loop means a closed simple cycle where nodes are not revisited except the starting and ending nodes. For instance, \( v_0 \to v_1 \to v_2 \to \cdots \to v_{L-1} \to v_L \) is a feedback loop of length \( L \geq 1 \) if there are links from \( v_{i-1} \) to \( v_i \) (\( i = 1, 2, \ldots, L \)) with \( v_0 = v_L \) and \( v_j \neq v_k \) for \( j \neq k \) (\( j, k \in \{0, 1, \ldots, L-1\} \)). Number of feedback loops (NuFBL) of a node \( v \) denotes the number of different feedback loops starting from \( v \). In addition, the sign of a feedback loop is easily determined by the parity of the number of negative relationships involved. If the parity number is even or zero, the feedback loop is positive; otherwise, it is negative.

3.2 Computational network models

A network is represented by a weighted-directed graph \( G = (V, A) \) where \( V \) is a set of continuous variables and \( A \) is a set of ordered pairs of the variables with weights. Each \( v_i \in V \) (\( i = 1, 2, \ldots, N \)) has a continuous value in the range \([-1, +1]\) which represents the expression state of \( v_i \). A directed link \( (v_i, v_j) \in A \) with a weight \( w_{ij} \) describes the regulatory effect of \( v_i \) on \( v_j \) with a weight \( w_{ij} \) where its sign represents either a positive ('activating') or a negative ('inhibiting') relationship from \( v_i \) to \( v_j \). We generate random networks as follows: \( N \) nodes are considered. In this case, there can be \( N^2 \) links. Among theses, we randomly choose (with a uniform distribution) a \( |V| \) links such that every node has at least one incoming and one outgoing link (\( a \) was set to 2.5 in this article). Each link is independently assigned with a weight which is an integer value randomly chosen (with a uniform distribution) from the range \( 1 \leq |w_{ij}| \leq 9 \).

In this article, \( v_i \) may denote the node itself or its expression value depending on context. Similarly, \( V \) can represent either the set of nodes or the set of node values.

\( \lfloor x \rfloor \) denotes the largest integer less than or equal to \( x \).

This weight range was determined in consideration of \( a \) and the sigmoidal function used in this article such that state trajectories can vary over a sufficiently wide scope.
A state $V(t)$ is a vector composed of the values of the continuous variables at time $t$, i.e., $v_1(t), v_2(t), \ldots, v_6(t)$. The expression of each variable at time $t+1$ is updated as follows:

$$v_i(t+1) = f\left(\sum_{j=1}^{6} w_{ij} v_j(t)\right)$$

where $f(x)$ is a sigmoidal function defined as $f(x) = 2/(1 + \exp(-x)) - 1$. Let us assume that a biological process starts from an initial state, $V(0)$, and proceeds through $T$ time steps. An equilibrium steady state is considered to be arrived if the following criterion is met:

$$\sum_{k=T-\theta}^{T} d(V(k), \overline{V}) < 10^{-3}$$

where $\overline{V}$ is the average state value in the time interval between $T - \theta$ and $T$, and $d(V(k), \overline{V}) = \frac{1}{T} \sum_{k=T-\theta}^{T} (v_i(k) - \overline{v}_i)^2$ ($T$ and $\theta$ were set to 50 and 10, respectively, in our simulations). For a given network, based on these definitions, we can consider a mapping from an initial state to a final state, $F : [-1, 1]^6 \rightarrow [-1, 1]^6 \cup \{\emptyset\}$ where $\emptyset$ means the case of a nonequilibrium final state.

### 3.3 Robustness and fragility

To define robustness and fragility, we randomly divide (with a uniform distribution) $V$ into two disjoint subsets, a set of perturbed variables ($F^\ast$) and the other set of nonperturbed variables ($V \setminus F^\ast$), and randomly generate an initial state, $V^\ast \in [-1, 1]^6$. We generate a set of
$$M$$ perturbed states, $$V', V'^2, \ldots, V'^M$$, with respect to $$V^0$$ such that

$$v_i = \begin{cases} \, s_1 \, & \text{if } j = k; \\ \, -s_2 \, & \text{if } j \neq k. \\ \end{cases}$$

The fragility by a point mutation at $$v_k$$, $$F_{p}^{(v_k)}$$, is then defined as follows:

$$F_{p}^{(v_k)} = 1 - \frac{\sum_{j \in \mathcal{V}'} F_{p}^{(v_j)}}{M}.$$ 

This is the mean probability with which the robustness can be lost by an inversion of the initial state value of any node belonging to $$V'/V^*$$.

To compute the fragility by a knockout mutation at $$v_k$$, $$F_{k}^{(v_k)}$$, we first set all the weights of the links around $$v_k$$ \( \in V'/V^\ast \) and consider a set of $$M$$ mutated states, $$V'^1, V'^2, \ldots, V'^M$$, as follows:

$$v_i = \begin{cases} \, v_i \, & \text{if } j = k; \\ \, 0 \, & \text{if } j \neq k. \\ \end{cases}$$

The fragility by a knockout mutation at $$v_k$$, $$F_{k}^{(v_k)}$$, is then defined as follows:

$$F_{k}^{(v_k)} = 1 - \frac{\sum_{j \in \mathcal{V}'} F_{k}^{(v_j)}}{M}.$$ 

So, the fragility of a network by knockout mutations is defined as follows:

$$F_{k} = \frac{1}{|V'|} \sum_{v_k \in V^-} F_{k}^{(v_k)}.$$ 

This is the mean probability with which the robustness can be lost by the deletion of any node belonging to $$V'/V^*$$ and its related links.

For instance, knockout can biologically represent the case where a gene vanishes from its chromosomal region and thereby it is not expressed at all. $$F_{k}$$ describes the effect of such a knockout mutation.

**Fig. 2.** Comparison of $$\text{NuFBL}$$, $$\text{NuFBL}_+$$ and $$\text{NuFBL}_-$$ between robust networks and random networks with $$|V| = 12$$ and $$|A| = 30$$. A random network (Random) is generated by a random selection (with a uniform distribution) of the specified number of links. A robust network (Robust) means a random network with unity robustness ($$F_{k} = 1$$) (see Materials and Methods for details). (a) Distributions of $$\text{NuFBL}$$. (b) Distributions of $$\text{NuFBL}_+$$. (c) Distributions of $$\text{NuFBL}_-$. (d) Variations of $$\text{NuFBL}$$, $$\text{NuFBL}_+$$, and $$\text{NuFBL}_-$$, with the ratio of $$|V'|$$ over $$|V|$$. In (a), (b) and (c), we examined the distributions of $$\text{NuFBL}$$, $$\text{NuFBL}_+$$, and $$\text{NuFBL}_-$$ over 10000 random networks and 10000 robust networks. In each figure, $$\mu$$ and $$\sigma$$ denote mean and SD, respectively. For each network, the robustness was evaluated as one node is perturbed (i.e., $$|V'| = 1$$). In addition, 1000 robust networks were chosen for each $$|V'| = 1, 2, \ldots, 11/2/|V|$$ in (d). All the y-axis values in (d) represent the averages with 95% confidence level. For other networks with different $$|V|$$ and $$|A|$$, we also observed similar results (see Fig. S1 of Supplementary Information).

**4 RESULTS**

We investigate the topological characteristics of robust networks in comparison with random networks by using a computational model. Here, a random network means a network that is generated by a random selection (with a uniform distribution) of the specified number of links. On the other hand, a robust network means a random network that is robust against perturbations in its initial state values (see Materials and Methods for details). We consider a network composed of a set of nodes, $$V$$, where $$V^0 \subset V$$ is subject to perturbations.

**4.1 Feedback loops in robust networks**

We examine the distributions of the $$\text{NuFBL}$$, the number of positive feedback loops ($$\text{NuFBL}_+$$) and the number of negative feedback loops ($$\text{NuFBL}_-$$) in random networks and robust networks, respectively (Fig. 2). In Figure 2a–c, the robustness of networks was evaluated by assuming that only one node is subject to perturbations (i.e., $$|V'| = 1$$). We find that there is little difference in the distributions of $$\text{NuFBL}$$ for random networks and robust networks ($$P$$-value > 0.10; Fig. 2a). This suggests that a larger number of feedback loops do not
necessarily improve the robustness of a network. We also find that the ratio of the number of robust networks to the number of random networks has little relation with \( \text{NuFLB} \) (see Fig. S2 in Supplementary Information). On the other hand, we find that the distributions of \( \text{NuFBL}_+ \) (\( P \)-value <10\(^{-6}\); Fig. 2b) and \( \text{NuFBL}_- \) (\( P \)-value <10\(^{-6}\); Fig. 2c) of robust networks differ from those of random networks. Robust networks tend to have a larger number of positive feedback loops but a smaller number of negative feedback loops than random networks. This result is also partially supported by the previous study showing that networks have a larger proportion of basins corresponding to fixed-point attractors as they have more positive feedback loops (Kwon and Cho, 2007) following the definition of robustness, all states in a robust network must converge to fixed-point attractors rather than limit-cycle attractors. We further investigate the variations in \( \text{NuFBL}_+ \), \( \text{NuFBL}_- \) and \( \text{NuFBL}_- \) of robust networks as the perturbation ratio \( |V^*|/|V| \) becomes larger (Fig. 2d). We find that \( \text{NuFBL}_+ \), \( \text{NuFBL}_- \) and \( \text{NuFBL}_- \) are negatively correlated with the perturbation ratio. This implies that the distributions of \( \text{NuFBL}_+ \), \( \text{NuFBL}_- \) and \( \text{NuFBL}_- \) of robust networks are slightly shifted to the left. Therefore, the number of positive feedback loops is consistently larger than the number of negative feedback loops in robust networks irrespective of the perturbation ratio. This is partially supported by some previous experiments. The network regulating flower morphogenesis in \( \text{Arabidopsis} \) has five positive and two negative feedback loops and the regulatory network is known to robustly control the flower developmental process (Mendoza, 1999). Moreover, the regulatory network of cell differentiation having seven positive and two negative feedback loops is found to robustly induce quiescence, terminal differentiation and apoptosis (Huang and Ingber, 2000). These biological networks can robustly induce their stable states through the relatively larger number of positive feedback loops.

### 4.2 Difference between perturbed and nonperturbed nodes

As shown in the above, a network having a larger number of positive feedback loops and a smaller number of negative feedback loops is likely to be more robust. In such networks, let us examine whether there is any difference between the nodes subject to perturbations \( (V^*) \) and those not exposed to any perturbation \( (V \setminus V^*) \). We compare \( \text{NuFBL}_+ \), \( \text{NuFBL}_- \) and \( \text{NuFBL}_- \) of the two node groups (Fig. 3). In random networks, as expected, there is no difference between the perturbed and nonperturbed nodes (Fig. 3a). We find, however, that both the numbers of positive and negative feedback loops of the perturbed nodes are smaller than those of the nonperturbed nodes in robust networks (Fig. 3b). It is intriguing that the perturbed nodes of robust networks are involved with a relatively smaller number of feedback loops. We further investigate whether such characteristics generally hold without regard to a specific perturbation ratio (Fig. 3c–e). It turns out that \( \text{NuFBL}_+ \), \( \text{NuFBL}_- \) and \( \text{NuFBL}_- \) of the perturbed nodes are less than those of the nonperturbed nodes irrespective of the perturbation ratio.

From the above result, we infer that the nodes involved with a relatively larger number of feedback loops might be more essential in carrying out certain biological functioning. Some examples support this hypothesis. For instance, the \( \text{Drosophila melanogaster} \) regulatory network for the segment polarity composed of eight genes robustly performs its role only if the initial states of two specific genes are not perturbed (Chaves, 2005). We note that the two genes are involved with 8 feedback loops while the other six genes are involved with 4.33 feedback loops on the average. Another example is the gene regulatory network of \( \text{Arabidopsis thaliana} \) for cell-fate determination during flower development (Espinosa-Soto \textit{et al.}, 2004). The network composed of 15 genes robustly leads to inflorescence meristem cell identity. In this case, there are all eight genes which must not be perturbed to keep the robustness of such functioning and these are involved with 22.75 feedback loops on the average. On the other hand, we note that the other seven genes are less important in maintaining the robustness and they are involved with only 2.57 feedback loops on the average.

### 4.3 Fragility of robust networks

The robustness of a biological network is fragile by unexpected mutations. In this article, we consider two types of such mutations: a point mutation and a knockout mutation.

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**Fig. 3.** Comparison of \( \text{NuFBL}_+ \), \( \text{NuFBL}_- \) and \( \text{NuFBL}_- \) between the nodes subject to perturbations \( (V^*) \) and the other nodes not subject to any perturbation \( (V \setminus V^*) \). (a) \( \text{NuFBL}_+ \), \( \text{NuFBL}_- \) and \( \text{NuFBL}_- \) in 10000 random networks. (b) \( \text{NuFBL}_+ \), \( \text{NuFBL}_- \) and \( \text{NuFBL}_- \) in 10000 robust networks with \( |V^*| = 1 \). (c) Variation of \( \text{NuFBL}_+ \) along with \( |V^*|/|V| \). (d) Variation of \( \text{NuFBL}_- \) along with \( |V^*|/|V| \). (e) Variation of \( \text{NuFBL}_- \) along with \( |V^*|/|V| \). In (c), (d) and (e), 1000 robust networks were chosen for each \( |V^*| = 1, 2, \ldots, 10 \). All the \( \gamma \)-axis values represent the averages with 95% confidence level. All the networks have \( |V| = 12 \) and \( |A| = 30 \), but we also observed similar results for other networks (see Fig. S3 of Supplementary Information).
We define the fragility caused by a point mutation \( (F_P) \) and the fragility caused by a knockout mutation \( (F_K) \) as the probability with which the robustness of a network is lost by the respective mutation.

Let us examine the relationship between the robustness and the fragility of a network. To this end, we plot the variation of fragility along with the perturbation ratio (Fig. 4). We found that there is little significant change in both \( F_P \) and \( F_K \) against the perturbation ratio. This implies that a network robust against perturbations of a considerably large number of nodes can still be fragile against some unexpected mutations. It might be explained as follows: as shown in Figure 3, a network acquires robustness as it involves a smaller number of feedback loops for the perturbed nodes (\( V^* \)) compared to those for the nonperturbed nodes (\( V \setminus V^* \)) irrespective of the perturbation ratio. If an unexpected mutation occurs at previously nonperturbed nodes, the robustness of a network becomes fragile since a relatively larger number of feedback loops are involved in those nodes. This can always happen as far as all the nodes are not subject to perturbations. To take a close look at such a phenomenon, we further investigate the relationship between the fragility and the feedback loops of each node. For each node \( v \in V \) of a network, we examine \( \text{NuFBL} \), \( \text{NuFBL}_+ \), and \( \text{NuFBL}_- \) that involve \( v \), which are denoted by \( \text{NuFBL}(v) \), \( \text{NuFBL}_+(v) \) and \( \text{NuFBL}_-(v) \), respectively. We plot the fragility by a point mutation at \( v \) (\( F_P(v) \)) and that by a knockout mutation (\( F_K(v) \)), respectively, against each of the ratios \( \text{NuFBL}(v)/\text{NuFBL}_+ \), \( \text{NuFBL}_+(v)/\text{NuFBL}_- \) and \( \text{NuFBL}_-(v)/\text{NuFBL}_- \) (Fig. 5). Interestingly, we found that there are strong positive correlations between the fragility and such ratios. In particular, \( \text{NuFBL}_+(v)/\text{NuFBL}_+ \) can better discriminate \( F_P(v) \) and \( F_K(v) \) than \( \text{NuFBL}_-(v)/\text{NuFBL}_- \). In summary, mutations at the nodes involved with a relatively larger number of feedback loops are more likely to make the network fragile.

We postulated that robust networks are fragile for mutations at the nodes involving a relatively large number of feedback loops. Although there had been to our knowledge no biological experiment directly proving this hypothesis, there are some examples and experimental evidences that partially support this. For instance, the hippocampal CA1 neuronal signaling network includes 137 lethal proteins and 202 nonlethal proteins (Liu et al., 2006). As shown in Figure 6, lethal proteins are involved with a relatively larger number of feedback loops than other proteins. Specifically, we found that the lethal proteins are involved with 179.36 feedback loops on the average while the nonlethal proteins are involved with 104.82 feedback loops on the average. Another example is the p53 regulatory network that exhibits a robust behavior for a seemingly indefinite period of time. At the center of the network, p53 is involved with at least eight feedback loops (Harris and Levine, 2005). The absence of the p53 gene or functional protein is known to predispose the organism to develop cancers at a young age (Malkin et al., 1990). Hypoxia-inducible-factor 1 (HIF1) which is a master regulator of tumor cell responses to oxygen is expected as a point of fragility for tumors. We note that the expression of HIF1 is also controlled by a number of feedback loops including hypoxia (Shen et al., 2006), HIF-3 (Maynard et al., 2005) and von Hippel-Lindau (VHL) (Blagoslavkloyn, 2001). In the muscle cell fate specification network, MyoD plays an important role in determining diaphragmatic contractile properties (Staab et al., 2002). We observe that this protein is involved with at least three feedback loops in the network...
5 DISCUSSION

In this article, we have investigated the robust but fragile characteristics of biomolecular regulatory networks in terms of feedback loops. Through extensive simulations, we discovered two interesting topological characteristics. One is that a network with a larger number of positive feedback loops and a smaller number of negative feedback loops is likely to be more robust against perturbations. This result is partially related with previous studies on the dynamical roles of feedback loops (Snoussi, 1998; Thomas et al., 1995). A positive feedback loop induces multistationarity whereas a negative feedback loop generates an oscillatory behavior. The other finding is about the cause of network fragility. A network acquires robustness as it involves a smaller number of feedback loops for the nodes subject to perturbations while involving a larger number of feedback loops for the nodes under no perturbation. However, the robustness of a network becomes fragile when unexpected mutations occur at the nodes subject to no perturbation. This is related to the recent study on the robustness-fragility trade-off system by Kitano (2007) where it is argued that any increase of robustness against a subset of perturbations will be off-set by the decrease of robustness against other perturbations. We showed that the number of feedback loops is an indicator of the fragility and exemplified this through previous experimental results showing that many lethal or essential nodes involve a relatively larger number of feedback loops.

The present study provides us with a new insight into the topological characteristics versus their functional importance in biomolecular regulatory networks. In particular, it is suggested that the robustness and fragility of networks can be measured by examining the underlying coupled feedback loops. This result can also be used for synthetic biological applications when we design or engineer biomolecular regulatory circuits such that robustness and fragility are controlled.

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