THE COLLEGE OF
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A BOOLEAN MODELING APPROACH TO UNDERSTANDING SYNERGIES BETWEEN CELL PHENOTYPES IN EARLY NEUROGENESIS

DEPARTMENTS OF BIOLOGY AND NEUROSCIENCE

INDEPENDENT STUDY THESIS

Nathan R. Sundheimer

Advisor: Dr. Erzsébet Ravasz Regan

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ABSTRACT

Neuronal development is an investigation that has yet to be fully understood. Different transcription factors and markers are expressed differently throughout the developmental cascade of a neuron, either in the form of presence/absence of environmental signals. In this study, a Boolean modeling framework was employed to develop a model of the early stages of the neurodevelopmental cascade starting with embryonic stem cell proliferation and differentiation. A Boolean model was useful in terms of synthesizing previous work and simplifying the neurodevelopmental cascade using *in silico* analysis. In this module, I looked at the proliferative and differentiative qualities of an embryonic stem cell in relation to the Leukemia Inhibitory Factor (LIF), Bone Morphogenetic Protein (BMP), 2i (serum containing LIF) and growth factor (GF) environments. Drawing on the extensive experimental literature on embryonic stem cell maintenance, I assembled a model that represents a naive embryonic stem cell, and the coupling between this system and the cell cycle and pluripotency network. Using Boolean sampling, live attractor states were acquired and box graphs and time course experiments were assembled to represent different molecular and phenotype changes that occur in different combinations of environmental stimuli. Unique, unexpected phenotypic transitions based on single environment manipulation were analyzed and studied, pointing out flaws in the model and postulating gaps in knowledge in current biology. The current model possibly may require the addition of other pathways to supplement and improve the results in terms of providing a further understanding of the present phenotypic transitions as well as providing a wider variety of phenotypes that can be modeled.

INTRODUCTION

Stem cell biology

Stem cells are one of the greatest discoveries of modern science and medicine. These cells have an incredible power, as they are able to serve as a tool to unearth a plethora of cures in medicine, but they also provide deeper insight to our development and adult function on the cellular level. There are three major stem cell types defined as embryonic stem cells, adult stem cells, and induced pluripotent stem cells. Embryonic stem cells originate in and make up the blastocyst, a small structure that plays a crucial role in development, where these cells are maintained in a fully pluripotent, undifferentiated state. These cells provide insight as to how we humans develop, as well as to how embryos differentiate into different cell types to make complete adult organisms. These cells are totipotent, meaning that they can become any type of cell our adult bodies contain. The potential of embryonic stem cells is second to none, as they give rise to the possibility that we can generate specialized cells on demand, to fix and/or replace damaged tissues and restore health (Vazin et al., 2010).

In contrast, adult stem cells are multipotent, but tissue-specific stem cells. This means that each adult stem cell type can only develop into a select subset of adult cells, specifically in their tissues of origin. For example, an adult stem cell in the brain cannot differentiate into cardiovascular tissue and vice versa. Stem cells have been found to be relevant to a variety of fundamental and dynamic processes in the brain (Gage, 2000). In the field of medical research, the usage of these stem cells could provide researchers new insights on how to treat and cure physical damage and other types of disease, such as diabetes (Maehr et al, 2009). These cells are not only crucial to our development, but their potential and power is necessary to reign in to

further develop advances in medicine for the overall quality of life for our species, and to learn more about ourselves.

The development and biology of neural stem cells

In the development of mammalian species, a zygote is formed at conception. This zygote evolves into a blastocyst. A repeated round of divisions then gives rise to totipotent cells capable of giving rise to a fully complete, normally functioning organism when it is implanted into a female host (uterus; Figure 1) (Gage, 2000). From there, the structure develops into a blastocyst, in which an outer trophoblast cell layer surrounds the embryonic stem cells (inner cell mass). A layer of these cells then gives rise to the ectoderm (Figure 1, pink), and subsequently the rest of the neural system lineage. These cells are still capable of giving rise to a healthy organism, except that they no longer receive the same morphogens from the female host as totipotent cells and thus no longer become trophoblasts (Gage, 2000).

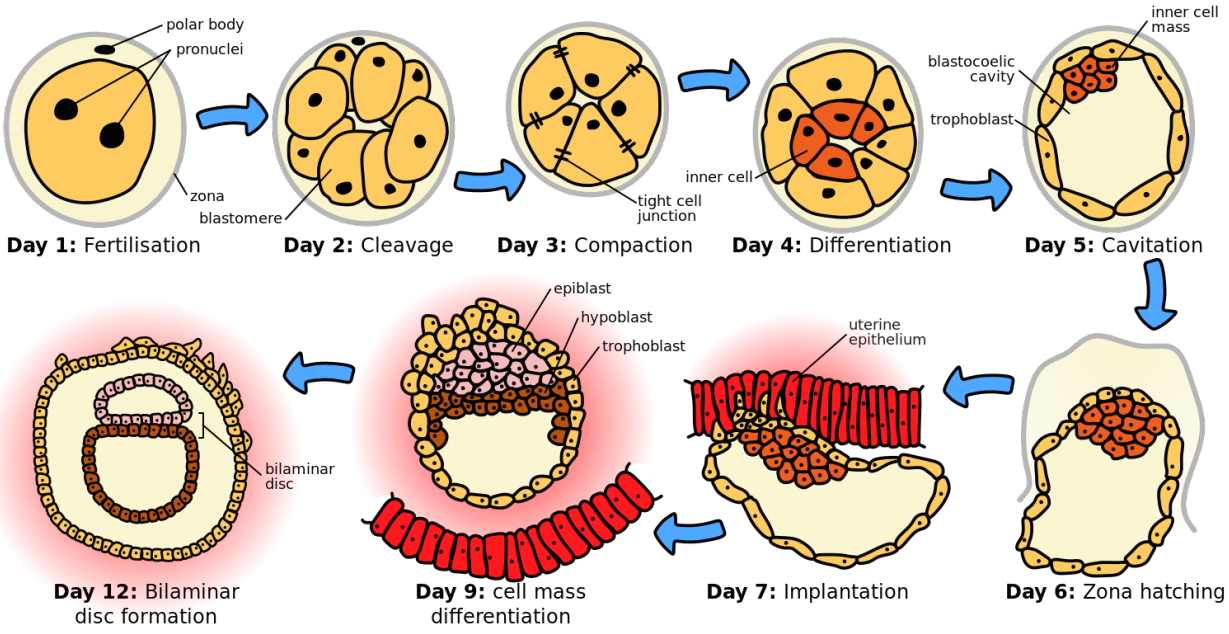

Figure 1. Early embryonic differentiation in space and time. *Pink: ectoderm.*(Zephyris. (2010). *The first few weeks of embryogenesis in humans. Beginning at the fertilised egg, ending with the closing of the neural tube.* Retrieved from https://commons.wikimedia.org/wiki/File:HumanEmbryogenesis.svg)

During development, three distinct layers arise in the blastocyst: the endoderm (Figure 1, maroon), the mesoderm (arising between the ectoderm and endoderm around day 12, not shown), and the ectoderm (Figure 1, pink).

Each specific layer differentiates into different cell types. The mesoderm typically differentiates into bone and cartilage, and the ectoderm differentiates into the brain and neural tissue (Vazin et. al., 2010).

Multipotent ectodermal cells later differentiate into neural progenitors. These cells then undergo a process that makes them committed neural progenitors, namely neuronal or glial progenitors. These cells proliferate little, and they are not functional in the brain. As they divide, they differentiate into completely differentiated, non-mitotic neurons or glia. In order to fully comprehend the origin and capabilities of each developmental stage of neurogenesis, it is important

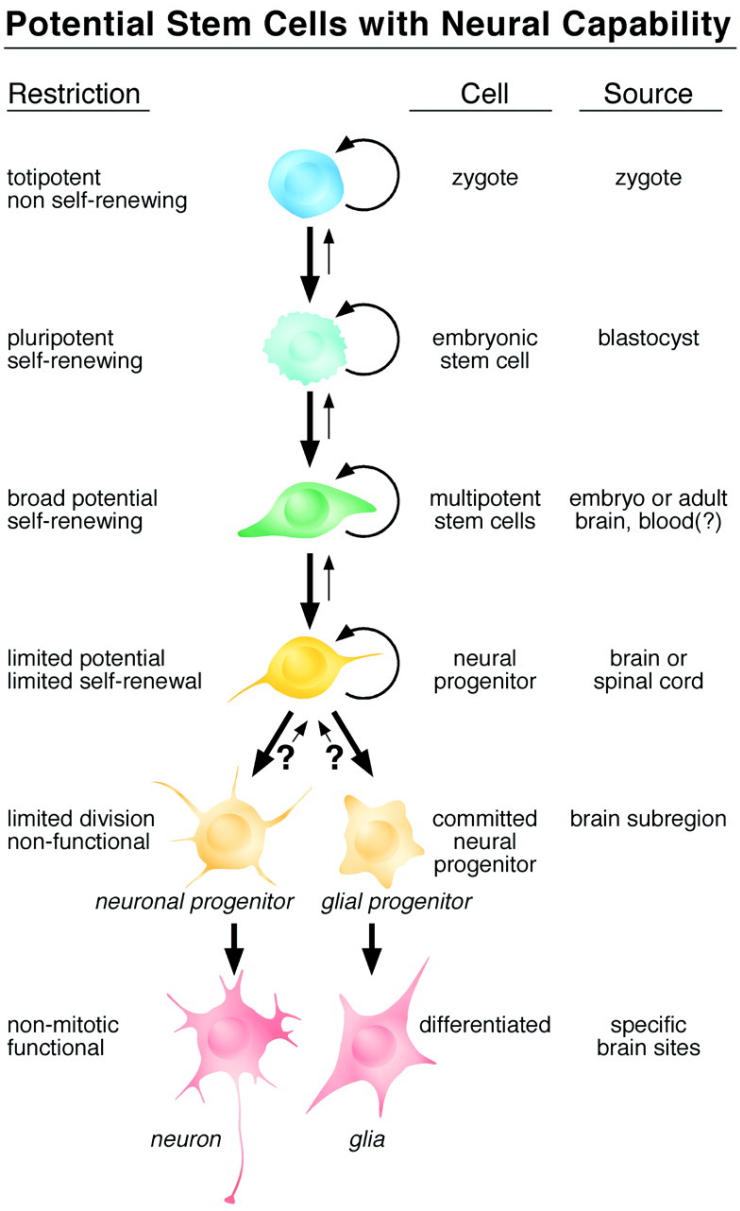

Figure 2. Hierarchy of stem cell stages leading to mature neurons. From [Gage, F. H. (2000). Mammalian Neural Stem Cells. *Science*, *287*(5457), 1433]. Reprinted with permission from AAAS. **(Gage, 2000)**

to be cognizant of the precise cell type cascade that leads to an adult neuron. Figure 2 shows each stage of the cascade, including the source and restrictions of neural capacity of each cellular stage.

In the early stages of neurogenesis, the central nervous system (CNS) arises from ectoderm of the neural plate (Herrup et. al., 2007). As the CNS completes its development and neurogenesis is finalized, all CNS neurons become postmitotic (Herrup et. al., 2007), meaning that they will no longer go through the cell cycle to further proliferate and differentiate. Beyond the neural tube, neural progenitors specific to each sub-region of the brain (Figure 3, teal) loose most of their renewal potential. Nevertheless, they can further differentiate into specific types of neurons at each location (Figure 3, green).

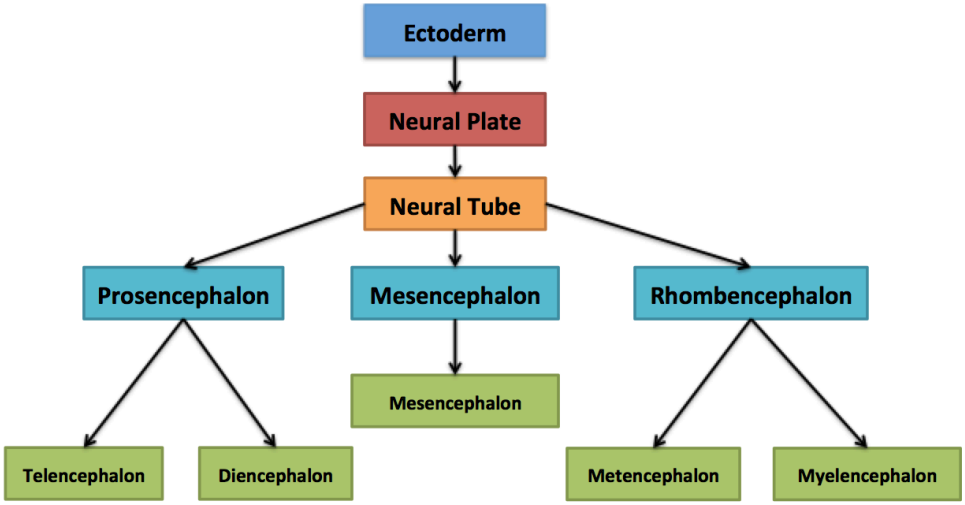

Figure 3. Tissue-specific differentiation of neuronal cells during neurogenesis.

The nervous system is unique because it does not require the rate of regeneration that is demanded by other functional systems in our body (Gage, 2000). However, recent discoveries have refined this assessment. Studies have found that neural stem cells exist in both development and adult brains of humans (Gage, 2000). Although neural stem cells have been discovered in

both stages, there is no one definite region where stem cells originate in the brain. The available locations, however, dramatically decrease in adulthood (Gage, 2000). Specifically, adult neural stem cells have been generally localized on the ventricular zone (VZ) and the subventricular zone (SVZ), which line the lateral ventricle and the denate gyrus by the hippocampus. During development, the subventricular zone develops after ventricular zone, which are both locations of final cell division (Herrup et. al., 2007). Interestingly enough, in the adult brain, the ventricular zone contains no mitotic cells, whereas subventricular zone does (Herrup et. al., 2007).

Even though there are a large number of neuronal types, neuroscientists developing experimental models of differentiation often only refer to generic neurons as the final products or steps in the neurogenesis cascade. Exactly how the differentiation cascade from ectoderm to distinct types of mature neurons unfolds is still not well understood. In this study, we focus on the first cell type-change along the differentiation path from embryonic stem cells to the neurons of the central nervous system (CNS); namely, the embryonic stem cell (ESC) → ectoderm transition. Our goal is to understand the molecular mechanisms that a) turn on the ectodermal lineage-specific transcriptional program, b) turn off the embryonic stem cell maintenance program and c) drive the cell cycle in different cell lineages.

Molecular mechanisms of neuronal proliferation and differentiation

There have been multiple studies on the proliferation and differentiation of neural stem cells. Most of these studies have focused on cell-to-cell signaling, cell polarity, and cell division symmetry (Farkas et al., 2008). Recently, there have been a few studies that investigate these focuses through the use of grafts. In grafts, neural stem cells differentiate based on the local environment rather than their inherent properties (Gage, 2000). These studies have shown that

differentiation is based on the local environment, which results in chimerism (Gage, 2000). In addition, scientists discovered that in the developing brain, neural stem cells tend to move to areas of damage and ultimately fix and/or replace damaged cells (Gage, 2000). In the adult brain neural stem cells placed into regions of damage can retain and perform the properties of normal adult neural stem cells. Interestingly, these studies also revealed that neural stem cells have different functions when implanted in different regions of the adult brain. For example, when the neural stem cells were implanted in the rostral migratory stream, they differentiated into olfactory bulb neurons (Gage, 2000). In contrast, when neural stem cells are implanted in regions of the brain that do not generate new neurons, the neural stem cells differentiate into glia (Gage, 2000).

In the adult brain, there are several characteristics of neural stem cells that help determine whether they should proliferate. There have been two areas of the adult brain that are specifically characterized by high levels of cell proliferation; the subventricular zone (SVZ) and the subgranular zone (SGZ) of the denate gyrus of the hippocampus (Gage, 2000). Further studies found other areas of proliferation as well. It was also discovered that a subpopulation of ependymal cells in lining of 3rd ventricle were stem cells (Gage, 2000). Intriguingly, these cells were shown to proliferate and differentiate into either neural or glial cells, depending on the symmetry or asymmetry of their division. One type of environmental signal leading to increased neural stem cell proliferation is the presence of glucocorticoids. Furthermore, the glutamatergic receptor antagonist MK-801 also increases proliferation (Gage, 2000). Finally, seizures in the temporal lobe have been found to increase proliferation & neurogenesis in denate gyrus (Gage, 2000).

In parallel with the response of other somatic cell types, the presence of certain growth factors has also proven to increase proliferation. For example, the growth factor EGF increased proliferation in SVZ, but not the SGZ (Gage, 2000). Interestingly, EGF also influenced neuronal cell fate in SVZ, as it lead to the production of more glia than neurons (Gage, 2000). Another growth factor, FGF-2, increased the number of neurons instead. The mechanism by which FGF-2 preferentially leads to neuronal production is still not known. Lastly, the presence of the brain-derived neurotrophic factor can also increase the number of neural stem cells that proliferated into neurons (Gage, 2000). Interestingly, if a factor increases the production of neurons, it will likely increase the size of the olfactory bulbs. As a result, olfactory bulb size can be used as a convenient readout for testing the effects of these factors on neuron production.

In addition to the signal – proliferation responses described above, the molecular pathways that control neural stem cells behavior have also been extensively studied (Poser et al., 2015). The STAT3, or signal transducer and activator of transcription 3 pathway, for example, is crucial for neural stem cell (NSC) function. The phosphorylation of STAT3 is essential for NSC survival (Poser et al. 2015). The activation and phosphorylation of STAT3 is caused by a plethora other factors that serve as a convergence point for STAT3 phosphorylation and Hes3 activation (Poser et al., 2015). Some of these factors are FGF, the Noncanonical Notch signaling branch, and the angiopoietin2/Tie2 system (Poser et al., 2015). These pathways are often blocked by the JAK pathway, which promotes the survival of pluripotent stem cells (PSCs) instead of NSCs (Poser et al., 2015).

In addition to neural progenitor cell function, both Stat 3 and Hes3 have been implicated in brain cancer. For example, hairy and enhancer on split 3 (Hes3), is known to regulate glioblastoma multiform cancer stem cells and adrenomedullary chromaffin regulators (Poser et

al., 2015). STAT3 mediates carcinogenesis in models of prostate cancer, which in turn is useful to study and treat similar cancer types (Poser et al., 2015). Lastly, Hes3 has been shown to turn non-neural stem cells into NSCs (Poser et al., 2015).

Molecular mechanisms of embryonic stem cell maintenance

Transcription factors play a crucial role in embryonic stem cells as well as at each subsequent stage of differentiation. These transcription factors also play a role in whether a cell decides to proliferate or not. The three main transcription factors required for embryonic stem cell maintenance are Oct4, Sox2, and Nanog (Figure 4). All three of these transcription factors play a role in cell self-renewal, proliferation and differentiation, as they all work together by forming a feedback loop (Amini, 2014).

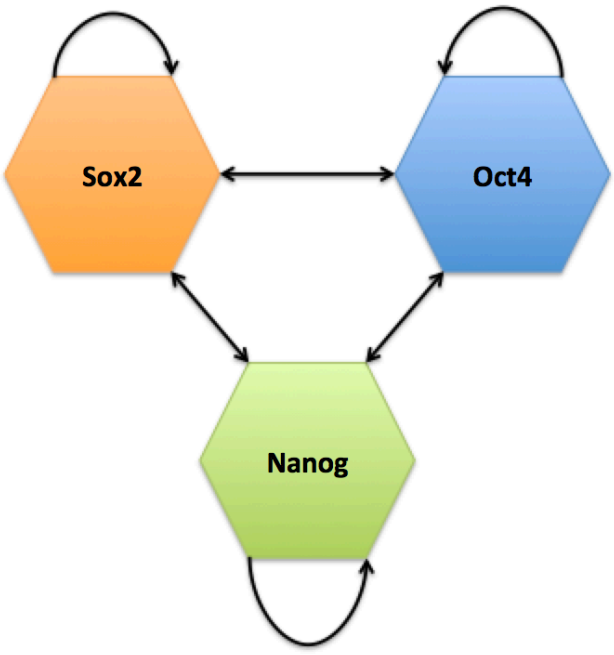

Figure 4. Pluripotency core circuit composed of Oct4, Sox2, and Nanog. All three transcription factors regulate one another, and they all have the ability to self-sustain.

The function of Oct4 is to regulate the expression of target genes that maintain ESC cell functions, and blocks lineage-specific transcription factors. It helps coordinate many cellular functions and it plays a big role in cell fate (Radzisheuskaya, 2013). It is also important in self-renewal, pluripotency and cell differentiation (Quinlan, 2011). Oct4 works together with Sox2 by forming a heterodimer. The function of Sox2 is to help activate target genes. Cells low in Sox2 tend to lose their pluripotency and differentiate rather quickly (Quinlan, 2011). The Oct4 / Sox2 complex also positively regulates Nanog expression (van den Berg et al, 2008), both directly and through Esrrb activation (Esrrb is a direct activator of Nanog) (Zhang et al., 2008). Lastly, the function of Nanog is to determine cell fate, as well as to maintain pluripotency and prevent differentiation (Chickarmane et al. 2012). Nanog overexpression is a key factor in driving embryonic cell proliferation (Hanna et al. 2009). In embryonic stem cells, the precise activity level of these three factors can determine what a cell differentiates into, or if the cell will stay in its undifferentiated state (Chickarmane et al. 2012). Finally, Nanog can also trigger apoptosis (Zernicka-Goetz et al., 2009).

It has also been discovered that overexpressing certain transcription factors can reprogram a somatic cell back to its pluripotent state (Hanna et al., 2009). It is clearly demonstrated that these three transcription factors play a crucial role in these cells. It has been discovered that inducing pluripotency is also possible using the Yamanaka factors Oct4, Sox2, c-Myc and Klf4 under embryonic stem cell culture conditions (Takahashi & Yamanaka, 2006). Interestingly, it has been further discovered that these induced pluripotent state (iPS) cells can be done solely using Oct4, Sox2, and Klf4. (Takahashi & Yamanaka, 2006)

In addition to these transcription factors, there are a series of signaling pathways and their target transcription factors that play a role in stem cell behavior. Primarily, previous literature

focuses on the STAT3, Lin28, LIF (Leukemia Inhibitory Factor), and CDX2 factors and the p53/p21, and Wnt pathways (Rosso & Inestrosa, 2013). In our study, we primarily focus on the LIF pathway, which is a direct activator of the Oct4 / Sox2 / Nanog network, and which is crucial to embryonic stem cell pluripotency (Niwa et al., 2009) and self-renewal (Dunn et al., 2014).

The Leukemia Inhibitory Factor (LIF) environment

Leukemia inhibitory factor, also knows as LIF, is an environmental stimulus that affects a cell by maintaining pluripotency (Figure 5). LIF also acts as a driver of the cell cycle, as it not only promotes cell growth by activating the PI3K / AKT pathway, but it also promotes cell cycle entry by activating Myc and Cyclin D via the STAT3 pathway, leading to Klf4 (LIF → STAT3 → Klf4 → Myc → Cyclin D).

When an ESC is exposed to LIF, it maintains its pluripotent qualities as well as ability to proliferate. While this specific environment is not easily studied *in vivo*, ESC can be placed in an environment containing LIF *in vitro.* These studies employ serum containing LIF-2i , where 2i is a double inhibitor cocktail that blocks MEK (MAPK/ERK kinase; one of the kinases in the MAPK cascade), as well as GSK3 (Glycogen synthase kinase 3; an AKT inhibitor) activity. This 2i inhibitor helps block ERK from inhibiting a key activator of Nanog, Tbx3, while maintaining AKT signaling. The net result is a stabilization of the embryonic stem cell state (Fig. 5).

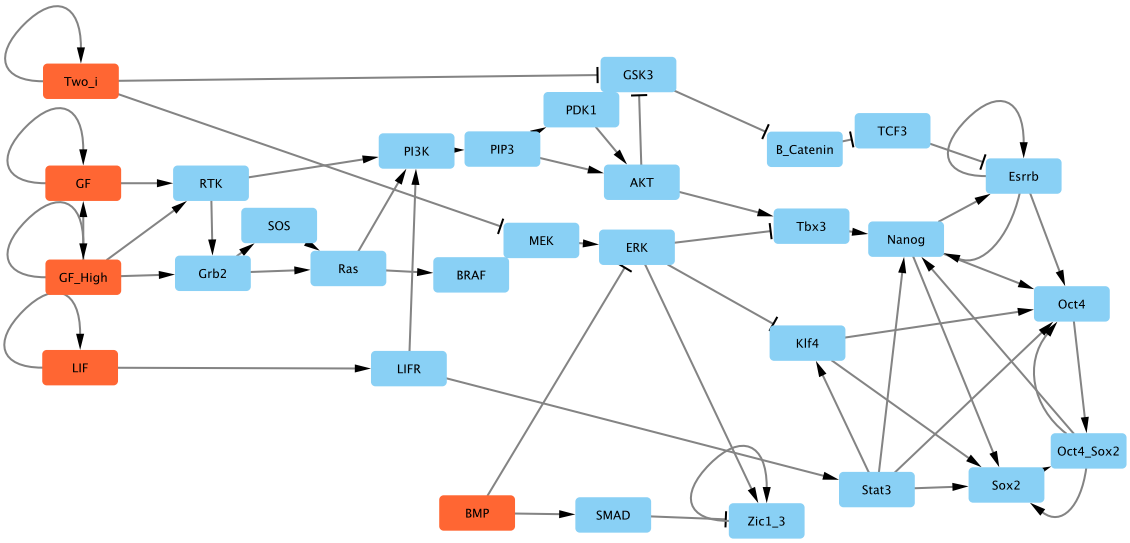

Figure 5. Pluripotency circuit in the presence of environmental stimuli. *Orange*: input signals; *blue:* regulatory molecules involved in pluripotency.

The Bone Morphogenetic Protein (BMP) environment

Bone morphogenetic protein (BMP) is an environmental morphogen that has an ever-changing role along the neuronal development cascade. After gastrulation, the ectoderm layer forms a thickened plate, and produces BMP in the areas on the edges, where the non-neural ectoderm develops. In contrast, the presence of Noggin towards the middle blocks BMP signaling and gives rise to neural ectoderm (Figure 6, top; see Figure 5 for BMP signaling). The neuroectoderm then invaginates, forming the neural tube but also bringing the BMP-

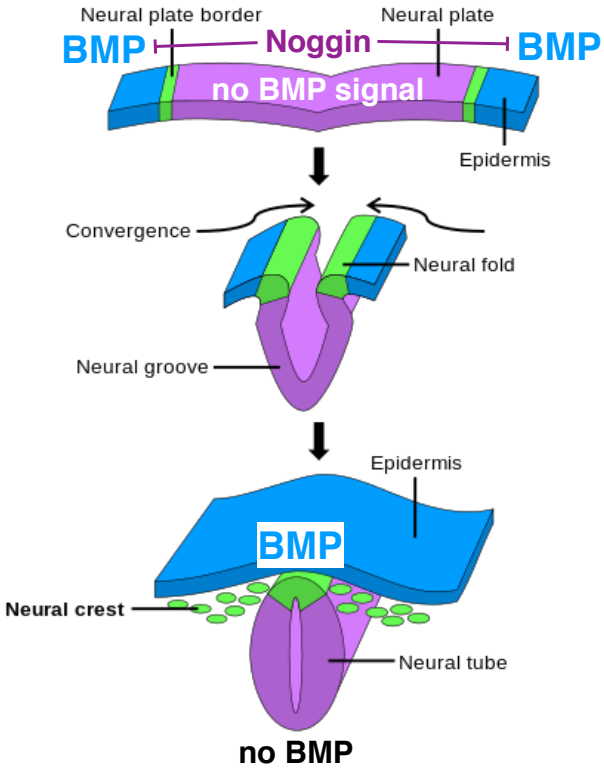

Figure 6. Formation of the neural tube , then the neural crest, driven by BMP gradients. *Blue*: ectoderm; *purple*: neuroectoderm; *green*: neural fold & neural crest.

producing epidermis in close proximity to the dorsal (top) side of the tube. The BMP released by these cells helps the cells in the top of the neural tube differentiate into neural crest cells. The remaining cells of the neural tube, exposed to intermediate to low levels of BMP, form the central nervous system (CNS).

Experimental investigations of neural stem cell specification

Neural stem cells (NSC) have been previously studied both *in vitro* and *in vivo*. Commonly, *in vitro* studies are divided into two stages. The first stage is obtaining the cells before they are forced to proliferate, and the second stage is studying the cells after they have proliferated. Neural stem cells are acquired by dissecting out a region of a fetal or adult brain that was shown to contain dividing cells (Gage, 2000). In the fetal brain, they can be obtained from nearly anywhere in the brain, whereas in the adult brain they are obtained from the subventricular zone (SVZ) and hippocampus (Gage, 2000). Once the neural stem cells have been separated from other neural tissue, they are exposed to high concentrations of growth factors in order to induce proliferation. The most commonly used mitogen is fibroblast growth factor–2 (FGF-2) and epidermal growth factor (EGF) (Gage, 2000). After the neural stem cells have gone through proliferation, mitogens are removed and the cells are induced to differentiate (Gage, 2000). This is accomplished by exposing them to other factors (such as FGF or Wnt) that induce specificity of their differentiation into neurons.

During *in vivo* studies, neural stem cells are detected via by using either thymidine uptake or bromodeoxyuridine (BrdU) labeling (Gage, 2000). Unfortunately, this mode of study is not as effective as *in vitro* studies. They do not provide adequate statistics, and the full spectrum of neuronal cell phenotypes are often underrepresented (Gage, 2000). Additionally, labeling studies

can be inefficient because the labeling solution becomes diluted as cells proliferate (Gage, 2000). Information from all these studies helped us formulate a general idea of the molecular mechanisms required to model the differentiation / proliferation changes from embryonic stem cells towards neuronal stem cell phenotypes. Our goal is to synthesize information, and overcome the disconnectedness apparent in this field of inquiry,

Computational modeling of neural stem cell differentiation

All of these pathways and transcription factors are brought into play when researchers formulate computational models to quantify and analyze the internal functions and behaviors of stem cells. According to Jacob Hanna, there are "Deterministic vs Stochastic" processes in modeling (Hanna et al. 2009). This refers to the fact that there is a lot of difficulty in formulating a model that accounts for what is expected versus what is random in cellular function.

In this study, we focused on the developmental pathway of a neuron from its embryonic state (Figure 7). Our first goal was to understand how embryonic stem cells maintain pluripotency, as well as how they proliferate. To this

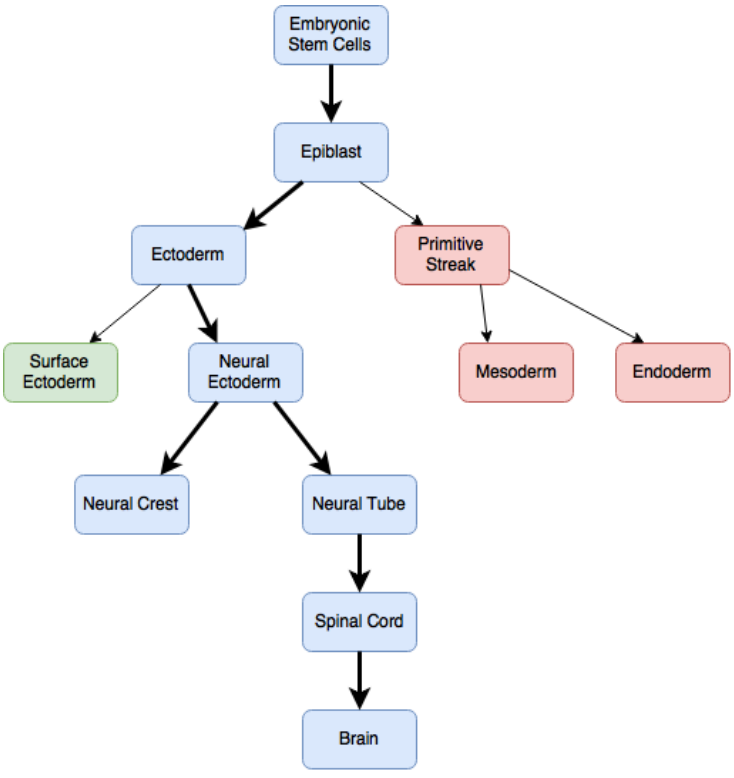

Figure 7: Embryonic stem cell lineage. Blue boxes connected by bold lines represent the lineage cascade that leads to neuronal specification. Red boxes represent lineages that have no ectodermal origin, while the green box represents the non-neuronal lineage of the ectoderm.

end, we have built a dynamic Boolean computational model to predict how maintenance and proliferation are coordinated, as well as how differentiation (loss of the ESC state) affects the signals that drive proliferation. Second, once we bridged the embryonic stem cells to the epiblast gap, we went on to analyze the molecular processes that ultimately determine the lineage of the embryonic stem cell; whether it becomes ectodermal, or enters a dermal layer of the primitive streak (Figure 7).

Once we have determined the cellular characteristics of the ectoderm, we know that it differentiates into two lineages as seen in Figure 7. The lineage that we focused our modeling efforts on is the neural ectoderm. These cells further differentiate into two lineages, the neural crest giving rise to the peripheral nervous system, and the neural tube giving rise to the central nervous system. In order to understand this cascade, we first need to focus on the developmental biology of the early stages of embryogenesis in the future.

We decided to model this concept through the use of a Boolean model. Boolean modeling is a qualitative technique of studying the dynamic regulatory and signaling networks of large systems, without the need of previously known synthesis and degradation rate parameter for every biochemical interaction (Wang et al., 2012). It also serves as a functional foundation of combining and correlating relationships. Boolean models are capable of predicting effective interventions that could impact these relationships. The effectiveness of this particular modeling method is dependent on the availability of dynamic information, the size of the systems, and the types of questions that are addressed. The Boolean model is limited in terms of predicting precise, quantitative information with respect to concentration changes in small systems. However, it makes up for this shortcoming by providing excellent qualitative information on large networks, where building detailed, quantitative models is rarely feasible. We chose this tool

to study all the intercellular relationships of a proliferating and differentiating embryonic stem cell, resulting in a Boolean regulatory model with 69 nodes.

In addition to building predictive regulatory models, Boolean modeling can also be used for reverse engineering regulatory relationships from cell-wide gene expression data (Wang et al, 2012). These models help bridge the gap between static network representations of regulation, and detailed, continuous dynamic models that track individual molecule concentrations as a function of time. In relation to our study, Boolean models are capable of modeling gene regulatory and signaling networks (Wang et al, 2012). More importantly, Boolean models have been previously attempted to study embryonic stem cell fate control (Herberg et al, 2015), as well as embryonic stem cell maintenance in the presence of the transcription factors Oct4 and Sox2 (Herberg et al, 2015). Lastly, there have been preliminary studies on the overall nature of embryonic stem cells in regards to the interaction network of ground state transcription factors (Martello et al, 2014).

METHODS

Characteristics of synchronous Boolean models

In order to systematically synthesize information about the mechanisms driving embryonic stem cell differentiation and proliferation, we used a mechanistic modeling approach. This refers to a series of steps in which we test the relationships among known regulatory connections as they work in concert. The first step is building a Boolean network model of a regulatory process. Once this has been completed, the next step is to simulate the model's dynamical behavior in different environments and under changing stimuli, and to test these results against known experimental data. Once the known cell states and behaviors conform well to the model, we are able to test scenarios that have not yet been tested, leading to new predictions and new proposed experiments.

As previously stated, each component in the model shares a unique relationship with all of the other components (also referred to as nodes), either providing direct effects or downstream effects to other nodes. In Boolean modeling, each node exhibits a binary behavior, which can be described as being 0 or 1. Components that are OFF (repressed or inactivated) are represented with a 0 whereas nodes that are ON (activated) are represented with a 1. Each node either represents a specific protein's availability (e.g. BMP), an active transcription factor (e.g. SMAD or Sox2), or the activation state of a protein (e.g. pRB or p27Kip1). The different expressions of activation and inactivation of these nodes represent a multitude of different cell states. However, only a few of these states are stable, in that the state of each node is consistent with the incoming regulatory signals from their upstream neighbors. These robust states (or cycles) correspond to cell phenotypes, such as Naïve ESC's or Primed ESC's. A subset of nodes in our model represent input stimuli, corresponding to different environmental factors that affect the behavior

of the core model. There are 4 such input stimuli in our model: BMP, LIF, 2i, and GF_High. These specific nodes are manipulated to stay in the ON or OFF state, whereas the other nodes change their expression (dynamic behavior) based on the activity of the input stimuli. The manipulation of the input stimulus nodes changes the expression of the core nodes in the rest of the model.

Our final Boolean model consists of a total of 69 nodes. The behavior of each node is dictated by a Boolean regulatory gate. Each Boolean gate consists of the actual singular node being influenced (output node) and all of the different nodes that influence that specific node's expression (input nodes). Each individual gate's input and output nodes, as well as their relationships and information sources, can be found in the *Supplementary Information Appendix*.

The precise effects on the expression of the input nodes based on the expression of the output nodes can be characterized using binary conditional statements expressed as AND, NOT, and OR relationships. Figure X shows how each of these conditional relationships combine the effect of input nodes (X and Y), and affect the output node (Z).

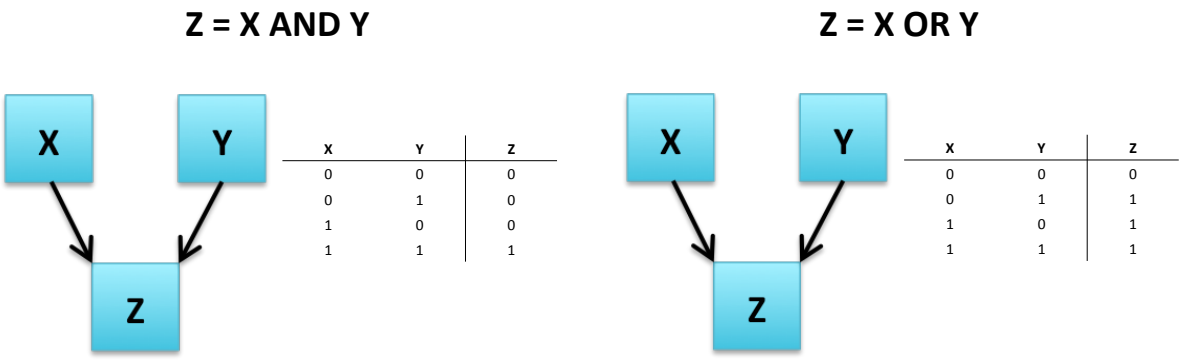

Figure 8. Simple Boolean gates using only AND or OR. These are two simple Boolean commands of 2 input nodes (X and Y) using only AND or OR which influences the activity of output node Z.

In this specific example, nodes A and B represent input nodes, while node C represents the output node. The relationship of node A and node B as well as their synergistic relationship with C affects the outcome in output node C. Looking at Figure 8, it is simple to discern the functional meanings of AND and OR. State AND signifies that both input nodes X and Y are needed to activate Z. State OR signifies that input node X and input node Y can both independently or work synergistically to activate output node Z

There are also four Boolean commands that combine the effects of AND and OR with repressive signals. These four specific complex commands are highlighted in Figure 9.

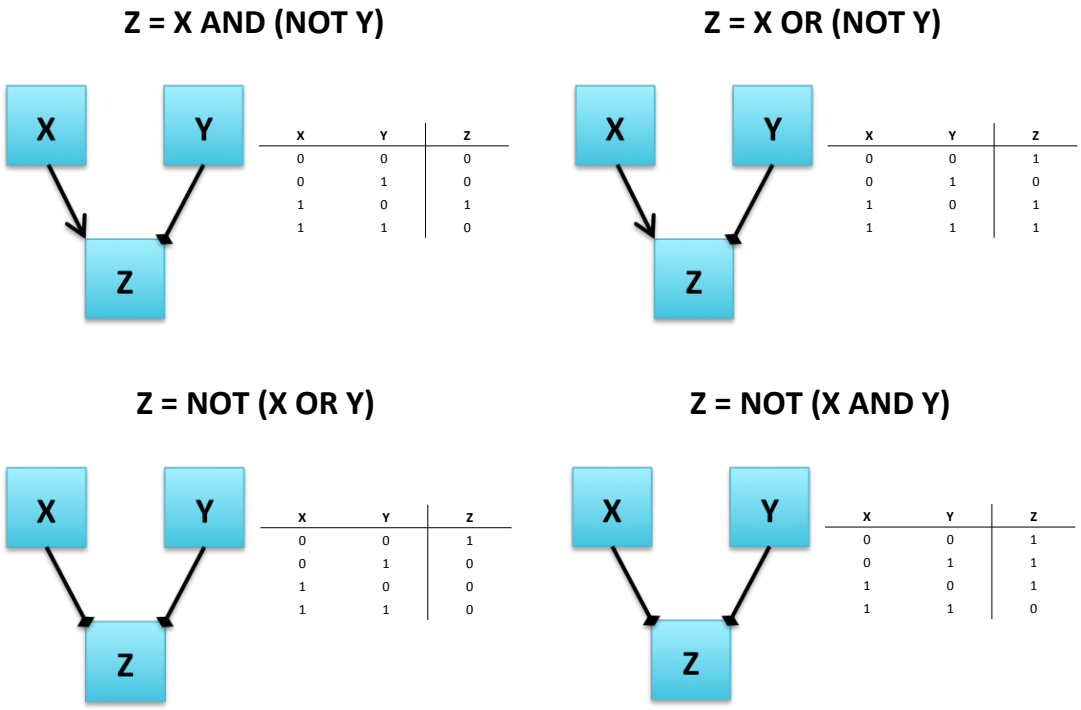

Figure 9. Complex Boolean involving NOT. Four complex Boolean commands of 2 input nodes (X and Y) using AND, OR, and NOT which influences the activity of output node Z.

When analyzing Figure 9, it is easy to discern the effect of adding NOT to the command. Any state including NOT signifies that there must be an absence of either input node X or input node Y, representing repressors, in order for output node Z to be activated.

When considering Boolean modeling, it is important to acknowledge the assumptions that are associated with this model. When a node is OFF (0), it means that the node is completely inactivated. On the other hand, if a node is ON (1), this means that it is completely activated. This concept would impact the predictions of a Boolean model by differentiating it from a laboratory experiment in the sense that a model node (component) could not exhibit partial activation or inactivation. In reality, the behavior of the same component often falls in between anywhere in the spectrum between complete activation and complete inactivation.

Construction of the Boolean model

Many interactions and regulatory relationships that provide the links of our model network can be found directly in scientific literature. Additionally, using the Boolean method, we determine the biochemical mode of activation of a node through the use of AND and OR. AND represents that the node requires the presence of two transcription factors to form a dimer while OR signifies that either input is known to be enough for the output activation. If the connection is not clear, we try both scenarios when manipulating the gate. The manipulations help formulate predictions of how the input and output nodes interact. Lastly, if no literature is present that describes the relationship between two nodes, a hypothetical relationship may be included, with the goal of showing that such a relationship is required for the system to remain consistent with experimentally observed cell behavior.

The overall procedure of Boolean model building is broken down into six main components. The first state is to formulate a network structure of the model (Wang et al, 2012). This is done by gathering previous research, and formulating a network of relationships between all the desired components of focus for the study. The next part is to code the state of each node

into its activation state in a binary fashion (Wang et al, 2012). Next, it is essential to study the causational information that leads to each node's activity or inactivity. This can then be further analyzed to examine differences between multiple states of the model. Once this is done, it is important to test the correctness of the model. This provides validity to the experiment and provides a control to the experiment (Wang et al, 2012). Once this is done, father manipulations can be made to the model to form experimental predictions. Finally, based on previous findings provided by the model, it is possible to derive new biological implications, serving as a basing for future predictions and experimental studies (Wang et al, 2012).

Identifying the stable states (phenotypes) of a Boolean model

Our first main goal was to match the phenotypes generated by the model to known biology. In order to do this, we used a random sampling procedure to find all stable states or cyclic behaviors the model can generate in the presence and absence of all its inputs, in every combination. As the full state space of possible initial conditions consists of 2^{69} different combinations of node ON/OFF states, we used the sampling algorithm from Dr. David Deretei (Deretei et al., 2016), provided as a program in C++ by Dr. Erzsébet Regan. Briefly, the algorithm places the Boolean model in a specific environment, then follows its dynamics in the presence of noise for $T = 5$ Boolean time-steps ($p_{\text{error}} = 0.02$). At every step, the algorithm checks the attractor (stable state or cycle) the model would converge to in the *absence* of noise, and keeps track of each attractor as it discovers them. This process is repeated $N_{\text{rnd}} = 100$ times in every ON/OFF combination of the input signals (environmental conditions) of the network. Once the sampling was finished, we tested the model against known biological behavior of cells, and attempted to match the model's stable states to those representing normal cellular function.

Simulating a change in the extracellular environment

The purpose of this part of our *in silico* experiment is to test the response of the cell to a specific environmental change. To do this, our program generates a visual representation of the time-course by which the activity of each node changes over time in response to changes in environmental signals. First, a specific initial attractor state of interest is chosen, corresponding to a cell with a particular phenotype, and then the code runs a simulation where all of the other nodes in the model change their activity or expression in response to signals triggered by the environment.

The extracellular inputs BMP, GF, GF_High, LIF, and 2i were each individually tested to see how their varying levels of expression for varying lengths of time alters the phenotype of the cell. The two cell phenotypes I was most interested in were naïve embryonic stem cells and neuroectodermal cells, exposed to signals that induce differentiation or attempt to reverse it. I also looked at whether these cells were in G0 or in the cell cycle. As a specific cell phenotype was tested, changes in its phenotype were monitored. The length of time where the cell either went thru apoptosis or exited the cell cycle into a G0 state was also monitored.

Using the results of time-course simulations, we compared the model's predictions to known behavior of cells under these environmental perturbations. Whenever we found discrepancies with the literature, or whenever the role of a node was not in accordance with its known activity pattern, we attempted to a change the regulatory gate of it, and restarted the sampling / validation process. Indeed, the misbehavior of the nodes can sometimes discredit some of the results of the model, so adjustments are needed to make the model as scientifically accurate as possible. If no plausible corrections or literature is present, assumptions need to be made about the expression of the node in its relationship to the expression of other nodes. This

can lead to novel predictions related to experimentally untested scenarios, but also point to severe gaps in the literature with respect to the mechanisms actually responsible for the behavior of cells.

Biological processes not included in the model

Many complex systems and relationships were either simplified or completely excluded from our model. For example, the BMP pathway is oversimplified by only including BMP, SMAD, and only one of many target genes repressed by SMAD, namely Zic1_3 (standing in for both Zic1 and Zic3). These components are crucial to the overall functional behavior of BMP activation and deactivation during neuroectoderm specification, but other contributing components critical for BMP signaling in other contexts were not included in the model.

Additionally, the molecular mechanisms responsible for differentiation into, and maintenance of cell states beyond the cell types present in early neurogenesis were also not included. The model specifically looked for phenotypes representing naïve embryonic stem cells, primed embryonic stem cells, ectodermal cells, or other (unspecified) differentiated cells. The precise mechanisms by which these other differentiated cell types sort into distinct lineages is outside the scope of this study.

Finally, details of processes such as DNA replication and the formation of the mitotic spindle apparatus (the execution of metaphase) are also not included. We know that DNA must be replicated in order to produce another cell, but we elected not to include the details of this mechanism, and encoded DNA replication through a Replication Node (denoting ongoing replication), and a 4N_DNA node denoting that Replication is complete (the cell has double the

normal amount of DNA). A similar method was used in accounting for the process of mitotic spindle assembly.

Lastly, we also elected not to include the input signals that typically trigger apoptosis, such as death receptor signaling or DNA damage signaling. The model does, however, include the internal switch capable of committing cells to apoptosis, and it requires survival signals to stay in its “alive” state. We can thus determine if a cell is dead by checking the behavior of certain nodes such as CAD (Caspase-activated DNase), whose activation signifies irreversible commitment to programmed cell death.

RESULTS

Modeling the embryonic stem cell state switch

The main goal of this project was to add an embryonic stem cell pluripotency circuit to the preexisting cell cycle model adapted from Dr. David Deretei (Deretei et al., 2016) and the Independent Study thesis of Andrew Hamel (Hamel, 2016). This pluripotency circuit is crucial not only to establishing embryonic stem cell states, but its alternate behavior can allow for the embryonic stem cell to differentiate into a more specific cell type. By incorporating this circuit, most notably involving Oct4, Sox2, and Nanog transcription factors, we were able to generate different phenotypes based on the different expressions of those specific transcription factors. The known biology that we were able to model were the naïve embryonic stem cell state and the neuroectodermal cell state.

There were a multitude of difficulties associated with the construction and modeling of this circuit. The first difficulty was simulating the behavior of Sox2 and Oct4's relationship. In the case of these transcription factors, neither really follows the binary logic of being completely activated to completely repressed. There are cases where Oct4 can be activated while Sox2 is inactivated and vice versa. This isn't necessarily congruent with scientific literature as neither factor is ever completely suppressed. To combat this difficulty, it was decided to create a node that shared the characteristics of each of those nodes. Therefore, the Oct4_Sox2 node was included to aid manipulation of Oct4 & Sox2 expression since neither one fully go away despite the binary characteristics of the nodes.

The next step was to figure out how to connect the pluripotency circuit with the rest of the preexisting model. We chose to include Klf4 in the model since it plays a role in cell proliferation and differentiation and it is directly linked with a few of the crucial nodes in the

core of the cell cycle model. In this model, Klf4 is connected and shares a relationship with Stat3, Myc, and the ERK pathway. Stat3 is critical for its roles in both cell growth and prevention of apoptosis. The Klf4 node was added in an interaction with Stat3, which tied in this circuit with the LIF input. This was critical because it tied in the LIF input stimulus with the rest of the pluripotency circuit, as well as the rest of the cell cycle model. Additionally, Esrrb was added in relation to Oct4 and Nanog to tie it in with B_Catenin and GSK3, involved with cell cycle regulation by controlling AKT activity.

There were a few possible exterior nodes associated with embryonic stem cell pluripotency circuit which were left out. We elected to only include Sox2 from the Sox family of transcription factors, as it had the largest influence in pluripotency in embryonic stem cells. This specific Sox was discussed in the literature significantly more than any other member of its Sox family, and its role is better understood. The case was the same for Klf4, as Klf4 has a much greater influence on the pluripotency circuitry than other member of the Klf gene family.

Lastly, ectodermal lineage commitment nodes such as Otx1, Ectodermin and Hoxb1 were not included because our focus was on distinguishing the neuroectoderm from other differentiated cell types. Additionally, Micah Auerbach's Independent Study project focused on the effect of lineage-specific factors such as Gata6 and Cdx2, allowing him to model commitment to the endodermal as well as trophoblast lineages. As the first step in neurogenesis is the specification of neuroectoderm by the absence of active BMP signaling in the central portions of the neural plate, our focus was modeling the effect of a BMP environment, and a lineage-specific factor downstream as readout for neuroectoderm specification. While there is scientific literature related to other nodes or factors that could influence the behavior of our ESC

module, we elected to stick with the core circuitry of ESC maintenance, and modeled its critical connections to other cellular functions such as the cell cycle and survival/ apoptosis.

Modeling Growth Factor (GF) signaling

Following the work of Andrew Hamel, we modeled the mitogenic influence of the fibroblast growth factor FGF by connecting it to its receptor (RTK, here denoting FGFR proteins) and the generic RTK-responsive Gbx2 adaptor. These nodes then simulate progression through the cell cycle by activating AKT as well as MAPK signaling. In order to model three distinct mitogenic environments, namely the absence of growth factors, a low level capable of sustaining survival but not proliferation, and a higher level that drives the cell cycle, the growth factor input was separated into two different Boolean input nodes, GF_High and GF. Thus, GF = ON represents the basic level of mitogens needed for a cell to survive, while GF_High represents the amount of mitogen needed for the cell to grow and proliferate. We modeled this by making GF self-sustaining, so that we could initialize it ON or OFF. In addition, GF_High activity (also self-sustaining) automatically turned the GF node ON.

The core of the growth factor signaling pathway modeled by Andrew Hamel (Hamel, 2016) was present (PI3K/AKT pathway as well as the MAPK/ERK pathway), but we chose to exclude the DNA damage signaling module, along its ability to arrest the cell cycle via Chk1 and Chk2 (the latter is also connected with apoptotic pathways).

The inclusion of the GF and GF_High inputs the rest of the growth factor signaling pathway worked as expected. GF ON was largely responsible for most cells being alive, although its activation was not always required for the cell to self-sustain (i.e., LIF could also provide the necessary survival signal; see below). GF_High ON, in contrast ensured that cells could undergo

the cell cycle. There were no attractor states where the cell was able to proliferate without the presence of either GF_High or LIF.

Modeling the Leukemia Inhibitory Factor (LIF) switch

The LIF input signal triggers the activity of its receptor, LIFR, which connects it to the PI3K/AKT and Stat3 pathways. LIF ON signifies the presence/activation of environmental LIF. When LIF is present, LIFR mediated PI3K/AKT activation contributes to survival, cell growth, cell cycle entry, and subsequent proliferation. LIFR ON also activates Stat3, which directly influences the Oct4/Sox2/Nanog embryonic stem cell pluripotency circuit. In this specific model, Stat3 directly influences Sox2 and Klf4. The link to Klf4 is critical, as Klf4 is involved in the regulation of proliferation by activating Myc and allowing cells to self-renew even in the absence of active MAPK signaling. Sox2, in contrast, is involved in maintaining pluripotency in embryonic stem cells.

We believed that the inclusion of LIF into the model would help explain how it can promote proliferation and inhibit differentiation at the same time. The goal of adding LIF to the model was to see if this same concept applied to naïve embryonic stem cells in terms of staying naïve or differentiating into the ectodermal lineage. Indeed, in our model, LIF OFF is associated with differentiation, and LIF ON is generally associated with cell proliferation without differentiation.

A few molecular connections were left out of the modeled LIF pathway. First, the JAK kinase was not explicitly included along the JAK/Stat pathway. JAK would have represented a single step between LIFR and Stat3, and thus its explicit inclusion wasn't necessary. The other biology that wasn't included relates to other factors that can inhibit LIF by blocking its receptor,

such as SOCS3 (White & Nicola, 2013). This could have potentially have altered the number of live attractor states created by the model. Additionally, we did not include the effect of LIF and LIFR on the Grb2 node. In our model, Grb2 activates the MAPK, which would inhibit Tbx3 and which is known to be OFF in embryonic stem cells. If Tbx3 was inhibited, then Tbx3 would be incapable of activating Nanog. This indicates that either the LIFR → Grb2 connection, or the Grb3 → MAPK connection is influenced and even blocked by unknown signals internal to ESCs. With the exception of Grb3, there were no difficult gates in constructing this specific module. LIF is self-sustaining, and LIFR is always activated as long as LIF is ON.

There weren't any redundancies with this specific module within the model. The only thing that I would have changed was to remove the LIFR node. The LIFR node's behavior models and parallels the behavior of the LIF node. The addition of this extra node was a bit unnecessary, especially since there wasn't an effort to include any other receptor nodes for the other stimuli in the model.

The inclusion of LIF and LIFR worked well with the rest of the model. When LIF was ON it prevented the cell from differentiating, but it mostly impacted if the cell could proliferate or not. LIF ON was able to drive the cell cycle in any environment where it was present. Essentially, the cell could live with LIF ON and GF OFF in terms of self-sustainment. On the other hand, if LIF was OFF, the probability that the cell could differentiate significantly increased.

Modeling the 2i switch

We included the 2i environment stimulus node because experiments clearly indicate that the presence of 2i adds additional robustness to the embryonic stem cell state. The goal of this

inclusion was to see how the presence of 2i would impact the cell when it is functioning on its own as a sole pluripotency factor, as well as how it works with other factors attempting to keep the cell pluripotent. Additionally, we wanted to see how 2i ON could influence differentiation triggered by other stimuli.

2i stands for a cocktail of 2 chemical inhibitors used in ESC cell culture in the laboratory, usually in conjunction with serum and LIF. 2i has the ability to inhibit the MEK and GSK3 kinases, which helps maintain the embryonic stem cell state. Thus, there were no difficulties with incorporating 2i into the model. The 2i gate is only connected to two nodes, MEK and GSK3, and it acts to inhibit both. The difference between 2i and LIF is that 2i prevents differentiation as well as growth-factor-driven proliferation, whereas LIF not only prevents differentiation, but it also promotes the cell cycle. This was due to the fact that in instances where 2i is ON and GF_High is also ON, the presence of 2i outweighs the presence of GF_High and MEK remains OFF.

Modeling the Bone Morphogenetic Protein (BMP) switch

BMP is a critical transcription factor needed for the onset of neurogenesis. We hypothesized that its absence would help trigger ESC differentiation into the neuroectodermal lineage. The goal was to simulate the biological scenario where BMP's activation triggers the cell differentiation cascade, pushing the cell to the ectodermal lineage, but then inactivating once the ectodermal cell forms for neuroectodermal cells to arise. The ectoderm is the default fate if LIF goes away and GF is ON, regardless of BMP expression. Once the BMP goes away, the ectoderm arises.

There were some difficulties with incorporating BMP signaling into our model, as different cell phenotypes and behaviors arise from varying levels of environmental BMP. High levels of BMP lead to the development of the ectoderm, while low levels of BMP allow for the development of the neuroectoderm. Additionally, BMP is needed once again later in neurogenesis within the neuroectoderm to produce a variety of neuronal types, while also suppressing neuroectodermal cells from falling in the oligodendroglial fate. Therefore, it was difficult to model BMP's phenotypic effects outside of its initial push from the embryonic stem cells towards neuroectodermal cells.

There was some nodes and biology that was left out in the making of this module. First of all, TGF-Beta was left out of the model. TGF-Beta not only connected with BMP, but it is also signals to activate the downstream effectors of BMP signaling; the SMAD complexes. Additionally, TGF-Beta also activates MAPK, leading to downstream activation of the ERK pathway. Since BMP is already connected to SMAD and BMP activates ERK in the model, it was not necessary to include this node. Furthermore, no BMP antagonists were included, such as Chordin and Follistatin. As BMP serves as an input environmental stimulus to the model, adding another environmental signal just to inhibit it would have been redundant. We also did not include any mechanisms that inhibit SMAD function, or of its targets outside its inhibitory effects on the transcription of two neuroectoderm-specific transcription factors Zic1 and Zic3.

Lastly, although we have a growth factor input in our model, we did not include fibroblast growth factor's (FGF) ability to down regulate BMP (Srinivasan et al., 2014), which would promote neural ectoderm formation. This would be an interesting complex/module to include in the model in the future, as it would serve as a direct link for BMP inhibition. This

could potentially make it clearer how BMP works with other factors. In our current model, we only looked at what would happen if BMP were completely active or completely inactive.

SMAD was included as a generic complex standing in of all of the SMAD proteins involved in the BMP pathway. Although there are multiple SMADs, they all work in unison to transmit the same signal, so it was unnecessary to add each individual SMAD. Its main influence considered here was its effect on Zic1 and Zic3 transcription. Since the two Zic proteins have a similar function in this cascade (Janesick et al., 2013), we kept the model simple by including a single node that represents both proteins. Zic1 and 3 impact neural proliferation and neural differentiation, as it activates Geminin, a replication origin licensing factor critical for cell cycle progression (Janesick et al., 2013).

In our model, BMP served as an input stimulus that inhibits ERK, activates SMAD and thus inhibits the Zic1_3 complex. In its absence, Zic1_3 can be activated by ERK. As BMP's activity directly blocks ERK, this means that BMP can indirectly inhibit Zic1_3 even in the absence of SMAD activation. BMP's activation also outweighs growth factor signaling in terms of its control on ERK.

The processes that lock in Zic1_3 activity and by extension, the neuroectodermal fate proved really difficult to model. The literature yielded no clues as to the molecular mechanisms could prevent a differentiated neuroectodermal cell, with Zic1_3 activity and *no* Oct4/Sox2/Nanog activity, from reverting to an embryonic stem cell state once the environmental signals of differentiation were removed. To remedy this, we attempted to make Zic1_3 self-sustaining. This is not only artificial, but it fails to block the ESC core circuit from reengaging. Overall, although we couldn't encapsulate all of BMP's behavior on the variety of phenotypes it is associated with, we were still able to model its main effect on embryonic stem

cells, leading to neuroectodermal cells. We were not able to capture a stable, primed embryonic stem state (primed ESC cells have Oct4/Sox2, but no Nanog), but we were able to model naïve embryonic stem cells and neuroectodermal cells.

The healthy embryonic stem cell phenotype

Our full network model can be seen on Figure 10, showing the naïve embryonic stem cell phenotype as it is finishing DNA replication, in an environment with a high level of mitogens (GF_High ON), LIF and 2i ON and BMP OFF. In general, state is dependent on pluripotency inducing input stimuli such as 2i and LIF, and the absence of BMP.

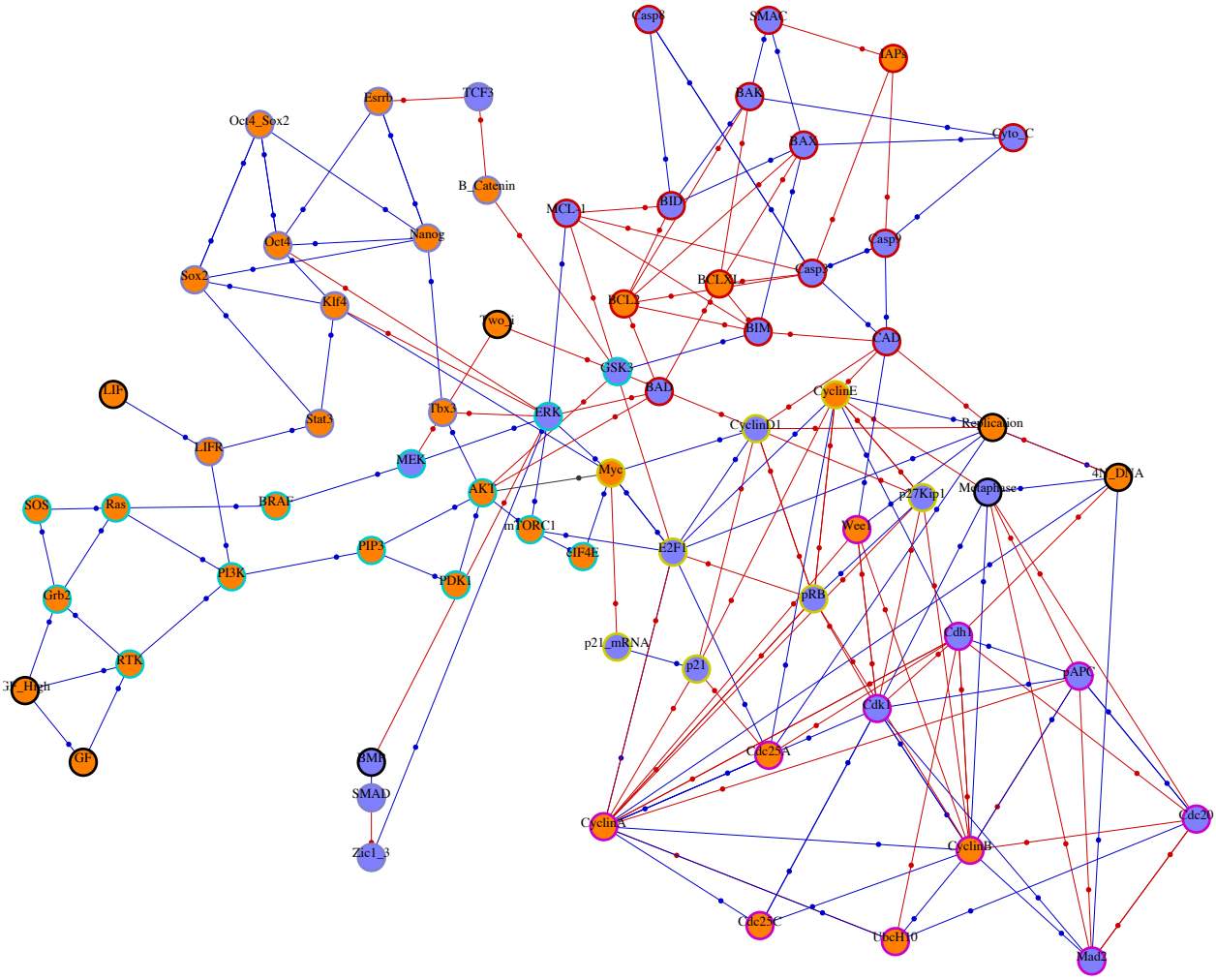

Figure 10. Phenotypic representation of a healthy Naïve ESC in cell cycle. (#83). All environments ON except for BMP. Orange circles signify activation and blue circles represent repression.

The healthy neuroectodermal phenotype

The healthy ectodermal state can be present when BMP is ON, which pushes ESCs towards the ectodermal lineage, but in order for the neuroectodermal lineage to stabilize, BMP needs to turn OFF. The phenotype shown on Figure 11 represents a neuroectodermal cell that lacks any pluripotency circuit activity. Here, GF_High is IN and LIF, 2i, and BMP are OFF.

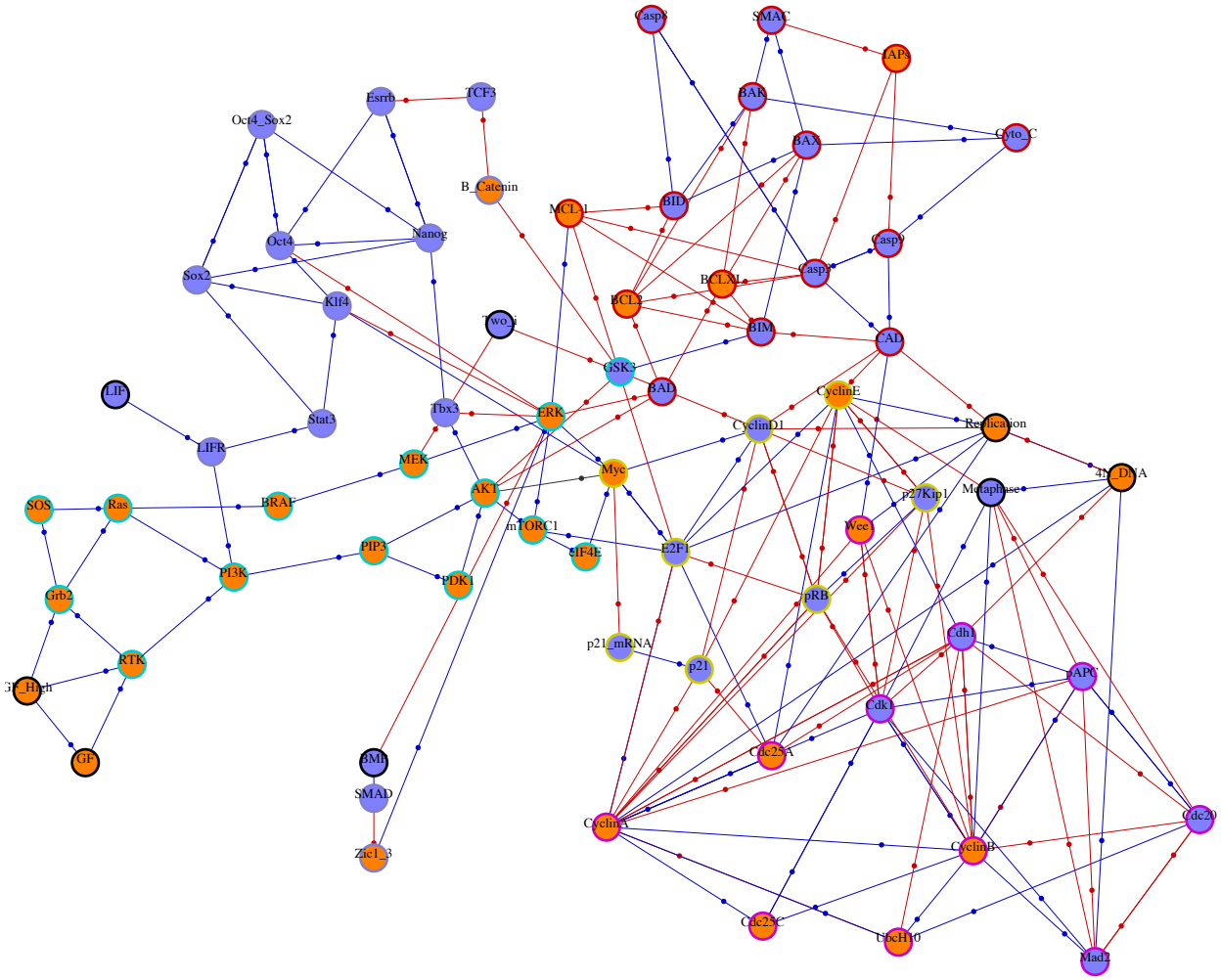

Figure 11. #45. Boolean model of a healthy neuroectoderm cell in cell cycle. GF & GF_High ON only. Orange circles signify activation and blue circles represent repression.

GF allows the cell to survive and self-sustain, and GF_High allows it go grow and proliferate. The absence of BMP allows the maintenance of the neuroectodermal lineage. Additionally, the absence of LIF and 2i block the phenotype from having any naïve ESC characteristics.

The apoptotic phenotype

In the model, apoptosis was triggered by the loss of all survival signals. This involved the inactivation of growth factor signaling. This source attributes to the most amount of deaths

among the stable attractors produced by the model. Although the cell could survive without GF if LIF was present, LIF could be overpowered by BMP, which would cause the cell to die.

The second source of apoptosis was the activation of apoptosis inducing agents such as CAD. If CAD or any other nodes in the apoptosis circuit was activated, the cell was certain to die regardless of other input environmental stimuli present and phenotype.

Sampling all stable phenotypes of the model

In a sample size of $N = 400$ initial conditions, a total of 236 stable states were produced. Of the 236 produced attractors, 95 represented naïve ESC's, 0 represented primed ESC's, 2 represented neuroectodermal cells, 21 represented neuroendodermal cells, and 118 were dead.

In order to test the environment-dependence of these phenotypes, we categorized them based on the combination of environments they were placed in. First, we separated them based on the state of BMP. Based on each BMP environment, different scenarios where the combinations of LIF, 2i, and GF/GF_High. First we looked at the different cell phenotypes that could arise in the BMP OFF condition, shown in Figure 13.

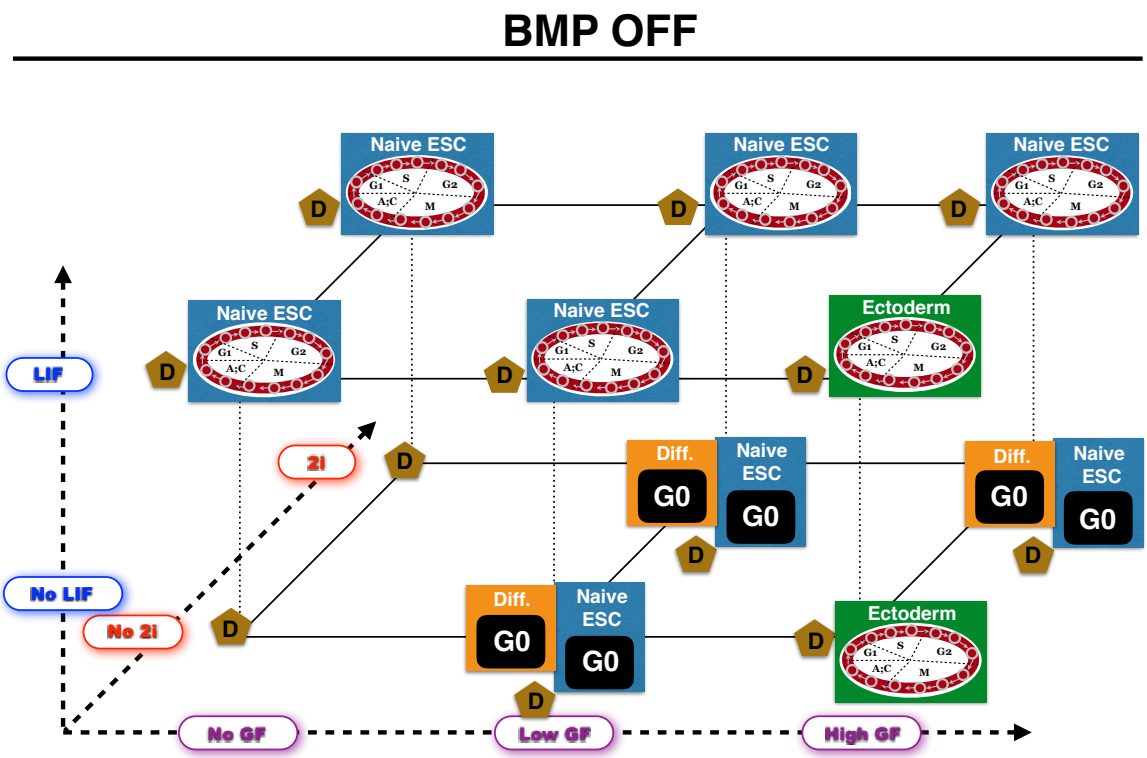

Figure 13. Stable phenotypes of the model in all possible BMP-OFF environments. The attractor states were determined by extensive sampling of cell phenotypes based on twelve possible combinations of inputs: absence or presence of LIF, absence or presence of 2i, and three levels of growth factor input.

The BMP OFF environment was able to sustain five different cellular phenotypes. The first and obvious one was cell death, or apoptosis (Figure 13, brown D). Theoretically, death could occur from any phenotype in any combination of the input environments. In addition, death was certain (the only option) if the cell was exposed to an environment of LIF OFF and GF OFF.

The next phenotype is the Naïve ESC undergoing cell cycle (Figure 13, blue with cell cycle). This phenotype is stable in 5 different conditions. Namely, each of these environments have LIF ON, with varying states of 2i and GF. The only scenario where a LIF ON environment could not sustain the Naïve ESC state was when GF_High was ON and 2i was OFF. This results

is in agreement with the experimentally documented role of 2i in stabilizing the ESC state in the presence of FGF signaling.

The next set of phenotypes represent Naïve ESCs in G0 (quiescent state) (Figure 13, blue with G0), as well as differentiated cells at G0 (Fig. 13, orange with G0). Both of these cell types are stable if LIF is OFF, or, alternatively, in either a GF/GF_High ON environment with 2i ON, or a GF ON/2i OFF environment. As our model does not explicitly includes the circuit required to commit to other lineages outside the neuroectoderm, the differentiated state is a stand-in for other cell types where the ESC circuit has been turned off.

The final phenotype of note is that of a neuroectodermal cell (Figure 13, green). This phenotype could arise in two conditions; in the presence of strong growth signaling with no ESC maintenance signals (GF_High ON, 2i OFF, and LIF OFF), in a GF_High, LIF ON, but 2i OFF condition. This is consistent with the fact that in the presence of active MAPK signaling (where the ESC circuit is destabilized), LIF is responsible for maintaining neural stem cells (i.e. neuroectoderm).

After we analyzed the BMP OFF condition, we studied the effect of turning BMP ON. After carefully sampling the model in the BMP ON condition, only three different phenotypes were stable across all other input environments, as shown in Figure 13.

Of the three stable phenotypes produced by the model in the BMP ON condition, the Naïve ESC (Figure 14, blue) was the most prevalent. With BMP ON, this cell type only required a minimal amount of survival signaling, either from LIF or GF. Additionally, these naïve ESCs proliferated in the presence of LIF or GF_High, and remained quiescent (G0) in no-LIF low GF conditions.

The next stable phenotype present was the differentiated cell in G0 (Figure 14, orange). This phenotype could not be maintained in the presence of LIF, pointing to the lack of adequate negative feedback from the differentiated state towards the ESC factors (thus, LIF can re-activate them). Lastly, cell death (Figure 14, brown D) could theoretically occur and is stable in any condition.

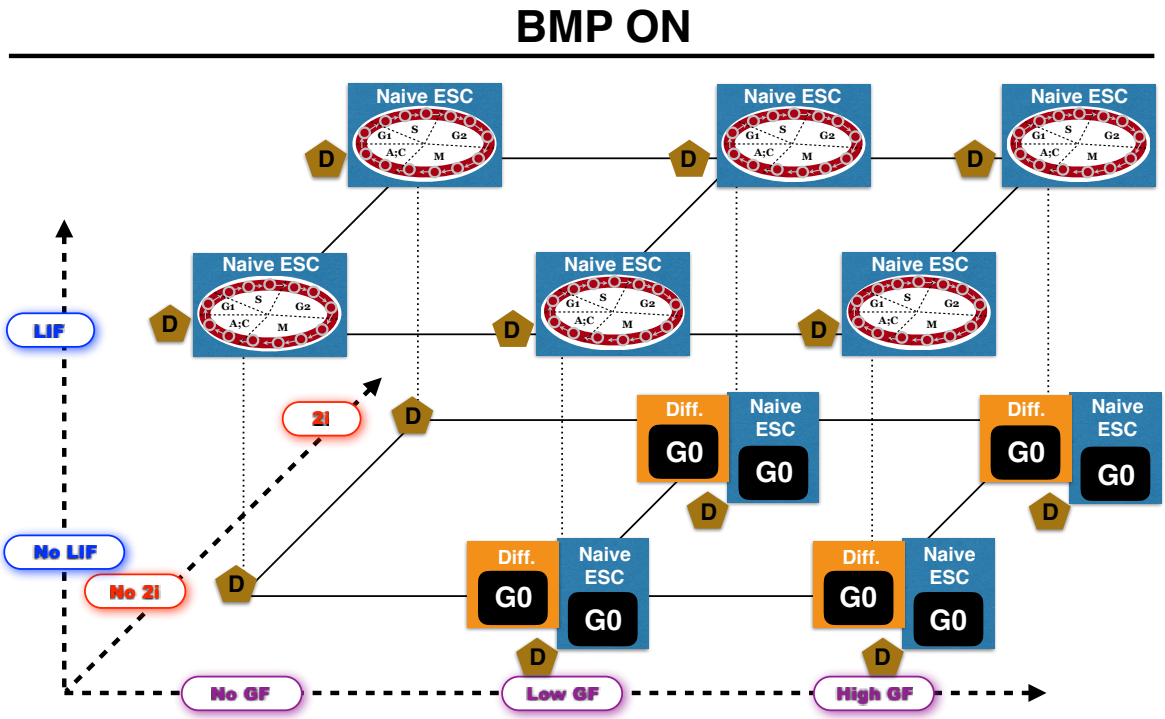

Figure 14. Attractor states of a naïve embryonic stem cell in a BMP ON environment. The attractor states were determined by extensive sampling of cell phenotypes based on twelve possible combinations of inputs: absence or presence of LIF, absence or presence of 2i, and three levels of growth factor input.

Phenotype transitions triggered by a change in the environment

In order to systematically break down the effects of each environmental input on a cellular phenotype, we created a series of box graphs to show the relationship of how a change in each specific input can alter the cell phenotype. We then followed by time course experiments to

look deeper into all the molecular changes that occurred due to change of expression downstream these environmental stimuli. Every time a time course experiment was conducted, a stable phenotype, such as Naïve ESC's in G0 or Ectodermal cells in cell cycle, was chosen first. This stable phenotype was then exposed to a pulse of GF, GF_High, 2i, LIF, or BMP. The resulting activation and inactivation of the rest of the nodes is then shown as a function of time. These time course plots show the coordinated dynamic changes that occur within the cell over time.

To deal with the combinatorial explosion from all possible environmental changes from every possible starting state, we first automated the identification of the initial-final state pairs for every change, then analyzed all of the biologically relevant phenotype changes in more detail. The aim of these experiments was to analyze how the dynamical behavior of the nodes within the cell can change the cellular phenotype, especially in response to transient stimuli.

Changing the 2i environment.

The first input we looked at was 2i. Figure 15 shows cell phenotype transitions from one attractor to another from each initial condition, in the presence of varying levels of LIF, 2i, and GF, as well as the presence or absence of BMP.

In the presence of BMP (BMP ON), a change in 2i does not result in any interesting cell phenotype transitions (thin red arrows). Regardless of high or low levels of 2i, cell phenotypes involved with Low GF or High GF and LIF remained the same. In the presence of NO GF and NO LIF, the cell dies regardless of the presence or absence of 2i.

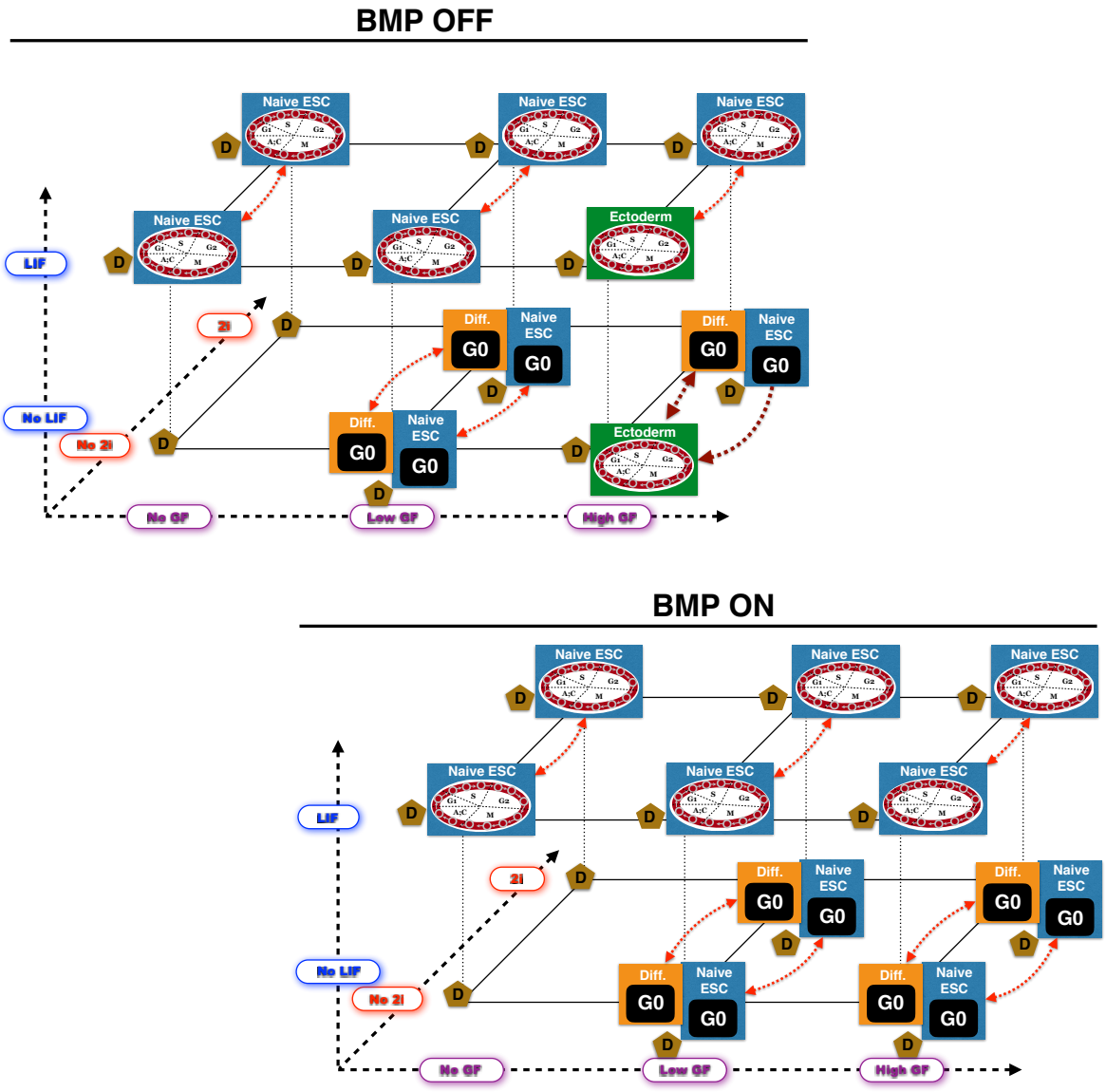

Figure 15. Changes in cell phenotypes dependent on 2i presence or absence. After extensive sampling, stable phenotypes were analyzed how they changes based on the addition or removal of 2i from the environment.

The same scenarios are present in the BMP OFF condition, with the exception of cell phenotypes in the GF_High and LIF environments. There were interesting results in two of these conditions. First, with LIF ON, 2i OFF and GF_High ON, a neuroectodermal cell in cell cycle is present. Unfortunately, the model shows that the neuroectodermal cell can toggle back and forth

into the Naïve ESC state, based on the expression of 2i. This is not a biological behavior; *in vitro* or *in vivo* differentiated neuroectodermal cells are incapable of reverting back to their embryonic stem cell state. In the model (and in the literature), however, there is no known feedback mechanism to prevent the Oct4/Sox2 circuit from re-engaging. Second, a neuroectodermal cell in cell cycle is present in the GF_High ON, 2i OFF and LIF OFF condition. If 2i is turned ON, this cell looses its neuroectoderm characteristics, but does not de-differentiate. Moreover, cells in a Naïve ESC and G0 state differentiate into neuroectoderm upon loss of 2i, but do not revert when 2i is turned back on. The behavior of the Naïve ESC is consistent with known biology.

To show the molecular dynamics driving this phenotype change, we took a deeper look at the 2i change from attractor state 229, the Naïve ESC, in response to a pulse of 2i deactivation (Figure 16). This time course starts with a Naïve ESC in G0 in a GF ON, GF_High ON, 2i ON, LIF OFF, BMP OFF environment. This Naïve ESC in G0 becomes a neuroectodermal cell when 2i is turned OFF. When 2i is subsequently turned back ON again, the neuroectodermal cell will become a non-neuronal (generic) differentiated cell in G0. Initially when 2i is ON, the Naïve ESC in G0 has an active pluripotency circuit; Oct4, Sox2, and Nanog are all ON. In the period where 2i is turned OFF, the pluripotency circuit is turned OFF, which pushes the cell to differentiate and there is an absence of any cycling behavior. BMP is blocking ERK and there is no LIF to compensate. Once the Naïve ESC in G0 commits to becoming a neuroectodermal cell, it is unable to revert to its naïve ESC via a change in 2i.

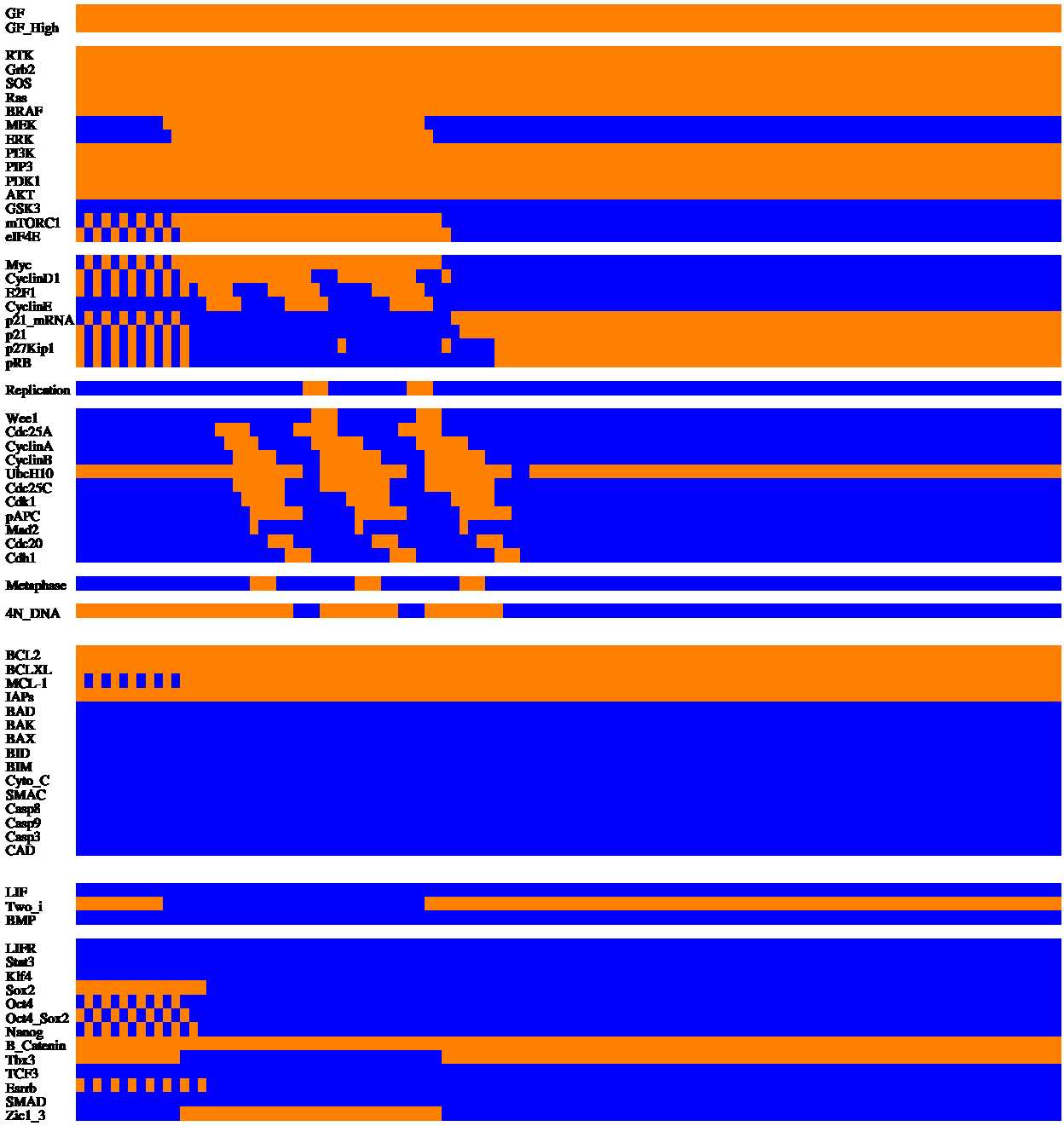

Figure 16. Attractor 229 - Phenotypic transitions from a Naïve ESC in G0 response to transient pulses of 2i. A 30 time step pulse of 2i affecting a Naïve ESC in G0. Orange blocks signify activation and blue blocks represent repression. Each column represents a time-point in the simulation.

Changing the LIF environment

The second input stimulus we looked at was the LIF environment. By looking at the figure, we compared cell phenotypes in each condition in the presence of varying levels of LIF, 2i, and GF in the presence or absence of BMP. Figure 17 exhibits how each phenotype can "flip" or is related in based on the presence or absence of LIF.

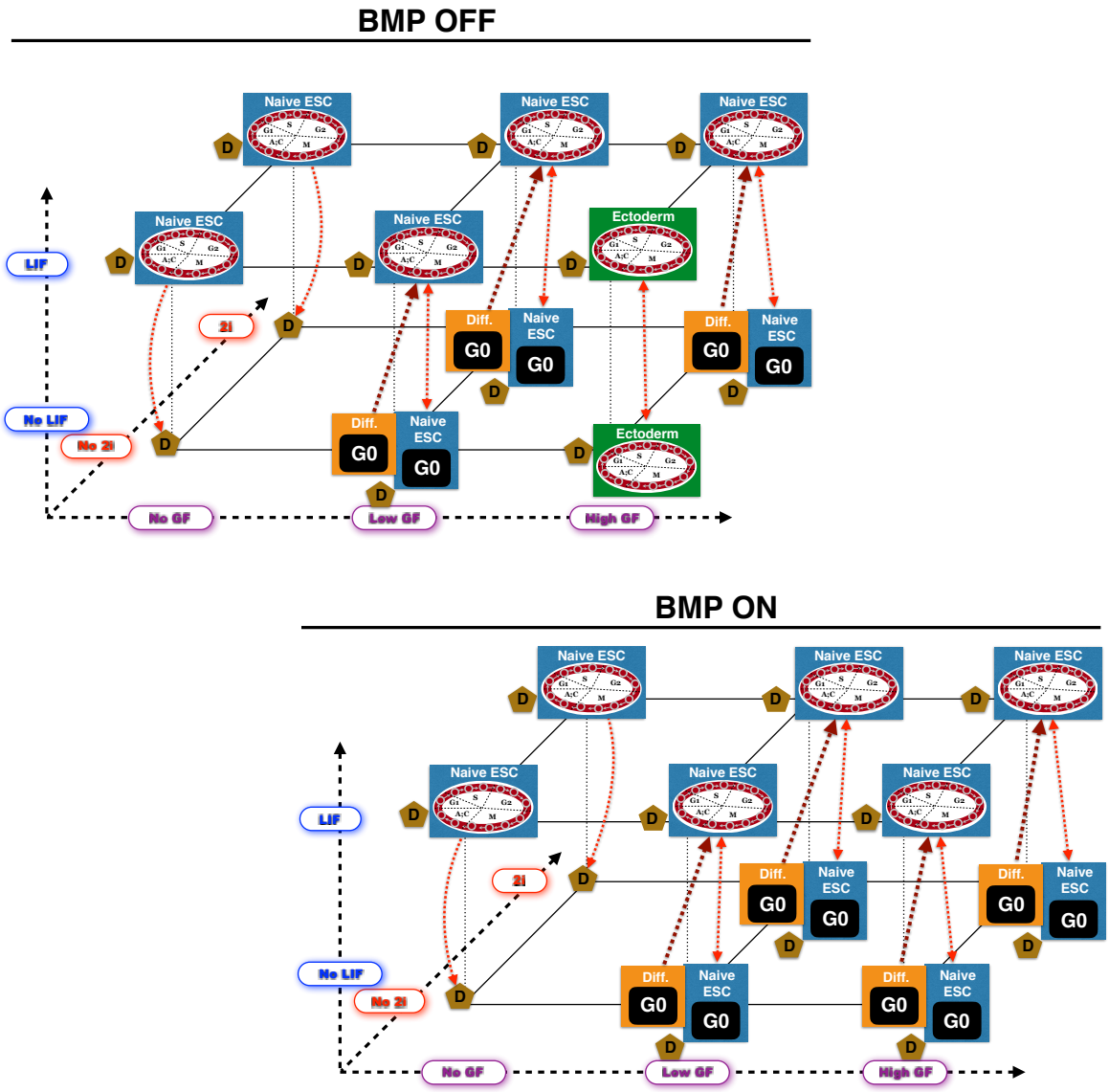

Figure 17. Changes in cell phenotypes dependent on LIF presence or absence. After extensive sampling, stable phenotypes were analyzed how they changes based on the addition or removal of LIF from the environment.

The models created in the presence of BMP ON and BMP OFF produced some expected results. In both BMP conditions, if LIF was removed from a Naïve ESC in cell cycle in a LIF ON GF OFF condition, the cell would die. Additionally, if a Naïve ESC in a LIF ON and GF ON (both GF and GF_High) condition were moved into a LIF OFF condition, the phenotype would change to a Naïve ESC in G0. Therefore, the presence or absence of LIF determines if a Naïve ESC is either in cell cycle or G0. These two phenotypes are completely capable of toggling back and forth.

When looking at LIF expression, the model also produced some interesting results. First of all, in the BMP OFF condition the presence or absence of LIF did not cause any effect on the ectodermal cells in cell cycle. This was inconsistent with the Naïve ESC's exposed to LIF variations if GF was ON, as LIF OFF in this condition would put the cell in G0. Additionally, the Differentiated ESC's in G0 acted improperly. The model shows these cells can revert back to their naïve state and initiate a cell cycle in the presence of LIF. This result was further analyzed using a transient pulse of LIF in attractor state 109 in Figure 18.

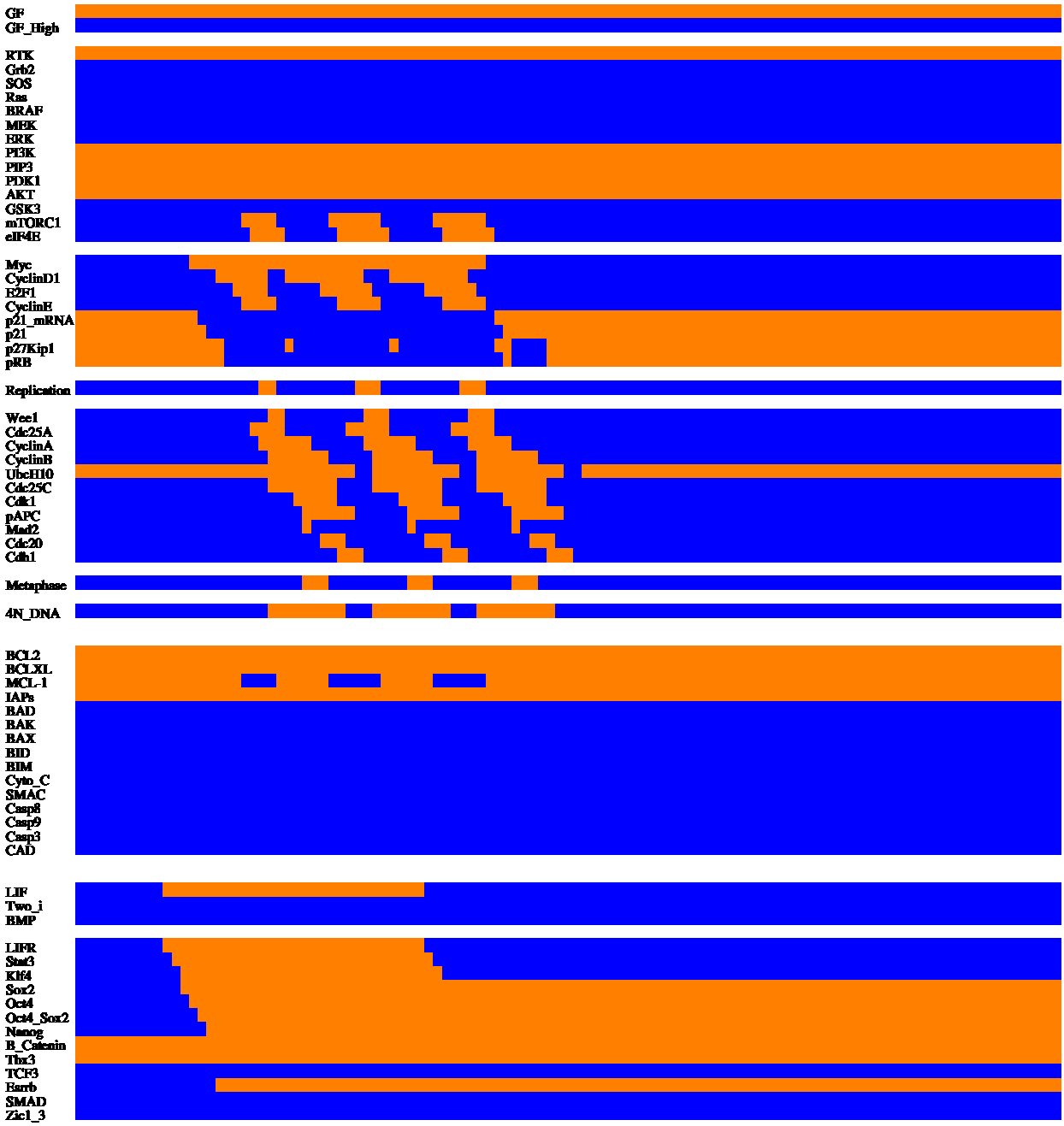

Figure 18. Attractor 109 - Phenotypic transitions from a differentiated ESC in G0 response to transient pulses of LIF. A 30 time step pulse of LIF affecting a differentiated ESC in G0. Orange blocks signify activation and blue blocks represent repression. Each column represents a time-point in the simulation.

This model represents a differentiated ESC in G0 in a GF ON, GF_High OFF, 2i OFF, LIF OFF, BMP OFF environment with a LIF transient pulse. The differentiated ESC in G0 will become a Naïve ESC in cell cycle if LIF is turned ON. Once this happens, if LIF is turned back OFF, the

cell will become a Naïve ESC in G0. The differentiated ESC in G0 starts cell cycling as soon as LIF is ON. This cell then completes three full cell cycles until LIF is turned OFF. Once LIF is turned OFF, the cell cycling stops but the cell then resembles a Naïve ESC in G0 in the absence of LIF. The Naïve ESC in G0 and the Naïve ESC in cell cycle are interchangeable based on LIF's expression. Once the differentiated ESC in G0 commits to a Naïve ESC in cell cycle, it can never become a differentiated ESC ever again.

Changing the GF environment

The third input stimulus we looked at was the GF environment. Once again, we compared cell phenotypes in each condition in the presence of varying levels of LIF, 2i, and GF in the presence or absence of BMP. Figure 19 exhibits how each phenotype can "flip" or is related in based on the presence or absence of GF.

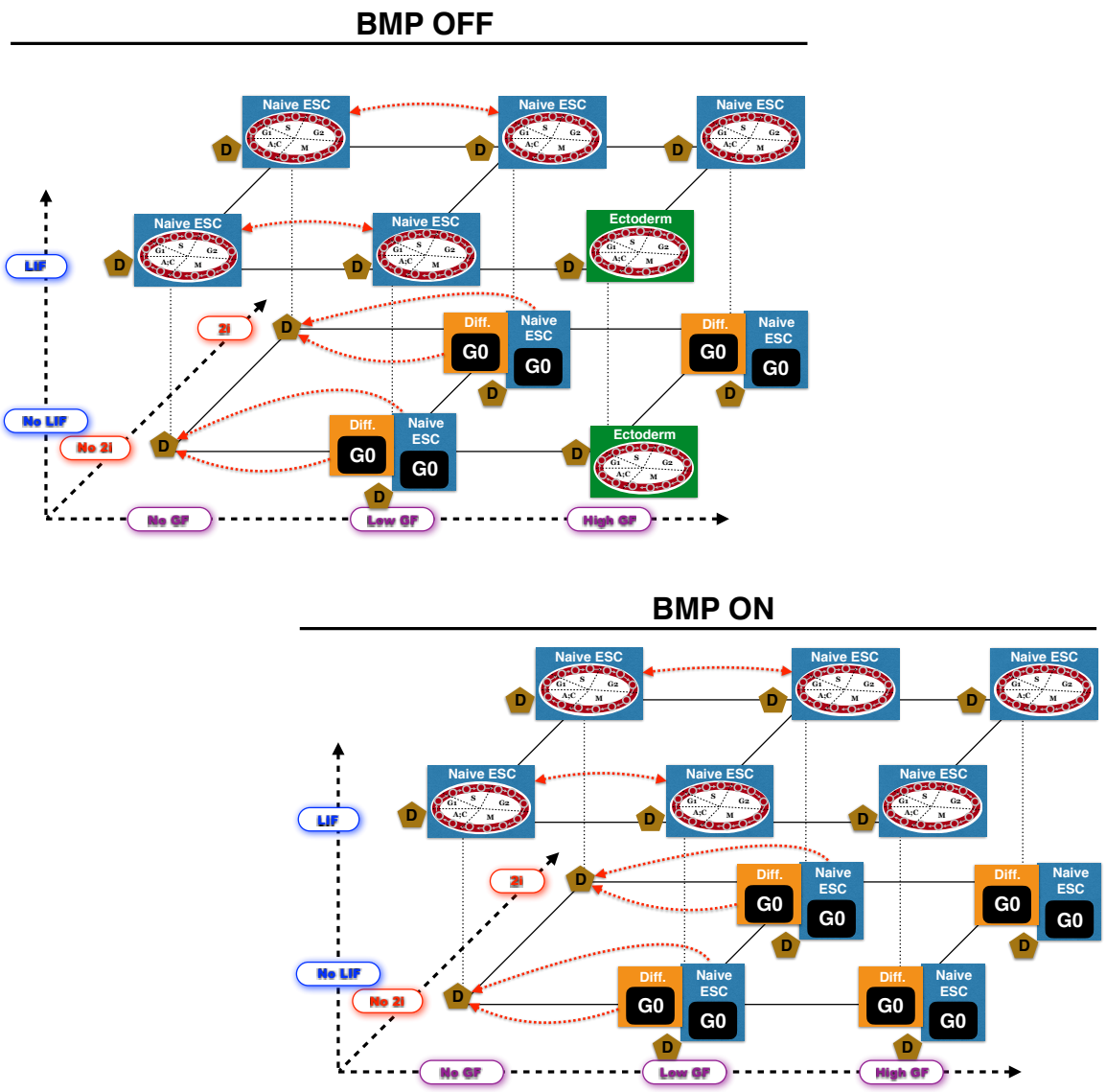

Figure 19. Changes in cell phenotypes dependent on GF presence or absence. After extensive sampling, stable phenotypes were analyzed how they changes based on the addition or removal of GF from the environment.

The model produced expected results when looking at changes in GF expression in both conditions. BMP OFF and BMP ON produced the same results. Cells that were in the LIF OFF GF ON conditions produced both Naïve ESC's in G0 / Differentiated ESC's in G0. If GF was removed, the cell had no factors to keep it alive and self-sustaining, so they ultimately die. There is no arrow pointing back in the opposite directions because dead cells cannot be brought back to life. Additionally, Naïve ESC's in cell cycle in the LIF ON condition could survive regardless if

GF was present or absent. This makes sense because the cell can still live and survive off of LIF. Adding or removing GF to a Naïve ESC in G0 would not alter its phenotypic expression.

The fourth input stimulus we looked at was the GF_High environment. We analyzed cell phenotypes in each condition in the presence of varying levels of LIF, 2i, and GF in the presence and absence of BMP. Figure 20 below exhibits how each phenotype can "flip" or is related in based on the presence or absence of GF_High.

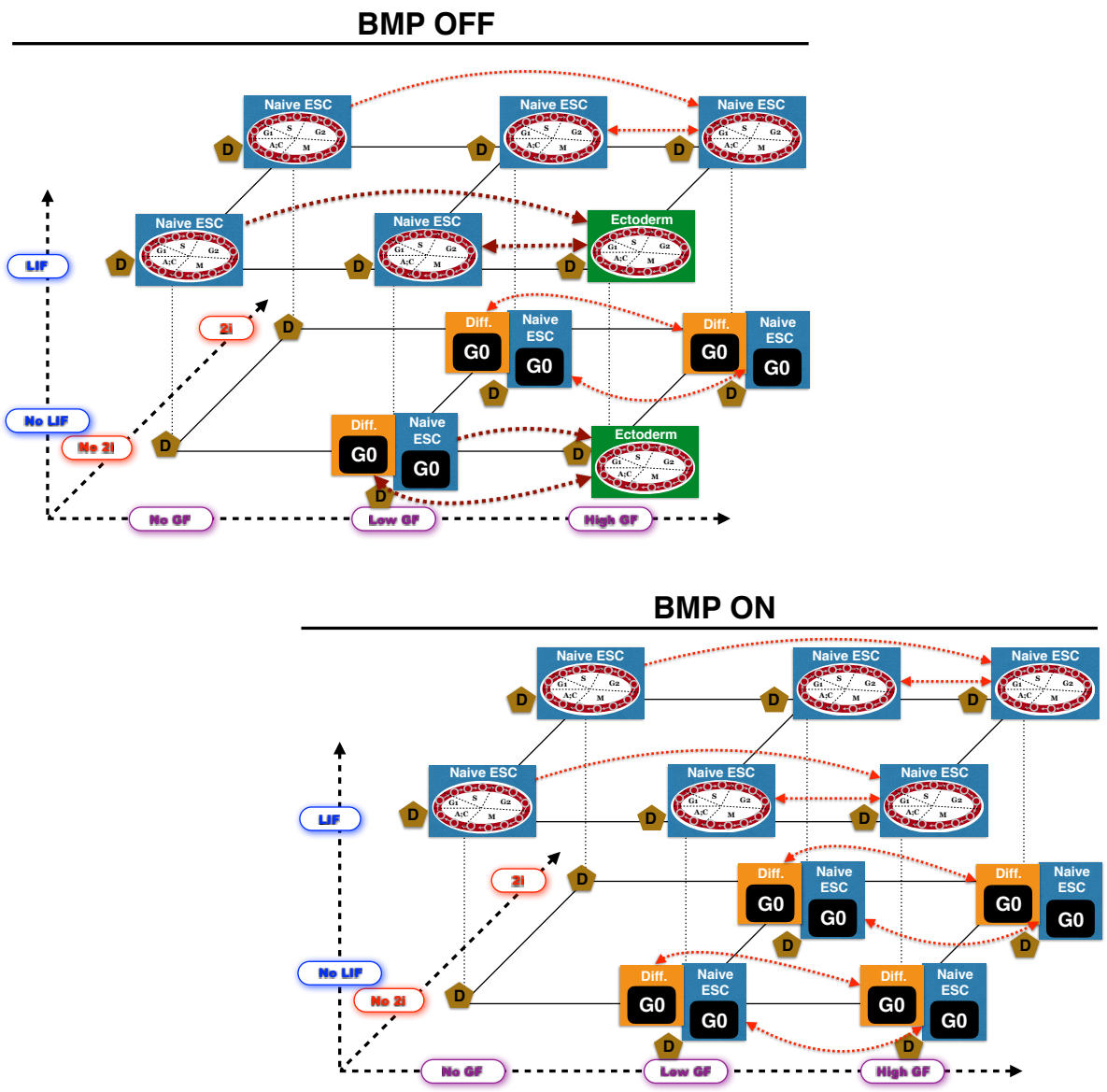

Figure 20. Changes in cell phenotypes dependent on GF_High presence or absence. After extensive sampling, stable phenotypes were analyzed how they changes based on the addition or removal of GF_High from the environment.

The model manipulating GF_High produced rudimentary results in the BMP ON condition. In this condition, Naïve ESC's in cell cycle remained the same regardless of 2i, GF, and GF_High conditions as long as LIF was ON. In the BMP ON LIF OFF conditions, Differentiated ESC's in G0 and Naïve ESC's in G0 toggled back and forth in the 2i ON and 2i

OFF conditions. The level of GF expression (GF and GF_High) did not impact the phenotypes of these cells.

The GF_High manipulation model also illustrated some interesting results in the BMP OFF condition. The results were the same as the BMP ON condition except for the conditions where 2i is OFF. In the 2i OFF LIF ON condition, the model shows that an ectodermal cell in cell cycle can toggle back and forth with a Naïve ESC in cell cycle based on the level of GF expression. This cannot happen as a Ectodermal cell is incapable of reverting back to its pluripotent ESC state. The other interesting result shown in this model is that a Naïve ESC in G0 and a Differentiated ESC in G0 in a 2i OFF LIF OFF GF ON condition can be pushed to becoming a ectodermal cell in cell cycle if GF ON moves to GF_High ON. Unlike the GF_High ON 2i OFF LIF ON condition, the ectodermal cell will not move back to its previous state in this condition, which is consistent with biology. To account for this unexpected change, we took a deeper look at attractor state 149 in Figure 21.

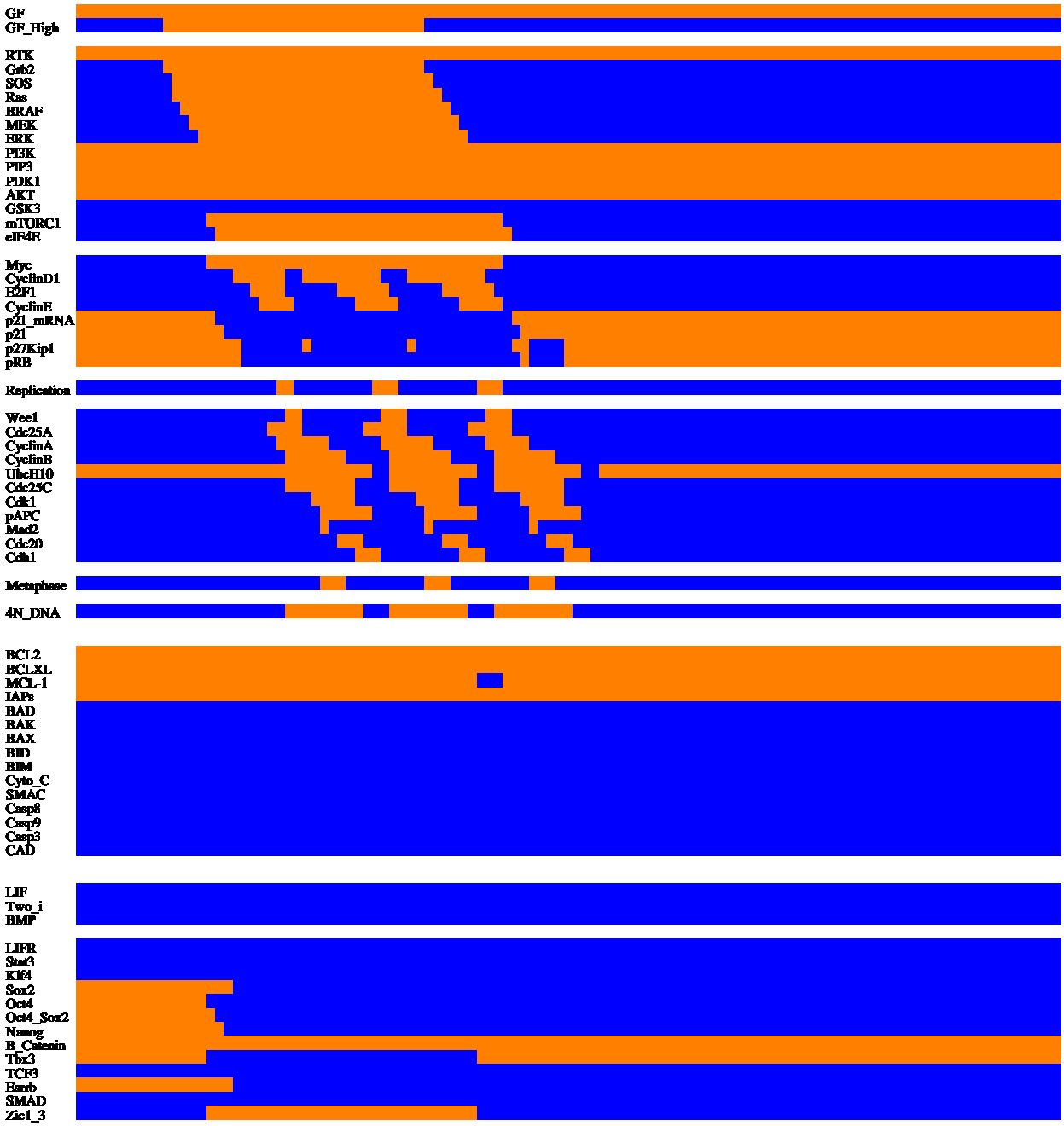

Figure 21. Attractor 149 - Phenotypic transitions from a Naïve ESC in G0 response to transient pulses of GF_High. A 30 time step pulse of GF_High affecting a Naïve ESC in G0. Orange blocks signify activation and blue blocks represent repression. Each column represents a time-point in the simulation.

This time pulse model shows a Naïve ESC in G0 in a GF ON, GF_High OFF, 2i OFF, LIF OFF, BMP OFF environment exposed to a GF_High transient pulse. The Naïve ESC in G0 becomes a neuroectodermal cell in cell cycle when GF_High is turned ON. Once the ectodermal cell in cell

cycle is established, if GF_High is removed the ectodermal cell in cell cycle becomes a differentiated cell in G0. Initially, GF_High is OFF and the Naïve ESC in G0 exhibits an ON pluripotency network and no cell cycle. Once the GF_High turns ON, the pluripotency circuit turns OFF, allowing the cell to differentiate into a Ectodermal cell in cell cycle. Additionally the cell is able to complete three full cell cycles. Once GF_High is turned back OFF, the Ectodermal cell in cell cycle becomes a differentiated ESC in G0. This is illustrated by showing that the pluripotency circuit and cell cycling nodes remain OFF. Once the Naïve ESC in G0 becomes an ectodermal cell in cell cycle, it cannot revert back to its naïve phenotype. However, the ectodermal cell in cell cycle and differentiated ESC in G0 phenotypes can toggle back and forth based on the expression of 2i.

Changing the BMP environment

The last input stimulus we looked at was the BMP environment. By looking at the figure, we compared cell phenotypes in each condition in the presence of varying levels of LIF, 2i, and GF in the BMP ON and BMP OFF conditions. Figure 22 exhibits how each phenotype can “flip” or is related in based on the presence or absence of BMP.

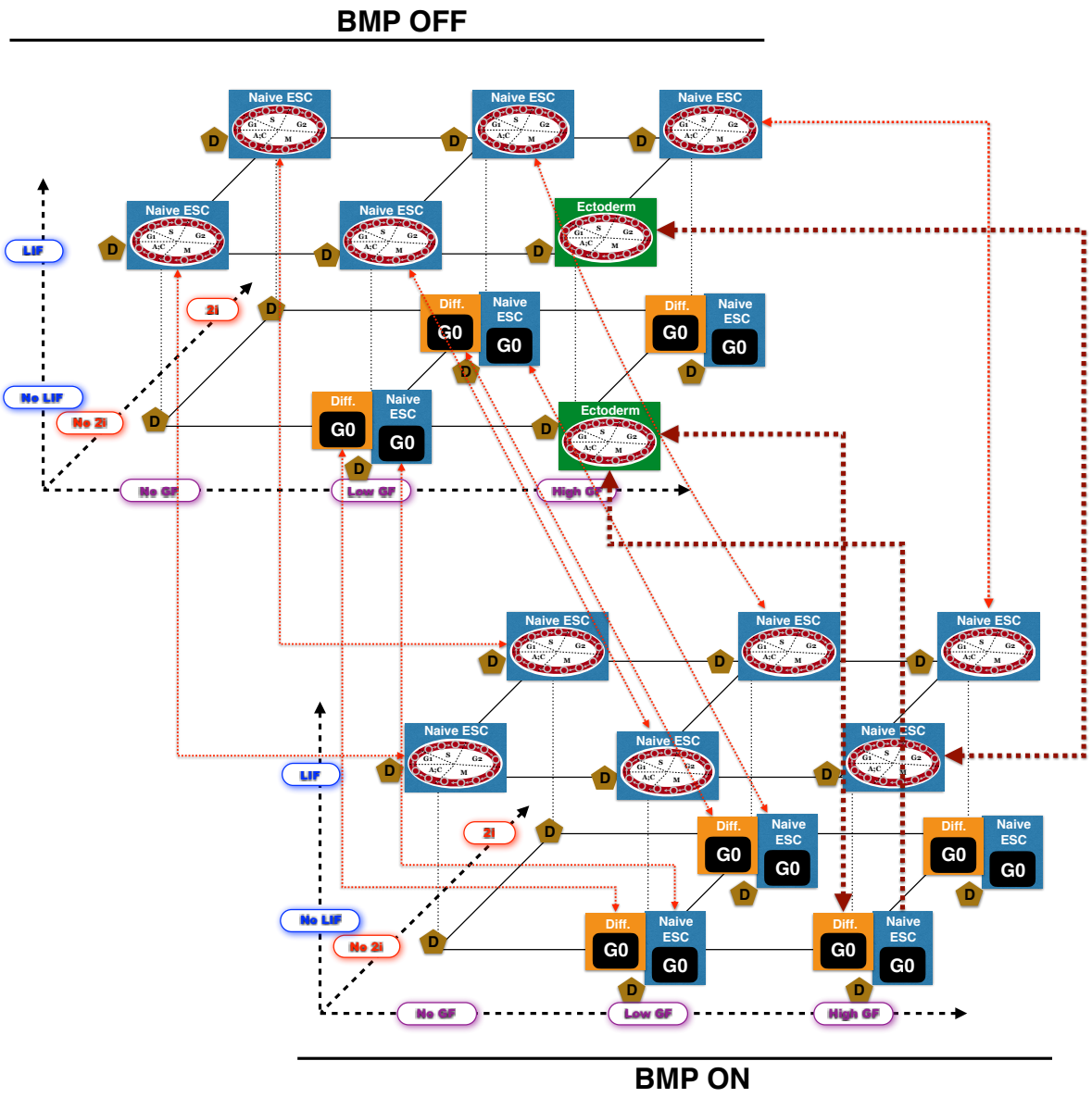

Figure 22. Changes in cell phenotypes dependent on BMP presence or absence. After extensive sampling, stable phenotypes were analyzed how they changes based on the addition or removal of BMP from the environment.

The BMP manipulation model shows how cell phenotypes can change based on the addition or removal of BMP from the environment. As seen in the figure, all cell phenotypes remain the same except for three conditions if they are flipped between BMP ON and BMP OFF. These unique changes occur in the 2i OFF GF_High ON LIF ON condition and the 2i OFF GF_High ON LIF OFF condition. In the 2i OFF GF_High ON LIF ON condition, the cell

phenotype is an ectodermal cell in cell cycle in the absence of BMP, and it is a Naïve ESC in cell cycle in the presence of BMP. The model shows that the two cell phenotypes are able to toggle back and forth based on the presence or absence of BMP. This cannot happen as a neuroectodermal cell should lock in and not allow itself to revert to the Naïve ESC state simply due to BMP being present. Lastly, in the 2i OFF GF_High ON LIF OFF condition, a Naïve ESC in G0 will only change to a neuroectodermal cell in cell cycle if BMP is removed, which is what we desired to see. On the other hand, a Differentiated ESC in cell cycle phenotype is able to toggle back and forth with an neuroectodermal cell in cell cycle based on BMP expression. This cannot happen, as the neuroectodermal cell cannot revert back to a differentiated ESC. To account for this unexpected change, we took a deeper look at BMP change in attractor state 216 in Figure 23.

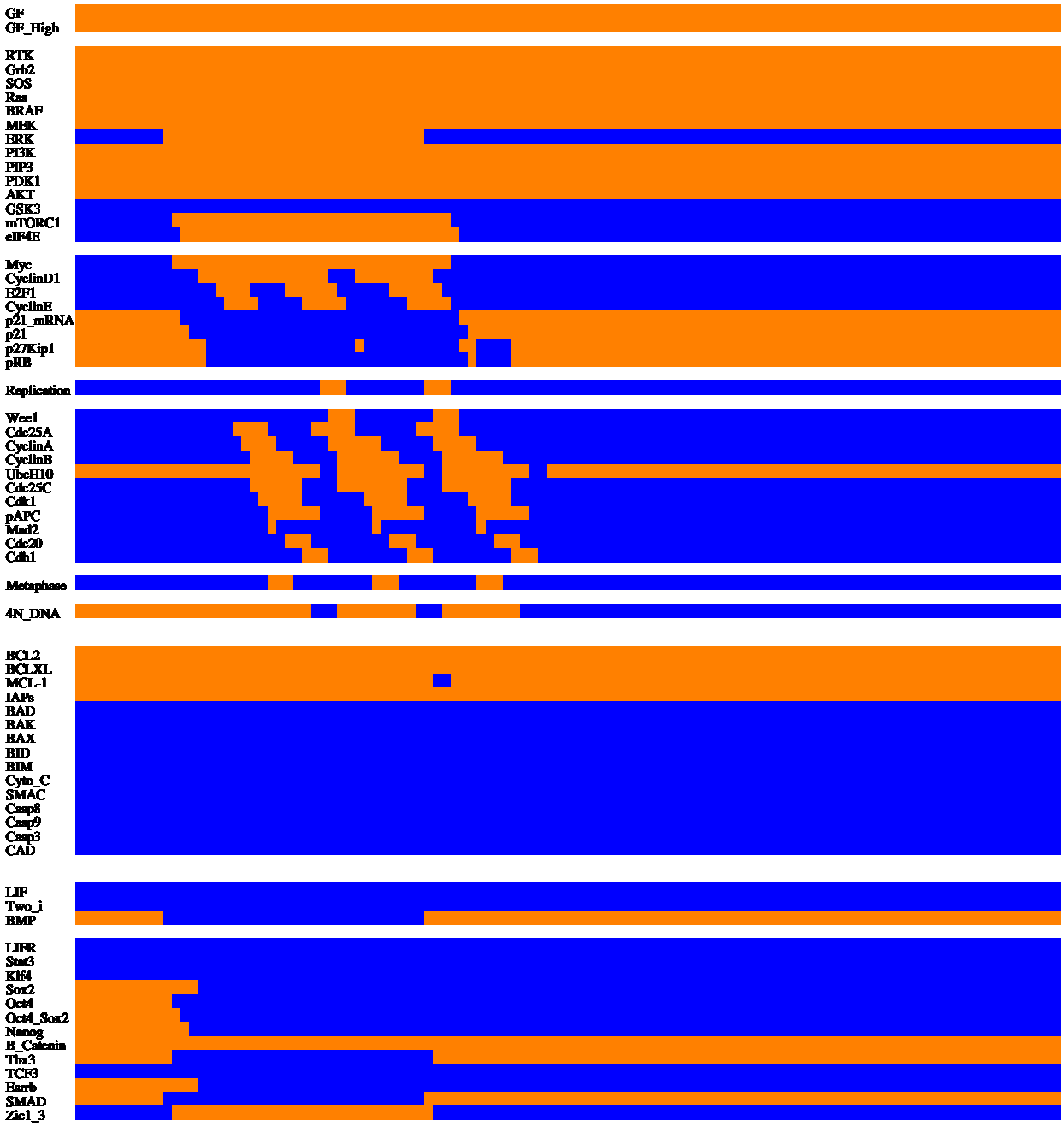

Figure 23. Attractor 216 - Phenotypic transitions from a Naïve ESC in G0 in response to transient pulses of GF_High. A 30 time step pule of BMP affecting a Naïve ESC in G0. Orange blocks signify activation and blue blocks represent repression. Each column represents a time-point in the simulation.

This time pulse model shows a Naïve ESC in a GF ON, GF_High ON, 2i OFF, LIF OFF, BMP ON exposed to a BMP transient pulse. Initially, the Naïve ESC in G0 exhibits no cell cycle nodes ON and the pluripotency circuit ON. Additionally, this Naïve ESC in G0 is in the presence of a BMP ON environment. Once the BMP is removed from the environment, the Naïve ESC in

G0 permanently moves to becoming an ectodermal cell in cell cycle. If BMP is turned back ON, the ectodermal cell in cell cycle becomes a differentiated ESC in G0. The model initially illustrates the Naïve ESC in G0 by having pluripotency nodes ON and having cell cycle nodes OFF. Once BMP is removed from the environment, the pluripotency circuit turns OFF, signaling differentiation, and the cell begins to cell. The cell cycles three times while BMP is OFF. Once BMP turns back ON, the pluripotency circuit cells remain off (signaling permanent differentiation) and the cell cycle nodes are also OFF, illustrating the G0 state. Once the Naïve ESC in G0 becomes a ectodermal cell in cell cycle, it is stuck in that phenotype. Nonetheless, the ectodermal cell in cell cycle and the differentiated ESC in G0 are not stuck in their phenotypes, as their states can toggle back and forth based on the presence or absence of BMP.

Stable phenotypes after changing the Zic1_3 Gate so that it sustains its own activity

Once this analysis was completed and results were obtained, we decided to change the gate of the Zic1_3 node so that it could autoregulate itself instead of having its activity solely dependent on the BMP node's activity (Figure 24). Originally, the Zic1_3 node could only be activated if SMAD was turned OFF by BMP and ERK was ON.

Original Zic1_3 Gate

SMAD	ERK	Zic1_3
0	0	0
0	1	1
1	0	0
1	1	0

Modified Zic1_3 Gate

Zic1_3	SMAD	ERK	Zic1_3
0	0	0	0
0	0	1	1
0	1	0	0
0	1	1	0
1	0	0	1
1	0	1	1
1	1	0	1
1	1	1	1

Figure 24. Functional change of the Zic1_3 gate.

In this change the Zic1_3 node will be ON in any scenario where Zic1_3 is present, regardless of SMAD or ERK expressions. Once this gate was changed, the phenotypes and connections between cell phenotypes in the BMP ON and BMP OFF conditions exhibit some changes.

The change in the Zic1_3 gate significantly changed the different phenotypes that could arise as a result of sampling this model. Primarily, we were able to model the ectodermal cell in G0, which we were unable to do in our previous model with the original Zic1_3 gate and seen in Figure 25.

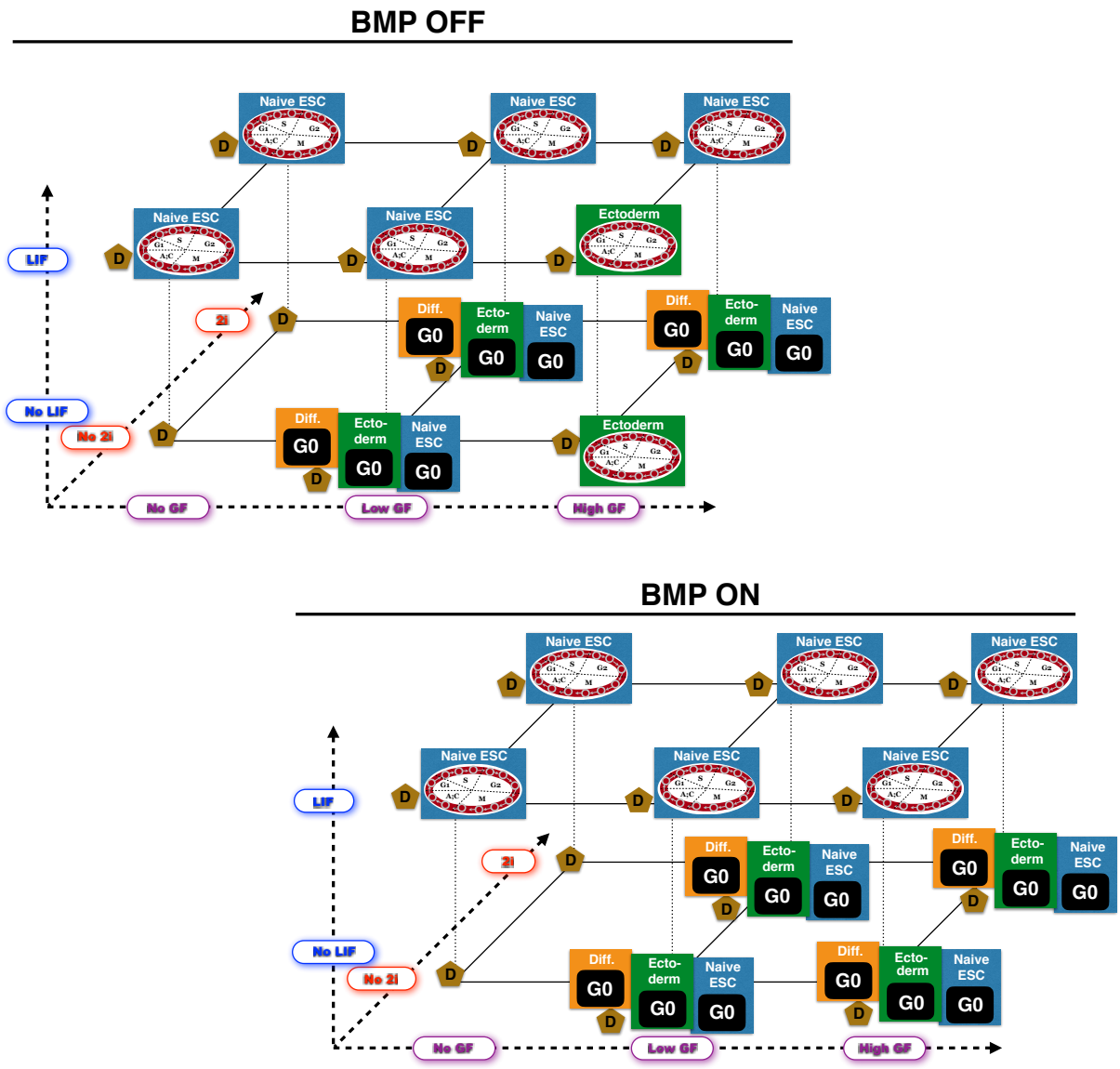

Figure 25. Attractor states of a healthy naïve ESC after adjustment of the Zic1_3 gate. The attractor states were determined by extensive sampling of cell phenotypes based on twenty-four possible combinations of inputs: absence or presence of BMP, absence or presence of LIF, absence or presence of 2i, and three levels of growth factor input.

In this new model we were able to show Naïve ESC's in cell cycle, neuroectodermal cells in cell cycle, dead cells, and Differentiated ESC/Ectodermal ESC/Naïve ESC in G0 states. In the BMP OFF condition, dead cells could have occurred in any condition, but they were guaranteed to die in the LIF OFF and GF OFF conditions, regardless of 2i expression. Naïve cells were produced in the BMP OFF condition in any scenario where LIF was ON, except for the LIF ON,

2i OFF, GF_High ON condition, which produced an neuroectodermal cell in cell cycle. In this BMP condition, neuroectodermal cells in cell cycle could arise if GF_High was ON and 2i was OFF, regardless of LIF expression. Lastly, Differentiated ESC/Ectodermal ESC/Naïve ESC in G0 states could arise in the BMP OFF condition as long as GF_High or GF was ON and no LIF was present.

In the BMP ON condition, once again dead states could occur in environmental combinatory condition, but occurred mostly in conditions where GF and LIF were absent. In the BMP ON condition, only two other phenotypes were present. The first phenotype, Naïve ESC's in cell cycle, could arise as long as LIF was on, regardless of 2i or GF expression. The other phenotype was Differentiated ESC/Ectodermal ESC/Naïve ESC in G0 states, which could arise in any BMP ON condition where LIF was OFF and some level of GF was present.

Then we analyzed how changes in expression between ON and OFF states for each of the environmental stimuli could impact the resulting cell phenotype. GF and GF_High changes between the BMP ON condition in the new model did not differ from the BMP ON condition in the original model. However, there was the presence of changes of GF and GF_High on the cell phenotypes in the BMP OFF condition of this model. Additionally, we did find noticeable changes between the BMP OFF and BMP ON states when analyzing LIF, 2i, and BMP conditions, as illustrated in Figure 26.

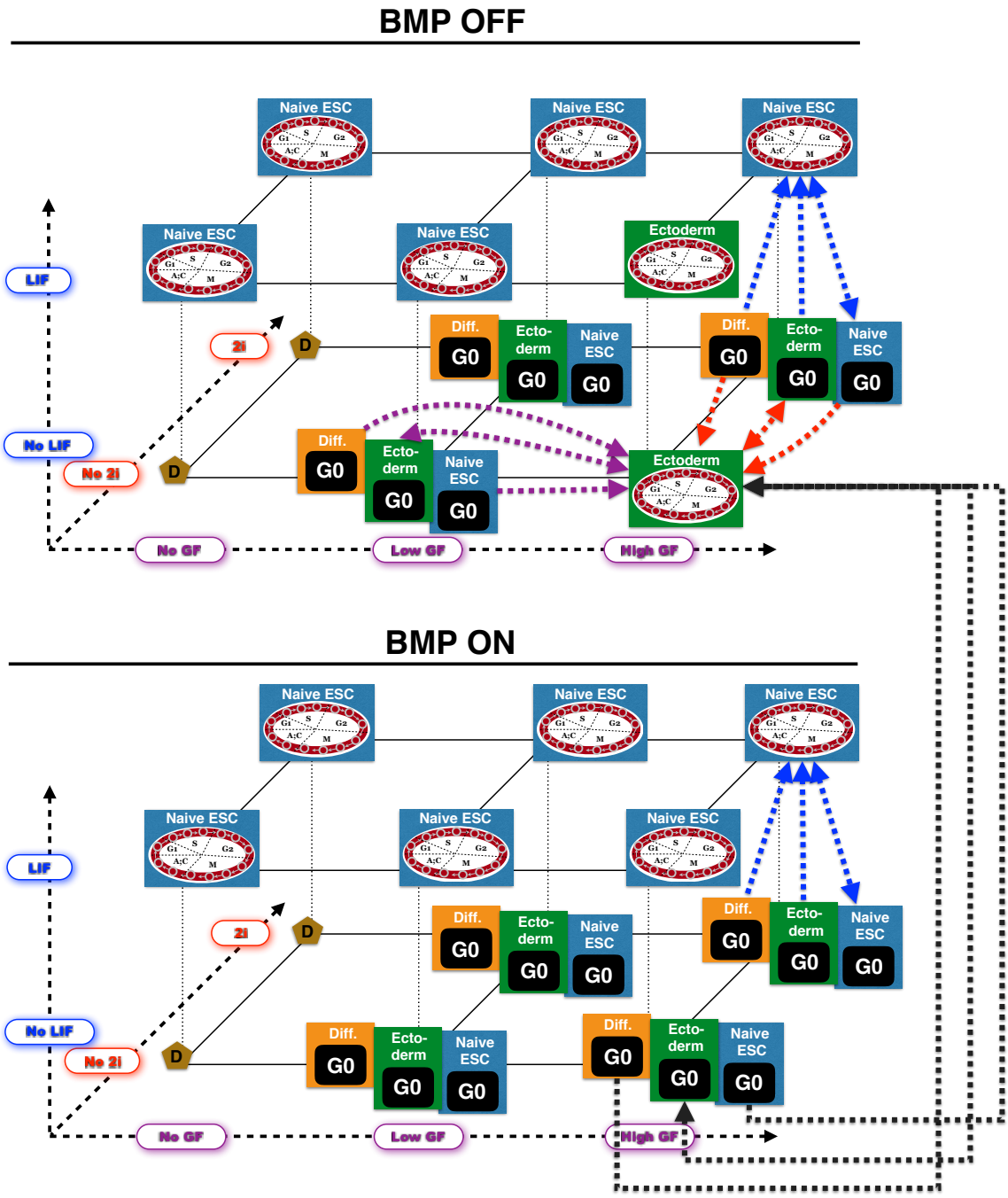

Figure 26. Unexpected changes in cell phenotypes dependent on presence or absence of specific environmental inputs. After extensive sampling, stable phenotypes were analyzed how they changes based on the addition or removal of 2i from the environment. Black lines signify BMP changes, blue lines signify LIF changes, red lines illustrate 2i changes, and purple lines represent GF_High changes.

After analyzing the changes in the original model, we analyzed odd changes in cell phenotypes after the new Zic1_3 gate was implemented in the new model. This change in the Zic1_3 gate alleviated a few issues viewed in the first model in terms of the differentiated ESC in G0. With this change, the ectodermal cell in G0 became a stable phenotype. Therefore, we could model that the Ectoderm cell in cell cycle and the ectoderm cell in G0 could toggle back and forth in terms of 2i while naïve ESC's in G0 and differentiated cells in G0 could only differentiate and be stuck in the ectoderm cell in cell cycle phenotype. However, we still developed issues with transient phenotypic changes involving LIF and GF_High. To account for this unexpected change, we looked at was GF_High change in attractor state 101 in Figure 27.

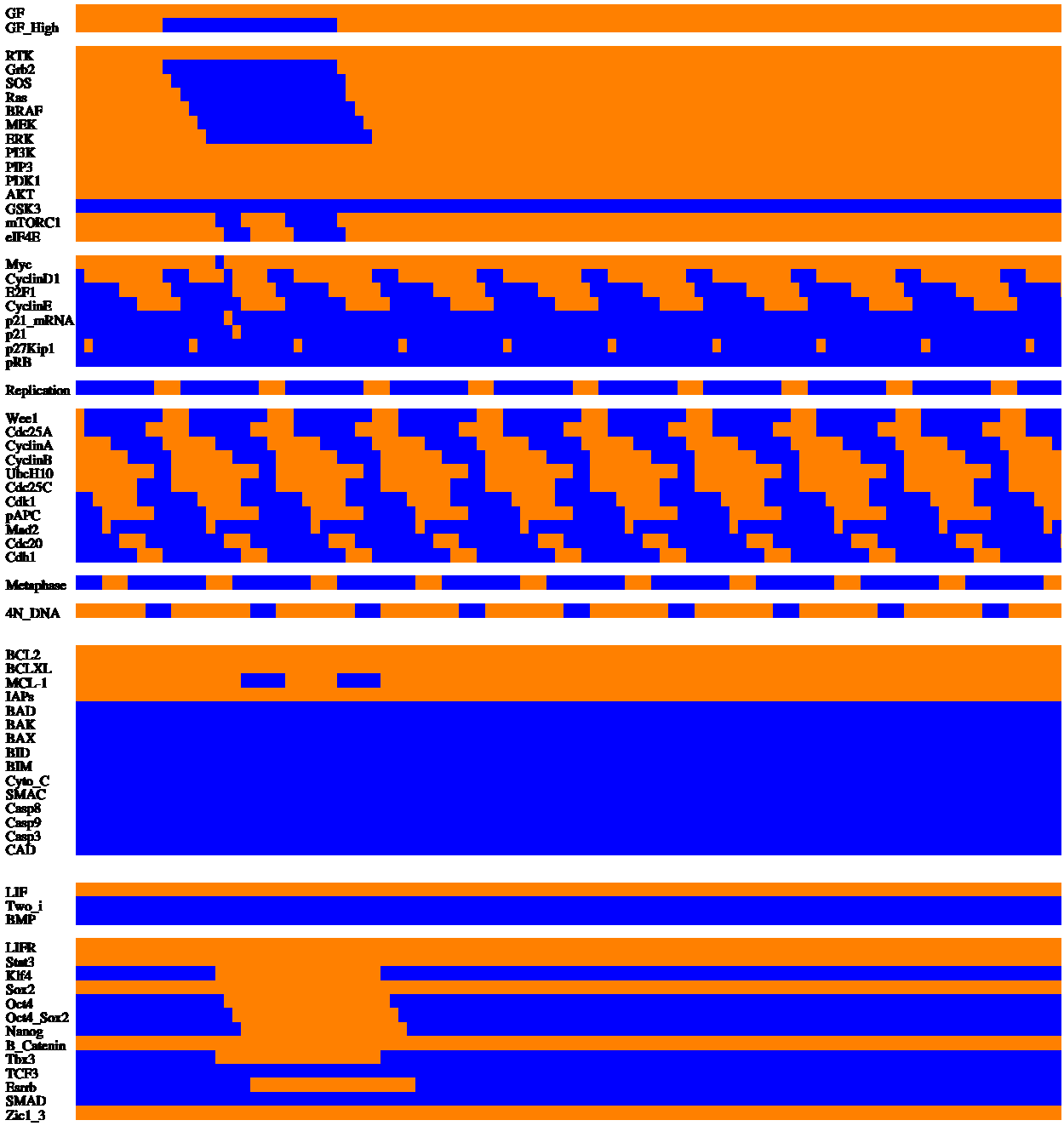

Figure 27. Attractor 101 - Phenotypic transitions from a neuroectodermal cell in cell cycle response to transient pulses of GF_High. A 30 time step pule of GF_High affecting an ectodermal cell in cell cycle. Orange blocks signify activation and blue blocks represent repression. Each column represents a time-point in the simulation.

This model illustrates an ectodermal cell in cell cycle in a GF ON, GF_High ON, 2i OFF, LIF ON, and BMP OFF environment exposed to a GF_High transient pulse. This model exhibits a change that is not possible in biology. Initially, the model shows that a Naïve ESC in cell cycle

exposed to no GF can differentiate into an ectodermal cell in cell cycle if GF_High is added to the environment. Once this Naïve ESC in cell cycle commits to this phenotypic change, it cannot go back to its initial state. Once the neuroectodermal cell in cell cycle in present, the model shows that if GF_High is OFF exhibits that this cell phenotype will revert back to a Naïve ESC in cell cycle state, which is not possible. However, if a Naïve ESC in cell cycle in a GF ON environment is moved in a GF_High ON environment, the Naïve ESC in cell cycle will become an ectodermal cell in cell cycle. This change comes as a result of manipulation of the pluripotency nodes, not the cell cycle nodes. The cell cycles continuously as soon as it is exposed to GF_High ON. However, the pluripotency nodes Oct4 and Nanog are OFF when GF_High is present and are ON when GF_High is absent. Sox2 stays ON regardless of GF_High expression.

In both the BMP OFF and BMP ON conditions, changes in LIF expression presented similar yet different results from the original model. The differentiated ESC in G0 and the ectoderm cell in G0 could become a Naïve ESC in cell cycle if they were exposed to LIF ON, yet, a Naïve ESC in cell cycle could not revert back to either previous state if LIF was removed. Interestingly, a Naïve ESC in G0 could become a Naïve ESC in cell cycle if it was in a LIF ON environment. Inversely, a Naïve ESC in cell cycle could become a Naïve ESC in G0 if LIF was removed. We took a deeper look at this unexpected change using a LIF pulse in attractor state 62, shown in Figure 28.

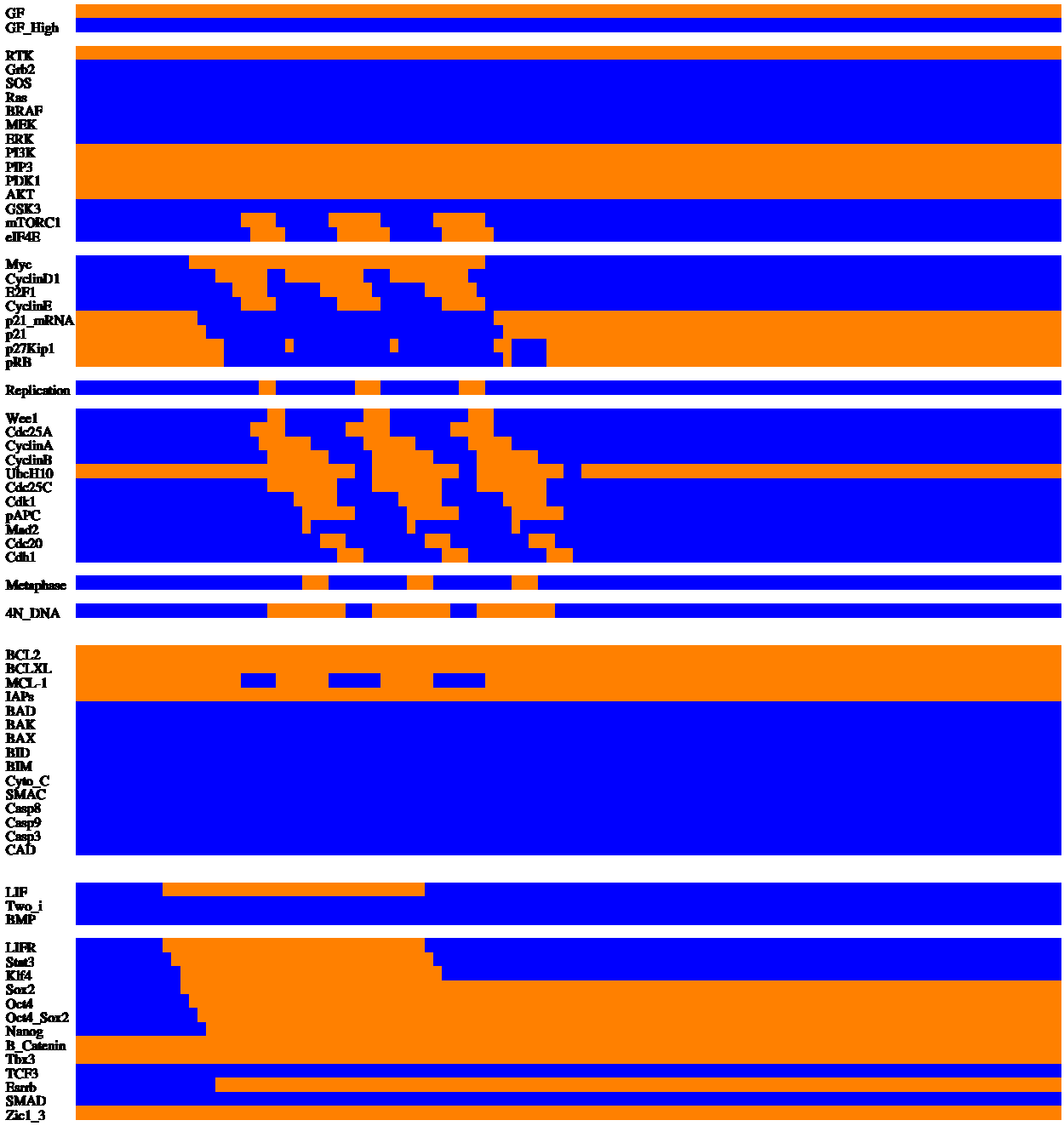

Figure 28. Attractor 62 - Phenotypic transitions from a neuroectodermal cell in G0 in response to transient pulses of LIF. A 30 time step pule of LIF affecting an ectodermal cell in cell cycle. Orange blocks signify activation and blue blocks represent repression. Each column represents a time-point in the simulation.

This time course model exhibits an ectodermal cell in G0 in a GF ON, GF_High OFF, 2i OFF, LIF OFF, and BMP OFF environment while exposed to a LIF transient pulse. The issue in this model is that an ectodermal cell in G0 can become a Naïve ESC in cell cycle if 2i is ON. The

same instance happens for the differentiated ESC in G0 in this specific start environment. Once the ectodermal cell in G0 becomes a naïve ESC in cell cycle, it is stuck and unable to become an ectodermal cell in G0 again. However, the naïve ESC in cell cycle is able to become a naïve ESC in G0 if LIF is OFF and the naïve ESC in G0 is able to become a naïve ESC in cell cycle if LIF is turned back ON. In the beginning of the time course model the ectodermal cell in G0 is exhibited by OFF pluripotency nodes and OFF cell cycle nodes. Once the ectodermal cell in G0 is placed in a LIF ON environment, the pluripotency nodes and cell cycle nodes turn ON, which accurately demonstrated the behavior of a naïve ESC in cell cycle. Once LIF turns back OFF, the pluripotency nodes remain ON while the cell cycle nodes turn OFF, illustrating a naïve ESC in G0.

A similar scenario occurred in the when analyzing changes in 2i in the BMP OFF condition. In this condition, if a LIF OFF GF_High ON 2i OFF environment produced an ectodermal cell in cell cycle and a LIF OFF GF_High ON 2i ON environment produced a Differentiated ESC/ Ectodermal ESC/ Naïve ESC in G0 state. A Differentiated ESC/ Naïve ESC in G0 state would become an Ectodermal cell in cell cycle if 2i was removed. The neuroectodermal cell in cell cycle is incapable of reverting back to a Differentiated ESC/ Naïve ESC in G0 state if 2i is reintroduced. However, the neuroectodermal cells can toggle back and forth by being in G0 if 2i is present and in cell cycle if 2i is absent.

The last major change between the model with the original Zic1_3 gate and the new Zic1_3 gate was illustrated by changes in cell phenotype based on BMP expression. If BMP was OFF, an ectodermal cell in cell cycle would arise in the GF_High 2i OFF LIF OFF environment. In that same condition, if BMP was on a Differentiated ESC/ Ectodermal ESC/ Naïve ESC in G0 phenotype would arise. If BMP was removed from the Differentiated ESC/ Ectodermal ESC/

Naïve ESC in G0 state in G0 phenotype, the cell would differentiate into a neuroectodermal cell in cell cycle, but the ectodermal cell in cell cycle could not revert back to the Differentiated ESC/ Naïve ESC in G0 phenotypes if BMP once again became present. However, if BMP was turned back ON in a neuroectodermal cell in cell cycle, it could revert back to an ectodermal cell in G0 phenotype.

DISCUSSION

This model exhibits an array of distinct, yet connected, phenotypes of neural as well as embryonic stem cells. Our model produced an array of information regarding phenotypic transitions triggered by changes in the cellular environment, some of which turned out to be somewhat unexpected. These unexpected phenotypic changes point to flaws or unexplored boundaries in our current understanding of the biology of neuronal specification. This model is the first stride in an effort to understanding how early specific cell types in the neuronal lineage can change their molecular and phenotypic behavior, as various signaling pathways and transcription factors contributing to proliferation and differentiation converge on them.

Modeling proliferation and differentiation of neurodevelopmental phenotypes

To test our model, we established a stable embryonic stem cell state to serve as the base phenotype for further experimentation. This ESC model was the sampled to produce an array of phenotypic attractor states of neurodevelopmental phenotypes (ESC, Differentiated, neuroectoderm), as well as distinct cell cycle behaviors (G0 or Cell Cycle) in different combinations of environmental inputs (BMP, LIF, GF, 2i). The model produced phenotypes that were consistent with our expectations from the experimental literature. When looking at the responses of these phenotypes to environmental change, however, there were a few interesting cases where the model was unable to correctly match the way certain cells differentiated, and locked into their final state. At the molecular level, the main root of these interesting differentiative anomalies is the lack of a locking mechanism/pathway that would prevent a differentiated cell from becoming either naïve or less differentiated in the presence of LIF and/or

2i. This lack reflects our inability to finds any hint of these molecular mechanisms in the literature.

We were able to produce these findings by looking at surprising connections across phenotypic changes, based on a manipulating the presence or absence of a single environmental stimulus. Taking a closer look at the time course of molecular changes produced by transient pulses of environmental input signals, we were able to understand the precise signal propagation by which phenotype changes occur in our model. We mainly looked at markers that are involved in pluripotency and the cell cycle. Here we were able to determine whether the cell was in a naïve or differentiated state, as well as whether it was in G0 or undergoing a cell cycle.

Using our model, we were also able to determine general rules or characteristics of each input stimulus's impact on a cell's molecular and phenotypic expression. For example, if growth factor was absent, the cell can only live if LIF is present, (which is consistent with known ESC biology). In these cases, the cell remains naïve regardless of 2i or BMP expression. However, although these ESCs were unable to become a terminally differentiated with no way back to the ESC state, they were able to undergo cell cycle as expected. Therefore, we could determine that the presence of growth factors has a prominent role in cell type determination, due to the fact that ESCs could only differentiate if mitogens were present to activate ERK signaling.

Furthermore, using only the known molecular connections leading to Zic1/3 activation (i.e., the original Zic1_3 gate), we were able to reproduce the fact that a self-renewing neuroectoderm (i.e., in cell cycle) was only able to arise in the NO BMP environment. In order for this to happen, GF_High needed to be ON and 2i needed to be OFF (LIF's expression was inconsequential). In the BMP ON environment, the cell could not phenotypically become anything more than a differentiated cell in G0. In both models, BMP needs to be absent for initial

entry kick into the neuroectodermal lineage. If BMP is present, only transitions of the naïve state or differentiation to a cell phenotype in G0 can happen. Moreover, GF_High needs to be present for transitions into the neuroectodermal phenotype to occur. When the Zic1_3 gate was a modified to sustain its own activity, the neuroectodermal cells in G0 could additionally be modeled, but only LIF and 2i were absent. This was due to the fact that we are still missing critical links that turn the ESC core circuitry off in the presence of neuronal lineage factors.

In order to further comprehend these intriguing phenotype changes, I propose a further series *in silico* analyses. I propose expanding the model and incorporating more nodes and mechanisms to further understand the pathways and environmental stimuli present, in order to obtain a more comprehensive molecular model of the phenotypes of interest. I predict that two critical links must be present to create more specific cell phenotypes, links we could not find in current literature. First, it would be beneficial to determine the mechanisms that sustain Zic1/3 expression in the presence of BMP at later points in development. Second, it would be important to determine what locks off the ESC pluripotency circuit, preventing its reactivation in ESC medium (i.e., LIF and 2i).

A modular approach to modeling embryonic stem cell intracellular regulation

The model was able to exhibit and synthesize previously established phenotypic and molecular characteristics of the early, cellular stages of neurodevelopment. As were modeling Naïve ESC's, differentiated cells and neuroectodermal cells in either G0 or in a cell cycle (or dead), it was intriguing to see how coordinated phenotypic changes could arise based on the manipulation of a single environment. Using the 4D graph analyses of each individual input's effect, we were able to see how the phenotypes are synergistically connected. Using the time course analyses, we could visually follow the molecular changes that occur within each module

of the network, giving rise to these phenotypic changes. Using these two types of analysis, we were able to categorize the model's time-dependent predictions in terms of cell type, cell cycle/G0 state, or apoptosis.

Weaknesses of the current ESC neuro-differentiation model

Before our model is ready to aid experimental research, some of its weaknesses need to be addressed. First, my model is completely unable to lock a neuroectodermal cell into its differentiated state. This was evident as the neuroectodermal cell phenotypes were able to revert back to naïve, or generic differentiated states. A cellular locking loop/mechanism to prevent phenotypic movement back to these previous states is sorely needed, but not covered by current literature. The inclusion of self-sustaining lineage specific factors could serve as a mechanism to prevent a phenotype from reverting back to a less differentiated state, as observed in a model studying differentiated endoderm and trophectoderm cells (Auerbach, 2017). Ideally, the pluripotency circuit should never be completely activated past the Naïve ESC state. The cell cycle circuit can remain engaged as the cell moves forward in its development towards a fully mature neuron, but the full pluripotency network should never be on again.

It is important to acknowledge that some of these issues are not due to flaws in the construction of the model. The literature regarding this locking mechanism is nonexistent. *In vitro*, experiments have been conducted where a differentiated cell could be forced to revert to previous cell types through the application of reprogramming transcription factors (Takahashi & Yamanaka, 2006), but there isn't a clear idea as to why, exactly, these cell don't revert back to a previous state in the first place.

Lastly, it is difficult to distinguish the precise signals that specify non-neural ectoderm vs. the neuroectoderm. We know that the ectoderm arises in the presence of BMP, and soon thereafter the neuroectoderm arises in its absence (Figure 6). In this model, we assumed that the neuroectoderm could be triggered from ESCs by the absence of BMP signaling, together with a loss of pluripotency-maintaining signals (LIF and 2i). In reality, there must be other factors present that further distinguish, and separately lock in these two cell lineages.

Future directions

Due to the unknowns detailed above, building on our current model to accurately reproduce other neural lineages such as neural crest cells is a considerable challenge. That said, expanding the model to include other neurodevelopmental signaling pathways such as Notch and Sonic Hedgehog (SHH) signaling could lead to a more comprehensive description of neuro-differentiation and provide the ability to model more cell types. Once the model is expanded (and its inability to maintain differentiation has been overcome), *in vitro* studies could be designed to track cellular responses to some of the transient stimuli where the model predicts irreversible phenotype changes.

In conclusion, Boolean modeling is a great instrument for testing assumptions about molecular and environmental changes, in terms of their ability to generate robust phenotypes and cell state transitions. It is also powerful for starkly exposing gaps in our current knowledge, especially regarding synergies between environmental inputs. In our case, the lack of lock-in mechanisms that keeps neuroectodermal cell from undifferentiating was a real surprise. It stands in sharp contrast with our knowledge of the mechanisms that lock endodermal or trophoblast cells into their final state (the subject of an Independent Study thesis by Micah Auerbach, 2017).

Moving forward, Boolean modeling lays the groundwork for future *in silico* models, and analyses leading to better integration between *in silico* and wet lab research. Furthermore it can push boundaries of current understanding and eventually help unearth non-intuitive behavior cells undergo through development.

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- Nathan Sundheimer

LITERATURE CITED

Amini, S., Fathi, F., Mobalegi, J., Sofimajidpour, H., & Ghadimi, T. (2014). The expressions of stem cell markers: Oct4, Nanog, Sox2, nucleostemin, Bmi, Zfx, Tcl1, Tbx3, Dppa4, and Esrrb in bladder, colon, and prostate cancer, and certain cancer cell lines. *Anatomy & Cell Biology*, *47*(1), 1–11.

Aruga, J., & Mikoshiba, K. (2011). Role of BMP, FGF, calcium signaling, and Zic proteins in vertebrate neuroectodermal differentiation. *Neurochemical Research*, *36*(7), 1286–1292.

Arnoult, D., Gaume, B., Karbowski, M., Sharpe, J. C., Cecconi, F., and Youle, R. J. (2003) Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J 22, 4385– 4399.

Auerbach, M. (2017) A Boolean Model of Early Stem Cell Fate Decisions. *College of Wooster*.

Berg, D. L. C. van den, Zhang, W., Yates, A., Engelen, E., Takacs, K., Bezstarosti, K., … Poot, R. A. (2008). Estrogen-Related Receptor Beta Interacts with Oct4 To Positively Regulate Nanog Gene Expression. *Molecular and Cellular Biology*, *28*(19), 5986–5995.

Beurel, E., and Jope, R. S. (2006) The Paradoxical Pro- and Anti-apoptotic Actions of GSK3 in the Intrinsic and Extrinsic Apoptosis Signaling Pathways. Prog Neurobiol 79, 173– 189.

Butz, K., Geisen, C., Ullmann, A., Zentgraf, H., & Hoppe-Seyler, F. (1998). Uncoupling of p21WAF1/CIP1/SDI1 mRNA and protein expression upon genotoxic stress. *Oncogene*, *17*(6), 781.

Cantley, L. C. (2002) The phosphoinositide 3- kinase pathway. Science 296, 1655– 1657.

Castellano, E., and Downward, J. (2010) Role of RAS in the regulation of PI 3- kinase. Curr. Top. Microbiol. Immunol. 346, 143– 169.

Chen, R. H., Waters, J. C., Salmon, E. D., and Murray, A. W. (1996) Association of spindle assembly checkpoint component XMAD2 with unattached kinetochores. Science 274, 242–246.

Cheng, J.-G., Chen, J. R., Hernandez, L., Alvord, W. G., & Stewart, C. L. (2001). Dual control of LIF expression and LIF receptor function regulate Stat3 activation at the onset of uterine receptivity and embryo implantation. *Proceedings of the National Academy of Sciences*, *98*(15), 8680–8685.

Cherepanova, O. A., Gomez, D., Shankman, L. S., Swiatlowska, P., Williams, J., Sarmento, O. F., … Owens, G. K. (2016). Activation of the pluripotency factor OCT4 in smooth muscle cells is atheroprotective. *Nature Medicine*, *22*(6), 657–665.

Chew, J.-L., Loh, Y.-H., Zhang, W., Chen, X., Tam, W.-L., Yeap, L.-S., … Ng, H.-H. (2005). Reciprocal Transcriptional Regulation of Pou5f1 and Sox2 via the Oct4/Sox2 Complex in Embryonic Stem Cells. *Molecular and Cellular Biology*, *25*(14), 6031–6046.

Chickarmane, V., Olariu, V., & Peterson, C. (2012). Probing the role of stochasticity in a model of the embryonic stem cell – heterogeneous gene expression and reprogramming efficiency. *BMC Systems Biology*, *6*, 98.

Clohessy, J. G., Zhuang, J., de Boer, J., Gil-Gómez, G., and Brady, H. J. M. (2006) Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor- mediated apoptosis. J. Biol. Chem. 281, 5750– 5759.

Connell-Crowley, L., Harper, J. W., and Goodrich, D. W. (1997) Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site- specific phosphorylation. Mol. Biol. Cell 8, 287–301.

Coqueret, O. (2003) New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol. 13, 65–70.

Coverley, D., Laman, H., and Laskey, R. A. (2002) Distinct roles for cyclins E and A during DNA replication complex assembly and activation. Nat. Cell Biol. 4, 523–528.

Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785– 789.

Croxton, R., Ma, Y., Song, L., Haura, E. B., and Cress, W. D. (2002) Direct repression of the Mcl- 1 promoter by E2F1. Oncogene 21, 1359– 1369.

Daksis, J. I., Lu, R. Y., Facchini, L. M., Marhin, W. W., and Penn, L. J. (1994) Myc induces cyclin D1 expression in the absence of de novo protein synthesis and links mitogen-stimulated signal transduction to the cell cycle. Oncogene 9, 3635–3645.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Akt phosphorylation of BAD couples survival signals to the cell- intrinsic death machinery. Cell 91, 231–241.

De Boer, L., Oakes, V., Beamish, H., Giles, N., Stevens, F., Somodevilla-Torres, M., … Gabrielli, B. (2008). Cyclin A/cdk2 coordinates centrosomal and nuclear mitotic events. *Oncogene*, *27*(31), 4261–4268.

DeGregori, J., Kowalik, T., & Nevins, J. R. (1995). Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Molecular and Cellular Biology*, *15*(8), 4215–4224.

Deibler, R. W., and Kirschner, M. W. (2010) Quantitative reconstitution of mitotic CDK1 activation in somatic cell extracts. Mol. Cell 37, 753–767.

Deritei, D., Aird, W. C., Ercsey-Ravasz, M., and Regan, E. R. (2016) Principles of dynamical modularity in biological regulatory networks. *Sci. Rep. 6*, 21957.

Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., … Martinou, J.-C. (1999). Bid-induced Conformational Change of Bax Is Responsible for Mitochondrial Cytochrome c Release during Apoptosis. *The Journal of Cell Biology*, *144*(5), 891–901.

Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) X- linked IAP is a direct inhibitor of cell-death proteases. Nature 388, 300–304.

Ding, Q., Huo, L., Yang, J.-Y., Xia, W., Wei, Y., Liao, Y., Chang, C.-J., Y ang, Y., Lai, C.-C., Lee, D.- F., Yen, C.-J., Chen, Y .-J. R., Hsu, J.-M., Kuo, H.-P., Lin, C.-Y., Tsai, F.-J., Li, L.-Y., Tsai, C.-H., and Hung, M.-C. (2008) Down- regulation of myeloid cell leukemia-1 through inhibiting Erk/Pin 1 pathway by sorafenib facilitates chemosensitization in breast cancer. Cancer Res. 68, 6109–6117.

Dlugosz, P. J., Billen, L. P., Annis, M. G., Zhu, W., Zhang, Z., Lin, J., Leber, B., and Andrews, D. W. (2006) Bcl-2 changes conformation to inhibit Bax oligomerization. EMBO J 25, 2287– 2296.

Domchek, S. M., Auger, K. R., Chatterjee, S., Burke, T. R., and Shoelson, S. E. (1992) Inhibition of SH2 domain/phosphoprot ein association by a nonhydrolyzable phosphonopeptide. Biochemistry 31, 9865–9870.

Domínguez-Kelly, R., Martín, Y., Koundrioukoff, S., Tanenbaum, M. E., Smits, V. A. J., Medema, R. H., Debatisse, M., and Freire, R. (2011) Wee1 controls genomic stability during replication by regulating the Mus81- Eme1 endonuclease. J Cell Biol 194, 567–579.

Dong, P., Maddali, M. V., Srimani, J. K., Thélot, F., Nevins, J. R., Mathey-Prevot, B., and You, L. (2014) Division of labour between Myc and G1 cyclins in cell cycle commitment and pace control. Nat Commun 5, 4750.

Donzelli, M., Squatrito, M., Ganoth, D., Hershko, A., Pagano, M., and Draetta, G. F. (2002) Dual mode of degradation of Cdc25 A phosphatase. EMBO J 21, 4875– 4884.

Dunn, S.-J., Martello, G., Yordanov, B., Emmott, S., & Smith, A. G. (2014). Defining an essential transcription factor program for naive pluripotency. *Science*, *344*(6188), 1156–1160.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) A caspase- activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391, 43–50.

Fan, J., and Bertino, J. R. (1997) Functional roles of E2F in cell cycle regulation. Oncogene 14, 1191–1200.

Farkas, L. M., & Huttner, W. B. (2008). The cell biology of neural stem and progenitor cells and its significance for their proliferation versus differentiation during mammalian brain development. *Current Opinion in Cell Biology*, *20*(6), 707–715.

Ferreira, K. S., Kreutz, C., Macnelly, S., Neubert, K., Haber, A., Bogyo, M., Timmer, J., and Borner, C. (2012) Caspase-3 feeds back on caspase-8, Bid and XIAP in type I Fas signaling in primary mouse hepatocytes. Apoptosis 17, 503– 515.

Festuccia, N., Osorno, R., Halbritter, F., Karwacki-Neisius, V., Navarro, P., Colby, D., … Chambers, I. (2012). Esrrb Is a Direct Nanog Target Gene that Can Substitute for Nanog Function in Pluripotent Cells. *Cell Stem Cell*, *11*(4), 477–490.

Foshay, K. M., & Gallicano, G. I. (2008). Regulation of Sox2 by STAT3 initiates commitment to the neural precursor cell fate. *Stem Cells and Development*, *17*(2), 269–278.

Frazer, C., and Young, P. G. (2012) Phosphorylation Mediated Regulation of Cdc25 Activity, Localization and Stability, in Protein Phosphorylation in Human Health (Huang, C., Ed.). InTech.

Fuentes-Prior, P., and Salvesen, G. S. (2004) The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem. J. 384, 201–232.

Gage, F. H. (2000). Mammalian Neural Stem Cells. *Science*, *287*(5457), 1433.

Gingras, A.-C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebersold, R., and Sonenberg, N. (1999) Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev. 13, 1422– 1437.

Guerrero, A. D., Schmitz, I., Chen, M., and Wang, J. (2012) Promotion of Caspase Activation by Caspase- 9-mediated Feedback Amplification of Mitochondrial Damage. J Clin Cell Immunol 3.

Hagenbuchner, J., Kiechl-Kohlendorfer, U., Obexer, P., and Ausserlechner, M. J. (2013) A novel Mcl1 variant inhibits apoptosis via increased Bim sequestration. Oncotarget 4, 1241– 1252.

Hahn-Windgassen, A., Nogueira, V., Chen, C.-C., Skeen, J. E., Sonenberg, N., and Hay, N. (2005) Akt Activates the Mammalian Target of Rapamycin by Regulating Cellular ATP Level and AMPK Activity. J. Biol. Chem. 280, 32081– 32089.

Hanna, J., Saha, K., Pando, B., van Zon, J., Lengner, C. J., Creyghton, M. P., … Jaenisch, R. (2009). Direct cell reprogramming is a stochastic process amenable to acceleration. *Nature*, *462*(7273), 595–601.

Hamel, A. (2016) A Dynamical Systems Approach to Understanding Synergies Between Thyroid Cancer Mutation. *College of Wooster*.

Harper, J. W., Burton, J. L., and Solomon, M. J. (2002) The anaphase- promoting complex: it's not just for mitosis any more. Genes Dev. 16, 2179– 2206.

Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L. H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., and Swindell, E. (1995) Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell 6, 387– 400.

Heald, R., McLoughlin, M., and McKeon, F. (1993) Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated cdc2 kinase. Cell 74, 463–474.

Helin, K. (1998) Regulation of cell proliferation by the E2F transcription factors. Curr. Opin. Genet. Dev. 8, 28–35.

Herberg, M., & Roeder, I. (2015). Computational modelling of embryonic stem-cell fate control. *Development*, *142*(13), 2250–2260.

Herrup, K., & Yang, Y. (2007). Cell cycle regulation in the postmitotic neuron: oxymoron or new biology? *Nature Reviews Neuroscience*, *8*(5), 368–378.

Hofmann, F., and Livingston, D. M. (1996) Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G1 exit. Genes Dev. 10, 851–861.

Hubbard, S. R., and Miller, W. T. (2007) Receptor tyrosine kinases: mechanisms of activation and signaling. Curr Opin Cell Biol 19, 117– 123.

Jackman, M. R., and Pines, J. N. (1997) Cyclins and the G2/M transition. Cancer Surv. 29, 47–73.

Janesick, A., Abbey, R., Chung, C., Liu, S., Taketani, M., & Blumberg, B. (2013). ERF and ETV3L are retinoic acid-inducible repressors required for primary neurogenesis. *Development*, *140*(15), 3095–3106.

Jiang, X., and Wang, X. (2000) Cytochrome c Promotes Caspase-9 Activation by Inducing Nucleotide Binding to Apaf-1. J. Biol. Chem. 275, 31199–31203.

Johnson, G. L., and Lapadat, R. (2002) Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases. Science 298, 1911– 1912.

Kalimuthu, S., and Se- Kwon, K. (2013) Cell Survival and Apoptosis Signaling as Therapeutic Target for Cancer: Marine Bioactive Compounds. Int J Mol Sci 14, 2334–2354.

Karaïskou, A., Cayla, X., Haccard, O., Jessus, C., and Ozon, R. (1998) MPF amplification in Xenopus oocyte extracts depends on a two-step activation of cdc25 phosphatase. Exp. Cell Res. 244, 491–500.

Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D- dependent kinase CDK4. Genes Dev. 7, 331–342.

Katsuno, Y., Suzuki, A., Sugimura, K., Okumura, K., Zineldeen, D. H., Shimada, M., Niida, H., Mizuno, T., Hanaoka, F., and Nakanishi, M. (2009) Cyclin A-Cdk1 regulates the origin firing program in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 106, 3184– 3189.

Kim, M. O., Kim, S.-H., Cho, Y.-Y., Nadas, J., Jeong, C.-H., Yao, K., … Dong, Z. (2012). ERK1 and ERK2 regulate embryonic stem cell self-renewal through phosphorylation of Klf4. *Nature Structural & Molecular Biology*, *19*(3), 283–290.

Kitagawa, M., Higashi, H., Takahashi, I. S., Okabe, T., Ogino, H., Taya, Y., … Okuyama, A. (1994). A cyclin-dependent kinase inhibitor, butyrolactone I, inhibits phosphorylation of RB protein and cell cycle progression. *Oncogene*, *9*(9), 2549–2557.

Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G., and Livingston, D. M. (1994) Negative regulation of the growth- promoting transcription factor E2F-1 by a stably bound cyclin A- dependent protein kinase. Cell 78, 161– 172.

Kuwahara, A., Sakai, H., Xu, Y., Itoh, Y., Hirabayashi, Y., & Gotoh, Y. (2014). Tcf3 Represses Wnt–β-Catenin Signaling and Maintains Neural Stem Cell Population during Neocortical Development. *PLOS ONE*, *9*(5), e94408.

Lawlor, M. A., and Alessi, D. R. (2001) PKB/Akt. Journal of Cell Science 114, 2903–2910.

Li, L., Sun, L., Gao, F., Jiang, J., Yang, Y., Li, C., … Jin, Y. (2010). Stk40 links the pluripotency factor Oct4 to the Erk/MAPK pathway and controls extraembryonic endoderm differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(4), 1402–1407.

Li, F. X., Zhu, J. W., Hogan, C. J., and DeGregori, J. (2003) Defective gene expression, S phase progression, and maturation during hematopoiesis in E2F1/E2F2 mutant mice. Mol. Cell. Biol. 23, 3607–3622.

Li, H., Zhu, H., Xu, C., and Yuan, J. (1998) Cleavage of BID by Caspase 8 Mediates the Mitochondrial Damage in the Fas Pathway of Apoptosis. Cell 94, 491–501.

Liang, Y., Nylander, K. D., Yan, C., and Schor, N. F. (2002) Role of caspase 3- dependent Bcl-2 cleavage in potentiation of apoptosis by Bcl-2. Mol. Pharmacol. 61, 142–149.

Lin, C.-J., Malina, A., and Pelletier, J. (2009) c-Myc and eIF4F Constitute a Feedforward Loop That Regulates Cell Growth: Implications for Anticancer Therapy. Cancer Res 69, 7491–7494.

Maehr, R., Chen, S., Snitow, M., Ludwig, T., Yagasaki, L., Goland, R., … Melton, D. A. (2009). Generation of pluripotent stem cells from patients with type 1 diabetes. *Proceedings of the National Academy of Sciences*, *106*(37), 15768–15773.

Magiera, M. M., Gueydon, E., and Schwob, E. (2014) DNA replication and spindle checkpoints cooperate during S phase to delay mitosis and preserve genome integrity. J Cell Biol 204,

165– 175.

Mailand, N., Falck, J., Lukas, C., Syljuâsen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000) Rapid destruction of human Cdc25A in response to DNA damage. Science 288, 1425–1429.

Manchado, E., Eguren, M., and Malumbres, M. (2010) The anaphase- promoting complex/cyclosome (APC/C): cell-cycle- dependent and - independent functions. Biochem. Soc. Trans. 38, 65– 71.

Marchal, L., Luxardi, G., Thomé, V., & Kodjabachian, L. (2009). BMP inhibition initiates neural induction via FGF signaling and Zic genes. *Proceedings of the National Academy of Sciences*, *106*(41), 17437–17442.

Margolis, B., and Skolnik, E. Y. (1994) Activation of Ras by receptor tyrosine kinases. J. Am. Soc. Nephrol. 5, 1288– 1299.

Martello, G., Sugimoto, T., Diamanti, E., Joshi, A., Hannah, R., Ohtsuka, S., … Smith, A. (2012). Esrrb Is a Pivotal Target of the Gsk3/Tcf3 Axis Regulating Embryonic Stem Cell Self-Renewal. *Cell Stem Cell*, *11*(4), 491–504.

Mendoza, M. C., Er, E. E., and Blenis, J. (2011) The Ras- ERK and PI3K- mTOR Pathways: Cross-talk and Compensation. Trends Biochem Sci 36, 320–328.

Mérino, D., Khaw, S. L., Glaser, S. P., Anderson, D. J., Belmont, L. D., Wong, C., Yue, P., Robati, M., Phipson, B., Fairlie, W. D., Lee, E. F., Campbell, K. J., Vandenberg, C. J., Cory, S., Roberts, A. W., Ludlam, M. J. C., Huang, D. C. S., and Bouillet, P. (2012) Bcl-2, Bcl-xL, and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. Blood 119, 5807–5816.

Metcalfe, C., & Bienz, M. (2011). Inhibition of GSK3 by Wnt signalling--two contrasting models. *Journal of Cell Science*, *124*(Pt 21), 3537–3544.

Mitchell, K. O., and El-Deiry, W. S. (1999) Overexpression of c- Myc inhibits p21W AF1/CIP1 expression and induces S-phase entry in 12-O- tetradecanoylphorbol- 13-acetate (TPA)-sensitive human cancer cells. Cell Growth Differ. 10, 223–230.

Mitra, J., & Enders, G. H. (2004). Cyclin A/Cdk2 complexes regulate activation of Cdk1 and Cdc25 phosphatases in human cells. *Oncogene*, *23*(19), 3361–3367.

Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A., and Pagano, M. (1999) Ubiquitination of p27 is regulated by Cdk- dependent phosphorylation and trimeric complex formation. Genes Dev 13, 1181–1189.

Muñoz Descalzo, S., Rué, P., Garcia-Ojalvo, J., & Martinez Arias, A. (2012). Correlations between the levels of Oct4 and Nanog as a signature for naïve pluripotency in mouse embryonic stem cells. *Stem Cells (Dayton, Ohio)*, *30*(12), 2683–2691.

Muñoz-Pinedo, C., Guío-Carrión, A., Goldstein, J. C., Fitzgerald, Newmeyer, D. D., and Green, D. R. (2006) Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. PNAS 103, 11573–11578.

Murphy, K. M., Ranganathan, V ., Farnsworth, M. L., Kavallaris, M., and Lock, R. B. (2000) Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. Cell Death Differ. 7, 102– 111.

Nezi, L., and Musacchio, A. (2009) Sister chromatid tension and the spindle assembly checkpoint. Current Opinion in Cell Biology 21, 785–795.

Novák, B., and Tyson, J. J. (2004) A model for restriction point control of the mammalian cell cycle. J. Theor. Biol. 230, 563–579.

Niwa, H., Ogawa, K., Shimosato, D., & Adachi, K. (2009). A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature*, *460*(7251), 118–122.

Ohtani, K., DeGregori, J., and Nevins, J. R. (1995) Regulation of the cyclin E gene by transcription factor E2F1. Proc. Natl. Acad. Sci. U.S.A. 92, 12146–12150.

Ohtsuka, S., Nakai-Futatsugi, Y., & Niwa, H. (2015). LIF signal in mouse embryonic stem cells. *JAK-STAT*, *4*(2).

Oswald, F., Lovec, H., Möröy, T., and Lipp, M. (1994) E2F- dependent regulation of human MYC: trans-activation by cyclins D1 and A overrides tumour suppressor protein functions. Oncogene 9, 2029–2036.

Pan, G., & Thomson, J. A. (2007). Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Research*, *17*(1), 42–49.

Peres, J., Mowla, S., & Prince, S. (2015). The T-box transcription factor, TBX3, is a key substrate of AKT3 in melanomagenesis. *Oncotarget*, *6*(3), 1821–1833.

Poser, S. W., Chenoweth, J. G., Colantuoni, C., Masjkur, J., Chrousos, G., Bornstein, S. R., … Androutsellis-Theotokis, A. (2015). Concise Review: Reprogramming, Behind the Scenes: Noncanonical Neural Stem Cell Signaling Pathways Reveal New, Unseen Regulators of Tissue Plasticity With Therapeutic Implications. *Stem Cells Translational Medicine*, *4*(11), 1251–1257.

Pucci, B., Indelicato, M., Paradisi, V., Reali, V ., Pellegrini, L., Aventaggiato, M., Karpinich, N. O., Fini, M., Russo, M. A., Farber, J. L., and Tafani, M. (2009) ERK-1 MAP kinase prevents TNF-induced apoptosis through bad phosphorylation and inhibition of Bax translocation in HeLa Cells. J. Cell. Biochem. 108, 1166– 1174.

Qiao, X., Zhang, L., Gamper, A. M., Fujita, T., and Wan, Y. (2010) APC/C- Cdh1: from cell cycle to cellular differentiation and genomic integrity. Cell Cycle 9, 3904– 3912.

Qin, S., & Zhang, C.-L. (2012). Role of Krüppel-Like Factor 4 in Neurogenesis and Radial Neuronal Migration in the Developing Cerebral Cortex. *Molecular and Cellular Biology*, *32*(21), 4297–4305.

Quinlan, L. (2011). Signaling pathways in mouse embryo stem cell self-renewal. In M. S. Kallos (Ed.), *Embryonic Stem Cells – Basic Biology to Bioengineering* (pp. 283-304). Rijeka, Croatia: InTech.

Radzisheuskaya, A., Le Bin Chia, G., dos Santos, R. L., Theunissen, T. W., Castro, L. F. C., Nichols, J., & Silva, J. C. R. (2013). A defined Oct4 level governs cell state transitions of pluripotency entry and differentiation into all embryonic lineages. *Nature Cell Biology*, *15*(6), 579–590.

Rape, M., and Kirschner, M. W. (2004) Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry. Nature 432, 588–595.

Real, S., Meo-Evoli, N., Espada, L., and Tauler, A. (2011) E2F1 Regulates Cellular Growth by mTORC1 Signaling. PLOS ONE 6, e16163.

Reddy, S. K., Rape, M., Margansky, W. A., and Kirschner, M. W. (2007) Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. Nature 446, 921–925.

Regan, E. R., and Aird, W. C. (2012) Dynamical systems approach to endothelial heterogeneity. *Circ. Res. 111*, 110–130.

Renault, T. T., Floros, K. V., and Chipuk, J. E. (2013) BAK/BAX activation and cytochrome c release assays using isolated mitochondria. Methods 61, 146–155.

Rodda, D. J., Chew, J.-L., Lim, L.-H., Loh, Y.-H., Wang, B., Ng, H.-H., & Robson, P. (2005). Transcriptional regulation of nanog by OCT4 and SOX2. *The Journal of Biological Chemistry*, *280*(26), 24731–24737.

Rosso, S. B., & Inestrosa, N. C. (2013). WNT signaling in neuronal maturation and synaptogenesis. *Frontiers in Cellular Neuroscience*, *7*, 103.

Russell, R., Ilg, M., Lin, Q., Wu, G., Lechel, A., Bergmann, W., … Kleger, A. (2015). A Dynamic Role of TBX3 in the Pluripotency Circuitry. *Stem Cell Reports*, *5*(6), 1155–1170.

Saito, Y., Furukawa, T., Arano, Y., Fujibayashi, Y., and Saga, T. (2010) Basic study on SH2 domain of Grb2 as a molecular probe for detection of RTK activation. Int. J. Oncol. 37, 281–287.

Saha, P., Eichbaum, Q., Silberman, E. D., Mayer, B. J., and Dutta, A. (1997) p21CIP1 and Cdc25A: competition between an inhibitor and an activator of cyclin-dependent kinases. Mol. Cell. Biol. 17, 4338–4345.

Sarosiek, K. A., Chi, X., Bachman, J. A., Sims, J. J., Montero, J., Patel, L., Flanagan, A., Andrews, D. W., Sorger, P., and Letai, A. (2013) BID preferentially activates BAK while BIM preferentially activates BAX, affecting chemotherapy response. Mol. Cell 51, 751–765.

Schmidt, R., & Plath, K. (2012). The roles of the reprogramming factors Oct4, Sox2 and Klf4 in resetting the somatic cell epigenome during induced pluripotent stem cell generation. *Genome Biology*, *13*, 251.

Shimizu, T., Ueda, J., Ho, J. C., Iwasaki, K., Poellinger, L., Harada, I., & Sawada, Y. (2012). Dual inhibition of Src and GSK3 maintains mouse embryonic stem cells, whose differentiation is mechanically regulated by Src signaling. *Stem Cells (Dayton, Ohio)*, *30*(7), 1394–1404.

Silva, J., Barrandon, O., Nichols, J., Kawaguchi, J., Theunissen, T. W., & Smith, A. (2008). Promotion of Reprogramming to Ground State Pluripotency by Signal Inhibition. *PLOS Biology*, *6*(10), e253.

Simon, J. A., and Schreiber, S. L. (1995) Grb2 SH3 binding to peptides from Sos: evaluation of a general model for SH3-ligand interactions. Chem. Biol. 2, 53–60.

Srinivasan, S., Hu, J. S., Currle, D. S., Fung, E. S., Hayes, W. B., Lander, A. D., & Monuki, E. S. (2014). A BMP-FGF Morphogen Toggle Switch Drives the Ultrasensitive Expression of Multiple Genes in the Developing Forebrain. *PLoS Computational Biology*, *10*(2).

Stacey, D. W. (2003) Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. Curr. Opin. Cell Biol. 15, 158–163.

Stennicke, H. R., Jürgensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J., and Salvesen, G. S. (1998) Pro-caspase-3 is a major physiologic target of caspase-8. J. Biol. Chem. 273, 27084–27090.

Sudo, T., Ota, Y., Kotani, S., Nakao, M., Takami, Y., Takeda, S., & Saya, H. (2001). Activation of Cdh1-dependent APC is required for G1 cell cycle arrest and DNA damage-induced G2 checkpoint in vertebrate cells. *The EMBO Journal*, *20*(22), 6499–6508.

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, *126*(4), 663–676.

Ueberham, U., & Arendt, T. (2013). The Role of Smad Proteins for Development, Differentiation and Dedifferentiation of Neurons.

Vazin, T., & Freed, W. J. (2010). Human embryonic stem cells: derivation, culture, and differentiation: a review. *Restorative Neurology and Neuroscience*, *28*(4), 589–603.

Vigo, E., Müller, H., Prosperini, E., Hateboer, G., Cartwright, P., Moroni, M. C., and Helin, K. (1999) CDC25A phosphatase is a target of E2F and is required for efficient E2F-induced S phase. Mol. Cell. Biol. 19, 6379–6395.

Vivanco, I., and Sawyers, C. L. (2002) The phosphatidylinositol 3- Kinase–AKT pathway in human cancer. Nat Rev Cancer 2, 489– 501.

Vucic, D., Deshayes, K., Ackerly, H., Pisabarro, M. T., Kadkhodayan, S., Fairbrother, W. J., and Dixit, V . M. (2002) SMAC Negatively Regulates the Anti- apoptotic Activity of Melanoma Inhibitor of Apoptosis (ML-IAP). J. Biol. Chem. 277, 12275–12279.

Wang, R.-S., Saadatpour, A., & Albert, R. (2012). Boolean modeling in systems biology: an overview of methodology and applications. *Physical Biology*, *9*(5), 55001.

Wei, Z., Yang, Y., Zhang, P., Andrianakos, R., Hasegawa, K., Lyu, J., … Lu, W. (2009). Klf4 interacts directly with Oct4 and Sox2 to promote reprogramming. *Stem Cells (Dayton, Ohio)*, *27*(12), 2969–2978.

White, C. A., & Nicola, N. A. (2013). SOCS3. *JAK-STAT*, *2*(4).

Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., … Huang, D. C. S. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes & Development*, *19*(11), 1294–1305.

Willmer, T., Hare, S., Peres, J., & Prince, S. (2016). The T-box transcription factor TBX3 drives proliferation by direct repression of the p21WAF1 cyclin-dependent kinase inhibitor. *Cell Division*, *11*.

Yam, C. H., Fung, T. K., and Poon, R. Y. C. (2002) Cyclin A in cell cycle control and cancer. Cell. Mol. Life Sci. 59, 1317– 1326.

Yang, K., Guo, Y., Stacey, W. C., Harwalkar, J., Fretthold, J., Hitomi, M., & Stacey, D. W. (2006). Glycogen synthase kinase 3 has a limited role in cell cycle regulation of cyclin D1 levels. *BMC Cell Biology*, *7*, 33.

Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) Bad, a heterodimeric partner for Bcl-xL and Bcl-2, displaces bax and promotes cell death. Cell 80, 285–291.

Yi, X., Yin, X.-M., and Dong, Z. (2003) Inhibition of Bid- induced Apoptosis by Bcl-2 tBid INSERTION, Bax TRANSLOCA TION, AND Bax/Bak OLIGOMERIZA TION SUPPRESSED. J. Biol. Chem. 278, 16992–16999.

Zephyris. (2010). *The first few weeks of embryogenesis in humans. Beginning at the fertilised egg, ending with the closing of the neural tube.*

Zernicka-Goetz, M., Morris, S. A., & Bruce, A. W. (2009). Making a firm decision: multifaceted regulation of cell fate in the early mouse embryo. *Nature Reviews Genetics*, *10*(7), 467–477.

Zhang, J., & Li, L. (2005). BMP signaling and stem cell regulation. *Developmental Biology*, *284*(1), 1–11.

Zhu, J., Blenis, J., and Yuan, J. (2008) Activation of PI3K/Akt and MAPK pathways regulates Myc-mediated transcription by phosphorylating and promoting the degradation of Mad1. Proc. Natl. Acad. Sci. U.S.A. 105, 6584– 6589.

SUPPLEMENTARY INFORMATION

Supplementary Table S1. Cell Growth Links

Node	Logic Gate	Link	Reference
AKT	PDK1 AND PIP3	PIP3 →	Lawlor, M. A., and Alessi, D. R. (2001) PKB/Akt. Journal of Cell Science 114, 2903–2910.
		PDK1 →	
BRAF	Ras	Ras →	Johnson, G. L., and Lapadat, R. (2002) Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases. Science 298, 1911–1912.
eIF4E	mTORC1	mTORC1 →	Gingras, A.-C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebersold, R., and Sonenberg, N. (1999) Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev. 13, 1422–1437.

ERK	MEK AND NOT BMP	BMP –|	Zhang, J., & Li, L. (2005). BMP signaling and stem cell regulation. *Developmental Biology*, *284*(1), 1–11.
		MEK →	Johnson, G. L., and Lapadat, R. (2002) Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases. Science 298, 1911–1912.
GF_High	GF_High	GF_High →	N/A
GF	GF OR GF_High	GF_High →	N/A
		GF →	N/A
Grb2	GF_High AND RTK	GF_High →	Saito, Y., Furukawa, T., Arano, Y., Fujibayashi, Y., and Saga, T. (2010) Basic study on SH2 domain of Grb2 as a molecular probe for detection of RTK activation. Int. J. Oncol. 37, 281–287.
		RTK →	

GSK3	NOT AKT AND NOT Two_i	Two_i –|	Shimizu, T., Ueda, J., Ho, J. C., Iwasaki, K., Poellinger, L., Harada, I., & Sawada, Y. (2012). Dual inhibition of Src and GSK3 maintains mouse embryonic stem cells, whose differentiation is mechanically regulated by Src signaling. *Stem Cells (Dayton, Ohio)*, *30*(7), 1394–1404.
		AKT –|	Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789.
MEK	BRAF AND NOT Two_i	Two_i –|	Silva, J., Barrandon, O., Nichols, J., Kawaguchi, J., Theunissen, T. W., & Smith, A. (2008). Promotion of Reprogramming to Ground State Pluripotency by Signal Inhibition. *PLOS Biology*, *6*(10), 253.

		BRAF →	Johnson, G. L., and Lapadat, R. (2002) Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases. Science 298, 1911–1912.
mTORC1	(AKT OR E2F1) AND (AKT OR ERK) AND (E2F1 OR ERK)	ERK →	Mendoza, M. C., Er, E. E., and Blenis, J. (2011) The Ras-ERK and PI3K-mTOR Pathways: Cross-talk and Compensation. Trends Biochem Sci 36, 320–328.
		E2F1 →	Real, S., Meo-Evoli, N., Espada, L., and Tauler, A. (2011) E2F1 Regulates Cellular Growth by mTORC1 Signaling. PLOS ONE 6, e16163.
		AKT →	Hahn-Windgassen, A., Nogueira, V., Chen, C.-C., Skeen, J. E., Sonenberg, N., and Hay, N. (2005) Akt Activates the Mammalian Target of Rapamycin by Regulating Cellular ATP Level and AMPK Activity. J. Biol. Chem. 280, 32081– 32089.

PDK1	PIP3	PIP3 →	Vivanco, I., and Sawyers, C. L. (2002) The phosphatidylinositol 3- Kinase–AKT pathway in human cancer. Nat Rev Cancer 2, 489– 501.
PI3K	LIFR OR RTK OR Ras	LIFR →	Ohtsuka, S., Nakai-Futatsugi, Y., & Niwa, H. (2015). LIF signal in mouse embryonic stem cells. *JAK-STAT*, *4*(2).
		Ras →	Castellano, E., and Downward, J. (2010) Role of RAS in the regulation of PI 3- kinase. Curr. Top. Microbiol. Immunol. 346, 143–169.
		RTK →	Domchek, S. M., Auger, K. R., Chatterjee, S., Burke, T. R., and Shoelson, S. E. (1992) Inhibition of SH2 domain/phosphoprotein association by a nonhydrolyzable phosphonopeptide. Biochemistry 31, 9865–9870.

PIP3	PI3K	PI3K →	Cantley, L. C. (2002) The phosphoinositide 3-kinase pathway. Science 296, 1655–1657.
Ras	Grb2 OR SOS	Grb2 →	Margolis, B., and Skolnik, E. Y. (1994) Activation of Ras by receptor tyrosine kinases. J. Am. Soc. Nephrol. 5, 1288– 1299.
		SOS →	
RTK	GF OR GF_High	GF_High →	Hubbard, S. R., and Miller, W. T. (2007) Receptor tyrosine kinases: mechanisms of activation and signaling. Curr Opin Cell Biol 19, 117–123.
		GF →	
SOS	Grb2	Grb2 →	Simon, J. A., and Schreiber, S. L. (1995) Grb2 SH3 binding to peptides from Sos: evaluation of a general model for SH3-ligand interactions. Chem. Biol. 2, 53–60.

Supplementary Table S2. Cell Cycle Links

Node	Logic Gate	Link	Reference
4N_DNA	NOT Cdh1 AND (4N_DNA OR CyclinA) AND (4N_DNA OR Replication)	4N_DNA →	N/A
		Cdh1 –|	Qiao, X., Zhang, L., Gamper, A. M., Fujita, T., and Wan, Y. (2010) APC/C-Cdh1: from cell cycle to cellular differentiation and genomic integrity. Cell Cycle 9, 3904–3912.
		CyclinA →	Katsuno, Y., Suzuki, A., Sugimura, K., Okumura, K., Zineldeen, D. H., Shimada, M., Niida, H., Mizuno, T., Hanaoka, F., and Nakanishi, M. (2009) Cyclin A-Cdk1 regulates the origin firing program in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 106, 3184–3189.
		Replication →	N/A

Cdc20	NOT Cdh1 AND NOT Mad2 AND pAPC	pAPC →	Manchado, E., Eguren, M., and Malumbres, M. (2010) The anaphase-promoting complex/cyclosome (APC/C): cell-cycle-dependent and -independent functions. Biochem. Soc. Trans. 38, 65–71.
		Cdh1 –|	
		Mad2 –|	Reddy, S. K., Rape, M., Margansky, W. A., and Kirschner, M. W. (2007) Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. Nature 446, 921–925.
Cdc25A	(CyclinA OR CyclinE) AND (CyclinA OR E2F1) AND (CyclinE OR E2F1) AND (E2F1 OR NOT Cdh1)	CyclinA →	Frazer, C., and Young, P. G. (2012) Phosphorylation Mediated Regulation of Cdc25 Activity, Localization and Stability, in Protein Phosphorylation in Human Health (Huang, C., Ed.). InTech.
		CyclinE →	

		E2F1 →	Vigo, E., Müller, H., Prosperini, E., Hateboer, G., Cartwright, P., Moroni, M. C., and Helin, K. (1999) CDC25A phosphatase is a target of E2F and is required for efficient E2F-induced S phase. Mol. Cell. Biol. 19, 6379–6395.
		Cdh1 –|	Donzelli, M., Squatrito, M., Ganoth, D., Hershko, A., Pagano, M., and Draetta, G. F. (2002) Dual mode of degradation of Cdc25 A phosphatase. EMBO J 21, 4875–4884.
Cdc25C	(Cdk1 OR CyclinA) AND (CyclinA OR CyclinB)	CyclinA →	Karaïskou, A., Cayla, X., Haccard, O., Jessus, C., and Ozon, R. (1998) MPF amplification in Xenopus oocyte extracts depends on a two-step activation of cdc25 phosphatase. Exp. Cell Res. 244, 491–500.
		Cdk1 →	
		CyclinB →	

Cdh1	NOT CyclinA AND (NOT Cdk1 OR NOT CyclinB) AND pAPC	pAPC →	Sudo, T., Ota, Y., Kotani, S., Nakao, M., Takami, Y., Takeda, S., & Saya, H. (2001). Activation of Cdh1-dependent APC is required for G1 cell cycle arrest and DNA damage-induced G2 checkpoint in vertebrate cells. *The EMBO Journal*, *20*(22), 6499–6508.
		CyclinA –|	Harper, J. W., Burton, J. L., and Solomon, M. J. (2002) The anaphase- promoting complex: it's not just for mitosis any more. Genes Dev. 16, 2179–2206.
		CyclinB –|	
		Cdk1 –|	
Cdk1	(Cdc25C OR Cdk1) AND (Cdc25C OR CyclinA) AND (Cdc25C OR CyclinB) AND (Cdc25C OR NOT Wee1) AND (Cdk1 OR NOT Wee1) AND (CyclinA OR CyclinB)	Wee1 –|	Heald, R., McLoughlin, M., and McKeon, F. (1993) Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated cdc2 kinase. Cell 74, 463–474.
		Cdk1 →	N/A
		Cdc25C →	Jackman, M. R., and Pines, J. N. (1997) Cyclins and the G2/M transition. Cancer Surv. 29, 47– 73.
		CyclinA →	
		CyclinB →	

CyclinA	NOT pAPC AND (Cdc25A OR CyclinA) AND (Cdc25A OR E2F1 OR NOT pRB) AND (CyclinA OR E2F1) AND (NOT Cdh1 OR NOT UbcH10)	pRB –|	Kitagawa, M., Higashi, H., Takahashi, I. S., Okabe, T., Ogino, H., Taya, Y., … Okuyama, A. (1994). A cyclin-dependent kinase inhibitor, butyrolactone I, inhibits phosphorylation of RB protein and cell cycle progression. *Oncogene*, *9*(9), 2549–2557.
		E2F1 →	DeGregori, J., Kowalik, T., & Nevins, J. R. (1995). Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Molecular and Cellular Biology*, *15*(8), 4215–4224.
		Cdc25A →	Katsuno, Y., Suzuki, A., Sugimura, K., Okumura, K., Zineldeen, D. H., Shimada, M., Niida, H., Mizuno, T., Hanaoka, F., and Nakanishi, M. (2009) Cyclin A-Cdk1 regulates the origin firing program in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 106, 3184–3189.

		CyclinA →	N/A
		pAPC –|	Yam, C. H., Fung, T. K., and Poon, R. Y. C. (2002) Cyclin A in cell cycle control and cancer. Cell. Mol. Life Sci. 59, 1317–1326.
		Cdh1 –|	Rape, M., and Kirschner, M. W. (2004) Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry. Nature 432, 588–595.
		UbcH10 –|	
CyclinB	NOT Cdh1 AND (Cdk1 OR CyclinA) AND (CyclinA OR CyclinB) AND (NOT Cdc20 OR NOT pAPC)	Cdk1 →	Mitra, J., & Enders, G. H. (2004). Cyclin A/Cdk2 complexes regulate activation of Cdk1 and Cdc25 phosphatases in human cells. *Oncogene*, *23*(19), 3361–3367.
		CyclinB →	N/A
		CyclinA →	De Boer, L., Oakes, V., Beamish, H., Giles, N., Stevens, F., Somodevilla-Torres, M., … Gabrielli, B. (2008). Cyclin A/cdk2 coordinates centrosomal and nuclear mitotic events. *Oncogene*, *27*(31), 4261–4268.

		pAPC –|	Harper, J. W., Burton, J. L., and Solomon, M. J. (2002) The anaphase- promoting complex: it's not just for mitosis any more. Genes Dev. 16, 2179–2206.
		Cdc20 –|	
		Cdh1 –|	
CyclinD1	NOT CAD AND NOT Replication AND (CyclinD1 OR E2F1 OR NOT GSK3) AND (CyclinD1 OR Myc OR NOT GSK3) AND (CyclinD1 OR NOT p21 OR NOT pRB) AND (E2F1 OR Myc) AND (E2F1 OR NOT p21) AND (Myc OR NOT p21) AND (NOT GSK3 OR NOT p21)	CAD –|	N/A
		Replication –|	Stacey, D. W. (2003) Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. Curr. Opin. Cell Biol. 15, 158–163.
		CyclinD1 →	N/A
		p21 –|	Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L. H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., and Swindell, E. (1995) Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell 6, 387–400.

		GSK3 –|	Yang, K., Guo, Y., Stacey, W. C., Harwalkar, J., Fretthold, J., Hitomi, M., & Stacey, D. W. (2006). Glycogen synthase kinase 3 has a limited role in cell cycle regulation of cyclin D1 levels. *BMC Cell Biology*, *7*, 33.
		pRB –|	Connell-Crowley, L., Harper, J. W., and Goodrich, D. W. (1997) Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol. Biol. Cell 8, 287–301.
		E2F1 →	Fan, J., and Bertino, J. R. (1997) Functional roles of E2F in cell cycle regulation. Oncogene 14, 1191–1200.

		Myc →	Daksis, J. I., Lu, R. Y., Facchini, L. M., Marhin, W. W., and Penn, L. J. (1994) Myc induces cyclin D1 expression in the absence of de novo protein synthesis and links mitogen-stimulated signal transduction to the cell cycle. Oncogene 9, 3635–3645.
CyclinE	E2F1 AND NOT CAD AND NOT p27Kip1 AND NOT pRB AND (Cdh1 OR NOT Metaphase)	CAD –|	N/A
		Cdh1 →	Qiao, X., Zhang, L., Gamper, A. M., Fujita, T., and Wan, Y. (2010) APC/C-Cdh1: from cell cycle to cellular differentiation and genomic integrity. Cell Cycle 9, 3904–3912.
		Metaphase –|	N/A
		P27Kip1 –|	Coqueret, O. (2003) New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol. 13, 65–70.

		pRB –|	(1) Helin, K. (1998) Regulation of cell proliferation by the E2F transcription factors. Curr. Opin. Genet. Dev. 8, 28–35.
		E2F1 →	Ohtani, K., DeGregori, J., and Nevins, J. R. (1995) Regulation of the cyclin E gene by transcription factor E2F1. Proc. Natl. Acad. Sci. U.S.A. 92, 12146–12150.
E2F1	NOT CyclinA AND NOT pRB AND (E2F1 OR Myc)	CyclinA –|	Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G., and Livingston, D. M. (1994) Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell 78, 161–172.
		E2F1 →	N/A

		Myc –|	Dong, P., Maddali, M. V., Srimani, J. K., Thélot, F., Nevins, J. R., Mathey-Prevot, B., and You, L. (2014) Division of labour between Myc and G1 cyclins in cell cycle commitment and pace control. Nat Commun 5, 4750.
		pRB –|	
Mad2	4N_DNA AND Cdk1 AND CyclinB AND NOT Metaphase AND (NOT Cdc20 |OR NOT pAPC)	4N_DNA →	Magiera, M. M., Gueydon, E., and Schwob, E. (2014) DNA replication and spindle checkpoints cooperate during S phase to delay mitosis and preserve genome integrity. J Cell Biol 204, 165– 175.
		Metaphase –|	Reddy, S. K., Rape, M., Margansky, W. A., and Kirschner, M. W. (2007) Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. Nature 446, 921–925.
		pAPC –|	
		Cdc20 –|	

		Cdk1 →	Chen, R. H., Waters, J. C., Salmon, E. D., and Murray, A. W. (1996) Association of spindle assembly checkpoint component XMAD2 with unattached kinetochores. Science 274, 242–246.
		CyclinB →	
Metaphase	4N_DNA AND Cdk1 AND CyclinB AND (NOT Cdc20 OR NOT pAPC)	pAPC –|	N/A
		4N_DNA →	Nezi, L., and Musacchio, A. (2009) Sister chromatid tension and the spindle assembly checkpoint. Current Opinion in Cell Biology 21, 785–795.
		Cdc20 –|	
		Cdk1 →	
		CyclinB →	
Myc	(E2F1 OR ERK OR Klf4) AND (ERK OR Klf4 OR Myc |OR eIF4E)	AKT →	Zhu, J., Blenis, J., and Yuan, J. (2008) Activation of PI3K/Akt and MAPK pathways regulates Myc-mediated transcription by phosphorylating and promoting the degradation of Mad1. Proc. Natl. Acad. Sci. U.S.A. 105, 6584–6589.
		ERK →	
		Myc →	N/A

		eIF4E →	Lin, C.-J., Malina, A., and Pelletier, J. (2009) c-Myc and eIF4F Constitute a Feedforward Loop That Regulates Cell Growth: Implications for Anticancer Therapy. Cancer Res 69, 7491–7494.
		Klf4 →	Schmidt, R., & Plath, K. (2012). The roles of the reprogramming factors Oct4, Sox2 and Klf4 in resetting the somatic cell epigenome during induced pluripotent stem cell generation. *Genome Biology*, *13*, 251.
		E2F1 →	Oswald, F., Lovec, H., Möröy, T., and Lipp, M. (1994) E2F-dependent regulation of human MYC: trans-activation by cyclins D1 and A overrides tumour suppressor protein functions. Oncogene 9, 2029–2036.

p21_mRNA	NOT Myc	Myc –|	Mitchell, K. O., and El-Deiry, W. S. (1999) Overexpression of c-Myc inhibits p21WAF1/CIP1 expression and induces S-phase entry in 12-O-tetradecanoylphorbol-13-acetate (TPA)-sensitive human cancer cells. Cell Growth Differ. 10, 223–230.
p21	NOT CyclinE AND (NOT Cdc25A OR NOT CyclinA) AND p21_mRNA	p21_mRNA →	Butz, K., Geisen, C., Ullmann, A., Zentgraf, H., & Hoppe-Seyler, F. (1998). Uncoupling of p21WAF1/CIP1/SDI1 mRNA and protein expression upon genotoxic stress. *Oncogene*, *17*(6), 781.
		Cdc25A –|	Saha, P., Eichbaum, Q., Silberman, E. D., Mayer, B. J., and Dutta, A. (1997) p21CIP1 and Cdc25A: competition between an inhibitor and an activator of cyclin-dependent kinases. Mol. Cell. Biol. 17, 4338–4345.

		CyclinA –|	Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L. H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., and Swindell, E. (1995) Inhibition of cyclin-dependent kinases by p21. Mol. Biol. Cell 6, 387–400.
		CyclinE –|	
p27Kip1	NOT CyclinD1 AND (NOT Cdk1 OR NOT CyclinB) AND (NOT CyclinA OR NOT CyclinE)	CyclinA –|	Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A., and Pagano, M. (1999) Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev 13, 1181–1189.
		Cdk1 –|	
		CyclinB –|	
		CyclinD1 –|	
		CyclinE –|	
pAPC	(Cdc20 OR Cdk1) AND (Cdc20 OR CyclinB) AND (Cdk1 OR pAPC) AND (CyclinB OR pAPC)	pAPC →	Qiao, X., Zhang, L., Gamper, A. M., Fujita, T., and Wan, Y. (2010) APC/C-Cdh1: from cell cycle to cellular differentiation and genomic integrity. Cell Cycle 9, 3904–3912.
		Cdc20 →	
		Cdk1 →	
		CyclinB →	

pRB	NOT CyclinD1 AND (NOT Cdk1 OR NOT CyclinB) AND (NOT CyclinA OR p27Kip1) AND (NOT CyclinE OR p27Kip1)	CyclinA –|	Hofmann, F., and Livingston, D. M. (1996) Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G1 exit. Genes Dev. 10, 851–861.
		Cdk1 –|	
		CyclinB –|	
		CyclinD1 –|	Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. 7, 331–342.
		CyclinE –|	Novák, B., and Tyson, J. J. (2004) A model for restriction point control of the mammalian cell cycle. J. Theor. Biol. 230, 563–579.
		p27Kip1 →	Coqueret, O. (2003) New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol. 13, 65–70.

Replication	NOT 4N_DNA AND NOT CAD AND (Cdc25A OR CyclinE OR E2F1) AND (Cdc25A OR Replication) AND (CyclinE OR Replication) AND (E2F1 OR Replication)	CAD –|	Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391, 43–50.
		4N_RNA –|	N/A
		Replication →	N/A
		E2F1 →	Li, F. X., Zhu, J. W., Hogan, C. J., and DeGregori, J. (2003) Defective gene expression, S phase progression, and maturation during hematopoiesis in E2F1/E2F2 mutant mice. Mol. Cell. Biol. 23, 3607–3622.
		CyclinE →	Coverley, D., Laman, H., and Laskey, R. A. (2002) Distinct roles for cyclins E and A during DNA replication complex assembly and activation. Nat. Cell Biol. 4, 523–528.

		Cdc25A →	Mailand, N., Falck, J., Lukas, C., Syljuâsen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000) Rapid destruction of human Cdc25A in response to DNA damage. Science 288, 1425–1429.
UbcH10	(Cdc20 OR CyclinA OR CyclinB OR NOT Cdh1) AND (NOT Cdh1 OR UbcH10)	Cdh1 –|	Rape, M., and Kirschner, M. W. (2004) Autonomous regulation of the anaphase-promoting complex couples mitosis to S-phase entry. Nature 432, 588–595.
		Cdc20 →	
		UbcH10 →	
		CyclinA →	
		CyclinB →	
Wee1	(CAD OR NOT Cdk1 OR NOT CyclinA) AND (CAD OR NOT Cdk1OR NOT CyclinB) AND (CAD OR Replication)	CAD →	Domínguez-Kelly, R., Martín, Y., Koundrioukoff, S., Tanenbaum, M. E., Smits, V. A. J., Medema, R. H., Debatisse, M., and Freire, R. (2011) Wee1 controls genomic stability during replication by regulating the Mus81-Eme1 endonuclease. J Cell Biol 194, 567–579.
		Replication →	

		Cdk1 –|	Deibler, R. W., and Kirschner, M. W. (2010) Quantitative reconstitution of mitotic CDK1 activation in somatic cell extracts. Mol. Cell 37, 753–767.
		CyclinA –|	
		CyclinB –|	

Supplementary Table S3: Apoptosis Links

Node	Logic Gate	Link	Reference
BAD	NOT AKT AND NOT ERK	ERK –|	Pucci, B., Indelicato, M., Paradisi, V., Reali, V ., Pellegrini, L., Aventaggiato, M., Karpinich, N. O., Fini, M., Russo, M. A., Farber, J. L., and Tafani, M. (2009) ERK-1 MAP kinase prevents TNF-induced apoptosis through bad phosphorylation and inhibition of Bax translocation in HeLa Cells. J. Cell. Biochem. 108, 1166–1174.
		AKT–|	Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91, 231–241.

BAK	(BID OR NOT BCL2) AND (BID OR NOT BCLXL) AND (NOT BCL2 OR NOT BCLXL)	BID →	Sarosiek, K. A., Chi, X., Bachman, J. A., Sims, J. J., Montero, J., Patel, L., Flanagan, A., Andrews, D. W., Sorger, P., and Letai, A. (2013) BID preferentially activates BAK while BIM preferentially activates BAX, affecting chemotherapy response. Mol. Cell 51, 751–765.
		BCLXL –|	Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., Adams, J. M., and Huang, D. C. S. (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 19, 1294– 1305.
		BCL2 –|	Dlugosz, P. J., Billen, L. P., Annis, M. G., Zhu, W., Zhang, Z., Lin, J., Leber, B., and Andrews, D. W. (2006) Bcl-2 changes conformation to inhibit Bax oligomerization. EMBO J 25, 2287–2296.

BAX	(BID OR BIM) AND (BID OR NOT BCL2) AND (BID OR NOT BCLXL)	BID →	Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., … Martinou, J.-C. (1999). Bid-induced Conformational Change of Bax Is Responsible for Mitochondrial Cytochrome c Release during Apoptosis. *The Journal of Cell Biology*, *144*(5), 891–901.
		BIM →	Sarosiek, K. A., Chi, X., Bachman, J. A., Sims, J. J., Montero, J., Patel, L., Flanagan, A., Andrews, D. W., Sorger, P., and Letai, A. (2013) BID preferentially activates BAK while BIM preferentially activates BAX, affecting chemotherapy response. Mol. Cell 51, 751–765.
		BCLXL –|	Muñoz-Pinedo, C., Guío-Carrión, A., Goldstein, J. C., Fitzgerald, Newmeyer, D. D., and Green, D. R. (2006) Different mitochondrial intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. PNAS 103, 11573–11578.

		BCL2–|	Murphy, K. M., Ranganathan, V ., Farnsworth, M. L., Kavallaris, M., and Lock, R. B. (2000) Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. Cell Death Differ. 7, 102–111.
BCL2	NOT BAD AND NOT Casp3	BAD –|	Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) Bad, a heterodimeric partner for Bcl-xL and Bcl-2, displaces bax and promotes cell death. Cell 80, 285–291.
		Casp3–|	Liang, Y., Nylander, K. D., Yan, C., and Schor, N. F. (2002) Role of caspase 3-dependent Bcl-2 cleavage in potentiation of apoptosis by Bcl-2. Mol. Pharmacol. 61, 142–149.

BCLXL	NOT BAD AND NOT Casp3	BAD –|	Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) Bad, a heterodimeric partner for Bcl-xL and Bcl-2, displaces bax and promotes cell death. Cell 80, 285–291.
		Casp3 –|	Liang, Y., Nylander, K. D., Yan, C., and Schor, N. F. (2002) Role of caspase 3-dependent Bcl-2 cleavage in potentiation of apoptosis by Bcl-2. Mol. Pharmacol. 61, 142–149.
BID	(Casp8 OR NOT BCL2) AND (Casp8 OR NOT MCL-1)	Casp8 →	Li, H., Zhu, H., Xu, C., and Yuan, J. (1998) Cleavage of BID by Caspase 8 Mediates the Mitochondrial Damage in the Fas Pathway of Apoptosis. Cell 94, 491–501.
		MCL-1 –|	Clohessy, J. G., Zhuang, J., de Boer, J., Gil-Gómez, G., and Brady, H. J. M. (2006) Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor- mediated apoptosis. J. Biol. Chem. 281, 5750–5759.

		BCL2 –|	Yi, X., Yin, X.-M., and Dong, Z. (2003) Inhibition of Bid-induced Apoptosis by Bcl-2 tBid INSERTION, Bax TRANSLOCA TION, AND Bax/Bak OLIGOMERIZA TION SUPPRESSED. J. Biol. Chem. 278, 16992–16999.
BIM	NOT BCL2 AND NOT BCLXL AND NOT CAD AND (GSK3 OR NOT MCL-1)	GSK3 →	Beurel, E., and Jope, R. S. (2006) The Paradoxical Pro- and Anti-apoptotic Actions of GSK3 in the Intrinsic and Extrinsic Apoptosis Signaling Pathways. Prog Neurobiol 79, 173– 189.
		MCL-1 –|	Hagenbuchner, J., Kiechl-Kohlendorfer, U., Obexer, P., and Ausserlechner, M. J. (2013) A novel Mcl1 variant inhibits apoptosis via increased Bim sequestration. Oncotarget 4, 1241–1252.
		CAD–|	Kalimuthu, S., and Se-Kwon, K. (2013) Cell Survival and Apoptosis Signaling as Therapeutic Target for Cancer: Marine Bioactive Compounds. Int J Mol Sci 14, 2334–2354.

		BCLXL–|	Mérino, D., Khaw, S. L., Glaser, S. P., Anderson, D. J., Belmont, L. D., Wong, C., Yue, P., Robati, M., Phipson, B., Fairlie, W. D., Lee, E. F., Campbell, K. J., Vandenberg, C. J., Cory, S., Roberts, A. W., Ludlam, M. J. C., Huang, D. C. S., and Bouillet, P. (2012) Bcl-2, Bcl-xL, and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. Blood 119, 5807–5816.
		BCL2–|	
CAD	Casp3 AND Casp9	Casp3 →	Yi, X., Yin, X.-M., and Dong, Z. (2003) Inhibition of Bid-induced Apoptosis by Bcl-2 tBid INSERTION, Bax TRANSLOCA TION, AND Bax/Bak OLIGOMERIZA TION SUPPRESSED. J. Biol. Chem. 278, 16992–16999.
		Casp9 →	Guerrero, A. D., Schmitz, I., Chen, M., and Wang, J. (2012) Promotion of Caspase Activation by Caspase- 9-mediated Feedback Amplification of Mitochondrial Damage. J Clin Cell Immunol 3.

Casp3	(Casp3 OR Casp8 OR Casp9) AND (Casp3 OR Casp8 OR NOT IAPs) AND (Casp3 OR Casp9 OR NOT IAPs) AND (Casp8 OR Casp9 OR NOT IAPs)	IAPs –|	Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388, 300–304.
		Casp8 →	Stennicke, H. R., Jürgensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J., and Salvesen, G. S. (1998) Pro-caspase-3 is a major physiologic target of caspase-8. J. Biol. Chem. 273, 27084–27090.
		Casp9 →	Fuentes-Prior, P., and Salvesen, G. S. (2004) The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem. J. 384, 201–232.
		Casp3 →	N/A

Casp8	Casp3	Casp3 →	Ferreira, K. S., Kreutz, C., Macnelly, S., Neubert, K., Haber, A., Bogyo, M., Timmer, J., and Borner, C. (2012) Caspase-3 feeds back on caspase-8, Bid and XIAP in type I Fas signaling in primary mouse hepatocytes. Apoptosis 17, 503–515.
Casp9	(Casp3 OR Cyto_C) AND (Casp3 OR NOT IAPs)	IAPs –|	Deveraux, Q. L., Takahashi, R., Salvesen, G. S., and Reed, J. C. (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388, 300–304.
		Cyto_C →	Jiang, X., and Wang, X. (2000) Cytochrome c Promotes Caspase-9 Activation by Inducing Nucleotide Binding to Apaf-1. J. Biol. Chem. 275, 31199–31203.
		Casp3 →	N/A
Cyto_C	BAK OR BAX	BAK →	Renault, T. T., Floros, K. V., and Chipuk, J. E. (2013) BAK/BAX activation and cytochrome c release assays using isolated mitochondria. Methods 61, 146–155.

		BAX →	Renault, T. T., Floros, K. V., and Chipuk, J. E. (2013) BAK/BAX activation and cytochrome c release assays using isolated mitochondria. Methods 61, 146–155.
IAPs	NOT SMAC	SMAC –|	Vucic, D., Deshayes, K., Ackerly, H., Pisabarro, M. T., Kadkhodayan, S., Fairbrother, W. J., and Dixit, V . M. (2002) SMAC Negatively Regulates the Anti-apoptotic Activity of Melanoma Inhibitor of Apoptosis (ML-IAP). J. Biol. Chem. 277, 12275–12279.
MCL-1	NOT Casp3 AND (ERK OR NOT E2F1)	Casp3 –|	Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., … Huang, D. C. S. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes & Development*, *19*(11), 1294–1305.
		E2F1–|	Croxton, R., Ma, Y., Song, L., Haura, E. B., and Cress, W. D. (2002) Direct repression of the Mcl-1 promoter by E2F1. Oncogene 21, 1359–1369.

		ERK →	Ding, Q., Huo, L., Yang, J.-Y., Xia, W., Wei, Y., Liao, Y., Chang, C.-J., Y ang, Y., Lai, C.-C., Lee, D.- F., Yen, C.-J., Chen, Y .-J. R., Hsu, J.-M., Kuo, H.-P., Lin, C.-Y., Tsai, F.-J., Li, L.-Y., Tsai, C.-H., and Hung, M.-C. (2008) Down- regulation of myeloid cell leukemia-1 through inhibiting Erk/Pin 1 pathway by sorafenib facilitates chemosensitization in breast cancer. Cancer Res. 68, 6109–6117.
SMAC	BAK OR BAX	BAK →	Arnoult, D., Gaume, B., Karbowski, M., Sharpe, J. C., Cecconi, F., and Youle, R. J. (2003) Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J 22, 4385–4399.
		BAX →	

Supplementary Table S4. Bone Morphogenetic Protein Links

Node	Logic Gate	Link	Reference
BMP	BMP	BMP →	N/A
SMAD	BMP	BMP →	Ueberham, U., & Arendt, T. (2013). The Role of Smad Proteins for Development, Differentiation and Dedifferentiation of Neurons.
Zic1_3	(ERK OR Zic1_3) AND (NOT SMAD OR Zic1_3)	SMAD –|	Aruga, J., & Mikoshiba, K. (2011). Role of BMP, FGF, calcium signaling, and Zic proteins in vertebrate neuroectodermal differentiation. *Neurochemical Research*, *36*(7), 1286–1292.
		ERK →	Marchal, L., Luxardi, G., Thomé, V., & Kodjabachian, L. (2009). BMP inhibition initiates neural induction via FGF signaling and Zic genes. *Proceedings of the National Academy of Sciences*, *106*(41), 17437–17442.
		Zic1_3 →	N/A

Supplementary Table S5. Leukemia Inhibitory Factor Links

Node	Logic Gate	Link	Reference
LIF	LIF	LIF →	N/A
LIFR	LIF	LIF →	Cheng, J.-G., Chen, J. R., Hernandez, L., Alvord, W. G., & Stewart, C. L. (2001). Dual control of LIF expression and LIF receptor function regulate Stat3 activation at the onset of uterine receptivity and embryo implantation. *Proceedings of the National Academy of Sciences*, *98*(15), 8680–8685.
Stat3	LIFR	LIFR →	Niwa, H., Ogawa, K., Shimosato, D., & Adachi, K. (2009). A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature*, *460*(7251), 118–122.

Supplementary Table S6. Pluripotency Links

Node	Logic Gate	Link	Reference
B_Catenin	NOT GSK3	GSK3 –|	Metcalfe, C., & Bienz, M. (2011). Inhibition of GSK3 by Wnt signalling--two contrasting models. *Journal of Cell Science*, *124*(Pt 21), 3537–3544.
Esrrb	Nanog AND NOT TCF3	TCF3 –|	Martello, G., Sugimoto, T., Diamanti, E., Joshi, A., Hannah, R., Ohtsuka, S., … Smith, A. (2012). Esrrb Is a Pivotal Target of the Gsk3/Tcf3 Axis Regulating Embryonic Stem Cell Self-Renewal. *Cell Stem Cell*, *11*(4), 491–504.
		Nanog –|	Festuccia, N., Osorno, R., Halbritter, F., Karwacki-Neisius, V., Navarro, P., Colby, D., … Chambers, I. (2012). Esrrb Is a Direct Nanog Target Gene that Can Substitute for Nanog Function in Pluripotent Cells. *Cell Stem Cell*, *11*(4), 477–490.

Klf4	NOT ERK AND Stat3	ERK –|	Kim, M. O., Kim, S.-H., Cho, Y.-Y., Nadas, J., Jeong, C.-H., Yao, K., … Dong, Z. (2012). ERK1 and ERK2 regulate embryonic stem cell self-renewal through phosphorylation of Klf4. *Nature Structural & Molecular Biology*, *19*(3), 283–290.
		Stat3 →	Qin, S., & Zhang, C.-L. (2012). Role of Krüppel-Like Factor 4 in Neurogenesis and Radial Neuronal Migration in the Developing Cerebral Cortex. *Molecular and Cellular Biology*, *32*(21), 4297–4305.
Nanog	(Esrrb OR Oct4_Sox2) AND (Esrrb OR Tbx3) AND (Oct4_Sox2 OR Tbx3)	Tbx3 →	Russell, R., Ilg, M., Lin, Q., Wu, G., Lechel, A., Bergmann, W., … Kleger, A. (2015). A Dynamic Role of TBX3 in the Pluripotency Circuitry. *Stem Cell Reports*, *5*(6), 1155–1170.

		Esrrb →	Festuccia, N., Osorno, R., Halbritter, F., Karwacki-Neisius, V., Navarro, P., Colby, D., … Chambers, I. (2012). Esrrb Is a Direct Nanog Target Gene that Can Substitute for Nanog Function in Pluripotent Cells. *Cell Stem Cell*, *11*(4), 477–490.
		Oct4_Sox2 →	Pan, G., & Thomson, J. A. (2007). Nanog and transcriptional networks in embryonic stem cell pluripotency. *Cell Research*, *17*(1), 42–49.
Oct4_Sox2	Oct4 AND Sox2	Oct4 →	Chew, J.-L., Loh, Y.-H., Zhang, W., Chen, X., Tam, W.-L., Yeap, L.-S., … Ng, H.-H. (2005). Reciprocal Transcriptional Regulation of Pou5f1 and Sox2 via the Oct4/Sox2 Complex in Embryonic Stem Cells. *Molecular and Cellular Biology*, *25*(14), 6031–6046.
		Sox2 →	

Oct4	(Esrrb OR Klf4 OR Oct4_Sox2) AND (Esrrb OR NOT ERK) AND (Klf4 OR Nanog OR Oct4_Sox2) AND (Klf4 OR NOT ERK) AND (Nanog OR NOT ERK) AND (NOT ERK OR Oct4_Sox2)	ERK –|	Li, L., Sun, L., Gao, F., Jiang, J., Yang, Y., Li, C., … Jin, Y. (2010). Stk40 links the pluripotency factor Oct4 to the Erk/MAPK pathway and controls extraembryonic endoderm differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(4), 1402–1407.
		Esrrb →	Berg, D. L. C. van den, Zhang, W., Yates, A., Engelen, E., Takacs, K., Bezstarosti, K., … Poot, R. A. (2008). Estrogen-Related Receptor Beta Interacts with Oct4 To Positively Regulate Nanog Gene Expression. *Molecular and Cellular Biology*, *28*(19), 5986–5995.
		Nanog →	Muñoz Descalzo, S., Rué, P., Garcia-Ojalvo, J., & Martinez Arias, A. (2012). Correlations between the levels of Oct4 and Nanog as a signature for naïve pluripotency in mouse embryonic stem cells. *Stem Cells (Dayton, Ohio)*, *30*(12), 2683–2691.

		Oct4_Sox2 →	Chew, J.-L., Loh, Y.-H., Zhang, W., Chen, X., Tam, W.-L., Yeap, L.-S., … Ng, H.-H. (2005). Reciprocal Transcriptional Regulation of Pou5f1 and Sox2 via the Oct4/Sox2 Complex in Embryonic Stem Cells. *Molecular and Cellular Biology*, *25*(14), 6031–6046.
		Klf4 →	Cherepanova, O. A., Gomez, D., Shankman, L. S., Swiatlowska, P., Williams, J., Sarmento, O. F., … Owens, G. K. (2016). Activation of the pluripotency factor OCT4 in smooth muscle cells is atheroprotective. *Nature Medicine*, *22*(6), 657–665.
Sox2	Klf4 OR Nanog OR Oct4_Sox2 OR Stat3	Stat3 →	Foshay, K. M., & Gallicano, G. I. (2008). Regulation of Sox2 by STAT3 initiates commitment to the neural precursor cell fate. *Stem Cells and Development*, *17*(2), 269–278.

		Oct4_Sox2 →	Chew, J.-L., Loh, Y.-H., Zhang, W., Chen, X., Tam, W.-L., Yeap, L.-S., … Ng, H.-H. (2005). Reciprocal Transcriptional Regulation of Pou5f1 and Sox2 via the Oct4/Sox2 Complex in Embryonic Stem Cells. *Molecular and Cellular Biology*, *25*(14), 6031–6046.
		Klf4 →	Wei, Z., Yang, Y., Zhang, P., Andrianakos, R., Hasegawa, K., Lyu, J., … Lu, W. (2009). Klf4 interacts directly with Oct4 and Sox2 to promote reprogramming. *Stem Cells (Dayton, Ohio)*, *27*(12), 2969–2978.
		Nanog →	Rodda, D. J., Chew, J.-L., Lim, L.-H., Loh, Y.-H., Wang, B., Ng, H.-H., & Robson, P. (2005). Transcriptional regulation of nanog by OCT4 and SOX2. *The Journal of Biological Chemistry*, *280*(26), 24731–24737.

Tbx3	AKT AND NOT ERK	AKT →	Peres, J., Mowla, S., & Prince, S. (2015). The T-box transcription factor, TBX3, is a key substrate of AKT3 in melanomagenesis. *Oncotarget*, *6*(3), 1821–1833.
		ERK –|	Willmer, T., Hare, S., Peres, J., & Prince, S. (2016). The T-box transcription factor TBX3 drives proliferation by direct repression of the p21WAF1 cyclin-dependent kinase inhibitor. *Cell Division*, *11*.
TCF3	Not B_Catenin	B_Catenin –|	Kuwahara, A., Sakai, H., Xu, Y., Itoh, Y., Hirabayashi, Y., & Gotoh, Y. (2014). Tcf3 Represses Wnt–β-Catenin Signaling and Maintains Neural Stem Cell Population during Neocortical Development. *PLOS ONE*, *9*(5), e94408.
Two_i	Two_i	Two_i →	N/A