A Unified Framework to Dissect Robustness, Plasticity, Evolvability and Canalisation of Biological Function

Debomita Chakraborty[2,3,4], Raghunathan Rengaswamy[*1,3,4,5], and Karthik Raman[*1,2,3,4]

[1]Department of Data Science and AI, Wadhwani School of Data Science and AI, Indian Institute of Technology (IIT) Madras, Chennai — 600 036, India
[2]Bhupat and Jyoti Mehta School of Biosciences, Department of Biotechnology, IIT Madras, Chennai - 600 036, India
[3]Centre for Integrative Biology and Systems mEdicine (IBSE), IIT Madras, Chennai — 600 036, India
[4]Robert Bosch Centre for Data Science and Artificial Intelligence (RBCDSAI), IIT Madras, Chennai — 600 036, India
[5]Department of Chemical Engineering, IIT Madras, Chennai — 600 036, India

Abstract

A unique balance of seemingly contradictory properties like robustness and plasticity, or evolvability and functional canalisation, characterises biological systems. To understand the basis of these properties, we look into gene regulation, which is at the core of biological function. We simulate dynamical models of over 190 million genetic circuits covering all possible three-gene circuit structures. We develop a computational pipeline to classify these circuits into functional clusters by matching the shape of their temporal responses. Thus, we generate a dataset containing circuit structure, parameters and a corresponding functional label. Our key finding is a finite list of 20 functions that three-node genetic circuits can perform. Moreover, the space of structure and parameters for these circuits tend to be primed for responses that stabilise over time following a perturbation. Every structure has the potential to exhibit multifunctionality with a range of 2–17 functions contingent upon parameters. We quantify network degeneracy and show that many structural changes can be made to a circuit without changing its function. We then define three quantities: structural, parametric, and functional diversities. For a pair of circuits in our generated dataset, we derive these diversities and present a unified framework that analyses the four key biological properties—robustness, plasticity, evolvability, and functional canalisation. Using this unified framework, we identify that only 6.5% of network structures are non-plastic, while it is always possible to find parameter sets for every three-node network to exhibit parametric robustness. We identify functionally canalised circuits from structure pairs that can be interchanged for a large number of parameter sets without a change in function. Overall, our framework offers insights into the fundamental organisation of biological networks by thorough analysis of three-node networks.

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[*]Corresponding authors. E-mail: raghur@iitm.ac.in, kraman@iitm.ac.in

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Significance Statement

Biological systems exhibit remarkable properties like robustness, plasticity, evolvability, and canalisation. This study presents a unified computational framework to understand these properties by exhaustively exploring the design space of three-node genetic circuits, identifying that only 20 functions are achievable, and revealing a natural bias toward stability. We uncover key principles of network degeneracy and multifunctionality, highlighting the versatility of genetic circuits. By analysing structural, parametric, and functional diversities, we identify structural and parametric changes that can transition a genetic circuit from robust behaviour to plasticity or from being canalised to becoming evolvable. Our work advances theoretical insights into biological function. It provides a method to identify alternate designs and parametric conditions for genetic circuits, paving the way for the design of novel synthetic genetic circuits.

1 Introduction

Biological systems demonstrate a fine balance of robustness, plasticity, evolvability and functional canalisation. Robustness is the ability of the system to maintain a function under changes in the structure and/or parameters of the system, or environmental changes. Over two decades back, Kitano [1] suggested the possible bases of robustness in biological systems to be network structure, alternative mechanisms like duplicate genes or multiple structures for the same function, modularity and decoupling. Furthermore, he argued that robustness is fundamental in evolvable systems and called for a unified framework with a strong mathematical basis to analyse robustness in all its possible forms. Plasticity is the property that allows organisms to dynamically tailor responses to internal or external cues. Evolvability may be defined as the potential to evolve or exhibit diverse phenotypes, whether at the organism or population level. Wagner [2] elaborates on the coexistence of robustness and evolvability, citing that mutations can unleash evolutionary innovations by inching towards novel phenotypes even when they do not affect a particular phenotype immediately due to the existence of a neutral mutational space that confers mutational robustness to biological systems. A comprehensive study of evolvability can be found elsewhere [3], along with methods to quantify this property from an evolutionary biology perspective. Waddington [4] coined the term canalisation to describe the low variance in phenotypes due to genetic and environmental variations in the wild-type, unlike strains of organisms manipulated in the laboratory.

In this work, we aim to construct a unified framework with a solid mathematical basis to analyse robustness, plasticity, evolvability, and canalisation at the level of gene regulation. For this, we consider a genetic circuit as a dynamical system whose response ("circuit function") to a stimulus depends on the circuit structure and parameters [5–8]. We assume that mutations and epigenetic or environmental changes can affect gene regulation by changing the structure and/or parameters of genetic circuits, thereby influencing the circuit function, i.e. phenotype. In our unified framework, we do not directly quantify parametric robustness, plasticity, evolvability, and functional canalisation but define conditions under which these properties are exhibited. These conditions are based on the changes in circuit structure and parameters, and in our analysis, we do not refer to their underlying biological cause, which

may be mutational, epigenetic, or environmental.

The space of all possible circuit structures and parameters constitutes the design space. We study the design space of all possible three-node genetic circuits based on the classic framework proposed by Tang and co-workers [9, 10], who studied three-node networks to identify the structural conditions necessary for perfect adaptation. Our focus, however, expands to exploring the global design space of three-node genetic circuits with the aim of finding all possible functions. Most existing works in the literature aim to design or fine-tune a genetic circuit to achieve only one functionality of interest. One notable work that delves into the design space is the Design Space Toolbox V3, which adopts a systems-theoretic approach [11], but its assumptions regarding the kinetic equations may not accurately model underlying mechanisms, and it cannot be used to explore multiple stable states [12].

To address these gaps, we develop a novel computational approach. Firstly, we map the circuit structures and parameters with the circuit functions. For this we simulate the mathematical models of the circuits over millions of parameter sets and pass the simulated data through our computational pipeline for clustering circuits with similar temporal responses. The output of this pipeline is a dataset with circuit structure and parameters along with a functional label. Secondly, we use this labelled dataset to construct and use our unified framework for analysing the four biological properties– parametric robustness, plasticity, evolvability, and functional canalisation.

In Section 2, we describe our methodology for generating the simulated dataset and the computational pipeline we have used to process the dataset to get the labels. In Section 3, we discuss our key findings. Further details of our methodology and validation of our findings are provided in Supplementary Methods.

2 Methodology

We develop a computational framework for studying the design space of genetic circuits. The main steps involved are the generation of time-course data, and creating a computational pipeline for processing these data as illustrated in Figure 1.

2.1 Data Generation

We represent a genetic circuit as a network with genes as the nodes. Each of the genes in the network expresses a transcription factor (TF) that either activates or inhibits the expression of the other genes in the network. These two types of interactions are represented by two types of edges in the network, *viz*., activation as arrowheads, and inhibition as bar heads. We label the three nodes as A, B, and C, where a step input is applied to node A, and hence, it is designated the input node, while node C is called the output node. The output gene expression over time for the given input, i.e. the expression of protein/TF from the output gene, C, is the focus of our analysis in this work. This framework is identical to that proposed by Ma *et al.* [9].

2.2 Setting up a Computational Pipeline

We simulate the ODE models to get each network's concentration dynamics for 100 time points over the parameter sets for which a steady-state greater than 0.001 could be determined, as given in Section § 1.5 in Supplementary Methods. We construct a pipeline that recursively performs two operations: Time Series Clustering and Barycenter Calculation. We calculate the pairwise distances for time courses

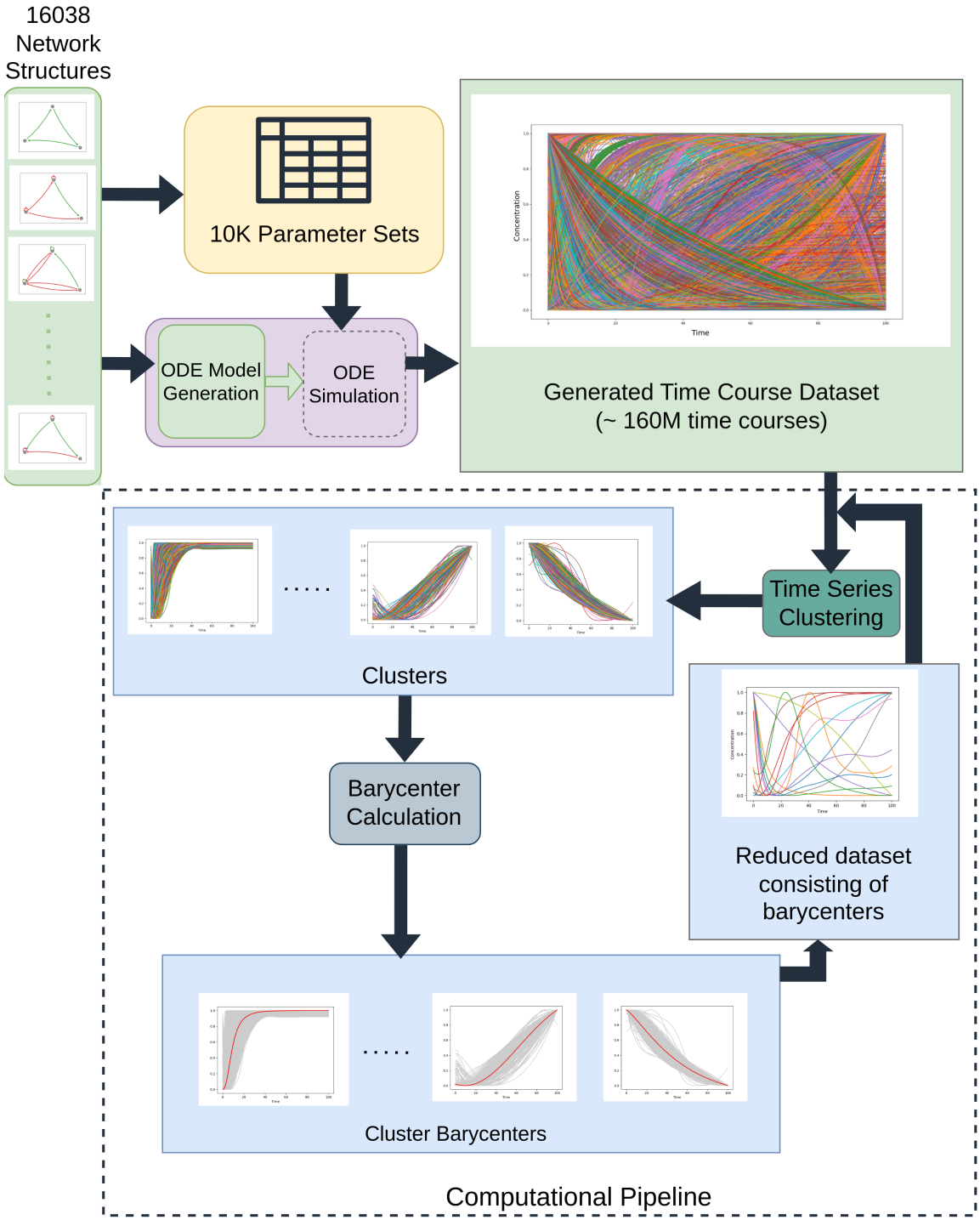

Figure 1: **Workflow Diagram.** We derive the ODE model of each of the 16038 networks from their adjacency matrices and simulate these models over three sets of 10,000 parameter sets each, sampled using Latin Hypercube Sampling. We pass the resulting time course dataset through a computational pipeline that performs time series clustering followed by barycenter calculation for each time course cluster. (The barycenter corresponding to a cluster is shown in red.) The barycenters constitute a reduced dataset that serves as the input to the computational pipeline in the next iteration.

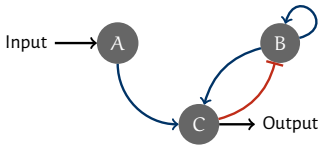

Figure 2: **A representative three-node genetic circuit.** A, B, C represent genes that interact by activation (shown as arrowheads) or inhibition (bar-heads). An activating input is applied to gene A, labelled as the *input gene* while C is labelled as the *output gene*.

(min-max scaled) in our dataset using Dynamic Time Warping Distance (DTW-Distance) [13]. Subsequently, we perform K-Means clustering based on pairwise DTW distance. The number of clusters is determined by locating the "elbow point" within a range of 2-100 clusters [14]. For each cluster identified by K-Means, the barycenter is the time series representative of the cluster. We calculate the barycenters of each of the clusters using Soft-DTW [15].

In the first iteration, we cluster the time courses obtained for each network over 10K parameter sets. Suppose a cluster with less than 10 time courses is found; we drop these time courses from further iterations as these represent network functionalities that cannot be achieved by even 0.1% of the parameter sets [9]. For all the remaining clusters for each network, the barycenters are calculated. The set of barycenters across all the networks then forms a reduced dataset for the second iteration of time series clustering. At the end of an iteration we check if the shapes of all the barycenters are distinct from each other and if so, we terminate the execution at this iteration.

3 Results

We systematically examine all possible three-node networks by sampling ten distinct sets, each comprising 10% of the networks. The sampling procedure (detailed in Supplementary Text § S1.2) guarantees structural representativeness within each set, effectively capturing the structural characteristics of the complete set of 16038 networks. We run two iterations of our computational pipeline with the time courses for each sampled set of networks. In the second iteration, the reduced dataset consists of about 15–16 clusters. We observe this across all the ten sampled sets of networks (Supplementary Information § S2). Furthermore, we repeat the complete application of our computational pipeline to three sets of 10,000 parameter sets each to check for consistency in the results obtained (Supplementary Figure S1) and again we find 15-16 distinct clusters after the second iteration. Finally, we integrate the findings across the three runs to arrive at our consolidated results below.

3.1 Three-node networks perform 20 circuit functions

Our analysis identifies a set of 20 functionalities achievable with three-node circuits over 30,000 parameter sets, detailed in Supplementary Table S2. These represent distinct time courses, each embodying a unique shape that we interpret as distinct circuit functionalities illustrated in Fig. 3. A noteworthy observation is that 17 out of 20 circuit functions consistently emerge across all three pipeline runs, as detailed in Supplementary Information section § S2. Furthermore, we map each time course within a functional cluster to the specific network that produced it to establish a clear connection between the temporal dynamics and their underlying network structures.

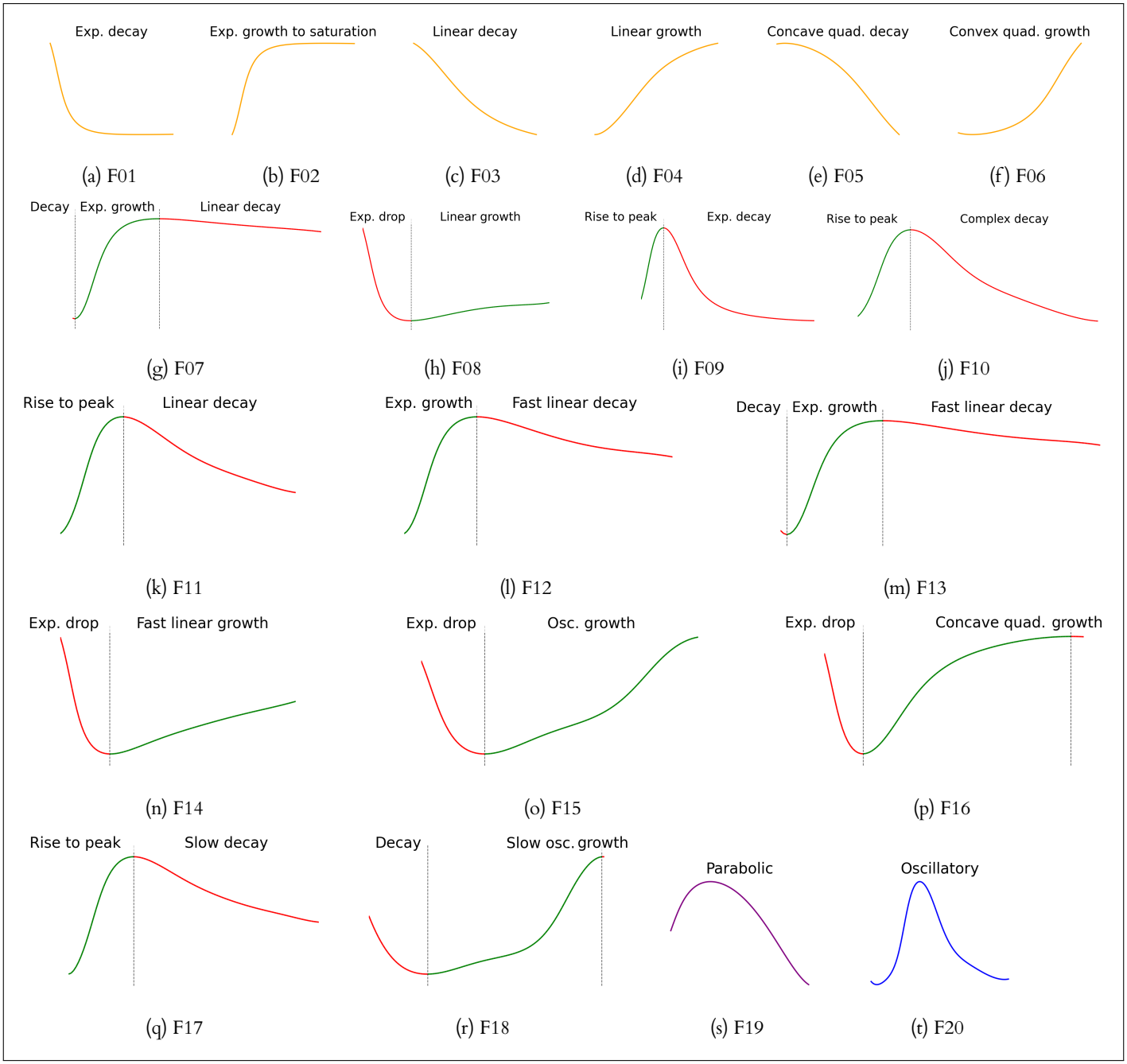

Figure 3: **Three-node genetic circuit functions list.** The 20 distinct time courses characterise the functional space (in response to a step input) of all three-node genetic circuits. The description of each function is given in Supplementary Table S2, along with the percentage of circuits exhibiting the function.

3.2 The design space is primed to produce circuits that stabilise over time

Since circuit function depends on the network structure and the parameters governing the interactions within the network, we analyse the prevalence of the 20 functions exhibited by three-node networks in two ways: (a) in terms of network structure alone (network distribution) and (b) in terms of network structure with parameters defined (circuit distribution). In Supplementary Table S2, we show the high occurrence of circuits showing exponential decay (28.36%) and the circuits that saturate after exponential growth (27.37%) compared to the other circuit functions. This suggests that the design space favours some functions over others and aligns intuitively with the observed tendency of biological systems to navigate toward stabilisation. The least frequent functions are characterised by oscillations (F20), a parabolic trajectory in time (F19), and decay followed by oscillating growth (F18). We note that the percentage of oscillatory networks represents only networks that are initially at a steady state and only respond to a step input with oscillations. From our analysis, we have eliminated any circuit that shows oscillations inherently when we determine the initial conditions for our models, explaining the small percentage of oscillatory networks. Moreover, we have eliminated any behaviour exhibited by a network for less than 10 out of 10000 parameter sets in the first iteration of our computational pipeline. These eliminated time courses may include oscillatory as well as adaptive behaviours. We separately analysed the simulated time courses to look for adaptive networks using criteria from existing works [9, 10]. The structural analysis of the adaptive networks reveals that using Hill kinetics, it is possible to achieve adaptation in three-node networks even without negative feedback loops or incoherent feedforward loops, unlike previously reported results by Shi [10]. The detailed findings on adaptive networks are given in the Supplementary Information section § S4.

3.3 Every network is multifunctional

A notable observation in our analysis of three-node genetic circuits is that none of the networks displayed mono-functional behaviour. That is, as we change the parameters of a given circuit, showing a particular function, it produces at least one new function at a different point in the parameter space. This finding highlights the intrinsic versatility of gene regulatory networks, showcasing their ability to exhibit diverse functionalities under varying parametric conditions. The number of distinct functions a three-node network performs spans an impressive range, from at least two to as many as 17 functions.

We further categorise the 20 circuit functions into 5 categories, *viz.*, (i) monophasic, (ii) biphasic, (iii) triphasic, (iv) oscillatory, and (v) complex (Supplementary Table S3) based on the number of phases a time response can be divided into. The distribution of networks across the five categories is depicted in Supplementary Figure S2.1, while that of the circuits is shown in Supplementary Figure S2.2. The monophasic category encompasses networks that show either growth or decay. The biphasic category includes time responses that exhibit a period of growth (or decay) followed by a period of decay (or growth). The triphasic category includes responses with three phases of interleaved growth/decay. We consider oscillatory circuits a different category as they exhibit periodicity. We define a complex dynamic as one that exhibits a general trend of growth or decay but with oscillations or multiphasic behaviours superimposed on it.

Supplementary Figure S2 allows a nuanced analysis of the design space, revealing that while 5% of the networks manifest oscillatory behaviour, merely 0.02% of circuits actually exhibit this oscillatory behaviour. The percentage of circuits that exhibit the functions corresponding to each function category is much smaller than that of networks that fall in the corresponding category. This underscores the pivotal role of parameters in biological regulation determining a network's function, complementing its structural characteristics. All 16038 networks are capable of exhibiting monophasic responses.

However, networks that exclusively exhibit monophasic dynamics constitute only 21.8% of all networks. Interestingly, 53.03% of the networks can exhibit all the categories of functions under different parametric conditions except for oscillations, suggesting that an oscillatory response to a step input is a rare function. Other combinations of function categories are exhibited by <5% of the networks, with the rarest combinations being monophasic (I) and oscillatory (IV), or monophasic (I), biphasic (II) and oscillatory (IV). The rarest category of circuit function is oscillatory, while more than 90% of the circuits exhibit monophasic responses.

3.4 Network degeneracy allows a large number of structural changes with no change in function

We define network degeneracy as a measure of structural changes (edge addition/removal/change in the sign of an edge) that can be made, with no subsequent parameter adjustment, without causing loss of function. To quantify network degeneracy, we leverage our method of using a superset of parameter sets in 21 dimensions (for fully connected networks) to derive parameter sets for all 16038 networks (Supplementary Text section § S1.4). For a 21-dimensional parameter set, removing an edge makes the corresponding parameters zero, whereas adding an edge introduces the same parameter values from the 21-dimensional set under consideration. For simplicity, a change in the sign of an edge does not change the corresponding parameter values, i.e. the activation (inhibition) threshold and cooperativity become the inhibition (activation) threshold and cooperativity. We track, for each parameter set in 21 dimensions, the number of structures that produce the same circuit function.

In Supplementary Figure S3, we show the median numbers of structures over all parameter sets for each of the 20 functional clusters. While the network degeneracy depends on the function under consideration, across all functions, we find a median of 60 structural changes.

3.5 A Unified Framework for Insights into Biological Function

We now describe a conceptual framework that unifies the analysis of the four properties of biological systems—parametric robustness, plasticity, evolvability and functional canalisation, using the results of our computational pipeline, as shown in Fig. 4. Considering a pair of circuits C_i and C_j with structures, parameter sets and functions given by (N_i, P_i, f_i) and (N_j, P_j, f_j), respectively, we define three quantities, *viz*., structural diversity (SD), parametric diversity (PD), and functional diversity (FD).

Structural diversity (SD_{ij}) between C_i and C_j is quantified by the Hamming distance between the adjacency matrices N_i and N_j and assumes 10 discrete levels $[0-9]$.

Parametric diversity (PD_{ij}) is quantified by the Euclidean distance between parameter sets P_i and P_j in the 21-dimensional parametric space.

Functional diversity (FD_{ij}) is quantified using equation 1. We encode each network function as a k-hot binary vector of length 20 corresponding to the list of 20 possible functions with k indicating the number of functions exhibited by the network and ranging between $2-17$. We calculate the Hamming distance between the k-hot function vectors for N_i and N_j to obtain HD_{k-hot}. Furthermore, we encode each circuit function as a *one*-hot binary vector of length 20 corresponding to the list of 20 possible functions. We define the Hamming distance between the function codes as HD_{1-hot}, which is 0 if f_i and f_j are the same; else, 1.

$$FD_{ij} = HD_{k-hot} + w_{ij} HD_{1-hot} \tag{1}$$

where w_{ij} is the weight quantifying the dissimilarity in functions depending on the function categories to which f_i and f_j belong, as given in Supplementary Table S4. FD is a discrete variable that assumes

37 unique values ranging between $0 - 18$. For circuit pairs that share the same network structure ($SD_{ij} = 0$ since $N_i = N_j$), $HD_{k-hot} = 0$, so FD can assume only 3 values $\{0.0, 0.5, 1.0\}$. This shows that constraining the structural diversity also constrains the functional diversity.

We have functionally labelled over 190 million three-node genetic circuits using our computational pipeline. Each data point in the conceptual space in Fig. 4 involves the calculation of structural, parametric and functional diversities for pairwise circuits. The number of computations involved in analysing all labelled circuit pairs is highly time-consuming and resource-intensive. We identify four properties of biological systems that can be analysed in terms of the three diversities: functional canalisation, parametric robustness, plasticity, and evolvability. These can be mapped to specific regions of the conceptual space in Fig. 4. Hence, we confine our analysis to these biologically relevant regions of this space and derive insights that will help us advance our understanding of the design space. The representation in Fig. 4 allows us to abstract the effect of mutations and/or epigenetic changes as changes in genetic circuit structure and/or parameters and enables us to dissect the role of structure and parameters in producing functional canalisation, parametric robustness, plasticity, and evolvability, in a unified framework.

3.6 Conditions for parametric robustness and plasticity

Suppose mutations or epigenetic changes lead to changes in parameters but not the circuit structure; the circuits before and after the change constitute a pair with $SD_{ij} = 0, PD_{ij} \neq 0$. For all such pairs of circuits, we calculate the parametric and functional diversity and find the overall mean parametric diversity $PD_{overall_mean}$.

Parametric Robustness: We consider the circuit pairs for which $FD_{ij} = 0$, and $PD_{ij} > PD_{overall_mean}$ to be robust as their functions/function categories remain unchanged for more than average changes in parameters.
Plasticity: Circuit pairs that have $FD_{ij} \neq 0$ and $PD_{ij} < PD_{overall_mean}$ are considered plastic as they exhibit diverse functions with relatively small changes in parameters.

We analysed the relationship between circuit structure and parametric robustness/plasticity in two ways for data points with zero structural diversity (SD). First, we identified the most frequently occurring network structures among robust and plastic circuit pairs. These predominant structures are illustrated in Supplementary Figures S4.(1) and S4.(3) for robust and plastic circuits, respectively. Second, we quantified the propensity of each network structure to generate robust or plastic behaviour. The most striking example, shown in Supplementary Figure S4.(2), exhibits robustness in 52.67% of all circuit pairs sharing this structure. We found 209 distinct network structures maintaining robust behaviour across more than half of their parameter sets. In contrast, even the most plastic-prone structure exhibits parameter-dependent behaviour in only 40.75% of cases. Notably, our analysis revealed a fundamental asymmetry: while every three-node network structure can achieve robust behaviour with appropriate parameter selection, 1,043 structures (6.5% of the total) never display plastic behaviour, regardless of their parameters. This finding implies better suitability of certain structures in synthetic biological applications where plasticity is not a desired property.

3.7 Conditions for functional canalisation and evolvability

Suppose a circuit undergoes structural and parametric changes ($SD_{ij} \neq 0, PD_{ij} \neq 0$) due to mutations or epigenetic change but retains its function; then the circuit pair before and after the change represent

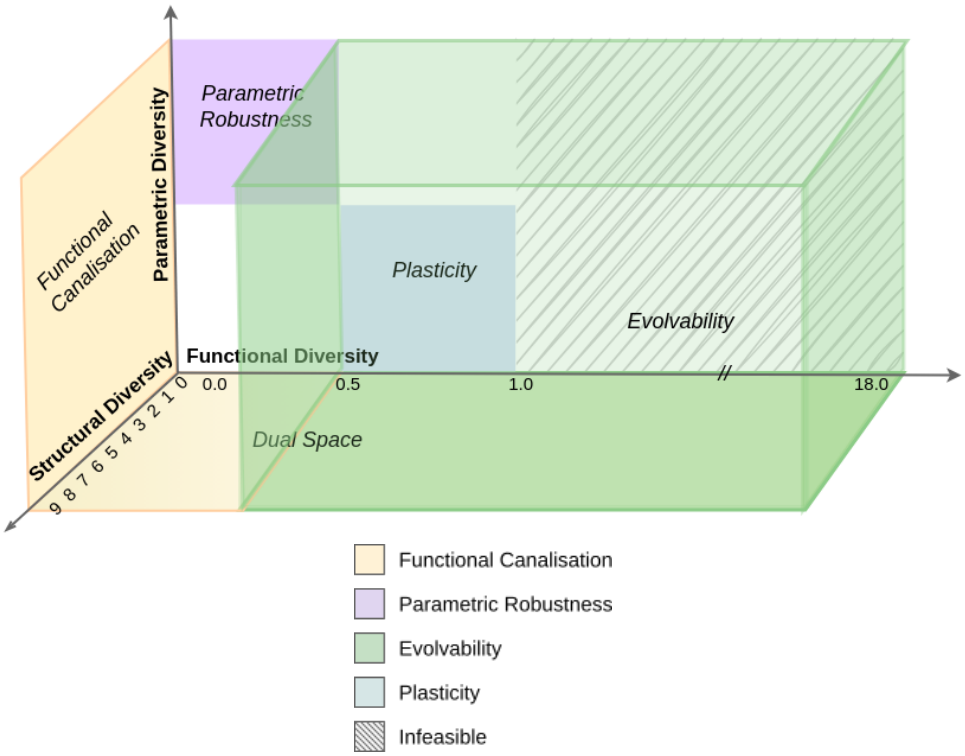

Figure 4: **A unified framework.** A conceptual framework for quantifying parametric robustness, plasticity, functional canalisation, and evolvability in terms of structural, parametric and functional diversities.

a data point on the $FD = 0$ plane. To ensure $PD_{ij} \neq 0$, we consider structural changes with at least one edge being added or removed (more details in Section 3.8). A nuance to consider in this context is that when a mutation leads to a change in the structure of a circuit from N_i to N_j, it inherently leads to changes in parameters corresponding to the edge changes while the parameters corresponding to the unaltered edges may or may not be affected. In our analysis, we do not distinguish between the two scenarios. Instead, we track only whether the circuit pair has zero functional diversity ($FD_{ij} = 0$).

Functional Canalisation: We interpret every point on the $FD = 0$ plane to be functionally canalised since they represent circuits that can maintain functionality even after changes in structure and parameters (Fig. 4). When a circuit pair having structures that share the same function vector ($HD_{k-hot} = 0$) exhibits the same function ($HD_{1-hot} = 0$), or it exhibits two distinct functions but of the same category ($w_{ij} = 0$), the functional diversity is zero (Eq. 1). We find that canalised circuit pairs tend to have a peak SD between 4-6, indicating that a medium level of structural diversity is conducive to canalisation. Canalised circuit pairs can provide reliable alternate design options for achieving the same function in synthetic biology applications.

Evolvability: Circuit pairs for which $FD_{ij} \neq 0$ and $SD_{ij} \neq 0$ are defined as evolvable since they represent circuit pairs with both structural and parametric changes leading to new functions. The space representing evolvability with $FD \neq 0$ is shown in Fig 4.

3.8 Dual Space

Given a circuit, when there is a change in the type of interaction from activation to inhibition or vice versa without introducing a new interaction or eliminating any existing interactions, we consider this a special case of structural change that produces *dual circuits*. If a circuit before and after such a structural change has at least one interaction type unchanged, then we term it a *partial dual* while a type change for all the edges results in a *complete dual*. The condition for duality is given below.

$$SD_{ij} \neq 0, \text{ and } PD_{ij} = 0 \tag{2}$$

In our sampling method, since we use parameter sets from a super-set, the numerical value of the parameters corresponding to the altered edges in duals do not change, i.e., although activation (inhibition) changes to inhibition (activation), the parameter values for these remain the same. PD_{ij} being the Euclidean distance between a pair of parameter sets, for all dual pairs, PD_{ij} is zero. If duals have zero FD_{ij}, they are functionally canalised duals, while duals with $FD_{ij} \neq 0$ fall in the evolvable category. Interestingly, functionally canalised duals produce the same function when one or more of its activating interactions is changed to an equal level of inhibiting interaction. When designing genetic circuits, understanding the dual space as defined in the proposed framework can help to identify design options for achieving a particular function, especially when the choice of some of the genes is constrained and only certain interactions can be manipulated. Notably, out of all possible ≈128 million pairs of 16038 three-node networks, we find 69174 pairs of networks that are complete duals. Moreover, we find 1343 parameter sets for which at least 10 (and a maximum of 44) complete duals exist for achieving the same function. Not all functions afford complete duals; however, for 16 out of the 20 possible functions, we could find at least one complete dual. We defer looking into the space of partial duals and constructing a library of alternate circuit designs using both complete and partial duals for the future.

4 Discussion

Our study presents the first comprehensive exploration of the three-node genetic circuit design space, analysing over 190 million circuits to reveal fundamental principles of biological regulation. This unprecedented scale of analysis has led to discoveries that reshape our understanding of biological circuit design and function. While computational models have long enabled synthetic biological circuit design [16, 17], previous approaches like Tang and co-workers' study of perfect adaptation [9] or Chiang's KMFA pipeline focused on optimising circuit parameters for single functionalities [18]. Our work transcends these limitations by providing a complete map of the achievable functional space and establishing a unified framework for understanding circuit behaviour. The discovery that only 20 distinct functions are achievable by three-node genetic circuits represents a fundamental insight into the constraints of biological regulation. This finding, emerged from analysing 30,000 parameter sets across all possible three-node network structures, suggests an inherent limit to the complexity achievable with three-node circuits. Remarkably, the distribution of these functions is highly non-uniform, with over 55% of circuits exhibiting either exponential decay or exponential growth to saturation. This strong bias toward stabilising responses to perturbation suggests an evolutionary preference for regulatory systems that can return to equilibrium over time. The implications of this finding extend beyond theoretical interest - it provides crucial guidance for synthetic biology efforts by defining the complete space of achievable functions with three-node circuits. Our analysis revealed two fundamental properties of genetic circuits that have profound implications for both natural and synthetic systems. First, every network structure examined exhibits multifunctionality, capable of producing between 2 and 17 distinct functions depending on parameter configurations. This universal multifunctionality suggests that natural circuits may have evolved to exploit this inherent versatility, potentially switching functions through parameter changes rather than structural modifications. Second, we discovered significant network degeneracy, where multiple structural changes often preserve circuit function without requiring parameter adjustments. This finding has immediate practical applications in synthetic biology, offering multiple design options for achieving desired functions and suggesting strategies for engineering robust circuits. The unified framework we developed, based on structural, parametric, and functional diversity metrics, provides a novel approach to analysing fundamental biological properties. Previous studies by von Dassow [19] quantified robustness of the segment polarity gene function through the count of random parameter samples that produce a phenotype. Wagner [20] suggested an antogonistic relationship between genetic robustness and evolvability but positive correlation between phenotypic robustness and evolvability due to large neutral spaces indicating a many-to-one map between genotype and phenotype. However, Mayer showed using Boolean maps between genotype and phenotype that Wagner's suggestion was valid only in special cases while in general, phenotypes are more likely to have a trade-off between robustness and evolvability [21]. Our framework moved away from the genotype-phenotype paradigm to the circuit level to simultaneously consider structural, parametric, and functional aspects. This approach revealed that while every three-node network can achieve robust behaviour with appropriate parameters, 6.5% of structures are fundamentally non-plastic - a finding that is significant for synthetic biological applications where plasticity is not a desired property. A particularly significant discovery is the existence of functionally canalised duals - circuits that maintain identical function despite having opposite interaction types (activation versus inhibition). We identified 69,174 pairs of complete duals among approximately 128 million possible network pairs, with some parameter sets supporting up to 44 different dual implementations of the same function. This finding has immediate practical applications in synthetic biology, offering multiple implementation strategies for desired functions and suggesting new approaches to circuit design. While well-characterised ex-

amples like the *lac* operon [22] and *kai* circadian genes [23] provide important functions arising from the interaction of three genes, our work reveals a much broader landscape of possible circuit behaviors. The practical relevance of our findings extends to critical areas like cancer research, as exemplified by the RAS gene family (KRAS, HRAS, and NRAS) [24, 25]. Our framework provides new ways to analyse such systems and identify potential therapeutic targets by understanding their regulatory dynamics within the complete functional space. Our approach complements experimental methods like scRNA-seq and ATAC-seq by providing a forward design perspective rather than inferring networks from data. The comprehensive data repository we have created serves as a valuable resource for synthetic biology, offering a complete map of structure-parameter-function relationships and enabling rational design of circuits with desired properties. This is particularly powerful when combined with our understanding of network degeneracy and dual implementations, as it allows designers to choose optimal implementations based on practical constraints. Looking forward, several exciting avenues exist for expanding this work. While we focused on step input responses, future investigations could explore other input types and incorporate noise, which has been shown to produce richer dynamics [18]. The framework could be extended to larger networks, though the computational challenges would increase significantly. Most importantly, our findings suggest specific experiments to validate the predicted rare functional categories and test the practical implementation of dual circuits. The key contribution of this study goes beyond providing a conceptual framework - it establishes fundamental limits on three-node circuit functionality, reveals universal properties like multifunctionality and network degeneracy, and provides practical guidelines for circuit design. By unifying the analysis of robustness, plasticity, evolvability, and canalisation, we bridge the gap between theoretical understanding and practical application, opening new avenues for both basic research and synthetic biology applications.

Code Sharing Plan

All the code used for the generation of datasets and subsequent analysis will be made publicly available in a GitHub repository upon publication. The repository will include detailed instructions for usage, a list of dependencies, and appropriate licensing to facilitate reuse. A link to the repository will be provided in the final manuscript.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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