Reprogramming cell fate: Systemic failures and their mitigation through feedback control

Domitilla Del Vecchio\textsuperscript{1,2}(\textsuperscript{*}), Hussein Abdallah\textsuperscript{3}, and James J. Collins\textsuperscript{2,4–8}

\textsuperscript{1} Department of Mechanical Engineering, MIT
\textsuperscript{2} Synthetic Biology Center, MIT
\textsuperscript{3} Department of Electrical Engineering and Computer Science, MIT
\textsuperscript{4} Institute for Medical engineering & Science, MIT
\textsuperscript{5} Department of Biological Engineering, MIT
\textsuperscript{6} Harvard-MIT Program in Health Sciences and Technology
\textsuperscript{7} Broad Institute of MIT and Harvard
\textsuperscript{8} Wyss Institute for biologically Inspired engineering, Harvard university
(\textsuperscript{*}) corresponding author: Domitilla Del Vecchio (ddv@mit.edu)

Abstract. After ten years of induced pluripotent stem cell (iPSC) reprogramming, the efficiency of the process remains dramatically low. While many factors contribute to such scarce success, some of these can be systematically investigated by mathematical analysis of the gene regulatory network (GRN) that controls pluripotency. Such investigations are largely missing, yet they can be valuable for identifying systemic bottlenecks and alternate reprogramming strategies. Here, we analyze the dynamical properties of the GRN that is known to control pluripotency and single out structural properties that may contribute to failures in current reprogramming strategies. We find that it is not possible, even theoretically, to guarantee successful reprogramming to pluripotency using pre-fixed overexpression of transcription factors, as is performed in current reprogramming strategies. This is because the core pluripotency network is constituted by positive regulatory interactions while the pluripotent state is associated with intermediate values of one of the factors’ concentration. Based on our findings, we propose an alternative method for iPSC reprogramming, which is theoretically proven to overcome these difficulties. In particular, reprogramming to pluripotency can be theoretically achieved by a feedback overexpression of factors, in which the overexpression level is iteratively adjusted on the basis of the outcome. Accordingly, we propose a synthetic genetic circuit that implements this feedback overexpression and demonstrate its performance through mathematical analysis and simulation. Our results provide a system-level explanation of the low efficiency of current reprogramming approaches. The alternative approach that we propose will possibly lead to a substantial improvement of the reprogramming success rate.

1 Introduction

Reprogramming differentiated cell types to pluripotency is a promising approach to perform basic research on cell development. At the same time, it offers tremendous potential for medicine, where iPSCs could be derived from patient-specific cells to cure a number of illnesses [1]. Reprogramming to pluripotency was first performed a decade ago by overexpressing four key transcription factors (the Yamanaka factors) in fibroblast cells [2]. Since then, intensive research has been conducted to improve the process through the use of different delivery methods, combinations of factors, as well as chemical stimuli and stoichiometric optimizations [3–7]. Although these results show that in principle reprogramming cell fate to the pluripotent state is possible, today iPSC reprogramming remains a very inefficient process. Even integrating methods, which typically are more efficient than non-integrating approaches [8, 9], have still efficiencies as low as 1-2% [10].

Identifying the reasons for such a low success rate and thus potential remedies is particularly difficult because of the large number of variables that play a role in the process of cell differentiation (and its reversal). At the epigenetic level, the impact of chromatin modification has been the subject of intense investigation and
small molecule compounds have been proposed that affect the chromatin state and improve reprogramming efficiency [11–14]. At the mRNA and protein level, specific microRNAs and protein modification pathways have been identified as possible means for improving efficiency of the process [15, 16]. At the gene regulatory network level, much of the core circuitry controlling pluripotency has been identified and experimentally well characterized [17–19]. Still lacking, however, is a systematic mathematical analysis of the dynamical properties of this core circuitry to assess differentiated cells’ potential for being reprogrammed. Yet, such an investigation can provide invaluable information on whether the pluripotency circuitry has any structural property that prevents a successful reprogramming. In fact, it is very well known from control theory that the properties of a system, among which the network’s structure, determine whether a desired state can be reached through suitable input stimuli [20].

Previous works proposed and studied mathematical models of the pluripotency network and its variants. These works performed bioinformatics studies to determine the probability of multistability [21], bifurcation analysis, and simulation to determine how specific network parameters affect the number of (and switching between) steady states [22–26]. Here, we build on these previous models and analyze the dynamical properties of the pluripotency network with respect to its ability to be reprogrammed to the pluripotent state. We find that the core molecular pluripotency circuit [17] falls into the class of cooperative monotone systems [27] due to the fact that it is constituted of positive regulatory interactions. Because of this, positive constant (in time) input stimulation is guaranteed to reprogram the system only to its maximal stable state. However, the pluripotent state is associated with intermediate values of one of the core transcription factors [14, 28]. Therefore, constant overexpression of transcription factors, independent of the delivery method, is not guaranteed to reprogram the network to the pluripotent state. These conclusions continue to hold for the extended pluripotency network, which encompasses additional transcription factors and regulatory links [23, 25].

Therefore, we propose an alternative approach to reprogramming, which is theoretically guaranteed to succeed independent of the network properties and specific parameters. Within this approach, the level of the factors’ overexpression is not fixed and is instead adjusted in time based on the proximity of the system’s state to the target pluripotent state in a negative feedback fashion. That is, as the network’s state approaches the pluripotent state, the overexpression level is decreased. This technique is consistent with previous experimental observations according to which the pluripotent potential of iPS cells depends upon subtle temporary differences in the overexpression levels of the reprogramming factors [4]. Accordingly, we propose a synthetic genetic circuit that implements this negative feedback strategy through simultaneous large overexpression and strong degradation of the reprogramming factors. This synthetic genetic feedback controller forces the reprogramming factors to approach a target concentration that is uniquely determined by the proportion between the overexpression level and the degradation strength, and is independent of the natural structure of the pluripotency network. As a result, the network becomes “frozen” at this target state. The factors’ concentrations in this target state are then compared to those in the pluripotent state and adjusted accordingly by controlling the rations between overexpression and degradation. Control of this ration can be easily accomplished by inducible overexpression of the factors. We demonstrate the performance of this genetic feedback controller through mathematical analysis of the system’s model, a detailed parametric study, and computer simulations.

This paper is divided into two parts. The first part (Section 2) illustrates the poor reprogramming properties of the core pluripotency network across different models and network structures considered in the literature. The second part (Section 3) shows that feedback control allows one to steer and freeze the network’s state to any desired state, independent of the network, which may not even be fully known. It then proposes a synthetic genetic circuit implementation of the feedback controller and demonstrates its performance mathematically and computationally.
Figure 1: The pluripotency network. (a) Network topology of the fully connected triad (FCT), in which Nanog, Oct4, and Sox2 mutually activate each other while self activating [17]. (b) The two-node network lumps in a single node both Sox2 and Oct4.

2 Reprogramming the pluripotency network

The gene regulatory network shown in Figure 1 (pluripotency network) has been identified as the core molecular circuit responsible for the maintenance of pluripotency [17]. It is composed of three key transcription factors, Oct4, Sox2, and Nanog, which mutually activate each other while also self-activating [3, 18]. The dynamics of the transcription factors' concentrations have been described by a set of coupled ordinary differential equations (ODEs), which capture the regulatory interactions through Hill functions [21–26]. Within these models, the process of cell differentiation is mathematically represented by transitions between different stable steady states, a conceptual framework for cell fate decision originally due to Waddington and developed further by a number of researchers [29–31]. Previous studies demonstrated that ODE models of the pluripotency network can admit a large number of stable steady states, each possibly corresponding to a different stable cell lineage that differentiates from pluripotent cells [21]. Experimental studies have discovered that this pluripotent (PL) stem cell state is characterized by an intermediate concentration of Oct4, since increased expression of Oct4 or repression of Oct4 in pluripotent cells leads to somewhat differentiated cells [14, 28]. For example, levels of Oct4 in pluripotent (PL) cells are higher than those in trophectoderm (TR) cells [32, 33] and lower than those in primitive endoderm (PE) cells [33, 34]. Therefore, in any ODE model of the pluripotency network, the pluripotent cell type will necessarily correspond to a stable steady state associated with an intermediate value of Oct4. Nearby stable states characterized by higher or lower Oct4 concentrations will instead correspond to somewhat differentiated cells, which we tag for illustration purposes as TR and PE, respectively. The analysis that we develop applies generally to the pluripotency network, independent on the specific differentiated cell states considered, as long as the PL state is characterized by an intermediate Oct4 concentration.

Here, we investigate whether and when this network can be reprogrammed to the pluripotent state through some fixed overexpression of the factors. To address this question, we analyze an ODE model of the pluripotency network, which is general enough to capture any specific molecular mechanisms of the regulatory interactions and is consistent with previously proposed models. Within this model, overexpression of a given factor is included as an external “input” to the system, which directly increases the rate of production of the factor. Specifically, letting $x_1$, $x_2$, and $x_3$ denote the concentrations of the network’s factors Oct4, Sox2, and Nanog, we write:

$$
\Sigma_u : \quad \frac{dx_1}{dt} = f_1(x, u_1), \quad \frac{dx_2}{dt} = f_2(x, u_2), \quad \frac{dx_3}{dt} = f_3(x, u_3), \quad f_i(x, u_i) = H_i(x) - \gamma_i x_i + u_i, \quad i \in \{1, 2, 3\}, \quad (1)
$$

in which $H_i(x)$ is the Hill function that captures the activation of factor $x_i$ by the networks’ factors [35, 36], $\gamma_i$ is the decay rate constant due to dilution (cell growth) and/or degradation, and $u_i \geq 0$ captures the externally imposed overexpression from the reprogramming process. When $u = 0$, the above system, which we refer to as $\Sigma_0$, describes the natural network’s dynamics without external intervention. Here, we have neglected the mRNA dynamics to simplify notation, assuming that mRNA quickly reaches its quasi-steady state [36]. This
assumption can be made without loss of generality, as the analysis and results that follow hold independent of it. If the transcription factors are cooperative, which is known to be the case in practice [37, 38], this model can have three or more stable steady states [21]. The state associated with an intermediate value of Oct4 concentration can model the pluripotent state, while two of the other stable states can correspond to somewhat differentiated cells, which we call TR and PE.

With the dynamical model representation of the network’s behavior, the process of iPSC reprogramming can be qualitatively described as in Figure 2. For illustration purposes, let us assume that the model with no input $\Sigma_0$ has exactly three stable steady states TR, PE, and PL. Since these are stable, they each have a region of attraction such that if the system’s state $x$ is initialized in the region of attraction of TR (respectively PE or PL), then the system’s trajectory $x(t)$ will approach TR (respectively PE or PL) after a sufficiently long time. When a constant input stimulation $u$ is applied, the landscape of steady states changes. Ideally, one would like the stimulated system $\Sigma_u$ to have a unique globally stable steady state PL' that lies in the region of attraction of the pluripotent state PL (center plot of Figure 2). In this case, sufficiently prolonged stimulation will lead the trajectory of the system starting from any initial state $x(0)$ to approach PL'. Since this state is in the region of attraction of PL, when the stimulation is removed the trajectory will ultimately converge to PL, leading to successful reprogramming of the system $\Sigma_0$ to PL. In cases such as this one, where the stimulated system has a unique stable steady state in the region of attraction of PL, we will say that the system is strongly reprogrammable to PL.

To determine whether the pluripotency network with ODE model as in equations (1) is strongly reprogrammable to the pluripotent state PL, consider the following facts about the model:

1. $\frac{\partial f_i}{\partial u_t} \geq 0$ (positive stimulation): increasing the input increases the production rate of the factors;
2. $\frac{\partial H_i(x)}{\partial x_j} \geq 0$ for $i \neq j$, since the factors mutually activate each other. Therefore, we also have that $\frac{\partial f_i}{\partial x_j} \geq 0$, for all $i \neq j$, implying that the system is a cooperative monotone system [27].

The set of stable steady states in a monotone cooperative system always has a maximal element, which is a stable steady state whose components are all greater than the components of all other stable steady states (see Supplementary Information, Section 1.1). Most importantly, a cooperative monotone system with positive stimulation is strongly reprogrammable only to this maximal stable state (see Supplementary Information, Section 1.1 for the mathematical proof). It follows that the pluripotency network is not strongly reprogrammable to PL because, as explained above, this state is characterized by an intermediate value of Oct4.
Figure 3: Large overexpression is always a losing strategy in reprogramming. Nullclines and vector field for the two-node pluripotency network model in equation (2). (a) System $\Sigma_0$. The black line corresponds to $\dot{x}_2 = 0$ while the blue line corresponds to $\dot{x}_1 = 0$. The light blue arrows indicate the direction and strength of the vector field ($\dot{x}_1, \dot{x}_2$). The highlighted intersections of the nullclines correspond to stable steady states: TR, PL and PE. Parameters are picked as: $a_1 = 0.6, b_1 = 3, c_1 = 1.95, a_2 = 0.0252, b_2 = 0.252, c_2 = 0.28, d = 1, \gamma_1 = 0.3$, and $\gamma_2 = 0.14$. The strength, $a_i$, of activation by $x_1^1$ should be smaller than the strength, $c_i$, of activation by $x_1^2 x_2$, in accordance with the experimental data showing that activation by Nanog is made stronger by cooperation with Oct4 and Sox2 [39]. (b) System $\Sigma_u$ with $u_1 = 0.045$ and $u_2 = 0.007$.

concentration and hence cannot be the maximal one. This result indicates that it is not possible to force all network’s states to the region of attraction of the pluripotent state PL through constant overexpression.

We therefore ask whether a weaker property than strong reprogrammability holds wherein the system can be reprogrammed to PL when starting at some specific state. In the Supplementary Information (Section 1.1), we illustrate that this is not possible if the system is started at a stable state greater than PL. If instead the system is started at a stable state that is lower than PL, an intermediate input may exist that reprograms the system to PL. However, whether this input stimulation exists and its specific value depend critically on the parameters of the Hill functions. We illustrate this point in detail in the next section through a two-dimensional model of the pluripotency network for which trajectories can be easily depicted in the plane.

2.1 Detailed parametric analysis of a 2D model

In this section, we graphically illustrate why the range of constant inputs that can reprogram the system from TR to PL may be very small or even empty. While we are modeling here for illustration purposes transitions to PL from TR, our findings hold in general for transitions to PL from any state in the landscape where the level of Oct4 is lower than that in the PL state, such as in fibroblasts cells [40].

To this end, we analyze in more detail the dynamics of the two-node network of Figure 1b, which also belongs to the class of monotone cooperative systems. Therefore, any conclusions drawn from this system on the difficulties of reprogramming carry to the three-node system analyzed in the previous section.

A general two-dimensional ODE model of the network in Figure 1(b) can be written as

$$\Sigma_u: \quad \dot{x}_1 = H_1(x_1, x_2) - \gamma_1 x_1 + u_1, \quad \dot{x}_2 = H_2(x_1, x_2) - \gamma_2 x_2 + u_2,$$

in which $x_1$ and $x_2$ represent the concentrations of the transcription factors. As before, $\gamma_i$ are the decay rate constants of the two factors, $u_i$ the additional production rate due to ectopic expression of the factors, and $H_i(x_1, x_2)$ are the Hill functions modeling transcriptional regulation. Consistent with the fact that Nanog
forms a homodimer [38, 41], that Oct4 forms a heterodimer with Sox2 [37], and that these two work together, we assume that $x_i$ both appear with a power of two. Under these conditions, we can write the Hill functions as

$$H_i(x_1, x_2) = \frac{a_i x_1^2 + b_i x_2^2 + c_i x_1^2 x_2^2}{1 + x_1^2 + x_2^2 + d x_1^2 x_2^2}, \quad i = 1, 2,$$

in which we have normalized the concentrations of the factors by their respective effective dissociation constants to decrease the number of parameters. The presence of the term $x_1^2 x_2^2$ is consistent with the fact that there is a cooperative action between the Oct4/Sox2 heterodimer and the Nanog homodimer [39]. Therefore, the resulting model that we study is given by

$$\dot{x}_1 = \frac{a_1 x_1^2 + b_1 x_2^2 + c_1 x_1^2 x_2^2}{1 + x_1^2 + x_2^2 + d x_1^2 x_2^2} - \gamma_1 x_1 + u_1,$$

$$\dot{x}_2 = \frac{a_2 x_1^2 + b_2 x_2^2 + c_2 x_1^2 x_2^2}{1 + x_1^2 + x_2^2 + d x_1^2 x_2^2} - \gamma_2 x_2 + u_2.$$

(2)

Figure 4: Constant intermediate overexpression of either factor leads to reprogramming failure. (Top: $u_1 > 0$) Progressive over expression of $x_1$ leads first to the disappearance of the stable steady state lying in the region of attraction of PL and then to the disappearance of the stable steady state lying in the region of attraction of TR. The values of $u_1$ from left to right are: 0.009, 0.0384, and 0.0054. (Bottom: $u_2 > 0$) Progressive over expression of $x_2$ leads as above to disappearance of the stable steady state in the region of attraction of PL and then to the disappearance of the one lying in the region of attraction of TR. The values of $u_2$ from left to right are: 0.00028, 0.00084, and 0.0126. All other parameters are the same as in Figure 3a.

Figure 3a shows a generic configuration of the nullclines of system $\Sigma_0$ in equations (2) with $u_1 = u_2 = 0$, which gives rise to exactly three stable steady states (indicated as TR, PL, and PE). The plot also depicts the vector field $(\dot{x}_1, \dot{x}_2)$, which shows stable and unstable steady states. From this vector field, for a trajectory to converge to PL, it must be initialized with a suitable intermediate value of $x_2$. Figure 3b demonstrates how the nullclines and the vector field change when one applies a constant and sufficiently large input $u$. The system will have a unique globally stable steady state in the region of attraction of PE. Therefore, the trajectories
starting in TR will approach this state and when \( u \) is set back to zero, the trajectory will ultimately converge to PE, leading to reprogramming failure. This is in accordance to what presented in the previous section, according to which the system is strongly reprogrammable only to its maximal stable steady state.

Since the system is not strongly reprogrammable to PL, we investigate whether it can be reprogrammed to PL when starting from a specific initial state, such as TR, for some intermediate stimulation. Figure 4 shows that this is not the case in general. In fact, when \( u_1 \) and/or \( u_2 \) are progressively increased, the equilibrium point near PL disappears before the one near TR. Therefore, if the system’s state is initially TR, a too low overexpression (\( u_1 \) small in Figure 4) will keep the trajectory of the system near TR, leading to reprogramming failure. Further increase of overexpression (intermediate \( u_1 \)) will lead to a system \( \Sigma_u \) with only two stable steady states: one in the region of attraction of TR and the other in the region of attraction of PE. Hence, the trajectory will once more remain near TR, leading to reprogramming failure once again. Increasing the overexpression further (large \( u_1 \)) will cause \( \Sigma_u \) to have a unique globally asymptotically stable steady state in the region of attraction of PE. As a consequence the trajectory starting from TR will approach this unique stable steady state, missing yet again the pluripotent state PL.

![Diagram](image)

**Figure 5:** Conceptual illustration of reprogramming failure through constant overexpression. A ball is rolling in a valley landscape under the force of gravity. If we pull up enough the left-hand side of the landscape to make the ball roll out of the TR valley, it will roll through the PL valley without stopping and land in the PE valley.

This example demonstrates the major difficulty encountered when reprogramming monotone cooperative systems to a state characterized by an intermediate value of at least one transcription factor. This difficulty is depicted in Figure 5 through a conceptual representation in which a ball moves through a landscape of valleys while under the force of gravity. Let the ball be initially in the TR valley when we start pulling up the left-hand side of the landscape. If we pull up too little, the ball will not move from the TR valley as this is still a stable configuration. If we pull just enough to make the TR valley almost disappear (we destabilize the TR configuration), the ball will roll out of the TR valley but will not land in the PL valley as this valley has also disappeared. That is, when we make the TR valley shallow we also (as a side effect) make the PL valley shallow to the point that it disappears before or at the same value of \( u \) at which the TR valley disappears. Hence, the ball rolling out of TR misses PL no matter what overexpression level \( u \) is employed.

Taken together, these findings suggest that excessive overexpression is always a losing strategy for reprogramming. Also, an intermediate overexpression level that reprograms the system from TR to the intermediate state PL may not exist. In this case, the state will be trapped in the region of attractions of steady states that are neighbors of PL.

However, despite its low efficiency in practice, reprogramming does occasionally succeed of course. We can explain this by using slightly different parameter conditions under which, upon increase of \( u_2 \) for example, the nullcline intersection near TR disappears before the one near PL. In such a case, there is an intermediate
value of inputs such that the system can be reprogrammed from TR to PL. This is illustrated in Figure 6, where the parameters of the system have been slightly changed compared to Figures 3 and 4. In particular, the parameters of $\Sigma_0$ have been modified from those of Figure 3a to have an increased $a_2 = 15$ and a decreased $a_1 = 0.0126$. In $\Sigma_u$, we have set $u_2 = 0.0091$. In this case, the equilibrium point in the region of attraction of TR has disappeared while an equilibrium point PL' in the region of attraction of PL is still present. This qualitative situation is found only for a narrow input set $u_2 \in [0.0063, 0.0091]$. The green area represents the region of attraction of PL', the light blue and dark blue plots are representative trajectories starting from initial condition $x(0)$.

The autocatalysis of $x_2$ is slightly stronger compared to that of Figure 3a; on the contrary, the autocatalysis of $x_1$ is slightly weaker compared to that of Figure 3a. In this condition, there is a narrow range of $u_2$ values that leads to two stable steady states: one in the region of attraction of PL, called PL' (Figure 6, middle plot), and the other in the region of attraction of PE. Only initial conditions belonging to the green shaded area in Figure 6 lead to trajectories approaching PL', while any other initial condition will lead to trajectories approaching the top-right steady state. After these trajectories have reached their corresponding steady states, removal of the stimulus (Figure 6, right-side) leads the trajectories initiated in the green area to approach PL, while the others approach PE.

Taken together, these findings indicate that the existence of a range of inputs that allows one to reprogram the system from TR to PL, and the range itself when it exists, is highly dependent upon the shape of the nullclines and in fact highly sensitive to it. The shape of the nullclines, in turn, is controlled by the parameters of the Hill functions $H_i$, which are poorly known and subject to large variations within one experiment and across different experiments. This suggests that the success of current reprogramming approaches that use estimated $a priori$ overexpression levels is extremely sensitive to parameter values. Since these values are largely unknown and subject to large variations, there is no guarantee that current approaches to reprogramming succeed.

In the next section, we demonstrate that this lack of robustness of the reprogramming process to parameter uncertainty persists when we incorporate additional known interactions into the model, which come from the extended network in which the pluripotency network is inserted.

### 2.2 Reprogramming in the extended pluripotency network

The pluripotency network of Figure 1a is embedded in a larger regulatory network that includes additional transcription factors and activation/repression interactions, some of which are well documented and have
Figure 7: The extended pluripotency network. (a) The core pluripotency network is embedded in a larger network depicted in grey. The net interactions with the network can be of two types: Type 1 (positive) and Type 2 (negative or undetermined). (b) Examples of Type 1 and Type 2 interactions.

been modeled in other studies [19, 23, 25, 42] (Figure 7a-7b). Specifically, the antagonisms between Nanog and Gata6 and between Oct4 and Cdx2, each forming a toggle-switch type of interaction [25], have been separately analyzed. In addition, there is evidence and related models for an effective repression from Oct4 to Nanog, which was reported to become apparent only at high Oct4 concentrations [23]. Other papers have modeled this effective repression through an activation from Oct4 to Gata6, which in turn represses Nanog [25].

Toward the goal of studying the effect of potential additional interactions on the reprogramming properties of the network, we make a distinction between two types of interactions: Type 1 and Type 2 (Figure 7a). In a Type 1 interaction, we have a simple directed path with positive sign that starts from Oct4, Sox2, or Nanog and goes through the nodes of the surrounding network before returning back to Oct4, Sox2 or Nanog. In a Type 2 interaction, this directed path can either be simple and have negative sign or can be undetermined. For example, referring to Figure 7b, there are two additional net interactions that arise due to the presence of the Gata6 node. One is due to the simple path starting at Nanog, going through Gata6 and coming back to Nanog, which is composed of two sequential repressions, leading to an overall positive sign of the interaction from Nanog back to Nanog. This interaction is therefore of Type 1. The second net interaction is due to the simple path starting at Oct4, going through Gata6 and ending at Nanog, which is composed of an activation followed by a repression, leading to an overall negative sign of the interaction from Oct4 to Nanog. This interaction is therefore of Type 2.

Type 1 interactions preserve the monotone cooperative structure of the system; Section 1.2 in the Supplementary Information has a formal proof and the precise mathematical definitions. Therefore, these interactions do not alter the reprogrammability properties of the network, which stay qualitatively unchanged with respect to what was illustrated in the two previous sections. Type 2 interactions, instead, do not necessarily preserve the monotone cooperative structure of the system. However, it is possible to mathematically demonstrate that the reprogramming properties of the network stay qualitatively unchanged with respect to what shown previously if these Type 2 interactions are sufficiently weaker than the interactions within the core pluripotency network. This fact is mathematically proven for a general monotone cooperative system in the Supplementary Information (Section 1.3). Here, we illustrate this point by considering again the two-dimensional model of the pluripotency network, which we now perturb by adding an additional repression from Oct4 to Nanog, leading to a Type 2 interaction (Figure 8a). We therefore take the following ODE model of the network of Figure 8a:

\[
\dot{x}_1 = \frac{a_1 x_1^2 + b_1 x_2^2 + c_1 x_1^2 x_2^2}{1 + x_1^2 + x_2^2 + d x_1^2 + e x_2^2} - \gamma_1 x_1 + u_1, \quad \dot{x}_2 = \frac{a_2 x_1^2 + b_2 x_2^2 + c_2 x_1^2 x_2^2}{1 + x_1^2 + x_2^2 + d x_1^2 + e x_2^2} - \gamma_2 x_2 + u_2, \quad (3)
\]

which differs from the system in equations (3) because of the term “ex2m” modeling the re a potential repres-
Figure 8: Type 2 interactions shift the steady states’ location. (a) The two-node model of the pluripotency network with an additional repression (Type 2 interaction). (b) Nullcline configuration and vector field for the case in which the parameters are the same as those given in Figure 3a with $e = 6$ and $m = 6$. The additional repression lowers the level of Nanog in PE to a value lower than that in PL [43–45].

sion from Oct4/Sox2 to Nanog. Since this is known to become apparent (only) for sufficiently large Oct4 concentrations, we will require that $m$ is sufficiently higher than 2. An instance of the nullclines along with the vector field when $u = 0$ is provided in Figure 8b. Figure 9 illustrates that progressive overexpression of either factor still leads as before to the disappearance of the pluripotent state PL before the TR state, leading to reprogramming failure. For large overexpression, the PE state persists and therefore, as before, large overexpression is a losing strategy and always leads the system’s trajectories to approach the region of attraction of the PE state.

Taken together, these findings indicate that the existence of a range of inputs that allows to reprogram the system from TR to PL, and the range itself when it exists, are highly dependent on the shape of the nullclines and in fact highly sensitive to it. The shape of the nullclines, in turn, is controlled by the parameters of the Hill functions $H_i$, which are poorly known and subject to large variations within one experiment and across different experiments. This suggests that the success of current reprogramming approaches that use a priori guessed over expression levels is extremely sensitive to parameter values and, since these are largely unknown and subject to large variations, there is no guarantee that currently employed approaches to reprogramming work. We therefore propose in the next section a different approach to reprogramming to overcome this limitation. The approach that we propose is specifically designed to guarantee reprogramming success independent of network structure and parameter values.

3 Reprogramming through closed loop feedback control

As demonstrated in the previous section, a constant (in time) amount of overexpression $u$ cannot guarantee reprogramming of differentiated cells to pluripotency due to the monotone network structure and the intermediate location of the PL state with respect to Oct4 concentration. We thus propose a design strategy that steers the network’s state to any desired location, and in particular to PL, independent of the network’s structure and parameters. This design strategy uses closed loop feedback control, wherein the system’s input (overexpression level) $u$ is iteratively adjusted based on the error between the actual state $x$ and a desired target state $x^*$, which should ideally be close to PL. This approach is in contrast to open loop control, in which the system’s input $u$ is pre-fixed and remains unchanged over time. In this sense, the approach to reprogramming described in the previous section, in which constant overexpression of factors is imposed, can be regarded as an open loop control strategy.
We therefore set \( u_i = G_i(x_i^* - x_i) \) with \( G_i > 0 \) a positive constant. Note that as \( x_i \) approaches \( x_i^* \), the control effort \( u_i \) decreases and reaches zero when \( x_i = x_i^* \). If we assume that \( G_i \) is sufficiently large such that \( G_i x_i^* \gg H_i(x) \) and \( G_i \gg \gamma_i \), the ODE model describing the rate of change of \( x_i \) with this input becomes

\[
\dot{x}_i = H_i(x) - \gamma_i x_i + G_i(x_i^* - x_i) \approx G_i(x_i^* - x_i),
\]

from which it is apparent that \( x_i(t) \) will approach its unique steady state given by \( x_i^* \) as \( t \) goes to infinity. More precisely, we have that \( \limsup_{t \to \infty} |x_i(t) - x_i^*| = (H_M + \gamma_i x_i^*)/(G_i + \gamma_i) \), in which \( H_M \) is an upper bound on \( H_i(x) \). As a consequence, the larger the value of \( G_i \), the smaller the error between the steady state of \( x_i \) and its desired value \( x_i^* \) (see Section 1.4 in the Supplementary Information for the mathematical details). This can be graphically appreciated from the nullcline plots of Figure 10. In particular, if \( G_1 \) and \( G_2 \) are sufficiently large the nullcline \( \dot{x}_1 = 0 \) morphs into a vertical line going through \( x_1^* \) and similarly the nullcline \( \dot{x}_2 = 0 \) morphs into a horizontal line going through \( x_2^* \). These nullclines intersect at one point only given by \( x^* = (x_1^*, x_2^*) \). Therefore, for sufficiently large \( G_i \) the feedback makes the controlled system globally asymptotically stable with unique equilibrium point given by \( x^* \). Hence, all trajectories starting from any initial condition will converge to \( x^* \). If \( x^* \) is further chosen to lie in the region of attraction of PL, the trajectories will approach PL upon shutting down the controller \( (u = 0, \text{Figure 10}) \). This result holds independent of the specific parameters of the Hill functions \( H_i \) and independent of the initial condition \( x(0) \). This approach is therefore robust to uncertainty in network structure, system parameters, and initial conditions.

We can interpret the effect of this controller as follows. Since \( u_i = G_i x_i^* - G_i x_i \), this control strategy simultaneously applies a large overexpression rate \( G_i x_i^* \) and a similarly large degradation rate \( -G_i x_i \). Qualitatively, the sole application of \( u_i = G_i x_i^* \) makes the system’s trajectories converge to the region of attraction of the
Figure 10: **Closed loop feedback control leads to successful reprogramming.** System $\Sigma_0$ has the same parameters as in Figure 3a and the pink area shows the region of attraction of PL. In system $\Sigma_u$, we use a control input in negative feedback form with $G_1 = 1.5$, $G_2 = 0.7$, and target state $x^*_1 = 4$, $x^*_2 = 0.2$. The nullclines morph into practically straight lines intersecting at a unique point given by $x^*$, so that the trajectory approaches $x^*$ while input $u$ is applied. Once the input is removed, the trajectory approaches PL.

maximal state of $\Sigma_0$, given by PE, as previously shown. By contrast, the sole application of $u_i = -G_i x_i$ makes the system’s trajectories converge to the region of attraction of the minimal state of $\Sigma_0$, which is given by TR. The simultaneous application of these large and opposing stimulations makes the system’s trajectories converge to their “ratio” given by $x^*$. The concept of this high-gain negative feedback controller is illustrated in Figure 11 using the extended analogy of a ball in a valley landscape as before.

Figure 11: **Conceptual illustration of the high-gain negative feedback controller.** (Left) Large overexpression destabilizes TR and makes PE globally stable. (Center) Large repression destabilizes PE and makes TR globally stable. (Right) Simultaneous large overexpression and large repression, in ratio given by $x^*$, makes $x^*$ the globally stable steady state.

In summary, for the high-gain negative feedback to successfully reprogram the system to PL, we need a way to implement the negative feedback control law $u_i = G_i (x^*_i - x_i)$ within the pluripotency network with $G_i$ large enough such that $x^*$ becomes the unique globally stable steady state of the system. Second, we need to set $x^*$ close enough to PL such that it lands in its region of attraction. We address these two requirements in the following two sections.
3.1 Synthetic genetic circuit implementation of the high-gain negative feedback control law

We propose to implement the high gain negative feedback control law on $x_i$ by simultaneously overexpressing and degrading the mRNA $m_i$ of factor $x_i$ (Figure 12). In particular, overexpression is achieved by placing gene $x_i$ under the control of an inducible promoter with inducer $I_{i,1}$. Degradation of $m_i$ is obtained by the use of a small interfering RNA (siRNA), denoted $s_i$, with perfect complementarity to the mRNA target $m_i$ [46]. The siRNA transcript is induced by $I_{i,2}$. In this section, we analyze the dynamics of the system by first assuming a one-step reaction model for the degradation of mRNA by siRNA to illustrate how this control realization can implement the feedback strategy. We then consider a more realistic two-step enzymatic reaction model for the interaction between mRNA and siRNA and demonstrate that the resulting ODE model structure still implements the high-gain negative feedback strategy under reasonable parameter conditions. We finally show through simulation the performance of this genetic circuit realization in reprogramming the pluripotency network.

Let the synthetic genetic circuit be encoded on DNA with concentration $D$. On the same DNA, a small interfering RNA $s_i$ is induced by $I_{i,2}$ (Figure 12). In particular, overexpression is achieved by placing gene $x_i$ under the control of an inducible promoter with inducer $I_{i,1}$. Degradation of $m_i$ is obtained by the use of a small interfering RNA (siRNA), denoted $s_i$, with perfect complementarity to the mRNA target $m_i$ [46]. The siRNA transcript is induced by $I_{i,2}$. In this section, we analyze the dynamics of the system by first assuming a one-step reaction model for the degradation of mRNA by siRNA to illustrate how this control realization can implement the feedback strategy. We then consider a more realistic two-step enzymatic reaction model for the interaction between mRNA and siRNA and demonstrate that the resulting ODE model structure still implements the high-gain negative feedback strategy under reasonable parameter conditions. We finally show through simulation the performance of this genetic circuit realization in reprogramming the pluripotency network.

Let the synthetic genetic circuit be encoded on DNA with concentration $D$ and let the inducers activate the target genes through functions $h_{i,j}(\cdot)$, whose specific form is usually of the Michaelis-Menten type [35] and is not relevant for the current treatment. Assuming a one-step reaction model for mRNA/siRNA interaction, the following reactions affect the mRNA $m_i$ and the protein $x_i$:

endogenous system reactions: $\emptyset \xrightarrow{H_i(x)} m_i, \ m_i \xrightarrow{\delta_i} \emptyset, \ m_i \xrightarrow{\kappa_i} x_i, \ x_i \xrightarrow{\gamma_i} \emptyset,$

synthetic circuit reactions: $\emptyset \xrightarrow{D_{hi,1}(I_{i,1})} m_i, \ \emptyset \xrightarrow{D_{hi,2}(I_{i,2})} s_i, \ s_i \xrightarrow{\beta_i} \emptyset, \ m_i + s_i \xrightarrow{k_i} s_i.$

While $\delta_i$ and $\gamma_i$ model natural degradation rates of mRNA and protein, respectively, $\beta_i$ models dilution due to cell growth. Since it is known that siRNAs are stable, we model removal of $s_i$ only through this dilution [46]. The ODE model of the system is given by

$$\dot{s}_i = D_{hi,2}(I_{i,2}) - \beta_i s_i, \quad \dot{m}_i = H_i(x) - \delta_i m_i + D_{hi,1}(I_{i,1}) - k_i s_i m_i, \quad \dot{x}_i = \kappa_i m_i - \gamma_i x_i. \quad (5)$$
Here, to make the steady state of $x_i$ the same as that of the model used in Section 2.1 given in equation (4), which was assuming mRNA at its quasi-steady state, we have set $\tilde{H}(x) = (\delta_i/k_i)H(x)$. For a fixed non-zero induction $I_{i,2}$, we define $\alpha_i = h_{i,2}(I_{i,2})$. After the siRNA has reached its equilibrium $s_i^* = D\alpha_i/\beta_i$, we can simplify the above system to

$$\dot{m}_i = \tilde{H}(x) - \delta_i m_i + Dh_{i,1}(I_{i,1}) - k_i s_i^* m_i, \quad \dot{x}_i = \kappa m_i - \gamma_i x_i,$$

which can be, in turn, re-written as

$$\dot{m}_i = \tilde{H}(x) - \delta_i m_i + G_i(m_i^* - m_i), \quad \dot{x}_i = \kappa m_i - \gamma_i x_i, \quad G_i = D \frac{k_i \alpha_i}{\beta_i}, \quad m_i^* = \frac{\beta_i h_{i,1}(I_{i,1})}{k_i \alpha_i}. \tag{6}$$

From here, we can see that if $G_i$ is sufficiently large such that $G_i m_i^* \gg \tilde{H}(x)$ and $G_i \gg \delta_i$, then we have that $\dot{m}_i \approx G_i(m_i^* - m_i)$ and therefore

$$m_i = m_i^*, \quad x_i = \frac{\kappa}{\gamma_i} m_i^* =: x_i^*$$

is the only steady state of the system, which is also globally asymptotically stable and can be tuned by changing $I_{i,1}$. The major structural difference with the ideal control system in equation (4) is that the negative feedback is applied to the mRNA and not to the protein. So, while we can tune this steady state through $I_{i,1}$, we cannot make the approach to this steady state arbitrarily fast as it was the case in equation (4). In fact, in the limit in which $G_i$ is very large, the mRNA will reach very quickly the target steady state $m_i^*$, but the protein concentration will still converge to $x_i^*$ with rate established by the time constant $\ln(2)/\gamma_i$.

These results stay qualitatively unchanged if we consider a more realistic two-step reaction model for mRNA degradation by siRNA, which has been experimentally validated [47]. However the parameters that contribute to the gain $G_i$ slightly change, as we now illustrate. In particular, letting $c_i$ denote the complex that siRNA forms with mRNA and assuming that it dilutes through cell growth just like siRNA, we have:

$$m_1 + s_i \xrightarrow{a_i} c_i \xrightarrow{d_i} s_i, \quad c_i \xrightarrow{b_i} 0.$$

This, along with the production and natural degradation of mRNA, lead to the new ODE model given by

$$\dot{s}_i = Dh_{i,2}(I_{i,2}) - \beta_i s_i - a_i m_i s_i + (d_i + k_i)c_i, \quad \dot{c}_i = a_i m_i s_i - (d_i + k_i)c_i - \beta_i c_i, \quad \dot{m}_i = \tilde{H}(x) - \delta_i m_i + Dh_{i,1}(I_{i,1}) - a_i m_i s_i + d_i c_i, \quad \dot{x}_i = \kappa m_i - \gamma_i x_i. \tag{7}$$

Exploiting the fact that the association and dissociation reactions are much faster than the others [35, 36], we can re-write the system in the slow variables $\bar{s}_i = s_i + c_i$ and $\bar{m}_i = m_i + c_i$ and approximate the complex to its quasi-steady state

$$c_i = \bar{s}_i - \frac{m_i}{K_m} = \frac{m_i}{1 + m_i/K_m}, \quad K_m = \frac{(d_i + k_i)}{a_i},$$

in which $K_m$ is the Michaelis-Menten constant of the mRNA/sRNA reaction. This leads to the new set of ODEs

$$\dot{\bar{s}}_i = Dh_{i,2}(I_{i,2}) - \beta_i \bar{s}_i, \quad \dot{\bar{m}}_i = \tilde{H}(x) - \delta_i m_i - k_i \bar{s}_i - \frac{m_i}{1 + m_i/K_m} + Dh_{i,1}(I_{i,1}), \quad \dot{x}_i = \kappa m_i - \gamma_i x_i,$$

in which, we have made the approximation $\beta_i \ll k_i$, since dilution is typically much slower than catalytic reactions [35] (see Table I for exact values). From [47], it is known that $m_i \ll K_m$ for physiologically relevant values of mRNA concentration. Also, with the synthetic genetic controller, we start from cells in the TR state that have very low concentration of the factors and hence of the corresponding mRNAs. We then approach from below the target mRNA concentration $m_i^*$, which should be close to the value in the PL state. Therefore,
we expect to keep the mRNA concentration within physiologically relevant values through the entire control operation. As a consequence, we can simplify the above system to

\[
\dot{s}_i = Dh_{i,2}(I_{i,2}) - \beta_i s_i, \quad \dot{m}_i = \bar{H}_i(x) - \delta_i m_i - \frac{k_i s_i}{K_m} m_i + Dh_{i,1}(I_{i,1}), \quad \dot{x}_i = \kappa_i m_i - \gamma_i x_i,
\]

in which, assuming that \(s_i\) has already reached its steady state given by \(D\alpha_i/\beta_i\), we can set

\[
G_i = \frac{k_i D\alpha_i}{K_m \beta_i}, \quad m_i^* = \frac{h_{i,1}(I_{i,1}) K_m \beta_i}{\alpha_i}, \quad \bar{x}_i = \frac{\kappa_i m_i^*}{\gamma_i}
\]

such that the above system finally becomes

\[
\dot{m}_i = \bar{H}_i(x) - \delta_i m_i + G_i(m_i^* - m_i), \quad \dot{x}_i = \kappa_i m_i - \gamma_i x_i,
\]

which has the same form as the system in equation (6), in which a one-step reaction was assumed. This leads to the same conclusion: we have a globally asymptotically stable system with unique equilibrium point given by

\[
m_i^* = \frac{h_{i,1}(I_{i,1}) K_m \beta_i}{\alpha_i}, \quad \bar{x}_i = \frac{\kappa_i m_i^*}{\gamma_i}
\]

for \(G_i\) sufficiently large.

**Parameter feasibility analysis and numerical simulations.** We next perform a feasibility study to determine what DNA concentrations \(D\) need to be used in order to ensure sufficiently large \(G_i\), that is, \(G_i \gg \delta_i\) and \(G_i m_i^* \gg \bar{H}_i(x)\), which is the only requirement for the control design to stabilize the system to the target state \(x^*\). From the half lives of transcription factors’ mRNA such as Nanog and Oct4, we can estimate \(\delta_i \approx [0.09, 0.17]\text{hrs}^{-1}\) [48]. Also, from the *in vitro* study of [47], we know that for completely complementary siRNA we can obtain \(k_i \approx 61\text{hrs}^{-1}\). We can estimate the maximal promoter induction, \(\alpha_i = h_{i,2}(I_{i,2})\), using the typical transcription initiation rate in mammalian cells. The initiation rate for transcription in mammalian cells was estimated to be about \(0.0216\text{s}^{-1}\), but only \(8.6\%\) of RNAP that arrive at the initiation step are estimated to result in an mRNA molecule product [49]. Therefore, we take an effective transcription initiation rate of \(0.0018\text{s}^{-1}\), or equivalently \(\alpha_i = 6.7\text{hr}^{-1}\) for a maximally induced promoter. We call this maximal level \(h_{\text{MAX}}\). Considering that dilution rate is about \(\beta_i \approx 0.05\text{hrs}^{-1}\), corresponding to a doubling time of 20 hours [50], in the worst case scenario when \(K_m = 1\) the gain is given by

\[
G_i \approx D \frac{61 \cdot 6.7}{1 \cdot 0.05} = 8,174D.
\]

Requesting that \(G_i \geq 10\delta_i\) with \(\delta_i = 0.17\text{hr}^{-1}\), then leads to

\[
D \geq 0.0002\text{nM} \iff D \text{ copy number} \geq 1.
\]

Similarly, we can find the copy number needed to make \(G_i m_i^* \gg \bar{H}_i(x)\). To this end, we estimate the maximal value of \(\bar{H}_i(x)\) from the maximal rate of transcription used above, \(\alpha_i = 6.7\text{hr}^{-1}\), and from the fact that this should be multiplied by the concentration of DNA. Since the endogenous system is on the chromosome, which is in one copy, it has a concentration of \(0.4 \cdot 10^{-3} \text{nM} [50]\), so that we estimate an upper bound of \(\bar{H}_i(x) \approx 2.68 \cdot 10^{-3}\). Given that \(m_i^* = \bar{x}_i^* \gamma_i/k_i\), we can estimate \(m_i^* = 3.3 \cdot 10^{-4}\) nM, given by \(m_i^* = \bar{x}_i^* \gamma_i/k_i\), as follows. The target value of \(x_i\) is given by

\[
\bar{x}_i = \frac{\kappa_i \beta_i K_m h_{i,1}(I_{i,1})}{\gamma_i k_i},
\]
which is essentially determined by the ratio of the two inducible promoter activities \( h_{i,1}(I_{i,1}) \) and \( \alpha_i = h_{i,2}(I_{i,2}) \). We assume that upon maximal induction through \( I_{i,1} \) the value of the production rate \( h_{i,1}(I_{i,1}) \) reaches at most \( \alpha_i \). From [51], we know that the time required for translation initiation is in the interval \([1, 1372]\) s. Taking the mean value of this, we obtain \( \kappa_i \approx 1, 800 \text{hr}^{-1} \), which, considering that \( \gamma_i \approx 0.3 \text{hr}^{-1} \) as estimated for Nanog [52], we obtain that

\[
x_i^* \in [0, 5] \text{nM}, \quad h_{i,1}(I_{i,1}) \in [0, \alpha_i],
\]

for which we pick \( x_i^* = 2 \text{nM} \) for the calculations. Since \( G_i \approx 8, 174D \), in order to have \( G_i m_i^* > 10 \cdot H_i(x) \), we need to require

\[
D \geq 0.009 \text{nM} \iff D \text{ copy number } \geq 25.
\]

Here, we have considered the fact that in a typical mammalian cell one molecule has a concentration of about \( 0.4 \cdot 10^{-3} \) nM [50]. Based on these calculations, it is guaranteed that with copy numbers of the synthetic circuit equal to or greater than 25, we will realize the high-gain negative feedback mechanism, leading to definite stabilization of the system at the target state \( x^* \). In practice, however, a much lower value of copy number may be sufficient as shown in simulations (Figure 13), since these calculations were based on a worst-case analysis.

<table>
<thead>
<tr>
<th>Table I: System Parameters</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_i = 61 \text{hr}^{-1} )</td>
<td>[47]</td>
</tr>
<tr>
<td>( K_m = 1 \text{nM} \in [1, 8] \text{nM} \rightarrow \alpha_i = 122, ; d_i = 61 )</td>
<td>[47]</td>
</tr>
<tr>
<td>( \beta_i = 0.05 \text{hr}^{-1} )</td>
<td>[50]</td>
</tr>
<tr>
<td>( \kappa_i = 1, 800 \text{hr}^{-1} )</td>
<td>[51]</td>
</tr>
<tr>
<td>( \alpha_i = 6.7 \text{hr}^{-1}, ; h_{i,1}(I_{i,1}) \in [0, 6.7] \text{hr}^{-1} )</td>
<td>[49]</td>
</tr>
<tr>
<td>( \gamma_i = 0.3 \text{hr}^{-1}, ; \gamma_2 = 0.14 \text{hr}^{-1} )</td>
<td>[52]</td>
</tr>
<tr>
<td>( D = 0.0002 \text{nM} )</td>
<td>our estimate</td>
</tr>
<tr>
<td>( \delta_1 = 0.17 \text{hr}^{-1}, ; \delta_2 = 0.09 \text{hr}^{-1} )</td>
<td>[48]</td>
</tr>
</tbody>
</table>

We simulated the full system of equations given in (7) using the parameters given in Table I, which have been estimated as described in the previous paragraph. In order to obtain a steady state \((x_1^*, x_2^*)\) close to PL such that it lands in its region of attraction, we choose \( x_1^* = 2 \text{nM} \) and \( x_2^* = 0.2 \text{nM} \). Thus we set

\[
h_{1,1}(I_{1,1}) = \frac{x_1^* \gamma h_{1,2}(I_{2,1}) k_1}{\kappa_1 K_m \beta_1} = 2.7 \text{hr}^{-1}, \quad h_{2,1}(I_{2,1}) = \frac{x_2^* \gamma_2 h_{2,2}(I_{2,2}) k_2}{\kappa_2 K_m \beta_2} = 0.12 \text{hr}^{-1}.
\] (10)

Note that concentrations of Nanog and Oct4 of about 2nM and 0.2 nM, respectively, correspond to about 2,000 and 200 copies of proteins in mammalian cells, consistent with physiological ranges of proteins [50].

Figure 13 shows simulation results where both factors \( x_i \) are controlled through the feedback controller circuit of Figure 12. In these simulations, the state of the network starts near the TR state with zero concentrations of the factors. After 5 hours, we apply maximal inducers concentrations for the siRNA such that \( h_{1,2}(I_{2,2}) = \alpha_i = 0.6 \text{hr}^{-1} \). As a consequence, the siRNA concentration in the feedback controller rises. After 80 hours, when the siRNA has reached the proximity of its steady state, we apply inducers for the factors such that \( h_{1,1}(I_{1,1}) = 2.7 \text{hr}^{-1} \) and \( h_{2,1}(I_{2,1}) = 0.12 \text{hr}^{-1} \), which were calculated above. The plots show that \( x_1 \) and \( x_2 \) reach their desired target values in the neighborhood of the PL state, leading to reprogramming the network to PL, which was not previously possible with only constant overexpression of the factors.

From these plots, one can also estimate the expected time for reprogramming if we can set in one shot the inducers such that \( x^* \) is close to PL. We first wait for the siRNA’s concentration to have approached its final value after its induction, such that the high-gain negative feedback is implemented. This time depends on the time that siRNA takes to build up. In these simulations, based on the physical parameters taken from the literature as discussed above, this time is about 70 hours (3 days) and is determined by the cell growth.
Figure 13: **The feedback controller reprograms TR into PL.** (a) Time trajectories of the transcription factors concentrations $x_1$ and $x_2$ and time trajectories of the siRNA concentrations $s_1$ and $s_2$. Initially the concentrations of the transcription factors are at zero (near the TR state), then at time $t = 5$ hr inducers $I_{1,2}$ and $I_{2,2}$ are applied to induce the production of the siRNAs (b). After the siRNA has approached its steady state, inducers $I_{1,1}$ and $I_{2,1}$ are applied in the specific amounts calculated in the text at time $t = 80$ hr. Light blue and green lines are not to scale. The parameters for the Hill functions are the same as those chosen in Figure 3a, where they were leading to reprogramming failure with open loop control.

rate $\beta_i$. However, note that it is not necessary to wait for the siRNA concentration to reach its final steady state to induce the factors as the system will approach anyway the same steady state independent of the timing of the siRNA and factors induction. A potential benefit of waiting for siRNA to build up before inducing the factors is to avoid that the factors’ concentration reach values higher than physiological ones, potentially causing DNA damage responses [53]. After siRNA has built up, one can apply the overexpression of the factors and wait for the new steady state, which is reached in less than 20 hours. As a consequence, the entire reprogramming process should take about 4 days. Of course, these estimates are based on a growth rate corresponding to a doubling time of 20 hours ($\beta_i = 0.05$ hr$^{-1}$) and on factors half lives between 4 hrs and 7 hrs ($\delta_i \in [0.09, 0.17]$ hrs$^{-1}$). Slower growth rate and longer half lives will increase (about proportionally) the time required for reprogramming.

Previously, we illustrated that it is not possible for a state starting in the region of attraction of PE to escape it under constant input stimulation. States starting in this maximal region are trapped by the PE state, no matter what constant control input is applied, because of the cooperative monotone dynamics of the pluripotency network. By contrast, the feedback controller can steer the factors’ concentration to their target values independent of the factors’ initial concentration. In particular, the factors can be steered to the neighborhood of the pluripotent state even when the initial state is in the PE region as shown in Figure 17a-17b in the Supplementary Information.

### 3.2 Outer loop control removes the need to know the synthetic control circuit’s parameters

In the previous section, under the assumption that parameters $\kappa_i, \beta_i, \gamma_i, k_i, K_m$ and $\alpha_i$ were known exactly as well as the form of $h_{i,1}$, we reached the desired concentration $x_i^*$ by setting the inducer concentration $I_{i,1}$ to

$$I_{i,1} = h_{i,1}^{-1} \left( x_i^* \frac{\gamma_i k_i \alpha_i}{k_i \beta_i K_m} \right).$$
This required knowledge of the synthetic control circuit’s parameters (not of the parameters of the network to be controlled, though), including the values of mRNA decay rates, dilution rates, Michaelis-Menten and catalytic constants of the siRNA/mRNA reaction, and mRNA/protein production rates. While we can have reasonable estimates for these parameters, even they may still be subject to significant uncertainty. We therefore seek an approach that does not require any knowledge of the synthetic genetic circuit parameters, leading to a design that functions robustly in the face of uncertainty even in the parts of the synthetic genetic controller.

To this end, we implement the outer-loop control architecture shown in Figure 14a. The basic idea is to progressively adjust the inducers concentrations $I_{1,1}$ based on the discrepancy between the actual steady state reached by the controlled network under such inducer level, that is $\bar{x}_1 := (k_1 \beta_1 K_{m} h_{i,1}(I_{1,1}))/\gamma_1 k_1 \alpha_1$, and the concentration $x^*_{i}$ in PL. Letting $T$ be the time that it takes for the controlled network to reach its unique steady state $\bar{x}$, letting $k$ denote a natural number, and defining $I = (I_{1,1}, I_{2,1})$, we can set the inducer level at time $t = (k+1)T$ based on its value at time $kT$ and the discrepancy between $\bar{x}$ and $x^*$. This leads to the proportional feedback law

$$I((k+1)T) = I(kT) + K(x^* - x(kT)),$$

with

$$K = \begin{pmatrix} K_1 & 0 \\ 0 & K_2 \end{pmatrix}, \quad k \in \mathbb{N},$$

in which $x(kT)$ is approximately equal to $\bar{x}$, where $\bar{x}_i = (k_i \beta_i K_{m} h_{i,1}(I_{i,1}(kT)))/\gamma_i k_i \alpha_i$. The comparison $x^* - x(kT)$ can be performed in a computer and the inducers can be adjusted as a consequence of the in silico computation as proposed by other works [54–56].

The key feature of this design is that this inducer adjustment does not need to take place while the controlled network is dynamically evolving, and in fact should take place only after it has reached the current steady state $\bar{x}$ (after $T$). This is an important aspect because the measurement of the actual concentrations of the factors, such as through immunostaining [57], is a process that takes time and therefore performing it while the network is evolving and using it for real-time feedback would break any guarantee of convergence. In particular, a strategy where the multistable network is directly controlled by an in silico controller without the synthetic feedback control circuit would not guarantee that the controlled network is monostable. Hence,
it would not overcome the major problem of this reprogramming task, which is the trapping of the state in local basins of attraction, a difficulty that we instead overcome with the synthetic feedback control circuit. Essentially, this circuit “freezes” the network at its unique steady state such that the in silico outer loop controller can take any required time for computing the inducer adjustment. This adjustment results in the change of the inducer level when the state of the network is still the same as the one used for computing the adjustment itself, removing any (otherwise present) delay-induced instabilities [20].

Figure 14b shows simulation results for different values of the feedback gains $K_1, K_2$. Independent of the feedback gain and of any parameters of the feedback control circuit or of the original network, the system will reach the desired steady state $x^*$. The only effect the specific control gain $K$ has is to possibly create an overshoot if it is too high or to take longer to converge to a neighbor of $x^*$ if it is too small. Intermediate values can make the system reach a close neighbor of the pluripotent state within about two iterations, corresponding to about 8 days. This time is largely determined by the decay rates of the factors and by cell growth (see previous section).

### 3.3 Shutting down the feedback controller

In the two previous sections, we have demonstrated mathematically and through simulations that our overall closed loop control strategy (Figure 14a) steers the pluripotency network’s state toward the pluripotent state PL independent of initial state, network parameters, and synthetic genetic circuit parameters. Once the state of the network has reached the proximity of the pluripotent state PL, it is important to be able to shut down the molecular feedback controller so that the reprogrammed cells can be used for subsequent applications.

This can be accomplished by setting the inducers $I_{i,1}$ and $I_{i,2}$ to zero while avoiding perturbation of the PL state just reached. Ideally, once overexpression of $m_i$ and $s_i$ are set back to zero and the state $x$ is close enough to PL, the state should continue to stay close to PL because in the original system with no feedback controller PL is stable. However, while setting $I_{i,1} = 0$ sets the overexpression to zero in equation (8), setting $I_{i,2} = 0$ does not result in an immediate decrease of the siRNA $\bar{s}_i$ to zero, because $s_i$ is removed from the system through dilution due to cell growth. This can be appreciated by the system dynamics obtained after setting the inducers’ levels to zero in equations (8):

$$\dot{m}_i = H_i(x) - \delta_i m_i - G_i(t)m_i, \quad m_i(0) = m^*_i \quad G_i(t) = \frac{k_i \bar{s}_i(t)}{K_m}, \quad \dot{s}_i = -\beta_i \bar{s}_i, \quad \bar{s}_i(0) = \frac{D\bar{h}_i\chi(I_{i,2})}{\beta_i}, \quad (11)$$

in which the term $-G_i(t)m_i$ will lead $m_i$ to decrease and to possibly push the system out of the region of attraction of PL. In this case, the system may converge back to the TR state.

In order to resolve this problem, we use mRNA sponges that quickly sequester the siRNA from its target $m_i$. It was computationally shown and experimentally verified that sufficiently large amounts of high affinity mRNA sponges could effectively upregulate the siRNA’s targets [58, 59]. Following these studies, we model this sponging effect by sequestration occurring through a reversible binding of the sponge with siRNA, which does not lead to degradation of the siRNA. Specifically, let $p_i$ be the sponge with perfect complementarity to the siRNA $s_i$, we thus have:

$$p_i + s_i \overset{\bar{d}_i}{\underset{d_i}{\rightleftharpoons}} c_{p_i}, \quad K_d = \frac{\bar{d}_i}{d_i}, \quad c_{p_i} \overset{\beta_i}{\rightarrow} 0$$

in which we overexpress $p_i$ through an inducible promoter with inducer $I_{i,3}$. Assuming a decay rate $\bar{\delta}_i$ for the sponge $p_i$, we have that system (7) after setting the inducers to zero, that is, $I_{i,1} = I_{i,2} = 0$ and employing a
nonzero inducer concentration \( I_{1,3} \neq 0 \) for the sponge, becomes

\[
\dot{s}_i = -\beta_i s_i, \quad \dot{s}_i(0) = \frac{Dh_{1,2}(I_{1,2})}{\beta_i}, \quad \dot{c}_i = a_i m_i s_i - (d_i + k_i) c_i - \beta_i c_i
\]

\[
\dot{m}_i = \dot{H}_i(x) - \delta_i m_i - a_i m_i s_i + d_i c_i, \quad \dot{x}_i = k_i m_i - \gamma_i x_i,
\]

\[
\dot{p}_i = \dot{D}h_{1,3}(I_{1,3}) - \delta_i p_i - a_i p_i s_i + d_i c_p, \quad \dot{c}_p = a_i p_i s_i - d_i c_p - \beta_i c_p,
\]

\[
s_i = \bar{s}_i - c_i - c_p,
\]

in which \( \dot{D} \) is the concentration of the DNA where the sponge is encoded and \( h_{1,3}(\cdot) \) is the inducer regulation function. By setting the complex dynamics to the quasi-steady state and re-writing the system in the slow variables \( \dot{\bar{m}}_i = m_i + c_i \) and \( \dot{\bar{p}}_i = p_i + c_p \), we have

\[
\dot{\bar{s}}_i = -\bar{\beta}_i \bar{s}_i, \quad \bar{c}_i = \bar{s}_i \frac{m_i / K_m}{1 + p_i / K_d}, \quad \bar{c}_p = \bar{c}_i \frac{p_i / K_d}{1 + p_i / K_d}
\]

\[
\dot{\bar{m}}_i = \bar{H}_i(x) - \delta_i \bar{m}_i - k_i \bar{c}_i, \quad \dot{\bar{p}}_i = \dot{D}h_{1,3}(I_{1,3}) - \delta_i \bar{p}_i - \beta_i \bar{c}_p,
\]

in which we have used the relations \( \beta_i \ll k_i \) and \( m_i / K_m \ll 1 \) before.

For this system, we can determine the smallest value of \( \dot{D} \) that is sufficient to guarantee that \( k_i c_i \) in the \( \dot{\bar{m}}_i \) equation becomes sufficiently small in a short time and thus it can be neglected compared to \( \delta_i m_i \) (see Section 1.5 in the Supplementary Information for the mathematical derivations). In particular, considering \( K_d \approx 0.004 \text{nM} \), which corresponds to one of the smallest values given by thermodynamic estimates [58] and using \( G_i = 10 \delta_i \) (\( D = 0.0002 \text{nM} \)) as before, we estimate that it is sufficient to have \( \dot{D} = 0.54 \text{ nM} \), corresponding to a DNA copy number of about 230. This number can be easily increased by increasing the number of sequences per DNA copy transcribed [59]. Figure 15 shows simulation results using the estimated

![Figure 15: siRNA sponges can be used to quickly shut down the feedback controller.](image)

The green plots show the induction of siRNAs, while the light blue plots show the induction of the mRNAs of the two factors \( x_1 \) and \( x_2 \). These inductions are both removed at \( t = 140 \) and at that time the siRNA sponges \( p_i \) are induced with \( \dot{D} = 0.86 \text{ nM} \), \( h_{1,3}(I_{1,3}) = 1 \text{hr}^{-1} \), \( \delta_i = \delta_3 \), \( a_i = 100 \text{ hr}^{-1} \text{ nM}^{-1} \) and \( d_i = 4 \text{ hr}^{-1} \) are set such that \( K_d = 0.04 \text{nM} \) (not the strongest possible binding). All other parameters are as before.

The copy number \( \dot{D} \) with a much weaker binding of the sponges to their target siRNA. Still, the feedback controller is shut down quickly and the network is reprogrammed to PL while being back to its original uncontrolled form \( \Sigma_0 \). In particular, once the inductions \( I_{1,1}, I_{1,2} \) of mRNA and siRNA are removed and the siRNA sponge \( p_i \) are induced, the concentrations of the \( x_1 \) and \( x_2 \) factors transiently decrease before the sponges \( p_i \) have had the time to build up enough to sequester all of the present siRNA. At this point, the system’s state moves toward the closest steady state, which is the PL state. As a consequence, the controller has been removed, that is, overexpression of the factors and overexpression of siRNA have both been removed, the system is in its original form (the uncontrolled network \( \Sigma_0 \)), and it has been reprogrammed to the PL state.
4 Discussion

We have found that reprogramming cell fate to the pluripotent state through constant overexpression of factors is an error-prone approach. This is because the gene regulatory network controlling pluripotency is constituted by all positive regulatory links (i.e., it is a monotone cooperative system) while the pluripotent state is intermediate in the landscape of states corresponding to stable cell lineages. Due to this combination of features, any constant overexpression of the factors that destabilizes a differentiated cell state can potentially destabilize also the pluripotent state (Figure 5), leading to reprogramming failure.

This discovery indicates that current reprogramming approaches have no guarantee of success. Hence there is a need for different methods that can ensure reprogramming to pluripotency, at least theoretically. We have proposed one such method, in which the overexpression of the factors is not pre-fixed to a constant value but it is adjusted in time based on the outcome. We realized this feedback overexpression strategy in two steps. First, a synthetic genetic circuit makes the multistable pluripotency network into a monostable system that remains frozen at a stable state where the concentrations of the factors are uniquely determined by the circuit’s inducers (Figure 11). Second, once the network is frozen at this state, we measure the discrepancy between the factors concentrations in this state and those in the target pluripotent state. We then adjust the inducers accordingly to decrease this discrepancy (Figure 14a). We have theoretically shown and demonstrated through simulation that this approach is guaranteed to reprogram the system to the pluripotent state independent of the network properties and of the synthetic genetic circuit’s parameters. The only information that the control strategy requires is the expected concentrations of the factors in the pluripotent state and the factors’ concentrations in the current state where the network is frozen. These, can, in turn, be obtained by a number of standard measurement techniques, such as immunostaining [60].

These findings suggest that current reprogramming practices may benefit from changing the current open loop control approach, in which the overexpression level is pre-set to a constant level, to the closed loop control approach, in which the overexpression level is instead adjusted based on the outcome. The latter theoretically ensures success, while the current open loop approach is not even theoretically guaranteed to succeed. While a decade ago the implementation of a closed loop control strategy may have been technologically daunting, today this implementation is within reach due to the advances of synthetic biology [61, 62]. We have proposed here an example of such an implementation, which relies on combining overexpression with strong siRNA-enabled repression. The capability of this implementation goes beyond the specific application of reprogramming a cell to pluripotency. In principle, it can be used to force any cell to switch into a desired lineage, whether this implies going in the direction of the natural differentiation process, jumping between different lineages (transdifferentiation), or reversing the natural process (reprogramming). It can, therefore, lead the way to a completely new approach to perform basic research in cell differentiation and to attack clinically relevant problems.

Our findings on the reprogramming properties of the pluripotency network are based on ordinary differential equations (ODE) models that capture the known genetic regulatory interactions among the core factors [63], but that neglect signaling pathways and chromatin structure [12, 16]. Since the synthetic genetic control circuit that we have proposed forces the pluripotency network to a desired state independent of the network’s structure, we expect that unmodeled dynamics such as those due to signaling or micro-RNA regulation pathways will have modest effect on the controlled system’s performance. Chromatin structure will instead likely play a role as important as in the current reprogramming approaches and, for a more accurate estimate of the closed loop control’s performance, it will need to be considered in future studies. In particular, epigenetics of DNA methylation and histone modification can be accounted for in the model by assuming slow binding of transcription factors to DNA as it has been proposed in a recent theoretical study [64]. This speed of binding can then, in principle, be controlled by using small molecule compounds that alter chromatin structure [11]. Synthetic genetic circuits constructed in mammalian cells have also demonstrated the ability of predictable epigenetic transgene control [65].
Another aspect that has not been accounted for by our models is stochasticity of gene expression and cell heterogeneity, which are believed to play a significant role in current reprogramming practices and in lineage decision making [66, 67]. Due to noise, the target factors’ concentrations in pluripotent cells are distributed about mean values [68]. Therefore, the outerloop feedback scheme will have to solve a stochastic control problem, in which both the current measured state and the target state are given by distributions as opposed to be given by a single value [69]. Stochasticity due to intrinsic noise can be readily included in our models by using, for example, weighted additive noise as in the chemical Langevin equation [70, 71]. Cell heterogeneity, instead can be included by considering distributions in the biochemical parameters representing differences across cells. In this case, the feedback control scheme will be able to force the mean value of the factors concentrations to a target state and reduce the variance due to intrinsic noise [72], but will not be able to control the variance due to cell heterogeneity unless inducers are selectively applied to each cell as shown in previous work [54]. Alternatively, a cell-cell communication module could be included in the synthetic genetic circuit to program directly the dynamics of the population despite differences among the single cells [73].

Finally, we recognize that efficiency is only part of the problem with reprogramming and that genomic stability of the reprogrammed cells is also a source of concern [53]. Different from the current open loop reprogramming practice where the transcription factors are overexpressed at high levels, we progressively increase their expression until their concentration reaches a value close to that found in the pluripotent state, at which point their concentration is held constant. This could, in principle, mitigate the severe DNA damage response often associated with too high overexpression levels of the factors in the open loop reprogramming approach.

Our future work will consider all these problems along with the experimental testing of the synthetic genetic controller. Ultimately, this line of research can create a ready-to-use synthetic biology tool that researchers and practitioners will be able to leverage for differentiation, transdifferentiation, and reprogramming research and for their potential clinical applications.

5 Acknowledgements

The authors would like to thank Prof. Eduardo Sontag and Prof. Ron Weiss for discussions on the monotone cooperative nature of the pluripotency network and on the siRNA technology for the implementation of the feedback controller, respectively. The authors would also like to thank Prof. George Daley for a number of useful discussions both on the reprogrammability property of the pluripotency network and on the feedback controller concept.

References


Supplementary Information

1.1 Reprogramming properties of monotone cooperative networks

We consider a system $\Sigma_u$ in the form $\dot{x} = f(x,u)$ with $x \in X = \mathbb{R}^n_+$ and $u \in U \subset \mathbb{R}^m_+$ a constant input vector. Let $S$ be the set of all stable steady states of $x = f(x,0)$, which we refer to as system $\Sigma_0$. Let $S \in S$ be one of the stable steady states. We let the flow of system $\Sigma$ starting from $x_0$ with input $u$ be denoted by $\phi_u(t,x_0)$ and we will write $\phi_0(t,x_0)$ for the flow of system $\Sigma_0$. Accordingly, we let $R_u(S)$ denote the region of attraction, or basin of attraction, of a stable steady state $S$ for system $\Sigma_u$. That is, $x_0 \in R_u(S)$ implies that $\lim_{t \to \infty} \phi_u(t,x_0) = S$. Also, we assume that for all $x_0 \in X, u \in U$, the omega-limit set $\omega_u(x_0)$ is finite.

Definition 1. We say that system $\Sigma_u$ is strongly reprogrammable to a steady state $S \in S$ provided there is an input $u \in U$ such that for all $x_0 \in \mathbb{R}^n_+$ the omega-limit set $\omega_u(x_0)$ is such that $\omega_u(x_0) \subset R_0(S)$.

From this definition, it follows that, starting from any initial condition $x_0$, after a sufficiently long application of control input $\bar{u}$, upon removal of such an input, that is, upon setting $u = 0$, the trajectory of $\Sigma_u$ approaches $S$. Qualitatively, this means that independent of the initial steady state in which system $\Sigma_u$ is found, we can force the state to transition to the stable steady state $S$ by a sufficiently long presentation and then removal of a suitable input. In this paper, we seek to determine conditions under which system $\Sigma_u$ is reprogrammable to a steady state $S \in S$.

In order to proceed, we assume that system $\Sigma_u$ is a monotone system. The reason of this assumption is double: first, many of the biological networks for which the reprogramming question is important are monotone; second, monotonicity allows to provide strong results about when a system is reprogrammable to a steady state given the rich geometrical properties of the system’s trajectories.

Definition 2. Let the state space $X$ be equipped with a partial order relation “≤” [74]. A system $\Sigma_u$ is monotone provided $x_0 \leq x_0' \Rightarrow \phi_u(t,x_0) \leq \phi_u(t,x_0')$ for all $t \geq 0$ and for all $u \in U$.

In the sequel, we consider the partial order established by component-wise ordering. That is, for all $x,y \in X$ we have $x \leq y$ if and only if $x_i \leq y_i$ for all $i$.

Assumption 1. System $\Sigma_u$ is monotone with component-wise partial order relation “≤”. Additionally, the system is cooperative, that is, $\partial f_i(x,u)/\partial x_j \geq 0$ for $i \neq j$ and for all $x \in X, u \in U$.

Note that a cooperative system is necessarily monotone with ordering on the state space established component-wise [27]. To keep the exposition of the theory simple, we chose the component-wise ordering. However, the results provided here naturally extend to any arbitrary partial order established according to a cone. Before giving the main results, we first provide some intermediate properties of the geometry of the stable steady states in a monotone dynamical system.

Proposition 1. Under Assumption 1, the set of stable steady states $S$ has a maximum and a minimum.

Proof. Let $\bar{x}$ be any element of $X$ such that $\bar{x} \geq S$ for all $S \in S$ and let us examine $\omega_0(\bar{x})$. Since $\omega_0(\bar{x})$ is bounded and the system is also cooperative, we have by Proposition 2.1 in [27] that $\omega_0(\bar{x})$ is an equilibrium, which in turn is an element of $S$. Let $y \in S$ be such an equilibrium. Since $S \leq \bar{x}$ for each element $S \in S$, it must be by the monotonicity property that $S \leq \omega_0(\bar{x})$, which, in turn, implies that $S \leq y$ for all $S \in S$. Therefore, $y = \sup(S)$ and since $y \in S$ we have that $y = \max(S)$. Hence, $S$ has a maximum. A similar proof holds for the minimum.

Now, we can state the first result. For a matrix $M$, we write $M \geq 0$ when $M_{i,j} \geq 0$ for all $i,j$.

Theorem 1. For system $\Sigma_u$ assume that $\partial f_i(x,u)/\partial u \geq 0$ for all $x \in X, u \in U$ (positive stimulation) and let Assumption 1 hold. Then, system $\Sigma_u$ is not strongly reprogrammable to any $S \neq \max(S)$.
Proof. First, we show that for all $u > 0$ system $\dot{x} = f(x, u)$ always admits a stable steady state $\bar{x}$ such that $\bar{x} \in S$ for all $S \in \mathcal{S}$. Using a similar approach as used in [75], we can consider the extended system

$$\dot{x} = f(x, u), \quad \dot{u} = 0,$$

which is also monotone with order on $U$ established component-wise. Consider two trajectories starting from the two initial conditions $(x_0, u_0) \leq (x_0', u_0')$ given by $x_0 = x_0' = \max(S)$ and $u_0 = 0, u_0' > 0$. Since $(x_0, 0)$ is a steady state of the above system, by the monotonicity property we have that $x_0 \leq \phi_{t}^u(t, x_0')$ for all $t$. Hence we have that in system $\dot{x} = f(x, u_0')$, $\omega_{u_0'}(\max(S))$ is greater than $\max(S)$ itself. In turn, consider $\dot{x} = f(x, 0)$ and an initial condition $z \geq \max(S)$. By the monotonicity property, we have that $\omega_0(z) \geq \max(S)$. Since there is no equilibrium of $\dot{x} = f(x, 0)$ in the cone $\{x \mid x \geq \max(S)\}$ and by Proposition 2.1 in [27] $\omega_0(\bar{x})$ is an equilibrium, we must have that $\omega_0(z) = \max(S)$. We conclude that for $\Sigma_u$ with $u > 0$ there is $x_0$ such that $\omega_u(x_0) \in R_0(\max(S))$, therefore $\Sigma_u$ cannot be reprogrammed to any of the steady states in $S$ that are different from the maximal one.

This result indicates that in a monotone (cooperative) system with only positive stimuli, it is not possible to strongly reprogram the system to any of the stable states that are not maximal. This indicates that in the Oct4/Sox2/Nanog network, the current approach to reprogramming that only uses overexpression of the factors cannot strongly reprogram the system to the pluripotent state since this one is characterized by an intermediate Oct4 concentration.

**Lemma 1.** Consider system $\Sigma_u$ satisfying Assumption 1 with $f_i(x, u_i) = H_i(x) + u_i - \gamma_i x_i$, $0 \leq H_i(x) \leq H_{IM}$ for all $x \in X$, and $u_i \geq 2H_{IM}$. Then, $\lim_{t \to \infty} x_i(t) \geq \max_{S \in \mathcal{S}}(S_i)$ independent of the initial condition.

**Proof.** Consider the system with $u = 0$ given by $\dot{x}_i = f_i(x, u) = H_i(x) - \gamma_i x_i$ for all $i \in [1, \ldots, n]$. Here, we can view $H_i(x)$ as a bounded disturbance and can therefore apply the robustness result from contraction theory [76] to obtain that $x_i(t) \leq Ae^{-\gamma_i t} + \frac{H_{IM}}{\gamma_i}$ for some positive $A$ depending on the initial condition. Letting $\bar{x}_i := \lim_{t \to \infty} x_i(t)$, we have that, $\bar{x}_i \leq \frac{H_{IM}}{\gamma_i}$. Since $\bar{x}_i$ is an unspecified equilibrium point of $\Sigma_0$, we have, in particular, that $\max(S_i) \leq \frac{H_{IM}}{\gamma_i}$.

Now, consider the pair of systems:

$$\dot{z}_i = u_i - \gamma_i z_i, \quad \dot{x}_i = H_i(\bar{x}) - \gamma_i \bar{x}_i + u_i,$$

in which we can view the first system as a nominal system and the second system as its perturbed version with disturbance $H_i(\bar{x})$, which is globally bounded by $H_{IM}$. Hence, we can apply again the robustness result from contraction theory to obtain

$$\lim_{t \to \infty} |\bar{x}_i(t) - \frac{u_i}{\gamma_i}| \leq \frac{H_{IM}}{\gamma_i}.$$

Letting $\epsilon := H_{IM}/u_i$ and re-arranging the terms, we obtain that $\lim_{t \to \infty} \bar{x}_i(t) \geq \frac{u_i}{\gamma_i}(1 - \epsilon)$. Since for $u_i \geq 2H_{IM}$, we have that $\frac{u_i}{\gamma_i}(1 - \epsilon) \geq \frac{H_{IM}}{\gamma_i}$, we also have that $\lim_{t \to \infty} \bar{x}_i(t) \geq \max(S_i)$. □

**Lemma 2.** Assume that system $\Sigma_u$ satisfies Assumption 1 and that it is in the following form: $\dot{x}_i = f_i(x, u_i) = H_i(x) - \gamma_i x + u_i$ with $u_i \in \mathbb{R}_+$ and $0 \leq H_i(x) \leq H_{IM}$ for all $x \in X$. Then, if $u_i \geq 2H_{IM}$ for all $i \in [1, \ldots, n]$, then $\omega_u(x_0) \geq \max(S)$ for all $x_0 \in X$.

**Proof.** By using Lemma 1, for system $\Sigma_u$ with $u_i \geq 2H_{IM}$ for all $i$ we have that $\lim_{t \to \infty} x_i(t) \geq \max_{S \in \mathcal{S}}(S_i)$ for all $i$ independent of the initial condition. Since this is true for any initial condition $x(0) = x_0$, we have that $\omega_u(x_0) \geq \max(S)$ for all $x_0 \in X$. □

**Theorem 2.** Assume that system $\Sigma_u$ satisfies Assumption 1 and that it is in the following form: $\dot{x}_i = f_i(x, u_i) = H_i(x) - \gamma_i x + u_i$ with $u_i \in \mathbb{R}_+$ and $0 \leq H_i(x) \leq H_{IM}$ for all $x \in X$. Then, system $\Sigma_u$ is strongly reprogrammable to $S$ if and only if $S = \max(S)$. In particular, a sufficiently large input will reprogram $\Sigma_u$ to $\max(S)$. 29
Proof. It follows from Theorem 1 and Lemma 2.

Strong reprogrammability of the system to \( S \) requires that all possible initial conditions can be steered to the region of attraction of \( S \) for some constant input \( u \). The system is not strongly reprogrammable to any intermediate state because initial conditions that are greater than the maximal element of \( S \) will be kept in the region of attraction of this maximal element independent of the input chosen. We therefore investigate whether a weaker reprogrammability to an intermediate state \( S \) holds, in which some initial condition not in the region of attraction of \( S \) can be steered to the region of attraction of \( S \) with constant input stimulation. We thus give the following definition.

**Definition 3.** We say that system \( \Sigma_u \) is weakly reprogrammable from steady state \( \bar{S} \in S \) to a steady state \( S \in S \) with \( S \neq S \) provided there is an input \( u \in U \) such that the omega-limit set \( \omega_u(\bar{S}) \) is such that \( \omega_u(\bar{S}) \subset \mathbb{R}_0(S) \).

The following result shows that if \( \bar{S} \neq S \), then the system cannot be weakly reprogrammed from \( \bar{S} \) to \( S \).

**Proposition 2.** Let \( S, \bar{S} \in S \) and let \( S < \bar{S} \). Then, system \( \Sigma_u \) is not weakly reprogrammable from \( \bar{S} \) to \( S \).

Proof. Systems \( \Sigma_u \) and \( \Sigma_0 \) are both monotone cooperative systems with \( f(x, 0) \leq f(x, u) \). It follows from Theorem VI (page 94) of [77] that \( \phi_0(t, S) \leq \phi_u(t, \bar{S}) \) for all \( t \). Also, we have that \( \phi_0(t, \bar{S}) = \bar{S} \). Therefore, we have that \( p := \omega_u(\bar{S}) \geq \bar{S} \). Since \( p \geq \bar{S} \), we have that \( \phi_0(t, p) \geq \bar{S} \) for all \( t \). This implies that \( \omega_0(p) \geq \bar{S} \), and therefore that \( p \) is not in the region of attraction of \( S \) since \( S < \bar{S} \).

The last result shows that if \( \bar{S} < S \) but the input is either too large or too small, the trajectory of \( \Sigma_u \) will not approach the region of attraction of \( S \).

**Proposition 3.** Let \( S, \bar{S} \in S \) and let \( \bar{S} < S \). There are inputs \( u_1 \) and \( u_2 \) such that if \( u \leq u_1 \) or \( u \geq u_2 \), then \( \Sigma_u \) is not weakly reprogrammable from \( \bar{S} \) to \( S \).

Proof. Consider \( \Sigma_u \) with \( u \) small. Since \( \bar{S} \) is a stable equilibrium for \( \Sigma_0 \), it follows that \( \partial f(x, u)/\partial x \mid_{x=0} \) is Hurwitz and hence non-singular. Since it is a continuous function of \( u \) and \( x \), it follows from the implicit function theorem that there is an open ball \( B \subset U \) about \( u = 0 \) such that \( \bar{x}(u) \) is a locally unique solution to \( f(x, u) = 0 \) for \( u \in B \); furthermore \( \bar{x}(u) \) is a continuous function of \( u \). Therefore, for small \( u \), we will have that \( \partial (u) \) is close to \( \bar{S} \). We can thus pick \( u \) small enough such that \( \bar{x}(u) \) is in the region of attraction of \( \bar{S} \). Also, we have that \( \bar{x}(u) \geq \bar{S} \) for the monotonicity property of the systems \( \Sigma_0 \) and \( \Sigma_u \). Therefore a trajectory \( \phi_u(t, \bar{S}) \) will asymptotically reach a point \( p \) that is always smaller than \( x(u) \) and hence in the region of attraction of \( \bar{S} \). Therefore, there is an input \( u_1 > 0 \) sufficiently small such that if \( u \leq u_1 \) the system is not reprogrammed from \( \bar{S} \) to \( S \).

Consider \( \Sigma_u \) with \( u \) large. The fact that there is \( u_2 \) sufficiently large such that if \( u \geq u_2 \) the system is not reprogrammed from \( \bar{S} \) to \( S \) follows from Lemma 2.

This result implies that system \( \Sigma_u \) with \( u \leq u_1 \) or \( u \geq u_2 \) is not weakly reprogrammable to any intermediate state \( S \in S \) from the minimum of \( S \). In other words, the system may be reprogrammed to the intermediate steady state \( S \) from the minimum one only if \( u \) takes values in an intermediate range \([u_1, u_2]\), which, however, may be empty since we may have \( u_2 < u_1 \).

### 1.2 Effect of a Type 1 interaction on a monotone cooperative network

In this section, we demonstrate that the addition of a type 1 interaction to a monotone cooperative network keeps the extended network monotone and cooperative in possibly new coordinates for the variables of the added interactions.
Specifically, let \( y \in \mathbb{R}^m \) represent the vector of concentrations of additional species added to the original network. The full system is now given by

\[
\dot{y} = g(y, x), \quad \dot{x} = \tilde{f}(x, y, u), \quad \text{with} \quad \tilde{f}(x, 0, u) = f(x, u).
\]

Consider any two nodes \( x_j \) and \( x_k \) and consider a path \( x_j \rightarrow y_{j_1} \rightarrow ... \rightarrow y_{j_p} \rightarrow x_k \) such that

\[
\begin{align*}
\frac{\partial g_{j_1}}{\partial x_j}, & \quad \frac{\partial g_{j_2}}{\partial y_{j_1}}, \quad ... \quad \frac{\partial g_{j_p}}{\partial y_{j_{p-1}}}, \quad \frac{\partial f_k}{\partial y_{j_p}}
\end{align*}
\]

are all not identically zero. Consider the restricted system in which the \( y \) dynamics take as “input” only \( x_j \) through only the interaction \( x_j \rightarrow y_{j_1} \) and the \( x \) dynamics take as input only \( y_{j_p} \) through only the interaction \( y_{j_p} \rightarrow x_k \). The dynamics of this system are given by:

\[
\begin{align*}
\dot{y}_{-k} &= g_{-j_k}(y, 0), \quad \dot{y}_{j_k} = g_{y_k}(y, (0, ..., x_j, ..., 0)), \\
\dot{x}_{-k} &= \tilde{f}_{-k}(x, 0, u), \quad \dot{x}_{k} = \tilde{f}_{k}(x, (0, ..., y_{j_p}, ..., 0), u),
\end{align*}
\]

(13)

in which for a vector \( v \), we have denoted by \( v_k \) its \( k \)th component and by \( v_{-k} \) the vector \( v \) with the \( k \)th component removed. In the sequel, for a vector \( v \) and a diagonal matrix with entries the vector’s coordinates \( M = \text{diag}(v) \) we denote by \( M_{-i} \) the \( n - 1 \times n - 1 \) diagonal matrix given by \( \text{diag}(m_{-1}) \).

We now consider interactions that do not change the monotone cooperative structure of the system. To this end, we make the following simplifying assumption.

**Assumption 2.** For system (13), we assume that each \( y_{j_i} \) in the path \( x_j \rightarrow y_{j_1} \rightarrow ... \rightarrow y_{j_p} \rightarrow x_k \) has only one parent and only one child, that is, the path is *simple*.

Under this assumption, we can re-write system (13) as the two decoupled systems:

\[
\begin{align*}
\dot{y}_{j_i} &= g_{j_i}(y_{j_i}, (0, ..., x_j, ..., 0)), \quad ... \quad \dot{y}_{j_{i+1}} = g_{j_{i+1}}(y_{j_i}, 0), \quad i \leq p - 1, \quad \dot{x}_k = \tilde{f}_k(x, (0, ..., y_{j_p}, ..., 0)),
\end{align*}
\]

(14)

and

\[
\dot{y}_{-} = g_{-}(y_{-}, 0),
\]

in which \( y_{-} \) is the vector \( y \) with the components \( y_{j_1}, ..., y_{j_p} \) removed. We now give the following definition of a type 1 interaction. Let \( \Lambda \) be a diagonal matrix with diagonal entries \( \lambda_i \in \{-1, 1\} \). We then give the following definition.

**Definition 4.** The simple path \( x_j \rightarrow y_{j_1} \rightarrow ... \rightarrow y_{j_p} \rightarrow x_k \) is a *type 1 interaction* provided there is a \( \Lambda \) such that system (14) in the new coordinates \( \bar{y} = \Lambda y \) is a cooperative monotone system.

This definition implies that a type 1 interaction extends the original \( x \) system to the larger system (given by (14)) that in the new coordinates \( \bar{y} = \Lambda y \) becomes

\[
\begin{align*}
\dot{y}_{j_i} &= \lambda_{j_i} g_{j_i}(\lambda_{j_i} \bar{y}_{j_i}, (0, ..., x_j, ..., 0)), \quad ... \quad \dot{y}_{j_{i+1}} = \lambda_{j_{i+1}} g_{j_{i+1}}(\lambda_{j_i} \bar{y}_{j_i}, 0), \quad i \leq p - 1, \quad \dot{x}_k = \tilde{f}_k(x, (0, ..., \lambda_{j_p} \bar{y}_{j_p}, ..., 0)),
\end{align*}
\]

(15)

which is still monotone and cooperative with the component-wise order \( x \leq x' \iff x_i \leq x_i' \quad \forall \ i \) according to which the isolated \( x \) system is also cooperative. It follows that this system is also not strongly reprogrammable to the intermediate state \( PL \) and may be weakly reprogrammable to it from a lower steady state, such as \( TR \), for some range of inputs.

With these premises, we can provide a check for when a simple path is a type 1 interaction.
Proposition 4. Consider system (14). If the condition
\[ \frac{\partial g_{j_1}}{\partial x_j} \lambda_{j_1} \geq 0, \quad \frac{\partial g_{j_2}}{\partial y_{j_1}} \lambda_{j_2} \geq 0, \quad \ldots, \quad \frac{\partial g_{j_p}}{\partial y_{j_{p-1}}} \lambda_{j_p} \geq 0, \quad \text{and} \quad \frac{\partial f_k}{\partial y_{j_p}} \lambda_{j_p} \geq 0. \] (16)
is satisfied, then the path is a type 1 interaction.

Proof. It is sufficient to prove that there are \( \lambda_{j_1}, \ldots, \lambda_{j_p} \) that each take value in \([-1, 1]\) such that
\[ \frac{\partial g_{j_1}}{\partial x_j} \lambda_{j_1} \geq 0, \quad \frac{\partial g_{j_2}}{\partial y_{j_1}} \lambda_{j_2} \geq 0, \quad \ldots, \quad \frac{\partial g_{j_p}}{\partial y_{j_{p-1}}} \lambda_{j_p} \geq 0, \quad \text{and} \quad \frac{\partial f_k}{\partial y_{j_p}} \lambda_{j_p} \geq 0. \]
This, in turn is the case if and only if we have
\[ \lambda_{j_1} = \text{sign}\left(\frac{\partial g_{j_1}}{\partial x_j}\right), \quad \lambda_{j_2} = \text{sign}\left(\frac{\partial g_{j_1}}{\partial x_j} \frac{\partial g_{j_2}}{\partial y_{j_1}}\right), \quad \ldots, \quad \lambda_{j_p} = \text{sign}\left(\frac{\partial g_{j_1}}{\partial x_j} \frac{\partial g_{j_2}}{\partial y_{j_1}} \cdots \frac{\partial g_{j_p}}{\partial y_{j_{p-1}}}\right), \]
and
\[ \lambda_{j_p} = \text{sign}\left(\frac{\partial f_k}{\partial y_{j_p}}\right). \]
This set of equations has a solution if and only if
\[ \text{sign}\left(\frac{\partial f_k}{\partial y_{j_p}}\right) = \text{sign}\left(\frac{\partial g_{j_1}}{\partial x_j} \frac{\partial g_{j_2}}{\partial y_{j_1}} \cdots \frac{\partial g_{j_p}}{\partial y_{j_{p-1}}}\right), \]
which is, in turn true by the assumed condition (16).

We will refer to a simple path where condition (16) is satisfied as a positive interaction. We will refer to a simple path where condition (16) is not satisfied as a negative interaction. In this case, by the same argument as those in the above proof, the system (14) does not admit a coordinate change \( \Lambda \) such that the system in the new coordinates is monotone and cooperative. If the path is not simple, the left-hand side of (16) looses meaning and we will refer to these paths as undetermined interactions. We will refer to negative or undetermined interactions as Type 2 interactions.

1.3 Reprogramming of Monotone Systems Subject to Undetermined Perturbations

Given system \( \Sigma_u \) monotone of the cooperative type
\[ \Sigma_u : \quad \dot{x} = f(x, u), \quad f_i(x, u) = H_i(x) - \gamma_i + u_i, \quad i \in \{1, \ldots, n\} \]
as before with set of partially ordered stable steady states for \( \Sigma_u \) given by \( \Sigma_u = \{S^1_u, \ldots, S^m_u\} \), in which we assume without loss of generality that \( S^1_u \) is the minimum and \( S^m_u \) is the maximum. We now consider an undetermined perturbation to this dynamics as follows:
\[ \Sigma^\epsilon : \quad \dot{x} = f(x, u) + \epsilon d(x), \quad \epsilon > 0, \quad ||d(x)|| \leq d_M, \quad \forall \ x \]
in which \( d(x) \) is a bounded perturbation that captures the effect of unmodeled interactions. Here, we assume that all functions are smooth. We also assume that the omega-limit set of any initial condition of \( \Sigma^\epsilon \) is a steady state.

Here, we seek to demonstrate that if \( \epsilon \) is sufficiently small, then we still have the reprogramming properties of \( \Sigma_u \). Namely, the system is not strongly reprogrammable to any stable steady state different from the continuation of \( S^m_u \) with \( \epsilon > 0 \) small. Furthermore, the system is not weakly reprogrammable from the continuation of \( S^1_u \) to any steady state that is the continuation of an intermediate steady state of \( \Sigma_0 \) with inputs that are either too large or too small.

The following theorem shows that for \( \epsilon \) small enough, the stable steady states of \( \Sigma^\epsilon \) lie within an \( \epsilon \) ball around the stable steady states of \( \Sigma_u \) (Figure 16).
Lemma 3. There is $\epsilon^* > 0$, smooth functions $\gamma_u^1(\epsilon), \ldots, \gamma_u^m(\epsilon)$, and $c > 0$ such that for $\epsilon < \epsilon^*$ we have

(i) $\|\gamma_u^i(\epsilon) - S_u^i\| \leq c \epsilon$;

(ii) $x = \gamma_u^i(\epsilon)$ is a stable steady state for $\Sigma_u^\epsilon$ for any $i$.

Proof. Let us call $F(x, \epsilon) := f(x, u) + ed(x)$ such that $F(x, 0) = f(x, u)$. Since $F(\cdot, \cdot)$ is a smooth function of its arguments and $\frac{\partial F}{\partial u}|(S_u^i, 0)$ is Hurwitz (because $S_u^i$ is a locally asymptotically stable equilibrium point), by the implicit function theorem there is $\epsilon^*_1 > 0$ and a locally unique smooth function $\gamma_u^i(\epsilon)$, such that $F(\gamma_u^i(\epsilon), \epsilon) = 0$ for all $\epsilon < \epsilon^*_1$. Also, $\frac{\partial F}{\partial u} = (\partial F/\partial x)^{-1}(\partial F/\partial \epsilon)$. Let $c$ be the supremum over $\epsilon \in [0, \bar{\epsilon}]$ with $\bar{\epsilon} < \epsilon^*_1$ of $\|\frac{\partial F}{\partial u}\|$, then $\|\gamma_u^i(\epsilon) - \gamma_u^i(0)\| \leq c \cdot \epsilon$, which leads to (i). The fact that $x = \gamma_u^i(\epsilon)$ is a steady state of $\Sigma_u^\epsilon$ follows from the fact that $F(\gamma_u^i(\epsilon), \epsilon) = 0$ for all $\epsilon < \epsilon^*$. The fact that it is stable follows from the following argument. Define the matrix $g(\epsilon) = \frac{\partial F}{\partial x}|(\gamma_u^i(\epsilon), \epsilon)$. By the problem definition, we have that the eigenvalues of $g(0)$ all have strictly negative real parts. Since $g$ is a smooth function of $\epsilon$ and the roots of the characteristic polynomial of $g$ depend continuously on its coefficients, there is $\epsilon'$ such that $g(\epsilon)$ has eigenvalues with strictly negative real part for all $\epsilon < \epsilon'$. Therefore, (ii) follows with $\epsilon^* = \min\{\epsilon', \epsilon^*_1\}$. \qed

In the sequel, we assume that $\epsilon$ is small enough such that this Lemma holds and also such that the balls $B_{\epsilon, u}(S_u^i)$ for $i \in \{1, \ldots, m\}$ are disjoint. Such an $\epsilon$ exists because the steady states in $S_u$ are isolated.

Lemma 4. Let $x(t, x_o)$ denote the trajectory of $\Sigma_u^\epsilon$: $\dot{x} = f(x, u) + ed(x)$, let $w(t, w_o)$ denote the trajectory of $\Sigma_u^M$: $\dot{w} = f(w, u) + ed_M$, and let $v(t, v_o)$ denote the trajectory of $\tilde{\Sigma}_u$: $\dot{v} = f(x, u) - \epsilon d_M$ starting from initial conditions $v_o \leq x_o \leq w_o$. Then, we have that $v(t, v_o) \leq x(t, x_o) \leq w(t, w_o)$ for all $t \geq 0$.

Proof. The result follows directly from Theorem VI (page 94) of [77] applied to the pairs $\dot{x}, f(x, u)$ and $\dot{x}, f(x, u) - ed_M$, in which the vector fields $f(x, u)$ and $f(x, u) - ed_M$ are each quasi-monotone according to the definition in [77]. \qed

This result says that the trajectories of $\Sigma_u^\epsilon$ are always comprised between those of $\tilde{\Sigma}_u$ and those of $\Sigma_u^M$.

Consider the set of stable steady states of $\tilde{\Sigma}_u$. For $\epsilon$ sufficiently small, the same arguments as those in Lemma 3 applied to $\Sigma_u^\epsilon$ apply and therefore this set will be given by $\tilde{\Sigma}_u: \{\tilde{S}_u^1, \ldots, \tilde{S}_u^m\}$, in which $\tilde{S}_u^i$ lies within a ball with radius proportional to $\epsilon$ centered at $S_u^i$. Also, since $\tilde{\Sigma}_u$ is monotone and cooperative, we have that the set of stable steady states has a maximum and a minimum. Without loss of generality, let $S_u^m$ be the maximum and $\tilde{S}_u^1$ be the minimum. Then, we have the following Lemma.

Lemma 5. Let $x_o$ be the initial condition of $\Sigma_u^\epsilon$. If $x_o \geq \tilde{S}_u^m$, then $\omega_u^\epsilon(x_o) \geq \tilde{S}_u^m$. 

Figure 16: Stable steady state landscape of $\Sigma_u^\epsilon$. When $\epsilon = 0$, the stable steady states of $\Sigma_u^\epsilon$ are equal to those of $\Sigma_u$, depicted in black. When $\epsilon$ is non-zero but small, the stable equilibria (depicted in pink) remain confined in small balls $B_{\epsilon, u}(S_u^i)$ (depicted in grey) around the stable steady states of $\Sigma_u$. 

33
Proof. By Lemma 4, we have that if \( v_o = x_o \) then \( v(t, v_o) \leq x(t, x_o) \) for all \( t \geq 0 \). This inequality continues to be true asymptotically, and therefore, we must also have that \( \omega_u^i(x_o) \geq \lim_{i \to \infty} v(t, v_o) \). In turn, since system \( \Sigma_u \) is monotone and cooperative and \( v_o \geq \tilde{S}_u^m \), then we must have that \( \lim_{i \to \infty} v(t, v_o) = \tilde{S}_u^m \), leading to the desired result. \( \square \)

This lemma implies that \( \Sigma^\infty_u \) for any \( u > 0 \) has a steady state that is greater than \( \tilde{S}_u^m \) (since \( \tilde{S}_u^m \) is greater than any of the stable steady states \( \tilde{S}_u^i \) by the monotonicity property along with positive stimulation).

**Theorem 3.** System \( \Sigma^\infty_u \) is not strongly reprogrammable to any stable steady state of \( \Sigma^\infty_0 \) different from \( \gamma^m_0(\epsilon) \).

**Proof.** By Lemma 5, any \( u > 0 \) for \( \Sigma^\infty_u \) will result for \( x_o \geq \tilde{S}_u^m \) in a stable steady state that is greater than \( \tilde{S}_u^m \) since \( \tilde{S}_u^m \) is greater than any of the stable steady states \( \tilde{S}_u^i \) by the monotonicity property along with positive stimulation. A trajectory \( x(t, x_o) \) of \( \Sigma^\infty_0 \) that starts with \( x_o \) greater than \( \tilde{S}_u^m \) will be such that (by Lemma 5) \( \omega^*_0(x_o) \geq \tilde{S}_u^m \). It will therefore converge to a stable steady state of \( \Sigma^\infty_u \) that is greater than or equal to \( \tilde{S}_u^m \). By Lemma 3, the only such steady state is \( \gamma^m_0(\epsilon) \). Since there are always initial conditions that give rise to trajectories approaching the region of attraction of \( \gamma^m_0(\epsilon) \), it follows that the system is not strongly reprogrammable to any other stable steady state. \( \square \)

**Theorem 4.** There are \( u_1, u_2 > 0 \) such that if \( u \leq u_1 \) or \( u \geq u_2 \) then \( \Sigma^\infty_u \) is not weakly reprogrammable from \( \gamma^m_0(\epsilon) \) to any \( \gamma^1_i(\epsilon) \) for \( i \neq m \).

**Proof.** From Lemma 2 with \( H_i(x) \) re-defined as \( H_i(x) + \epsilon d_i(x) \), we have that for all \( x_o \), the trajectory of \( \Sigma^\infty_u \) with \( u_i \geq 2H_iM \) for all \( i \) will result into \( \omega_u(x_o) \geq \tilde{S}_u^m \). As a consequence, \( \omega_u(x_o) \in \mathcal{R}_0(\gamma^m_0(\epsilon)) \) for all \( x_o \) and in particular for \( x_o = \gamma^m_0(\epsilon) \). Reversely, if \( u \) is too small, by continuity arguments \( x_o = \gamma^m_0(\epsilon) \) will approach a steady state that still lies in the region of attraction of \( \gamma^m_0(\epsilon) \). \( \square \)

### 1.4 Properties of High-gain Negative Feedback

Consider the system
\[
\dot{x}_i = H_i(x) - \gamma_i x_i + G_i(x^*_i - x_i),
\]
in which \( |H_i(x)| \leq H_iM \) for all \( x \). Now consider the error \( e = x_i - x^*_i \) and re-write the above dynamics in error coordinates:
\[
\dot{e} = H_i(x) - \gamma_i x^*_i - e(G_i + \gamma_i).
\]
In this system \( H_i(x) - \gamma_i x^*_i \) can be viewed as a bounded disturbance such that \( |H_i(x) - \gamma_i x^*_i| \leq H_iM + \gamma_i x^*_i \). Since this system is contracting with contraction rate \( G_i + \gamma_i \), we can use the robustness result from contraction theory [76] to conclude that
\[
\limsup_{t \to \infty} = \frac{H_iM + \gamma_i x^*_i}{G_i + \gamma_i}.
\]

### 1.5 Calculation of sponge’s DNA copy number sufficient for quick siRNA removal

We consider the system with the sponge expression and no overexpression of the factors or of their siRNA, given by
\[
\begin{align*}
\dot{s}_i &= -\beta_i \bar{s}_i, \\
c_i &= \bar{s}_i \frac{m_i/K_m}{1 + p_i/K_d}, \\
c_{p_i} &= \bar{s}_i \frac{p_i/K_d}{1 + p_i/K_d}, \\
\dot{m}_i &= H_i(x) - \delta_i m_i - k_i c_i, \\
\dot{p}_i &= \tilde{D} H_i(x) - \delta_i p_i - \beta_i c_{p_i}.
\end{align*}
\]
(17)

For this system, we seek to determine how large \( \tilde{D} \) must be to guarantee that \( c_i = \bar{s}_i(m_i/K_m)/(1 + p_i/K_d) \) in the \( \dot{m}_i \) equation becomes sufficiently small in a short time such that it becomes negligible. Specifically, we
request that by the time $T(\epsilon)$ at which $m_i(t)$ has decreased by $\epsilon \times 100\%$ with respect to $m_i^*$, the term $-k_i c_i$ has become negligible compared to $-\delta_i m_i$. If this is the case and $\epsilon$ is sufficiently small, at time $T$ the state of the system will still be in the region of attraction of PL and since $\bar{s}_i(m_i/K_m)/(1 + p_1/K_d)$ can be neglected, the $m_i$ dynamics (and hence those of the full system) are approximately the same as those of the original system without feedback controller. Since PL is stable for this system and the state at time $T$ is in its region of attraction, the state will ultimately converge to PL.

First, we find a lower bound for $T(\epsilon)$ from analyzing the dynamics of $m_i$. To this end, since $\dot{m}_i = \dot{m}_i + \dot{c}_i$, $\partial c_i/\partial p_i \leq 0$, $\partial c_i/\partial \bar{s}_i \geq 0$, and $\dot{p}_i \geq 0$, we have that $m_i(1 + \partial c_i/\partial m_i) \geq \dot{m}_i$. Since $\partial c_i/\partial m_i \leq \bar{s}_i/K_m$ and $\dot{m}_i \geq -\delta_i m_i - k_i(\bar{s}_i/K_m)m_i$, we finally have that

$$\dot{m}_i \geq -G_i m_i, \quad m_i(0) = m_i^*$$

in which we have used $\delta_i \ll G_i$. From this, it follows that $m_i(t) \geq m_i(0)e^{-G_i t}$ and therefore that $m_i(t) \geq (1 - \epsilon)m_i(0)$ as long as

$$t \leq \frac{1}{G_i} \ln \left(\frac{1}{1 - \epsilon}\right) = T(\epsilon).$$

Second, we determine for what values of the copy number $\bar{D}$ of the sponge sites, we can guarantee that $k_i c_i(t) \leq 0.1 \delta_i m_i$ for all $t \geq T$, so that the term $k_i c_i$ can be neglected after this time. To this end, consider that $\bar{s}_i(t) = e^{-\beta_i t} \bar{s}_i(0)$ with $\bar{s}_i(0) = \frac{D_{ai}}{\beta_i}$ from equation (11), so that

$$k_i c_i(t) = k_i \frac{e^{-\beta_i t} D_{ai} m_i}{K_m} \frac{m_i}{1 + p_1/K_d}, \quad G_i = \frac{k_i D_{ai}}{K_m \beta_i}.$$  

It is therefore sufficient to request that

$$G_i e^{-\beta_i t} \frac{1}{1 + p_1(t)/K_d} \leq 0.1 \delta_i, \quad \forall \ t \geq T. \quad (18)$$

To determine when this is the case, we analyze the $\dot{p}_i$ dynamics and determine the smallest value that $p_1(t)$ takes for $t \geq T$. From $\dot{p}_i = \dot{p}_i + \dot{c}_p$, with $\partial c_p/\partial p_1 = (\bar{s}_i/K_m)(1/(1 + p_1/K_d)^2)$, and using the expressions for $\dot{\bar{s}}_i$ and $\dot{\bar{p}}_i$ in equations (17), we finally obtain

$$\dot{p}_i = (\bar{D} h_{i,3}(I_{i,3}) - \bar{\delta}_i p_1) \left(\frac{1}{1 + (\bar{s}_i/K_m)(1/(1 + p_1/K_d)^2)}\right).$$

From this, employing differential inequalities, we obtain that

$$p_i(t) \geq \bar{D} h_{i,3}(I_{i,3}) \frac{1 - e^{-\lambda t}}{\bar{s}_i} \lambda = \frac{\bar{s}_i}{1 + \bar{s}_i(0)/K_m}.$$  

For $\epsilon$ sufficiently small, we can use Taylor expansion of $T(\epsilon)$ to obtain that $T \approx \epsilon/(G_i(1 - \epsilon))$. Letting $h_{i,3}(I_{i,3}) = \alpha_i$ as performed for the siRNA expression rate, we therefore have that

$$p_i(t) \geq \frac{D_{ai}}{\delta_i} \left(\frac{\lambda \epsilon}{G_i(1 - \epsilon)}\right), \quad \forall \ t.$$  

Substituting this expression into the left-hand side of (18), we finally obtain

$$\frac{D_{ai}}{K_d} \epsilon \geq 10 \frac{G_i^2}{\delta_i} \left(\frac{\bar{s}_i(0)}{K_m}\right).$$

Here, we consider $K_d = 0.004$ nM, which corresponds to one among the smallest values given by thermodynamic estimates [58]. Using $\epsilon = 0.1$, $G_i = 10 \delta_i$ ($D = 0.0002$ nM) as before, $\bar{s}_i(0) = D_{ai}/\beta_i$, $\delta_i = \delta_2 = 0.09$ hr$^{-1}$, it is sufficient to have $\bar{D} \approx 0.54$ nM, corresponding to a DNA copy number of about 230. This number can be easily increased by increasing the number of sequences per DNA copy transcribed [58].
Figure 17: The feedback controller reprograms PE into PL. (a) Time trajectories of the transcription factors concentrations $x_1$ and $x_2$ and time trajectories of the siRNA concentrations $s_1$ and $s_2$ (b). Initially the concentrations of the transcription factors and mRNAs are at steady state in the PE state, then at time $t = 5\text{hr}$ inducers $I_{1,1}$, $I_{2,1}$, $I_{1,2}$, $I_{2,2}$ are applied to induce the production of the siRNAs (b). After the siRNA has approached its steady state, inducers $I_{1,1}$ and $I_{2,1}$ are applied in the specific amounts calculated in the text at time $t = 80\text{hr}$. Light blue and green lines are not to scale. The parameters for the Hill functions are the same as those chosen in Figure 3a, where they were leading to reprogramming failure with open loop control.