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Title	Distinct noise-controlling roles of multiple negative feedback mechanisms in a prokaryotic operon system
Authors(s)	Nguyen, Lan K., Kulasiri, D.
Publication date	2011-03-01
Publication information	Nguyen, Lan K., and D. Kulasiri. "Distinct Noise-Controlling Roles of Multiple Negative Feedback Mechanisms in a Prokaryotic Operon System" 5, no. 2 (March 1, 2011).
Publisher	Institution of Electrical Engineers
Item record/more information	http://hdl.handle.net/10197/5020
Publisher's version (DOI)	10.1049/iet-syb.2010.0020

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Title:

Distinct Noise-controlling Roles of Multiple Negative Feedback Mechanisms in a Prokaryotic Operon System

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Keywords:

Multiple negative feedbacks, stochastic modelling, noise control, operon system

Abbreviations:

Ext	=	Extrinsic
Int	=	Intrinsic
Pro	=	Propagated
Tot	=	Total
FL	=	Feedback Loop
NFL	=	Negative Feedback Loop
SNFL	=	Single Negative Feedback Loops
MNFL	=	Multiple Negative Feedback Loops
MIBE	=	Multi-loop Inhibition Balancing Effect
Min	=	minute

Abstract

Molecular fluctuations are known to affect dynamics of cellular systems in important ways. Studies aimed at understanding how molecular systems of certain regulatory architectures control noise therefore become essential. The interplay between feedback regulation and noise has been previously explored for cellular networks governed by a single negative feedback loop. However, similar issues within networks consisting of more complex regulatory structures remain elusive. In this study, we investigate how negative feedback loops manage noise within a biochemical cascade concurrently governed by multiple negative feedback loops, using the prokaryotic tryptophan (*trp*) operon system in *E.coli* as the model system. To our knowledge, this is the first study of noise in the *trp* operon system. We show that the loops in the *trp* operon system possess distinct, even opposing, noise-controlling effects despite their seemingly analogous feedback structures. The Enzyme Inhibition loop, although controlling the last reaction of the cascade, was found to suppress noise not only for the tryptophan output but also for other upstream components. In contrast, the Repression loop enhances noise for all systems components. Attenuation poses intermediate effects by attenuating noise for the upstream components but promoting noise for components downstream of its target. Regarding noise at the output tryptophan, Repression and Attenuation can be categorised as noise-enhancing loops while Enzyme Inhibition as a noise-reducing loop. These findings suggest novel implications in how cellular systems with multiple feedback mechanisms control noise.

1 Introduction

It has been well established over the last decade that molecular noise is inherent and inevitable within cellular networks [1]. Genetically identical cells under the same environmental conditions can impose significant variations in their phenotypic characteristics. Such variations have been observed in organisms of various complexity ranging from bacteria to mammals, and are hypothesised to play important roles in the evolution as well as development of many living organisms [2]. More interestingly, accumulating evidence supports that noise can be subjected to selection, suggesting it might be an important physiological trait [3, 4]. It is thus of great interest to study how noise arises and how it is managed using different evolved mechanisms of cellular regulation.

Novel leaps in experimental molecular biology (e.g. fluorescent reporters and single-cell imaging techniques) in recently years have allowed stochastic gene expression to be quantified *in vivo* [1, 5-7]. These elegant experiments, along with theoretical studies [8], have greatly facilitated our understanding of the sources and consequences of molecular noise. Noise in cells has commonly been characterised as intrinsic or extrinsic, mainly motivated by the experiments conducted in gene expression context [1]. Intrinsic noise originates from the randomness in the biochemical processes that take place in gene expression whereas extrinsic noise comes from fluctuations in other factors that influence gene expression. Effort has since been carried out by different groups aiming to separate one type of noise from another experimentally [5, 6] and theoretically [8, 9].

More recent work on transcriptional cascades indicate that noise can propagate from upstream to downstream components, further decomposing the internal noise of a specific molecular species into an intrinsic noise part and a propagated noise part [10-12]. The intrinsic noise part is primarily determined by the component's copy number, while its propagated counterpart is determined by the total noise of upstream, internally connected components within the cascade [13]. Due to noise propagation, noise behaviour within a network would depend greatly on its topology and can be quite complex.

The interplay between feedback regulation and noise has been previously explored. Negative feedback was shown to be among the most common means for noise attenuation [14, 15]. However, this noise-suppressing feature of negative feedback was often drawn from studies of systems governed by a single negative feedback loop in isolation [16-18]. Cellular systems, on the other hand, are much more complex and often contain multiple feedback mechanisms operating simultaneously.

In this study, we investigate how noise behaves within a biochemical cascade concurrently governed by multiple negative feedback loops (Figure 1a), using the prokaryotic tryptophan (*trp*) operon system in *E.coli* as the model system. Our main goal is to understand how the negative feedback loops, alone and together, control noise and what the outcomes might mean for the system as a whole. Using stochastic modelling and numerical simulation, we examine whether the negative feedback loops retain the noise-suppressing property when operate in number and investigate possible effects of having additional negative feedback regulations on the noise characteristics of the cascade's components.

The *trp* operon system regulates the production of tryptophan in *E.coli* cells using three negative feedback loops [19]. We showed elsewhere that the evolution of three seemingly redundant loops in the *trp* operon system provided it with stability and robust responses to perturbations [19]. We also demonstrated earlier that multiple negative feedback loops (MNFL) gave rise to a richer repertoire of dynamical behaviour in comparison to single negative feedback loop (SNFL) [20]. This work naturally follows the previous work by asking whether the MNFL architecture would play any roles in controlling the noise behaviour of the harbouring system.

For the remaining of the paper, we call the molecular species, which is a product of a reaction channel on which a loop acts, the target of the loop. Furthermore, within a cascade, a molecular species is considered downstream or upstream of a loop if it is downstream or upstream relative to the target of that loop. We refer to a system's noise profile as the collective set of noise levels exhibited by all species within the system. The paper is organized as follows: section 2 gives an overview of relevant studies on the issue of noise propagation in connection with feedback regulation. The molecular basis and model development of the *trp* operon system are presented in section 3. We examine noise propagation in a negatively regulated two-stage cascade in section 4, which serves as a general framework for an extended analysis of multiple feedback loops in section 6. The inhibition balancing effect for MNFL systems is introduced in section 5. Finally, section 7 discusses potential implications of the results.

2 Noise Propagation and Feedback Control: an Overview

Attempts from both experimental and theoretical aspects have been made to probe the issues of noise propagation and how negative feedback regulation affects noise within cellular networks [10-13, 16, 18]. Typically in these studies, simple and easily manipulated gene

circuits were artificially engineered, often accompanied by stochastic models to explain observed data and furthermore, to give predictions.

Pedraza et al. [10] investigated how noise propagates through a gene regulatory cascade by constructing a synthetic network in single *E.coli* cells. Three genes were arranged so that the first gene regulates the expression of the second gene which, in turn, modulates the expression of the third gene (Figure 1b). Fluorescent reporter genes were fused and co-expressed with the genes of interest in order to quantify expression variability. Correlations between genes in the cascade and correlations with a constitutive gene were measured which revealed that noise in a gene is determined by three sources: intrinsic fluctuations, transmitted noise from upstream genes and global noise affecting all genes [10]. Stochastic modelling further demonstrated that expression variability of a target gene in the cascade was influenced mostly by transmitted noise from upstream regulator. This finding means that noise of gene expression can be more strongly affected by the underlying network connectivity than by its own intrinsic fluctuation. Austin et al. [21] lent more support to this hypothesis by showing that noise frequency content (spectra) is determined by the underlying gene circuit structure, establishing a mapping between the two. In a similar vein, synthetic transcriptional cascades of varying length were constructed in *E.coli* (Figure 1c) to explore the effect of cascade length on noise and sensitivity of the network's response [11]. It was shown that the longer cascades, which were more sensitive, amplified cell-to-cell variability, particularly at intermediate output states. On the other hand, the length of the cascade has minor effect on noise propagation for low and high output states [11]. Subsequent computational work on similar cascade motifs adding negative auto-regulation to one of the cascade components (Figure 1d) reported that this incorporation did not reduce noise for the new motifs, which was contrary to the widely believed noise-suppressing feature of negative feedback loops [12].

A number of theoretical studies also explored the effects of feedback regulation on noise of different types within various network motifs, among which transcriptional auto-regulation received frequent attention [16, 17]. Using the Fano factor as a measure of fluctuation, intrinsic noise at the mRNA level was found to be enhanced under auto-regulation regardless of whether it was negative or positive. Noise at the downstream protein level, however, behaved differently depending on the type of regulation, with negative feedback imposing decreasing effect while positive feedback imposing promoting effect [16]. This suggests that the sign of the feedback loop affects the propagation of intrinsic noise between the mRNA and the protein in different ways. Regarding external noise, negative auto-regulation could increase it for the protein level while positive auto-regulation could decrease it instead [17].

Figure 1. (a) Schematic diagrams of the cascade motifs investigated in this study. (b) Gene cascade studied by Pedraza et al. [10]. (c, d) Gene cascade studied in [11, 12]. S_i denotes the molecular species. Hereafter, the normal arrows indicate activation effect while arrows with bar-end indicate repression.

3 Molecular Basis and Stochastic Modelling of the Tryptophan Operon System

3.1 Molecular Basis of the Trp Operon System

Figure 2. (a) Schematic diagram of the tryptophan operon system. Five genes are denoted as E (AS), D, C, B and A. P, O, L denotes the promoter, operator and leader region, respectively. (b) Schematic diagram with parameters notation for the *trp* operon system. Three negative feedback loops are denoted Rep (Repression), Att (Attenuation), and EI (Enzyme Inhibition). The tryptophan level is depleted due to degradation as well as consumption for cellular proteins synthesis.

The *trp* operon system in *E. coli* controls the production of the tryptophan amino acid inside the cell. Key molecular processes include transcription, translation and synthesis of tryptophan. To regulate these processes, the *trp* operon system utilises three negative feedback mechanisms: transcriptional repression, attenuation, and enzyme inhibition [19, 22].

The transcription process is initiated as the RNA polymerase binds to the promoter. However, when the activated form of repressor induced by the attachment of two tryptophan molecules become abundant, it binds to the operator site and blocks RNA polymerase from binding to the promoter, thereby, repressing transcription and forming the first feedback loop. Furthermore, transcription can also be attenuated depending on the level of intracellular tryptophan and is controlled by the leader region sitting between the operator and the genes (Figure 2a). This attenuation makes up the second negative feedback loop. The tryptophan operon consists of five structural genes positioned consecutively after the leader region. These genes code for five polypeptides that make up the enzyme molecules in the form of tetramers, which in turn catalyse the synthesis of tryptophan from chorismates [19, 22-24]. Anthranilate synthase (AS) is the enzyme that catalyses the first reaction step in the tryptophan synthesis pathway. Tryptophan is feedbacked to inhibit anthranilate synthase activity if tryptophan level is high. Enzyme inhibition therefore forms the third negative feedback loop in the *trp* operon system.

3.2 Stochastic Model of the Trp Operon System

A stochastic differential equations model following Gillespie's derivation of the Langevin equations was developed for the *trp* operon system [25]. We identified four key molecular species which are the free operator, mRNA, enzyme, and intracellular tryptophan with corresponding concentrations denoted as *Op*, *mRNA*, *Enz* and *Trp* (Figure 2b). The system can be described using a set of 8 reactions involving the production and loss (including degradation and dilution due to cell growth) of these four species (Table 1, SI). Detailed derivation of the model development and discussion on choice of the values of model parameters are given in the section 1 and 2 of the Supplementary Information (SI). Note that the developed model and the parameter set used throughout our analysis were validated against a range of experimental data (Section 3, SI).

3.3 Quantification of Noise

Noise can be quantified in a number of ways. Autocorrelation has been used to summarize both the magnitude and frequency of fluctuations [26]. However, most models so far have focused on exploring the steady-state statistics of gene regulatory networks. Two most important characteristics are the mean and the variance of the number of molecules of each species within the networks. The advantages of these system properties are that they are fundamental and simple to understand, provide clear interpretations and more importantly, they are easily accessible experimentally [27]. Thattai and Oudenaarden [27] suggested to use the Fano factor (ratio of the variance to the mean) to measure the relative size of noise in gene expression. However, the Fano factor can be misleading for multivariate random

processes and only works well for univariate discrete random processes [26]. We adopted a preferred alternative measurement for noise, the coefficient of variation, formulated as the standard deviation over the mean [26]. Using this noise measure throughout the paper, we systematically quantify noise level for all the molecular species in the tryptophan operon system under various feedback structures. Elaborate description is provided in the following sections.

4 Noise propagation in a two-stage biochemical cascade

Figure 3. Schematic diagrams of two-stage, negatively regulated cascades. (a) A two-stage protein cascade (b) A two-stage transcriptional cascade (c) A two-stage signalling cascade (d) Generalised scheme of two-stage biochemical cascades that captures the systems in panels (a), (b) and (c).

To provide a general framework for the following parts of the paper, we examine noise propagation in simple two-stage biochemical cascades, whose schematic diagram is illustrated in Figure 3d. Under this scheme, the upstream molecular species A is constitutively synthesised from its precursor S , which is assumed fixed due to buffering by the cell. The downstream molecular species B is in turn synthesised from or activated by A . Moreover, this step is governed by a negative feedback loop imposed by B . Of note is that A is not transformed into B and is depleted only due to its degradation process.

Such common motif can be found in a number of scenarios in the cells. Examples include:

(1) a two-stage protein cascade shown in Figure 3a where protein A acts as a transcriptional activator for protein B [28]; (2) a transcriptional cascade in Figure 3b where transcript A serves as a template for the synthesis of protein B [29, 30]; or (3) a signalling cascade in

Figure 3c where the input ligand S activates the receptor protein A which then phosphorylates its substrate protein B . In examples (1) and (3), B may act as an inhibitor of A , forming a negative feedback loop whereas in example (2), B may interfere with its own translation by competing with the RNA polymerase or co-activators.

The simple cascade described above can be modelled by the following equations in which a Hill function is used to represent feedback inhibition:

$$\frac{dA}{dt} = k_1 \cdot S - k_{d1} \cdot A$$

$$\frac{dB}{dt} = k_2 \cdot A \cdot \left(\frac{K_1^n}{K_1^n + B^n} \right) - k_{d2} \cdot B$$

where k_1 and k_2 denotes the maximal rate of A and B production, k_{d1} and k_{d2} denotes the degradation rate of A and B , n is Hill coefficient and K_1 represents the half-saturation constant whose reciprocal indicates the strength of the negative feedback loop (Feedback Strength (F.S) = $1/K_1$).

Figure 4. Noise propagation in the two-stage cascade. (a) Stochastic time-course (in minutes) of the concentration of B at feedback strength varying from very weak ($K_1=10 \mu M$)

to very strong ($K_1=0.001\mu\text{M}$). Parameter values used are $S = 1\mu\text{M}$, $k_1 = 1 \text{ min}^{-1}$, $k_2 = 1 \text{ min}^{-1}$, $k_{d1} = 1 \text{ min}^{-1}$, $k_{d2} = 1 \text{ min}^{-1}$. **(b)** Total noise of A and B at increasing feedback strength (displayed in logarithmic scale to the base 10). The total noise $\eta_{\text{tot}}(B)$ was normalised to its maximal value; $\eta_{\text{tot}}(A)$ was adjusted accordingly. $[B_{\text{ss}}]$ denotes the steady-state level of B . Total noise for the unregulated case, where no loop is present, is indicated by the black point. **(c)** Normalised total noise, propagated noise and intrinsic noise of B at increasing feedback strength (displayed in logarithmic scale to the base 10) when its steady-state level is fixed by modulating B 's degradation rate k_{d2} . **(d)** Normalised total noise of B at increasing degradation rate of B . These values of k_{d2} correspond to the data points in panel (c).

Previous studies have showed that noise can be transmitted from upstream components to downstream ones in a biochemical cascade [10-12]. This means in the absence of extrinsic noise, the total noise of a downstream molecule is made up of its intrinsic noise (primarily as a consequence of its low copy number) and propagated noise coming from its immediate upstream species. The magnitude of this propagated noise part is determined by the intrinsic noise of the upstream component as well as its own propagated noise part which in turn comes from the total noise of its own preceding, upstream neighbour. Under this framework, the total noise at B can be expressed as:

$$\eta_{\text{tot}}(B) = \eta_{\text{int}}(B) + \eta_{\text{pro}}(B)$$

where $\eta_{\text{tot}}(B)$, $\eta_{\text{int}}(B)$ and $\eta_{\text{pro}}(B)$ respectively denote the total noise, intrinsic and propagated noise part of B .

In an unregulated cascade (no feedback loop), $\eta_{\text{pro}}(B)$ is entirely dependent on $\eta_{\text{tot}}(A)$. However, in a regulated cascade (with negative feedback loop), how much noise is propagated from A to B should also be determined by the ‘‘capacity’’ of the $A \rightarrow B$ reaction channel, which is modulated by the feedback loop. A strong loop would result in less B for the same level of A , thereby limiting the channel's capacity and allowing less noise to be propagated from A to B ; whereas a weak loop would enhance $\eta_{\text{pro}}(B)$ given the same $\eta_{\text{tot}}(A)$. Moreover, the feedback loop does not directly control B 's intrinsic noise level although it affects $\eta_{\text{int}}(B)$ indirectly through influencing the number of copies of B molecules. In the regulated case, the total noise of A is also expected to be independent of the feedback loop. Our numerical simulations indeed confirm these predictions, as discussed below. Details of the numerical computation are presented in section 4 of the Supplementary Information.

First, we found that at weak feedback strength, increasing the loop's strength does not appear to affect the total noise of B . Interestingly, the negative feedback loop increases noise of the output component only when the feedback is sufficiently strong, indicated by the blue arrow in Figure 4b. The stochastic temporal evolution of B at weak and strong feedback displayed

in Figure 4a consistently shows that it is more noisy at high feedback strength (low K_1). As expected, the total noise of component A does not appear to vary with feedback strength.

Since total noise is measured at steady state, to explain the observed increasing trend of $\eta_{\text{tot}}(B)$, we looked at the steady-state level of B . Interestingly, steady-state level of B consistently decreases as the feedback strength increases (Figure 4b, dashed curve). Generally, smaller level of B renders change in individual molecule more significant which results in B being more intrinsically noisy. However, Figure 4b shows that at weak feedback strength, the total noise of B is not affected even when its intrinsic noise part is sharply increasing (caused by the sharp decreasing of $[B_{\text{ss}}]$). This indicates that $\eta_{\text{tot}}(B)$ cannot be explained solely by the intrinsic noise and confirms that noise is indeed propagated from A . It is this propagated noise part, $\eta_{\text{pro}}(B)$, which decreases with stronger feedback, that balances the increase of the intrinsic noise and results in a non-increasing total noise of B observed at weak feedback strength. At increasingly high feedback strength, it is most likely that the increase in intrinsic noise outweigh the decrease in propagated noise which leads to an overall enhanced total noise of B .

To further test our framework, we keep the steady-state level of B unchanged by modulating its degradation rate k_{d2} aiming to fix the intrinsic noise component while varying the feedback loop's strength. Since B 's degradation process does not involve the reaction channel $A \rightarrow B$, $\eta_{\text{pro}}(B)$ is not affected by the modification of k_{d2} . According to our framework, a stronger (weaker) loop would result in a reduced (enhanced) level of propagated noise, leading to a reduced (enhanced) total noise level since the intrinsic noise part is kept fixed. This is exactly what we observed in the simulations (Figure 4c, blue curve).

Furthermore, $\eta_{\text{pro}}(B)$ can be made negligible by super-tightening the $A \rightarrow B$ reaction channel with extremely strong feedback loop while balancing B 's steady state level by a particularly long half life (very low k_{d2}). In this case, $\eta_{\text{pro}}(B) \sim 0$ yielding $\eta_{\text{int}}(B) \sim \eta_{\text{tot}}(B)$ which can be measured. Subtract this intrinsic noise part from the total noise; we can calculate the propagated noise at varying feedback strength. Following this, we calculated the propagated noise part for the component B at increasing feedback strength, indicated by the red curve in Figure 4c. To our expectation, the propagated noise decreases as the loop becomes stronger.

We therefore showed that noise is indeed transmitted from upstream to downstream components in a cascade, and this propagated noise is directly controlled by the feedback loop acting on the respective reaction channel.

We were also interested in the relative magnitude between the intrinsic and propagated noise. To this end, we modified A 's degradation rate to vary its concentration thereby affecting its noise level but keeping the loop unaffected. As expected, we observed noise of B is changed accordingly due to its propagated noise component. It turns out that neither the intrinsic noise or the propagated noise part outweighs the other universally; rather this relationship is parameters dependent. In certain cases, propagated noise could significantly dominate intrinsic noise.

5 The Multi-loop Inhibition Balancing Effect as an Inherent Feature of MNFL Systems.

Figure 5: Illustration of the Multi-loop Inhibition Balancing Effect (MIBE) between the loops in the *trp* operon system. Here, MIBE is demonstrated when each loop is alternatively made stronger (similar effects are seen when loops are made weaker). Inhibition level for each loop is calculated using the corresponding Hill functions whose values are between 0 and 1. Wild-type and mutant loops are represented by solid and dashed lines respectively. (A) When L_1 (red) is made stronger (K_1 reduced 5 times), its inhibition curve is seen shifted to a lower level. This tightening of L_1 leads to less output Trp (inbox plot). L_2 (blue) and L_3 (green) consequently compensate the decrease L_1 's inhibition curve by both lifting their inhibition curves higher, making them weaker. The second inbox plot is a zoom out of the inhibition curve of L_2 . (B) Similar to (A) except L_2 is now actively made stronger (K_2 reduced 5 times - inbox), both L_1 and L_3 are seen to be weaker. (C) Similar to (A) except L_3 is now actively made stronger (K_3 reduced 5 times - inbox), both L_1 and L_2 are seen to be weaker.

Within systems with MNFL we observed a feature that is not present in system with SNFL, which we termed the Multi-loop Inhibition Balancing Effect (MIBE). Essentially, when any loop's inhibition level is actively increased or decreased (e.g. by changing its strength due to mutations), all remaining loop will compensate this by changing their inhibition level in opposite direction, leading to negative correlations between inhibition levels of the actively modified loop and the remaining loops (Figure 5). Because MIBE is exhibited while all remaining loops are kinetically conserved (their feedback strengths and sensitivity are unchanged), this feature is parametrically independent and thus a consequence of the

topological structure of the networks. Moreover, MIBE is found not only in systems with steady state but also in oscillating systems.

The cause of MIBE is due to a shared feedback signal (or signals) between the involved negative feedback loops. In the *trp* operon cascade, all loops act in response to the concentration of tryptophan inside the cells, making tryptophan a shared signal. When one loop is lifted (reduced in strength), the output level of tryptophan becomes higher both in the transient state and the steady state. Elevated level of tryptophan subsequently lowers the intensity of corresponding Hill functions of the other loops, tightening their repression levels. Similar argument applies when any loop is tightened. MIBE demonstrates the ability of inter-compensating and self-adjusting between multiple loops in a MNFL system when being perturbed. Compared with SNFL systems, MIBE effectively curbs potential rapid plunge (or surge) in intracellular tryptophan level resulting from sudden perturbations, which might be detrimental to the cell. It is necessary to note that MIBE is not just a manifestation of mathematical representation of the negative feedback loops, but an inherent property of the system. If a different representation was used instead of the Hill kinetic rate law, MIBE should still be observed, due to the same regulatory structure of the network. MIBE has interesting implication to the behaviour of noise of the molecular species within a MNFL system, which we investigate below.

6 Characterisation of noise in the *trp* operon system

Although noise-related functions of negative feedback in systems with single loop have been explored in previous studies as discussed earlier, investigations of such kind of the systems in which multiple negative feedback loops interplay and possibly influence each other has been limited. An important goal of this study is to characterise the roles that each negative feedback loop might plays in the control and management of noise in the *trp* operon system. To achieve this, we employ an approach of systematically designed simulations and noise computations based on stochastic models of the *trp* system. Such computations are conducted not only for the wild-type *trp* system but also for a variety of its *in silico* mutants, ranging from those with modified strength of individual feedback loops to systems with structurally different network connections.

6.1 Distinct noise-controlling roles revealed for the individual loops in the *trp* system

Figure 6. Noise profile of all molecular components of the *trp* operon system in response to increasing strength of the individual feedback loops.

Three different sets of *in silico* experiments were performed in which the feedback strength of the Repression (Rep) loop, Attenuation (Att) loop and Enzyme Inhibition (EI) loop is varied, respectively. In each set, the loop subjected to change has its strength altered to both sides of its basal level, with the lower end of 2^{-10} times the basal value (signifying extremely weak feedback) and the higher end of 2^{10} times the basal value (signifying extremely strong feedback). Since the feedback strength is represented by the reciprocal of the respective half-saturation constant (K), this is done by simply varying the parameters K_1 , K_2 , and K_3 accordingly. It is noted that all noise were measured at steady state, all the mutant systems were thus simulated for long enough to reach the steady state (typically 500 mins).

The results displayed in Figure 6 present interesting observations. Despite the seemingly similar topological structure of the three loops, they affect noise levels in remarkably different manners. Rep acts completely opposite to EI in that it increases noise for all of the *trp* system's components whereas EI reduces noise for these components (Figure 6a-d, i-l). The Att loop, however, behaves somewhat in the middle: it reduces noise for the most upstream component (*Op*) but increases noise for other components downstream of the

reaction on which it acts (Figure 6e-h). Moreover, with respect to the noise level in the tryptophan output, Rep and Att can be categorised as the noise-inducing loops while EI as the noise-reducing loop (Figure 6d,h,l).

These distinguished noise-controlling mechanisms suggest that the position of a negative feedback loop may play decisive role in the way it affects the noise behaviour of a system with MNFL. Moreover, with increasing supporting evidence that noise is an important physiological trait [3, 4], it is conceivable that having multiple loops of distinct noise-related functions provides greater flexibility in the ability of the system to tune noise towards desired levels. Such feedback design also confers the system to a more diverse repertoire of noise profile. Our findings also suggest that one should be careful when generalising the conclusions with respect to the effects of negative feedback loops on noise in single-loop systems to those in systems with more than one loop operating concurrently.

Notwithstanding quantitative change in noise amplitude, the noise patterns in Figure 6 retain the qualitative nature at various levels of noise coefficients (presented in Figure S2, SI). Below we further investigate whether these noise characteristics are dependent on other system parameters or rather a property of the regulatory structure.

6.2 The wild-type *trp* system exhibits near optimal noise performance

Figure 7. (a) Total noise measured for the wild-type *trp* operon system (noise coefficient used is 0.005). **(b)** Stacked histograms for comparison of noise levels at four system components at basal, low and high level of feedback strength for each negative feedback loop.

The noise profile of the wild-type *trp* system is tabulated in Figure 7a. It is also highlighted by red points in all panels of Figure 6 in comparison to other mutant systems. Figure 7 shows that noise levels of the output tryptophan (Trp), enzyme (Enz) and the free operon (Op) are comparable but about an order of magnitude lower than that of the mRNA transcripts. Clearly, the cascade does attenuate noise from high levels of the upstream components, Op and mRNA, to much lower levels of the downstream components, Enz and Trp. Since tryptophan is an important ingredient of a multitude of cellular proteins, less variability in its levels allows for more stable production of these proteins in cells. This result is in line with recently proposed ideas, backed up by evidence in yeast, that noise might be kept low to prevent harmful stochastic variation in the levels of dosage-sensitive genes [3, 4].

We further compared the relative magnitudes of noise between the systems components at various specifications of the loops, and consistently observed the same pattern with attenuated noise in the tryptophan (Figure 7b). The high noise level at mRNA is probably due to the low steady-state concentration of mRNA relative to that of other systems components.

Notably, although the Rep loop tends to increase noise in levels of all system components, such behaviour becomes significant only when feedback strength higher than the basal level (Figure 6a-d). Repression feedback, weaker than the wild-type strength, results in comparable noise level to that of the wild-type system. Moreover, with respect to noise level of the tryptophan output, the wild-type E.I loop displays relatively low noise among the tested strengths, whereas increasingly weaker E.I results in sharp rise in tryptophan fluctuation (Figure 6l). Similarly, the wild-type Att loop performs relatively well at suppressing noise in the tryptophan levels (Figure 6h). Taken together, these findings clearly show that the wild-type *trp* operon system achieves a near-optimal performance with respect to keeping low noise in the tryptophan levels.

6.3 Noise propagation underlies noise behaviour in the *trp* operon system

In this section, we aim to substantiate the noise behaviour observed previously in the *trp* operon system. For each systems component, the total noise measured is made up of the intrinsic and propagated noise part, as discussed earlier. The intrinsic noise is primarily determined by its amount in cells. Specifically, it scales with the inverse square root of the

copy number [31]. Steady-state concentrations of the components of the same sets of *trp* mutants in Figure 6 are calculated in response to varying feedback strengths and displayed in Figure S3 in the SI.

Generally, low steady-state concentration leads to high steady-state noise level and such correlation can be consistently observed in almost all panels (Figure S3a-k, and Figure 6a-k). However, in the last panel of Figure 6, EI was shown to have a decreasing effect on tryptophan's total noise despite the fact that it also imposes a decreasing effect on the tryptophan concentration (Figure S3, panel l). To explain this phenomenon, noise propagation must be accounted for as an argument based solely on concentration levels (intrinsic noise) does not hold. Moreover, an explanation that fails to consider the interplay between the loops would have difficulty in elucidating the reduced noise observed in the levels of *Op*, *mRNA* and *Enz* despite that these components are not directly controlled by the EI loop. Below, we argue that the effect of *inhibition balancing* discussed earlier between the multiple feedback loops Rep, Att and EI and the propagation of noise can be exploited to account for the observed noise characteristics exhibited by the *trp* operon.

Enzyme Inhibition suppresses noise of all system components

When EI gets stronger, the Rep and Att loops become weaker according to the mechanism of MIBE. The weakening of Rep's inhibition strength releases (elevates) the *Ot*→*Op* reaction channel and allows more *Op* to be produced. $\eta_{\text{int}}(\textit{Op})$ is therefore decreased due to higher *Op* concentration. Since *Ot* is fixed, no noise is propagated from *Ot* to *Op*, making $\eta_{\text{pro}}(\textit{Op}) = 0$ and $\eta_{\text{tot}}(\textit{Op}) = \eta_{\text{int}}(\textit{Op})$ which is reduced as observed.

Regarding noise level at *mRNA*, we have: $\eta_{\text{tot}}(\textit{mRNA}) = \eta_{\text{int}}(\textit{mRNA}) + \eta_{\text{pro}}(\textit{mRNA})$. Because the Att loop becomes weaker, the steady-state concentration of *mRNA* increases, rendering $\eta_{\text{int}}(\textit{mRNA})$ to decrease. More intricately, $\eta_{\text{pro}}(\textit{mRNA})$ is dictated/controlled/influenced by opposing forces/effects. It is bound to decrease due to a reduced $\eta_{\text{tot}}(\textit{Op})$, at the same time expected to increase due to the enhanced capacity of the *Op*→*mRNA* reaction channel caused by weaker Att loop. Depending on the relative intensity of these effects, and the relative significance of the propagated noise to the intrinsic noise part, total noise at *mRNA* could either decrease or increase. Here, the intrinsic noise of *mRNA* is likely to dominate its propagated noise counterpart owing to a very low steady-state concentration of *mRNA*. This agrees with the observed decreasing total noise of *mRNA*.

Next, we consider noise level at the *Enz*. An increase in the steady-state concentration of *mRNA* leads to higher steady-state concentration of *Enz*. This lowers $\eta_{\text{int}}(\textit{Enz})$ as well as

$\eta_{\text{pro}}(Enz)$ since the reaction channel $mRNA \rightarrow Enz$ in the *trp* operon system is not regulated, therefore rendering total noise at the *Enz* to be lower. This is largely consistent with what was observed (Figure 6j), except at low feedback strength of the EI loop, a slight lift in the total noise of *Enz* was displayed. Finally, for noise level observed at tryptophan: $\eta_{\text{pro}}(Trp)$ is reduced due to a lower total noise at *Enz* as well as a stronger EI. This reduction of the propagated noise from the upstream components must outweigh the rise in $\eta_{\text{int}}(Trp)$, resulting in a decreasing total noise of *Trp*.

Repression promotes noise of all system components

Interestingly, it is observed that the Repression loop enhances noise at all the components of the *trp* system (Figure 6a-d). When Rep becomes stronger, the steady-state concentration of *Op* is decreased, resulting in reduced steady-state level of *mRNA*, *Enz* and *Trp* and leading to higher intrinsic noise for all these species. Moreover, the mechanism of ICE renders Att and EI weaker, further promoting the propagated noise of *mRNA* and *Trp*. Taken together, the total noise levels at *Op*, *mRNA* and *Trp* should be enhanced at stronger Repression feedback, as observed.

Attenuation promotes noise of its downstream species while suppresses noise of its /upstream species.

Unlike the Rep loop, stronger Att loop increases fluctuation level of only the components downstream of its targets molecule (*mRNA*) but instead promotes noise at the upstream species *Op*. A negative feedback loop therefore can influence noise level of an upstream species within a cascade with multiple loops. Once again, this observation was attributed to MIBE since for a system lacking the Att loop, changing Rep strength would not result in any change in the noise level of *Op*.

To substantiate the noise behaviour in this case, we note that the Rep loop loses its strength owing to the MIBE mechanism, which leads to a higher steady-state level of *Op* and resulting in a lower intrinsic noise at *Op*. Because the propagated noise component at *Op* is null, *Op*'s total noise decreases as a whole. On the other hand, as the Att loop becomes stronger, the steady-state concentration of *mRNA* is reduced, leading to lower steady-state concentration of *Enz* as well as of *Trp*. This means $\eta_{\text{int}}(mRNA)$, $\eta_{\text{int}}(Enz)$ and $\eta_{\text{int}}(Trp)$ are all promoted. Similarly, the MIBE mechanism renders EI weaker, which enables a higher level of $\eta_{\text{pro}}(Trp)$ therefore pushing up the total noise at *Trp*. $\eta_{\text{pro}}(Enz)$ should also become higher due to the promoted $\eta_{\text{int}}(mRNA)$, yielding a larger $\eta_{\text{tot}}(Enz)$. For noise at the *mRNA*, its intrinsic part increases while its propagated part decreases due to a stronger Att. However,

because the intrinsic noise part of *mRNA* in the *trp* operon system is dominant due to its low steady-state concentration, the total noise of *mRNA* is thus enhanced as a result.

6.4 Effects of the multiple-loop structure on noise characteristics of the *trp* operon system

Figure 8. Comparison of noise profiles between the wild-type *trp* system (blue) with mutant systems with only Rep loop, only Att loop and only EI loop (red) in response to increasing strength of the relevant loops.

To further understand how the multiple negative feedbacks structure employed by the *trp* operon system shapes its noise profile, we quantified noise for modified *trp* systems which consist of only a single negative feedback loop and compared them to the wild-type system. Figure 8 and S4 in the SI show remarkable discrepancies in the patterns of noise levels between the single-loop systems and that of the multiple-loop system.

Notably, in the single-loop systems, increasing the feedback strength appears not to control noise in the tryptophan output in consistent (increasing or decreasing) trends, as seen in the multiple-loop system (Figure 8, fourth column). Particularly, we observed that stronger Att in the Att-only system leads to increased tryptophan noise only at low feedback strength, but decreases tryptophan noise at higher feedback strength. The Att-only system consequently displays highest tryptophan noise at Att strength around its basal value (Figure 8h). Such a peak in tryptophan noise pattern is missing in the wild-type *trp* system. On the other hand, Figure 8l and S4l shows a bell-shape pattern for noise in the tryptophan level with respect to increasing EI in the EI-only system. Similarly, such noise-controlling behaviour was not

observed in the system with all three negative feedback loops. Taken together, these observations suggest that the noise characteristics obtained by the wild-type *trp* system was underpinned by its multiple negative feedback loops structure.

We also quantified noise for mutants having one of the loops completely turned off. These mutants behaved very differently in terms of responding to noise. Turning off the EI loop results in higher noise not only for tryptophan but for all other components in the cascade. In contrast, turning either the Rep or Att loop off actually decreases noise in the tryptophan level (data not shown). These results are consistent with the previous findings that EI acts as the noise-reducing loop while Rep and Att act as noise-inducing loops.

6.5 Effects of model parameters on noise pattern of the *trp* system

Given an initial state, a system's behaviour is generally characterised by both its regulatory structure (connections) and the intensity of these connections (parameter values). To further understand how the noise-managing roles of the *trp* system's loops might depend on model parameters, we performed a range of simulations wherein relevant parameters were subject to variations.

Beside degradation, tryptophan is consumed for the production of cellular proteins. This process affects the intrinsic noise of tryptophan directly and is mediated by the tryptophan uptake kinetic rate g (see model equations, section 1, the SI). We tested for the case for lower g and when no tryptophan is consumed for protein production ($g=0$). Figure S5 shows that the noise profile in these cases generally retains the qualitative pattern recorded for the wild-type *trp* system. Surprisingly, even when no tryptophan uptake is considered, the distinct noise-related functions for the loops are, although less pronounced for Rep and EI, still clearly visible.

We were interested to find out if higher concentrations of the systems components at steady state would affect the observed qualitative noise-related features of the loops. To this end, we increased the initial concentration of the operator (Op) to 100 times of its basal level. Overall, we observe reduction in noise levels in most cases due to increased steady-state concentrations of the components (Figure S6). Nevertheless, all the loops retain their noise-related characteristics, particularly regarding noise at the tryptophan outputs.

Next, we examined the effects of variations in the synthesis and degradation rates of each component on the systems noise pattern. We alternatively lower and upgrade the rate of free operator synthesis (k_1), transcription rate (k_2), translation rate (k_3), tryptophan synthesis rate

(k_4), rate of free operator degradation (k_{d1}), and mRNA degradation rate (k_{d2}) to five times below and above their basal values. Systematic computational experiments were similarly carried out and their results are displayed in Figure S7-9 in the SI. Consistently, we observed the exact same patterns of total noise measured for each component, suggesting that these patterns are independent of the synthesis and degradation rates examined. Furthermore, we explored if variation in the cell growth rate (μ) which potentially affect steady-state levels of all components might affect the systems noise pattern (Figure S10). A 10 times higher μ enhances noise in tryptophan in all cases, most likely owing to downgraded steady-state concentration, but the same noise patterns were observed.

Finally, it has been known that higher Hill coefficients make a negative feedback more switch-like (sensitive) [19]. Here we explored how raising the Hill coefficients would affect the noise-mediating roles of the negative feedback loops. In this regard, we alternatively doubled each Hill coefficient and assessed the systems components' noise levels in response to varying feedback strengths. Interestingly, all the loops appear to enhance their own noise-mediating functions when they are more sensitive (Figure S11d - green curve; Figure S11h, red curve and Figure S12l, red curve). A more sensitive Rep (or EI) loop appears to have little impact on the tryptophan-noise-mediating function of the other loops (Figure S11, 4th column, green curves). However, the noise-enhancing feature of the wild-type Rep loop becomes dramatically less visible with a more sensitive Att loop (Figure S11d, red curve). Similarly, the noise-reducing feature of the EI loop was also attenuated by a more sensitive Att loop (Figure S11l, red curve).

7 Discussion

Molecular fluctuations are known to affect dynamics of cellular systems in important ways [31]. Studies aimed at understanding how cellular systems of certain regulatory structures controls noise thus become essential. Such understanding would provide insights into how noise arises in the first place and shed light on the evolution of commonly encountered regulatory mechanisms, such as feedback regulation. More importantly, it might illuminate how systems like signalling cascades can efficiently process and reliably deliver signals originated from cell surface to the nucleus amidst constant stochastic cellular events. Deeper understanding of hidden connections between noise and regulatory mechanisms would also be particularly useful for areas such as synthetic biology, where artificial circuits are designed with desired properties [32].

The interplay between feedback regulation and noise has been previously explored for cellular networks governed by a single negative feedback loop [16-18] (summarised in

section 2). However, similar issues within networks employing more complex regulatory structures remain elusive. Moreover, since noise is thought to propagate between components, how it behaves depends largely on the topology of the studied network, yielding it hard to generalise any conclusions drawn from isolated work. In this study, we set out to investigate how negative feedback loops manage noise within a biochemical cascade concurrently governed by multiple negative feedback loops, using the tryptophan operon system in *E.coli* as a model system. To our knowledge, this is the first study of noise in the *trp* operon system.

To set the scene, we have demonstrated that noise indeed propagates in the two-stage, negatively regulated biochemical cascades. Systems of such hypothetical motifs can be found in cells ranging from protein networks to signalling cascades (Figure 3). We found that the negative feedback loop increases noise at the output component only when the feedback is sufficiently strong. This result agrees qualitatively with those from Stekel and Jenkins [33] wherein they showed that negative auto-regulation increases intrinsic noise for strong repressors. It should be noted that in their work, they did not distinguish between the intrinsic and propagated noise parts. Thus, the “intrinsic noise” measured by them is equivalent to our “total noise”. We showed that an explanation for the non-increasing behaviour of noise at low feedback strength is not satisfactory without taking into account the propagated noise part, hinting at its existence.

Interestingly, when we assessed effect of the negative feedback while keeping the steady-state level of the output component unchanged, the negative feedback displays opposite behaviour. Stronger feedback now results in weaker total noise. Closer examination showed that such reduction is largely attributed to the decrease in the propagated noise part as a result of a stronger feedback loop. Similar observation was demonstrated analytically and computationally in gene expression [34]. Therein, under the same condition of fixed steady-state protein level, transcriptional and translational negative feedbacks both tend to reduce protein noise. Taken together, it is clear that noise does transmit within cascades and the negative feedback loop acting on an upstream reaction can mediate how much noise propagates from an upstream to a downstream component.

How noise behaves, however, becomes more intricate when multiple negative feedback loops operate together. We revealed that the loops in the *trp* operon system possess distinct, even opposing, noise-controlling effects that might have been overlooked given their seemingly analogous feedback structures. The Enzyme Inhibition loop, although controlling the last reaction of the cascade, was found to suppress noise not only for the tryptophan

output but also for other upstream components. In contrast, the Repression loop enhances noise for all systems components. Attenuation poses intermediate effects by attenuating noise for the upstream component but promoting noise for components downstream of its target.

Although it remains to be confirmed experimentally whether or not noise has any physiological relevance in the *trp* operon system, it is reasonable to argue that low variability at the tryptophan level is required for stable production of proteins in the cell whose composition contains this amino acid. This argument is supported by our analysis of noise exhibited by the wild-type *trp* operon system. First, despite noise was high at the mRNA levels, it is efficiently downgraded at the tryptophan levels, indicating the noise-suppressing property of the *trp* cascade. Secondly, we found that the wild-type *trp* system achieves a near-optimal performance at keeping noise small at the tryptophan levels. Comparative work with systems of alternative feedback intensities and systems lacking the multi-loop structure suggested that the low noise feature obtained by the wild-type system is primarily attributed to its multi-loop regulatory arrangement.

Furthermore, at the tryptophan level, the Repression and Attenuation loops display noise-inducing characteristics, while Enzyme Inhibition imposes noise-reducing effect. Interestingly, these features are missing in single-loop systems, implying that their cause must be a result of the interplay between the loops. One such mechanism of feedback interplay was described here which we termed the multi-loop inhibition balancing effect (MIBE). Essentially, within a system with multiple negative feedback loops imposed by a common inhibitor, increasing strength of one loop would result in decreasing strength of remaining loops and vice versa. We showed that this mechanism coupled with noise propagation underpin the disparate noise-controlling functions of the different loops.

The notion that noise in cellular networks is subject to selection is supported by accumulating evidence [3, 4]. Various noise-controlling mechanisms have been thought to be employed by cells to tune noise in RNA and protein levels. For examples, increasing gene copies number through events such as gene duplication or polyploidy could effectively reduce noise [31]. Inefficient translation coupled with fast transcription was also thought to be adopted by some bacteria to curb protein fluctuations [5]. Other mechanisms include integral feedback [35] and regulatory checkpoints [36]. Our findings indicate that acquiring counteracting noise-controlling mechanisms through evolving multiple negative feedback loops might provide yet another way for cellular systems to fine-tune noise. Such feedback design also confers the system to a more diverse repertoire of noise characteristics which,

coupled with the tunability, offer enhanced robustness to the system. This might rationalise why negative feedback regulation appears to be a recurring theme in cells.

The dynamics of a system depends not only on its regulatory structure but can also depend on how it is parameterised [20]. To assess the parametric effects on noise pattern in the *trp* system, we carried out thorough parameter sensitivity analysis wherein we systematically varied individual parameters around their basal values. In most cases, we observed little impact to the noise pattern as well as the noise-controlling functions of each feedback loops. Interestingly, all the loops appear to enhance their own noise-mediating functions when they are induced to be more sensitive by having higher Hill coefficients. Altogether, these findings suggest that the observed noise-related behaviours in the *trp* are largely parameters independent.

It is important to note that the *trp* system considered here is fundamentally different to the gene expression cascades studied by most previous work [10, 12, 16-18]. First, the *trp* system described in our study integrates the transcriptional and metabolic levels of tryptophan production. It not only accounts for the expression of the catalytic enzymes (e.g. anthranilate synthase) but also describes the subsequent step of how such enzymes catalyse the metabolic synthesis of tryptophan. Secondly, in the *trp* system, upstream steps are feedback inhibited by tryptophan (a small molecule) rather than usually by proteins as in the case of gene expression cascade. Such differences may underline possible discrepancies in noise-related observations between the *trp* systems and those of the gene expression levels.

This study opens up several lines of research worth pursuing in future work. For example, it would be worthwhile to extend similar investigation for generalised cascades to see to what extent the conclusions here are parametrically dependent. Furthermore, it would also be useful to understand possible effects that variation of cascade length might have on the interplay between feedback regulation and noise.

Acknowledgement

The first author would like to thank Lincoln University for the writing scholarship that supports this work.

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