Explore Rb/E2F Activation Dynamics to Define the Control Logic of Cell Cycle Entry in Single Cells

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John Harer

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Graduate Program in Computational Biology and Bioinformatics in the Graduate School of Duke University

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ABSTRACT

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Abstract

Control of E2F transcription factor activity, regulated by the action of the retinoblastoma tumor suppressor, is critical for determining cell cycle entry and cell proliferation. However, an understanding of the precise determinants of this control, including the role of other cell cycle regulatory activities, has not been clearly defined.

Recognizing that the contributions of individual regulatory components could be masked by heterogeneity in populations of cells, we made use of an integrated system to follow E2F transcriptional dynamics at the single cell level and in real time. We measured and characterized E2F temporal dynamics in the first cell cycle where cells enter the cell cycle after a period of quiescence. Quantitative analyses revealed that crossing a threshold of amplitude of E2F transcriptional activity serves as the critical determinant of cell-cycle commitment and division.

By using a developed ordinary differential equation model for Rb/E2F network, we performed simulations and predicted that Myc and cyclin D/E activities have distinct roles in modulating E2F transcriptional dynamics. Myc is critical in modulating the amplitude whereas cyclin D/E activities have little effect on the amplitude but do contribute to the modulation of duration of E2F transcriptional activation. These predictions were validated through the analysis of E2F dynamics in single cells under
the conditions that cyclin D/E or Myc activities are perturbed by small molecule inhibitors or RNA interference.

In an ongoing study, we also measured E2F dynamics in cycling cells. We provide preliminary results showing robust oscillatory E2F expression at the single-cell level that aligns with the progression of continuous cell division. The temporal characteristics of the dynamics trajectories deserve further quantitative investigations.

Taken together, our results establish a strict relationship between E2F dynamics and cell fate decision at the single-cell level, providing a refined model for understanding the control logic of cell cycle entry.
To my wife, Nan Zhang, my reverse complement,

To my parents, Quancai Dong and Ying Sun, who raised me up

and have always supported my dream
Contents

Abstract ......................................................................................................................................... iv

List of Tables ................................................................................................................................ xii

List of Figures ........................................................................................................................... xiii

List of symbols and abbreviations ........................................................................................ xiv

Acknowledgements ................................................................................................................... xvi

1. Introduction ............................................................................................................................... 1

  1.1 Overview of eukaryotic cell cycle control ..................................................................... 3
  1.2 Mammalian cell cycle control ......................................................................................... 5
  1.3 Rb/E2F network ................................................................................................................ 8
  1.4 Modeling the cell cycle .................................................................................................. 15
  1.5 Single-cell analysis ......................................................................................................... 19
  1.6 Thesis roadmap ............................................................................................................... 25

2. Measurement of E2F dynamics at the single-cell level ...................................................... 28

  2.1 Introduction ..................................................................................................................... 28
  2.2 Results .............................................................................................................................. 30
    2.2.1 Construction of E2F1 transcriptional reporter ...................................................... 30
    2.2.2 Measurement of E2F1 dynamics at the single-cell level ...................................... 32
    2.2.3 Characterization of E2F1 dynamics .......................................................................... 34
  2.3 Discussion ........................................................................................................................ 36
  2.4 Methods ........................................................................................................................... 37
2.4.1 E2F1 transcriptional reporter system ................................................................. 37
2.4.2 Cell culture ............................................................................................................. 39
2.4.3 Live cell imaging ................................................................................................. 39
2.4.4 Image analysis ..................................................................................................... 40
2.4.5 qRT-PCR analysis .............................................................................................. 41
2.4.6 Western blot analysis ......................................................................................... 41

3. Amplitude of E2F accumulation as the predictor of commitment and division .... 43
3.1 Introduction ........................................................................................................... 43
3.2 Results .................................................................................................................. 44
3.2.1 Amp as the predictor of commitment .............................................................. 44
3.2.2 Amp as the predictor of division ..................................................................... 47
3.2.3 Correctness as predicted by different E2F1 dynamics metrics ................. 49
3.3 Discussion ............................................................................................................ 51
3.4 Methods ................................................................................................................. 52
3.4.1 EdU staining ..................................................................................................... 52
3.4.2 Analysis of E2F dynamics trajectories ........................................................... 53

4. Distinct roles of Myc and G1 cyclin/CDKs revealed by modeling analysis .... 55
4.1 Introduction .......................................................................................................... 55
4.2 Results .................................................................................................................. 57
4.2.1 Modularity revealed by sensitivity analysis ................................................... 57
4.2.2 Distinct roles of Myc and G1 cyclin/CDKs revealed by simulation analysis .... 61
4.2.3 Distinct roles of Myc and G1 cyclin/CDKs interpreted by system analysis ..... 63
4.3 Discussion ........................................................................................................................ 65
4.4 Methods ........................................................................................................................... 66
  4.4.1 Mathematic modeling ............................................................................................... 66
  4.4.2 Simulation analysis ................................................................................................... 71
  4.4.3 Log-sensitivity analysis ............................................................................................ 73
5. Roles of Myc and G1 cyclin/CDKs confirmed by analysis of E2F dynamics.............. 74
  5.1 Introduction .................................................................................................................. 74
  5.2 Results .......................................................................................................................... 75
    5.2.1 Control of timing but not commitment by G1 cyclin/CDKs ............................... 75
    5.2.2 Control of commitment into cell cycle entry by Myc ........................................... 79
  5.3 Discussion ..................................................................................................................... 82
  5.4 Methods ....................................................................................................................... 83
    5.4.1 Cell culture ................................................................................................................. 83
    5.4.2 Western blot analysis ............................................................................................... 84
    5.4.3 RNA interference ....................................................................................................... 84
6. Measurement of E2F dynamics in cycling cells ............................................................. 86
  6.1 Introduction .................................................................................................................. 86
  6.2 Results .......................................................................................................................... 87
    6.2.1 Measurement of E2F dynamics in cycling cells .................................................. 87
    6.2.2 $t_{c1}$ primarily contributes to the variation of cell cycle length ................. 90
    6.2.3 Correlation of E2F dynamics between sibling cells ........................................... 92
  6.3 Discussion ..................................................................................................................... 93
List of Tables

Table 3.1: Accuracy of prediction for cell commitment or division as assessed by different E2F1 dynamics metrics ...........................................................................................................50

Table 4.1: Description of variables...........................................................................................................67

Table 4.2: Description of reaction terms...................................................................................................67

Table 4.3: Equations for the full ODE model that describes Rb/E2F network.................................69

Table 4.4: Values of model parameters..................................................................................................70

Table 5.1: shRNA sequences against CDK2 and c-Myc.................................................................85
List of Figures

Figure 1.1: The mammalian cell cycle .............................................................................................................. 7
Figure 1.2: Rb/E2F network and cell cycle entry ............................................................................................ 12
Figure 2.1: E2F reporter constructs ................................................................................................................... 30
Figure 2.2: Confirmation of hE2F1p::4NLS-d4Venus reporter ...................................................................... 32
Figure 2.3: Measurement of E2F transcriptional dynamics in single cells ................................................. 33
Figure 2.4: Distribution of different metrics ................................................................................................... 35
Figure 2.5: Pairwise correlation between E2F1 dynamics metrics ................................................................. 35
Figure 3.1: A schematic diagram links E2F dynamics to phenotypes at the single-cell level .................................... 44
Figure 3.2: Amp as the predictor of commitment .............................................................................................. 46
Figure 3.3: Commitment as determined by relevant metrics ........................................................................... 47
Figure 3.4: Representative E2F trajectories in different sub-groups ............................................................. 48
Figure 3.5: Amp as the predictor of division .................................................................................................... 49
Figure 3.6: Division as determined by relevant metrics ................................................................................... 49
Figure 3.7: Probability of cell commitment or division as function of Amp .................................................... 51
Figure 4.1: Simulated temporal trajectories of all the factors in Rb/E2F network ........................................ 58
Figure 4.2: Sensitivity analysis of Amp, t, k and S to different model parameters ....................................... 60
Figure 4.3: Distinct roles of Myc and G1 cyclin/CDKs revealed by simulation analysis .................................. 62
Figure 4.4: Distinct roles of Myc and G1 cyclin/CDKs interpreted by system analysis .................................. 64
Figure 5.1: G1 cyclin/CDKs have little effect on commitment decision .......................................................... 76
Figure 5.2: G1 cyclin/CDKs control timing of cell cycle progression .............................................................. 78
Figure 5.3: Myc controls the commitment into cell cycle entry ........................................... 81

Figure 6.1: Measurement of E2F transcriptional dynamics in cycling cells ......................... 89

Figure 6.2: $t_{c-1}$ primarily contributes to the variation of cell cycle length ..................... 91

Figure 6.3: Correlation of E2F dynamics between sibling cells ........................................... 92
List of Symbols and Abbreviations

**Symbols**

- $Amp$ amplitude of E2F accumulation
- $Amp_{th}$ threshold of $Amp$ for commitment decision
- $t_1$ initial delay (first cell cycle)
- $t_2$ activation time (first cell cycle)
- $t_3$ post-activation time (first cell cycle)
- $k$ slope (first cell cycle)
- $s$ area (first cell cycle)
- $T$ cell cycle length (first cell cycle)
- $t_{c-1}$ initial delay (cycling cells)
- $t_{c-2}$ activation time (cycling cells)
- $t_{c-3}$ post-activation time (cycling cells)
- $T_c$ cell cycle length (cycling cells)

**Abbreviations**

- G0 quiescent state
- G1 gap1 phase
- G2 gap2 phase
- M mitosis phase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CycD</td>
<td>cyclin D/CDK4/6 complex</td>
</tr>
<tr>
<td>CycE</td>
<td>cyclin E/CDK2 complex</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma gene</td>
</tr>
<tr>
<td>G1-pm</td>
<td>post-mitotic interval</td>
</tr>
<tr>
<td>G1-ps</td>
<td>pre-S phase interval</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescence protein</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization sequence</td>
</tr>
<tr>
<td>BGS</td>
<td>bovine growth serum</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-20-deoxyuridine</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>ODE</td>
<td>ordinary differential equation</td>
</tr>
<tr>
<td>SDE</td>
<td>stochastic differential equation</td>
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the progression and direction of this study. I would like to express my gratitude to Dr. Joseph R. Nevins, who provided insightful suggestions to improve the manuscript that I was preparing for publication.

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Introduction

Where a cell arises, there must be a previous cell, just as animals can only arise from animals and plants from plants.

– [Rudolf Virchow, 1821 - 1902]

As suggested in the statement issued by the German pathologist Rudolf Virchow in 1858, the principle underlying the continuity of life lies in the process in which an individual cell is the descendant of another cell and will give rise to a newborn cell. This fundamental process of multiplication (“omnis cellula e cellula”) is known as “the cell cycle”. Although details of the cell cycle may vary among different organisms, cells have to replicate by completing a series of universal steps, such as duplication of genetic information, synthesis of organelles and increase of mass and size (Harashima et al., 2013). Moreover, cells have to appropriately integrate and coordinately respond to external and internal regulatory signals, such as growth stimuli, nutrient status and cellular stress, which fluctuate according to the environment and the cellular state (Cai and Tu, 2012; Hartwell and Weinert, 1989). Disruption of cell cycle regulation can result in developmental defects and diseases, particularly in the case of carcinogenesis (Kastan and Bartek, 2004; Kim and Zhao, 2005; Malumbres and Barbacid, 2001, 2009; Sherr, 1996). Therefore, understanding the mechanisms underlying cell cycle control is critical and remains a challenging problem in biological and medical sciences.
In this thesis, I will first introduce the fundamental concepts and molecular mechanisms underlying cell cycle control that have been established over the last four decades, and highlight specific questions that have not been solved. I will also cover some of the conventional approaches that have been used and discuss new methods recently developed to investigate cell cycle control. In the following part, I will focus on my own contribution to the field and present my work on the characterization of E2F dynamics in single cells to define the control logic of cell cycle entry. My experimental workflow will be divided into three parts. The first describes the establishment of an integrated platform that I developed to measure in real time E2F transcriptional dynamics. In the second part, I will discuss how I was able to use unprecedented single-cell data derived from my analysis of E2F transcriptional dynamics and monitoring of cell fates to establish a precise relationship between particular characteristics of such dynamics and the decision of the cell to divide or not in response to mitogenic signals. In the third part, I will describe using a mathematical model built from ordinary differential equations (ODE) that reflect the state of the Retinoblastoma/E2F (Rb/E2F) network was able to generate unanticipated theoretical predictions, which I was able to validate experimentally at the single-cell level. Taken together, my results provide a revised model for understanding the design principles of the Rb/E2F network and the molecular logic underlying cell cycle entry. This work was published in *Nature Communications* (Dong et al., 2014). The overarching theme of this thesis presents a novel
methodology for quantitative study of cell cycle control as well as a refined model for understanding cell cycle entry, with a special emphasis on single-cell analysis.

1.1 Overview of eukaryotic cell cycle control

Depending on the events observed during cell cycle progression, the eukaryotic cell cycle is traditionally divided into four phases (Fig. 1.1) (Harashima et al., 2013). Mitosis (M), during which a cell splits into two daughter cells, is the most prominent phase that can be followed under the microscope. S phase is the specific stage within a single cell cycle during which DNA synthesis occurs to achieve duplication of the genetic information before cell division can occur. Two gap phases (G1 and G2) take place between the S and M phases. The G1 phase spans the period after completion of the M phase and the beginning of the S phase, whereas the G2 phase resides between S phase completion and the start of the M phase. During the G1 phase, cells have to respond to a variety of external signals from the environment, such as growth stimuli and nutrients, in order to determine if they will commit to a new cell cycle and start initiating DNA synthesis. During the G2 phase, cells initialize molecular events in preparation for mitosis after they assure that the genetic information has been successfully replicated.

Eukaryotes have evolved a highly conserved machinery to coordinate these molecular events and ensure that each phase is proceeding normally before the next phase can take place. In particular, the existence of “checkpoints” was proposed to
tightly license the timely progression of the cell cycle at critical junctions (Hartwell and Weinert, 1989; Kastan and Bartek, 2004). By controlling the ability of a cell to halt progression at specific points in the cell cycle, checkpoints ensure that critical events have been appropriately completed before the next phase can begin. Three distinct checkpoints have been proposed to control the ordered transition of the cell cycle: the G1/S checkpoint (also known as “start point” in yeast or “restriction point” in mammalian cells), G2/M checkpoint (also known as “DNA replication checkpoint”) and the spindle checkpoint in M phase (Ciosk et al., 1998; Coller, 2007; Doncic et al., 2011; Guardavaccaro and Pagano, 2006; Pardee, 1974; Yang et al., 2013). At the molecular level, each checkpoint control is mediated by the activity of specialized proteins, which are under complex and dynamic regulation.

Owing to over decades of efforts, genes that participate in this essential control have been identified and cloned. Importantly, extensive studies in cell and molecular biology have uncovered detailed molecular interactions among these regulatory units. The current dogma describes that two groups of genes, cyclins and cyclin-dependent kinases (CDKs), play the central role in cell cycle control (Hochegger et al., 2008; Malumbres and Barbacid, 2009; Morgan, 1997). Cyclins and CDKs can form different complexes, which have specific functions and substrate specificities. The stage-specific activation of different cyclin/CDK complexes can drive a set of periodic molecular events, thereby promoting the cell cycle progression (Bloom and Cross, 2007). Given the
fact that genes for cyclins and CDKs are evolutionary conserved throughout the eukaryotic kingdom and that they function in similar regulatory networks, it was generally proposed that their involvement in cell cycle control universally applied to different settings (Harashima et al., 2013; Malumbres, 2014).

1.2 Mammalian cell cycle control

Although mammals adopt many aspects of the cell cycle control system that are used by other eukaryotic species, their complicated developmental program from a fertilized oocyte to different germ layers and eventually to a mature adult dictates that the mammalian cell cycle control must include robust mechanisms to either drive continuation or halt of cell cycle progression (Budirahardja and Gonczy, 2009; Ciemerych and Sicinski, 2005). Indeed, mammalian cells rely on cell cycle control for maintaining tissue homeostasis. Normal tissues carefully control the proliferation of their component cells through the fine tuning of growth-promoting and -suppressive signals that instructs cell cycle entry or halt, in order to maintain proper cell number and tissue architecture. If this balanced regulation is broken, one consequence is that normal cells lose their ability to appropriately respond to the environmental control and become the masters of their own fate, leading to malignant transformation and cancer development (Green and Evan, 2002; Hanahan and Weinberg, 2000, 2011).

A remarkable characteristic of mammalian cell cycle control is that they are only responsive to growth regulatory signals during a short time window in the early S phase.
(Fig. 1.1). Over the last four decades, multiple lines of evidence suggested that there was a critical time point in the G1 phase that governed whether a cell entered the cell cycle or not (Blagosklonny and Pardee, 2002; Pardee, 1974; Zetterberg and Larsson, 1985). In 1974, Pardee characterized this time point and coined it the “restriction point” (Pardee, 1974). The restriction point separates the G1 phase into two functional temporal domains: G1-pm (post-mitotic interval) and G1-ps (pre-S phase interval) (Fig. 1.1) (Zetterberg and Larsson, 1985). During G1-pm, deprivation of external growth signals leads mammalian cells to a quiescent state (G0), whereas re-stimulation with external growth signals promotes quiescent cells into cell cycle entry. In contrast, cells in G1-ps (past the restriction point) will continue to complete the ongoing cell cycle regardless of whether or not growth stimuli are present in the environment. Importantly, the operational restriction point is a critical checkpoint for programmed cell cycle, as it was found to be often lost in malignant, transformed cells.
FIGURE 1.1: The mammalian cell cycle. A new cell cycle begins immediately after the previous mitosis and progresses orderly through G1, S, G2 and M phases. Deprivation of growth factors leads cells to enter the quiescent state G0, while re-exposure to growth stimuli releases cells back into the cell cycle.

After cells have passed the restriction point, they are committed to progress through the cell cycle. They initiate events such as DNA replication, cell growth, chromosomal segregation and eventually go on to divide. Additional checkpoints provide safeguard at specific critical times of the cell cycle to ensure safe and accurate progress. The G2/M checkpoint is activated once DNA replication errors occur, providing cells with an opportunity to repair damaged DNA before entering mitosis (Guardavaccaro and Pagano, 2006). During mitosis, the spindle checkpoint prevents separation of the duplicated chromosomes until each chromosome is properly attached to the spindle apparatus (Ciosk et al., 1998).
1.3 Rb/E2F network

The molecular mechanisms underlying cell cycle entry were not understood until the discovery of retinoblastoma tumor suppressor gene (Rb). Rb was originally identified in the early 1980s through the pedigrees of families whose children developed retinoblastoma (Burkhart and Sage, 2008; Dick and Rubin, 2013; Weinberg, 1995). Its function in cell cycle control was clearly highlighted by the fact that Rb overexpression led to G1 phase cell cycle arrest (Dyson, 1998). Rb belongs to the pocket protein family together with two other proteins, p107 and p130, which are induced at different cell cycle stages. Together, they coordinate the response to upstream signals and play a critical role in causing cells to either enter a quiescent state or exit the cell cycle (Sage et al., 2000).

A few years later, the mechanism by which Rb exerted its activity was shown to be through potent inhibition of the transcription factor activators E2F1-3 (Bagchi et al., 1991; Burkhart and Sage, 2008; Chellappan et al., 1991; Nevins, 2001; van den Heuvel and Dyson, 2008; Wong et al., 2011a). The E2F transcription factors are a family of proteins that share a related DNA binding domain and can either activate or repress transcription. Most E2Fs form heterodimer with a DP protein (DP1 or DP2) for function (Girling et al., 1993). The activator E2Fs, including E2F1, E2F2 and E2F3a, are found in complex with Rb during G1 phase. The direct binding of Rb to activator E2Fs not only inhibits their intrinsic transcriptional activity, but also actively represses transcription by
recruiting a silencer complex containing chromatin modifiers and remodeling factors to the promoter of E2F target genes (Brehm et al., 1998; Luo et al., 1998). Once these E2Fs are released from Rb, they can initialize the transcription of a large number of genes essential for cell cycle progression (Fig. 1.2a) (Trimarchi and Lees, 2002; van den Heuvel and Dyson, 2008). Other types of E2Fs (E2F4-8) play different roles. For example, E2F4 and E2F5 are repressor E2Fs, which are found in complex with p130 to mediate stable transcriptional repression in G0 (Balciunaite et al., 2005; Beijersbergen et al., 1994). Unlike the other E2Fs, E2F7/8 do not interact with DP but form heterodimer with each other to repress transcription of target genes in late S phase (Moon and Dyson, 2008). The dynamic balance of E2Fs and the regulation of E2F1-3 activity by Rb primarily dictate whether a cell will enter the cell cycle or not, but it can also influence whether a cell differentiates, senesces or commits apoptosis. Deletion of all three activator E2Fs completely abolishes the ability of mouse embryonic fibroblasts to enter S phase (Wu et al., 2001), whereas targeted disruption of three Rb-related genes results in loss of G1 control and cellular immortalization (Sage et al., 2000).

How is Rb regulated? Extensive research shows that the Rb function is primarily regulated through its phosphorylation by the G1 cyclin/CDK complexes (Bloom and Cross, 2007; Hochegger et al., 2008; Nevins, 2001). There are two types of G1 cyclin/CDK complexes in mammalian cells: the complex formed by cyclin D and CDK4 or CDK6, and the complex of cyclin E/CDK2 (Bloom and Cross, 2007; Musgrove et al., 2011).
Activity of cyclin D/CDK4/6 is controlled by extracellular growth signals through activation of the mitogen-activated protein kinase (MAPK) pathway. In quiescence, hypo-phosphorylated Rb tightly binds to E2F and represses its activity. The canonical view suggests that, upon the increase of cyclin D/CDK4/6 activity in the G1 phase, Rb is gradually getting phosphorylated. The increase in phosphorylation alters the configuration of Rb protein, causing some release of E2F from Rb sequestration. Free E2F then activates transcription of its many target genes linked to cell cycle progression. Cyclin E is among these target genes. Its transcription leads to the activation of cyclin E/CDK2 activity, which creates a positive feedback loop by further phosphorylating Rb, resulting in the full release and activation of E2F (Fig. 1.2b) (Bloom and Cross, 2007; Malumbres and Barbacid, 2009). Interestingly, a recent study showed that, at the molecular level, the cyclin D/CDK complex only led to mono-phosphorylation of Rb in early G1, which served then as a substrate to the cyclin E/CDK complex leading to quantum hyper-phosphorylation of Rb, suggesting distinct roles for these two types of cyclin complexes (Narasimha et al., 2014). Nonetheless, it has been generally assumed that hyper-phosphorylation of Rb by successive rounds of G1 cyclin/CDK activity is the primary determinant for cell cycle entry.

However, the validity of this central dogma was questioned by recent studies relying on gene knockout mouse models, which indicated that most cyclins and CDKs were surprisingly dispensable for the completion of the entire cell cycle (Berthet et al.,
2003; Geng et al., 2003; Kozar et al., 2004; Malumbres et al., 2004). These somewhat conflicting data raised a critical question as to whether or to what extent mammalian cells relied on cyclin/CDK activities for cell cycle control. Indeed they raised the possibility that alternative models might better describe the control mechanism for cell cycle entry.
FIGURE 1.2: Rb/E2F network and cell cycle entry. (a) A schematic diagram shows that Rb/E2F network controls cell cycle entry. Red bar indicates the restriction point. (b) Topology of the Rb/E2F network.

Apart from the central regulators including Rb, E2F and G1 cyclin/CDKs, a number of other genes have been identified to directly contribute to the regulation of Rb
and E2F expression and/or activities. The potent oncogene, Myc, dramatically affects E2F activity, presumably through modulation of G1 cyclins expression as well as CDK activities (Adhikary and Eilers, 2005; Dang, 2012). Moreover, it was recently showed that Myc is also required for interaction of E2F protein with E2F gene promoters, suggesting a direct and Rb-independent regulatory role of Myc on E2F activation through facilitating E2F auto-regulation (Fig. 1.2b) (Johnson et al., 1994; Leung et al., 2008). Beside Myc, factors including E2F7/8, cyclin A, Skp2 and microRNAs are also known to directly interact with E2F1 (the main E2F activator) to modulate its activity or alter its steady state expression during the cell cycle. E2F7/8 functions as a transcriptional repressor of E2F1 that can bind directly to the E2F1 promoter region, while cyclin A forms a stable complex with E2F1 during S phase, thereby preventing E2F1 to activate target genes (Carvajal et al., 2012; Krek et al., 1995). Skp2 is the cell-cycle-regulated component of the ubiquitin-protein ligase SCFSKP2 that recognizes E2F1 and promotes its degradation (Marti et al., 1999). Moreover, miR-17 and miR-20, which are directly transcribed by Myc, were shown to inhibit E2F1 protein accumulation through post-transcriptional regulation (O'Donnell et al., 2005). In addition to miR-17 and -20, E2F7/8, cyclin A and Skp2 are also targets of E2F1 transcriptional activation, suggesting the existence of a negative feedback structure within the network that contributes to the highly dynamic expression of E2F1 protein and activity throughout the cell cycle (Fig. 1.2b) (DeGregori et al., 1995; Liu et al., 2013; Zhang and Wang, 2006).
Likewise, other effectors in the Rb/E2F network are subject to a multilayered regulation. A group of genes, namely CDK inhibitors (CKIs), can inhibit cyclin/CDK activities through direct binding, providing the regulation of cell cycle from a different layer (Besson et al., 2008; Chu et al., 2008). These CKIs includes the INK4 gene family and Cip/Kip gene family. The INK4 family encodes p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c}, and p19\textsuperscript{INK4d}, all of which bind to CDK4 and CDK6 and inhibit their kinase activities by interfering with their association with D-type cyclins. In contrast, the Cip/Kip family inhibitors, p21\textsuperscript{Cip1/Waf1/Sdi1} (p21, encoded by \textit{cdkn1a}), p27\textsuperscript{Kip1} (p27, \textit{cdkn1b}) and p57\textsuperscript{Kip2} (p57, \textit{cdkn1c}), bind to both cyclin and CDK subunits and can regulate the activities of cyclin D-, E-, A-, and B-CDK complexes. CKIs are critical mediators of external growth-inhibitory signal and internal stress signal into the cell cycle control machinery. For example, p21 is a critical effector of the DNA damage-induced cell cycle arrest, while p16 and p21 are often transcriptionally activated when telomere-shortening triggers cellular senescence (Besson et al., 2008). All the above genes sense and transduce internal and external cues relayed by diverse signaling pathways to the core Rb/E2F network, ultimately controlling cell cycle progression or other responses.

Given its central role in cell cycle progression, it is not surprising that the Rb/E2F network is commonly deregulated in various types of cancers through genetic or epigenetic mechanisms, resulting in E2F activation. Factors within this network are well-known oncogenes or tumor suppressors, and have been implicated in the development
of most cancer types. The classical example is a genetic loss of Rb in neural precursors in the eye, which leads to the occurrence of retinoblastoma. Viral proteins from human papilloma virus (e.g., E7) have been shown to bind E2F1 and release it from Rb sequestration, and they contribute to over 90% cases of human cervical carcinoma (Kim and Zhao, 2005; van den Heuvel and Dyson, 2008). Myc is a sensor of various signaling pathways (e.g. MAPK, Akt, Wnt, Notch and TGF-β) that regulate cell proliferation. Increases in Myc gene copy numbers or altered expression are commonly found in many types of cancer (Dang, 2012; He et al., 1998; Pomerantz et al., 2009). Aberrant Myc expression in tumors is not only driving cell growth but can also promote metastasis, vasculogenesis as well as genomic instability (Adhikary and Eilers, 2005; Baudino et al., 2002; Dang, 2012; Felsher and Bishop, 1999). Finally, over-expression of cyclin D or cyclin E, or loss of CKIs have been frequently observed in breast cancer, melanoma and oral squamous cell carcinoma, presumably contributing to tumorigenesis through the induction of E2F activity (Besson et al., 2008; Keyomarsi et al., 1995; Musgrove et al., 2011).

1.4 Modeling the cell cycle

A gene regulatory system contains two types of design principles. One is the design principle for individual components that relates to gene sequence and protein structure, and determines the specific function of each component. Conventional approaches based on biochemistry and molecular biology have contributed greatly to
our understanding at that level. The second aspect in the design principle at the system level, deals with connectivity or topology, which accounts for the dynamic expression of component genes at different levels and the corresponding system-level emergent properties. For example, a positive feedback loop comprised of two genes is able to generate bistability in gene expression that can act as a switch. In contrast, a negative feedback loop comprised of two genes has a property of repressing gene expression overshoot and constraining noise (Ferrell, 2013; Hart and Alon, 2013). Moreover, the incoherent feed-forward loop comprised of three factors can generate non-monotonic input functions for genes (Kaplan et al., 2008). A number of these small modules can constitute a system with even more complex connectivity for multiple functions. The emergent properties are mechanistically determined by the dynamic motion of the entire system, which is governed by the laws of chemical kinetics as the orbital motion of planets follows the law of gravitation. To explore these emergent properties, we turn to mathematic modeling, which applies the theory of non-linear dynamics for generating system-level insights.

Approaches for modeling biological systems include Boolean models, ordinary differential equations (ODEs) and stochastic models. Boolean models are simple and qualitative, but lack the functionality of capturing non-linearity under biochemical reactions. In contrast, ODEs are able to quantitatively capture chemical kinetics. Stochastic models not only formulate non-linearity but also recapitulate the stochastic
nature in each reaction step, providing the most accurate tool for modeling biological systems. Stochastic models are rigorously formulated through the chemical master equation but often approximated into the chemical Langevin equation in the form of stochastic differential equations (SDEs) (Gillespie, 1992, 2000; Lee et al., 2010). Under some circumstances in complex biological networks, the stochasticity is critical, because it can lead to the so-called “deviant effect”, which is the discrepancy between the manifestations of behaviors and the predictions from continuous deterministic classical chemical kinetics (Samoilov and Arkin, 2006). The deviant effect might be dramatic for systems with complex dynamics and strong non-linearity and/or in a small reaction volume with limited number of molecules. Therefore, if significant discrepancy was observed between measurement and simulation results from ODE models, a switch to stochastic modeling is likely to be an effective alternative. However, given the fact that stochastic models are usually computationally intensive, a majority of modeling work for the cell cycle control has focused on ODE models.

The ultimate goal in modeling biological systems is to develop correct models that have predicting power. To be useful a model must be correct in two aspects – description and model parameters. The correctness of description depends on how reliable or exhaustive our knowledge of the system is. If, for instance, the overall structure of the topology is incomplete because of connections that have not been identified or recognized, one can image that the generated predictions might diverge far
from the truth. In other words, mathematic modeling is more suitable for analyzing networks for which we have substantial knowledge at the biological and biochemical levels. However, even when we do, choosing parameter values within a biologically relevant range remains a big challenge in modeling analysis, particularly with models using ODEs or SDEs. The situation becomes increasing complicated for modeling complex networks, which may include tens or hundreds different parameters. In many cases, only a small number of parameter values can be fixed from either well-known biochemical constants or results from published data. For those unknown parameters, one possible way is to infer them retrospectively through biophysical measurements such as static light scattering, surface plasmon resonance and fluorescence correlation spectroscopy (Attri and Minton, 2005; Elson, 2011; Homola et al., 1999). Moreover, the recent advances in super-resolution microscopy indicate that the stoichiometry, or the relative composition of protein complexes, can be quantified from state-of-the-art super-resolution imaging data (Leake et al., 2006). An alternative way to resolve this parameter uncertainty is to perform sensitivity analysis. Through perturbation of all the parameters within a wide range, it is possible to narrow down the parameter space to those essential ones that significantly affect system outputs.

During the past forty years, modeling the cell cycle has received a great deal of interest largely fueled by knowledge accumulated in studies relying on biochemical approaches. Hundreds of models have been published that partially or completely
describe the cell cycle in a variety of organisms from *S. cerevisiae* to mammals (Ferrell et al., 2011; Tyson et al., 2001). Most relevant to my work, Guang Yao working with Lingchong You, established a set of ODEs based on the existing knowledge of the Rb/E2F network structure to model the specific stage of cell cycle entry (Yao et al., 2008). The model includes a set of ODEs describing the major interactions within the network. Simulation analyses indicate that the positive feedback loop within the network is able to generate a bistable E2F output, which corresponds to quiescence or proliferation state, a prediction that was experimentally validated. These results provided a novel system-level insight in how the Rb/E2F network behaves collectively in specifying commitment to the cell cycle. Later, Lee et al. further improved the above ODE model but turning it into an SDE model. This new version successfully reconciled two apparently contradictory phenomenological models proposed four decades ago (Lee et al., 2010). However, because the Rb/E2F network structure includes multiple feedback loops, the model does not resolve which positive feedback loop takes precedence and how other nodes in the network affect it.

**1.5 Single-cell analysis**

Predictions obtained from mathematical modeling have to be validated through well-controlled biological experiments. However, conventional experimental approaches based on population analysis suffer from two important limitations that undermine the ability to confirm predictions in many circumstances. First, the assumption in
population analysis is that cells are equal or at least similar in terms of behavior and gene expression under experimental conditions. However, this fundamental assumption has been challenged by unequivocal evidence from single-cell level analysis. For example, there is significant heterogeneity in behavior and gene expression among cells that are otherwise genotypically and phenotypically identical (Altschuler and Wu, 2010; Eldar and Elowitz, 2010). Second, cell fate decision in many cases depends on the cumulative effect of gene expression, which includes the “memory” of the entire “history” of gene expression (Purvis and Lahav, 2013; Spiller et al., 2010). As such, a snapshot of gene expression at a single time point may miss essential information encoded in the temporal scale. These problems become even more acute in the study of cell cycle control, because the cell cycle is a periodic process. To normalize as much as possible the state of the cells, a synchronization procedure is often performed to get them to enter a quiescent state, either through the use of serum starvation or the addition of small molecules that arrest cells at specific stages of the cell cycle. However, due to the stochastic nature of the biochemical processes, individual cells become gradually unsynchronized after release from their quiescent state. Moreover, synchronization is not an option or is of little help when studying the cell cycle in cells actively cycling.

Single-cell analysis provides the opportunity to follow the dynamics of signaling networks that reflect how an individual cell encodes and decodes information to adopt a
particular cellular outcome (Purvis and Lahav, 2013; Spiller et al., 2010). In particular, the live-cell imaging technique permits analysis at the single-cell resolution and in real-time, successfully overcoming the major defects in traditional approaches. In this case, results from single-cell level measurement can be directly used to validate predictions from mathematical models. Thus, the combinational use of both theoretical and experimental tools, including modeling-guided experimental design and data-driven model refinement turns out to be a powerful approach for understanding the design principles underlying different gene circuits.

An important prerequisite for single-cell analysis is the development of proper fluorescence reporters. Owing to the fact that gene expression is regulated at multiple levels, reporters can be generated to reflect different types of regulation. For instance, to study the transcriptional regulation of an endogenous gene of interest, a construct consisting of a gene encoding a fluorescent protein, linked to the promoter of the endogenous gene under study is usually sufficient for practice. Tradeoffs exist in the selection of the promoter region, because genomic insertion of long DNA fragments is always inefficient whereas a short promoter without distal enhancers sometimes cannot mimic the endogenous regulation. Alternatively, if the regulation mediated by protein degradation or translocation is critical, a protein-fusion reporter can be constructed by replacing the gene encoding a fluorescent protein with one encoding a fusion protein consisting of the protein of interest linked in frame to a fluorescent protein either at its
N- or C-terminus (Loewer et al., 2010; Nelson et al., 2004). There are several caveats for building such protein reporters. First, the fusion protein may fold improperly, resulting in low fluorescence, loss of function or inability to be normally regulated. Second, the integration of these protein reporters into genome increases the gene copy number and perhaps the gene expression dosage as well, thereby altering cellular homeostasis. Likewise, reporters to probe other types of regulation such as post-transcriptional inhibition by microRNAs can be constructed if the corresponding regulatory elements are included within the 3′ end of the reporter gene (Mukherji et al., 2011). Moreover, strategies for developing reporters capable of assessing the activity of specific kinases inside of cells have been recently obtained. It was achieved by incorporating a cognate phosphorylation site into a nuclear export sequence linked to a fluorescent marker, resulting in a gated, phosphorylation-dependent nucleo-cytoplasmic shuttling of the marker (Regot et al., 2014).

As single-cell analysis measurements in real time must be obtained in a non-invasive fashion, the detection method of choice has been traditionally based on collecting microscopy images during the course of the experiment. However, for this approach to be practical, one needs to overcome the limitation of the labor-intensive task of image analysis. To address this issue, computer programs for automatic image processing must be developed. Time-lapse experiments usually generate a huge amount of images in time series. Automatic processing tools are demanding for efficient data
analysis. A useful tool should be able to combine the functions of cell segmentation, signal quantification and cell tracking over a series of frame. These tasks are always challenging, because cells have irregular shapes and move over time under normal culture conditions. Although commercial packages are available, they tend to be rather basic and are often onerous. As a result, several academic groups have been developing open platform software (e.g. CellProfiler from Broad Institute and CellTracer from Duke University) for use (Carpenter et al., 2006; Wang et al., 2010). However, these programs are still in their infancy and do not always fulfill the specific needs of a particular experimental approach.

In the past decade, single-cell analysis has been applied to the study of cell signaling transduction, including those of the p53, NFκB, MAPK, TGF-β and apoptosis-associated caspase pathways (Albeck et al., 2013; Aoki et al., 2013; Loewer et al., 2010; Nelson et al., 2004; Sorre et al., 2014; Spencer et al., 2009). For example, it was shown that p53 level in single cells shows spontaneous pulses in normal conditions, but oscillates continuously when cells are exposed to stress signals such as DNA damage (Loewer et al., 2010). Similarly, the use of a fluorescent fusion protein reporter revealed that NFκB translocation dynamics show asynchronous oscillations following cell stimulation that decreased in frequency with increased IκBα transcription (Nelson et al., 2004). In comparison, the TGF-β signaling pathway exhibited a distinct regulatory logic. SMAD4 translocation signal dramatically increased in response to transiently increased TGF-β
β1 concentration but kept constant when cells were exposed to slowly increased TGF-
β1 concentration, enabling cells to extract positional information from an environment
with gradient morphogen (Sorre et al., 2014).

To date, few studies have been done at the single-cell level to investigate the
mechanisms underlying cell cycle control. Most of them were performed in the model
organism *S. cerevisiae*. By using a yeast, G1 cyclin *CLN2* promoter-driven reporter,
Skotheim et al. demonstrated that the positive feedback mediated by G1 cyclins ensures
coherent cell cycle entry (Skotheim et al., 2008). A following study using a Whi5 (Rb
homolog in in *S. cerevisiae*)-GFP reporter revealed that nuclear Whi5 precisely predicts
whether a yeast cell can commit into cell cycle and divide (Doncic et al., 2011). Recently,
live-cell imaging approaches have been applied to study mammalian cell cycle control.
Through the measurement of CDK2 activity, Spencer et al. indicated that, after the
previous mitosis, mammalian cells can either continue cycling or go into a G0-like state
(Spencer et al., 2013). Interestingly, these two cell fate decisions correlated with a
bifurcation in CDK2 activity at the mitotic exit. In all, results from single-cell analysis not
only revealed quantitative measures of the regulatory mechanisms, but also provided
mechanistic insights about how signals are shaped by the network structure.

Probing E2F dynamics during cell cycle progression at the single-cell level and in
real-time is likely to contribute to our understanding of the design principles of Rb/E2F
network. Indeed, E2F is the most downstream component of the network structure,
which integrates multiple upstream signals for the initialization of G1/S transcriptional program. In a pioneering study that relied on the use of an E2F1 transcriptional reporter, Yao et al. established the relationship between bistable E2F activation and the crossing of the restriction point (Yao et al., 2008). However, their analysis was limited by the fact that they obtained their measurements through flow cytometry, which only provide a snapshot of gene expression at a particular time, rather than inform on the temporal dynamics. In additional, their developed reporter was not optimized for time-lapse microscopy, because it produced a diffused and weak fluorescence signal throughout the cells, which was hard to quantified accurately. Therefore, before I initiated my studies, the development of a proper E2F reporter for live-cell imaging remained a central challenge for applying single-cell approaches for the study of cell cycle entry.

1.6 Thesis roadmap

In the present study, I sought to develop an E2F reporter and experimental platform suitable for the analysis of E2F dynamics at the single-cell level. Moreover, I describe how I combined mathematical modeling and single-cell analysis to define the molecular logic underlying the control of cell cycle entry by Rb/E2F network.

The overall study aims to address the following questions about E2F dynamics: Do E2F dynamics determine the commitment to cell cycle entry in individual cells? If so, what aspects of E2F temporal dynamics are the major determinants of cell cycle entry? How do Myc and G1 cyclin/CDKs affect different aspects of E2F temporal dynamics?
How do their effects manifest themselves in the ability of a single cell to enter and pace the cell cycle?

Chapter 2 describes the design and use of an E2F transcriptional reporter that enables real-time measurements of E2F transcriptional dynamics in individual mammalian cells. I then describe an integrated experimental platform that was used throughout my experiments. Rat fibroblast (REF52) cells stably integrated with the reporter construct were cultured in microfluidic slides and monitored under a time-lapse fluorescent microscope for live-cell imaging, for periods of up to 96 hours. E2F transcriptional dynamics were extracted and quantified from raw data images collected from single cells (50-200 cells monitored per experiments). These dynamics trajectories were characterized by using a set of quantitative metrics.

Chapter 3 establishes quantitative relationship between E2F dynamics and the adopted cell fate (commitment, division or quiescence). The data revealed that a single metric, the amplitude of E2F accumulation ($Amp$), is capable of precisely predicting cell cycle entry.

Chapter 4 discusses the theoretical analysis based on a developed full ODE model for Rb/E2F network. The simulation results predicted distinct effects on E2F dynamics that are exerted by Myc and G1 cyclin/CDKs. Myc level is critical for modulating $Amp$ and thereby controls the commitment into cell cycle, whereas G1 cyclin/CDKs activity has little effect on $Amp$ but contributes to the regulation of the
timing of E2F activation and thereby influences the pace of cell cycle progression. These predictions were also met after reducing the network to a functional central motif, suggesting that the distinctive modes of control over E2F dynamics are an intrinsic property of the core network structure.

Chapter 5 provides evidence for experimental validation of the predictions derived in Chapter 4. Under the perturbations of Myc or G1 cyclin/CDKs activities by using specific small molecule inhibitors as well as RNA interference (RNAi), the E2F dynamics trajectory in each individual cell was analyzed and mapped to its corresponding cell fate.

Chapter 6 details ongoing progress on using the E2F transcriptional reporter to study E2F dynamics in cycling cells. I present preliminary data on my measurements and characterization of E2F dynamics in these cells, and discuss how they are consistent with the observations made by Spencer et al. in their study.

Chapter 7 summarizes the major discoveries of this study, draws conclusions and highlights potential directions that can be taken in future studies.
Measurement of E2F dynamics at the single-cell level

2.1 Introduction

Conventional approaches for detecting and quantifying E2F activation are population-based methods used in molecular biology, including quantitative reverse transcription polymerase chain reaction (qRT-PCR), northern blot and western blot. Other methods have been developed to measure gene expression in individual cells. These include fluorescence immuno-staining, RNA fluorescence in situ hybridization (FISH) and newly developed tools such as single-cell qRT-PCR and single-cell western blot (Hughes et al., 2014; Phillips and Lipski, 2000). However, all the above approaches require lysing or fixing cells before they can be performed. As such, they can only reveal a snapshot measurement at a specific time point, at the expense of recording the history of gene expression over the course of an entire biological process. In order to follow gene expression both at the single-cell level and in real time, the method of choice is to perform live-cell imaging with a fluorescence reporter that can act as a proxy for the gene of interest.

To measure E2F1 activation at the single-cell level, Yao et al. originally developed a fluorescence reporter (Yao et al., 2008). The reporter was constructed by
inserting the proximal human E2F1 promoter upstream of a sequence encoding destabilized green fluorescence protein (GFP). They integrated this reporter into rat fibroblast REF52 cells and screened single clones. Cells were released from serum starvation at different serum levels for a period of time before being collected for flow cytometry analysis. Their data uncovered a bimodal distribution for reporter signal in cells, suggesting that the Rb/E2F pathway functions as a bistable switch to convert graded serum inputs into all-or-none E2F responses. However, despite its usefulness for flow cytometry analysis, that reporter was not suitable for live-cell imaging in single cells.

In this Chapter, I describe how I was able to improve the design of the E2F1 transcriptional reporter to be useful for time-lapse microscopy, taking into account the issues of repeated exposure for image acquisition, ease of image analysis and signal quantification. The improved reporter was introduced into REF52 rat fibroblasts and NIH3T3 mouse fibroblasts, and single cell clones were isolated. Temporal dynamics of E2F1 expression were determined at the single-cell level. Acquired dynamics trajectories in individual cells show cell-to-cell differences but adopted a consistent pattern, which can be characterized by a set of quantitative metrics.
2.2 Results

2.2.1 Construction of E2F1 transcriptional reporter

To measure E2F1 transcriptional dynamics in single cells, we re-engineered the reporter construct from Yao et al. to generate a brighter fluorescent signal that is largely localized to the nucleus. We substituted a destabilized Venus (~4 hour half-life) for destabilized GFP, since the former one is brighter and has a shorter folding time. To constrain the signal to the nucleus, we fused four repeats of the nuclear localization sequence (NLS) at the N-terminus of the fluorescent protein (Fig. 2.1a). As an alternative confirmation, we constructed a mouse E2F1 promoter-driven reporter using the same strategy and integrated it into the mouse fibroblast NIH3T3 cells (Fig. 2.1b).

The improvements facilitated segmentation and signal extraction in individual cells, as nuclei are well separated from each other in the field of observation. Individual
cell clones (REF52-hE2F1p::4NLS-d4Venus) expressing the reporter construct were isolated and used for experimental validation. Consistent with our expectations, the majority of the Venus fluorescence signal in independent clones was restricted to the nucleus, with a small amount of the signal localized around the nucleus (Fig. 2.3a). We confirmed that the reporter recapitulated E2F1 dynamics at the population level by comparing the individual trajectories and endogenous E2F1 mRNA expression over time (Fig. 2.2a). Furthermore, we found that the accumulation of Venus and endogenous E2F1 proteins were also concordant, although minor differences can be seen, particularly in later time points (Fig. 2.2b). Altogether, the dynamic expression of the reporter correlated with endogenous E2F1 expression throughout the time course of the experiment.
FIGURE 2.2: Confirmation of hE2F1p::4NLS-d4Venus reporter. (a) Alignment of E2F dynamics trajectories to endogenous E2F1 mRNA expression. Gray curves indicate 53 smoothened E2F dynamics trajectories measured after REF52-hE2F1p::4NLS-d4Venus cells were released from serum starvation after the addition of 10% BGS. The black curve indicates endogenous E2F1 mRNA expression (normalized to the peak value) measured by qRT-PCR at different time points. (b) Western blot of Venus and endogenous E2F1 protein expression following serum stimulation in a single cell clone of REF52-hE2F1p::4NLS-d4Venus cells integrated with the reporter.

2.2.2 Measurement of E2F1 dynamics at the single-cell level

We next used this proxy system to examine the real-time dynamics of E2F in single REF52 rat fibroblasts held in G0 through serum starvation and released into the cell cycle by serum stimulation. For each time point, we quantified the level of fluorescence in individual cells, which could be accurately measured up to early M phase, when the nuclear membrane starts to fall apart (Fig. 2.3a). The temporal E2F dynamics in individual cells were highly variable (Fig. 2.3b), but exhibited a consistent pattern: after an initial delay, the E2F1 signal increased from a basal level to a maximum value and then slowly decreased (Fig. 2.3c). Moreover, the same overall pattern was
observed in single cells isolated from other clones transduced independently with our reporter gene.

**FIGURE 2.3: Measurement of E2F Transcriptional dynamics in single cells.** (a) Time-lapse microscopy images of REF52 rat fibroblasts expressing hE2F1p::4NLS-d4Venus reporter released from serum starvation by adding 10% bovine growth serum (BGS). Upper panel, phase channel; low panel, Venus channel. Scale bar, 50 μm. (b) E2F1 dynamics trajectories of the five individual cells shown in (a). Time-series raw data (sampled per hour) were smoothened in a 3-hour time window. Arrows indicate the cell division time point of corresponding cells. (c) Characterization of E2F1 dynamics trajectory in divided (green) cells with defined metrics: \( t_i \), initial delay; \( t_2 \), activation time; \( t_3 \), post-activation time; \( \text{Amp} \), amplitude increase; \( S \) (shadow area), E2F1 work during activation time; \( k \), slope; \( T \), the entire cell cycle length. Orange triangle indicates E2F1 signal upturn time point and the blue one indicates cell division time point.
2.2.3 Characterization of E2F1 dynamics

We then defined a set of metrics to quantify the observed E2F dynamics (Fig. 2.3c). These include maximum amplitude ($Amp$), initial delay ($t_1$), activation time ($t_2$) and post-activation time ($t_3$, for cells that undergo division only). $Amp$ and $t_2$ can be combined to define two additional metrics – the slope ($k = Amp/t_2$) and the area under the curve ($S = Amp \times t_2$); $k$ corresponds to the rate increase, which reflects the strength of positive feedback loop in the regulation, whereas $S$ correlates with the total transcription work of the network directed at the E2F1 promoter (Fig. 2.3c).

To evaluate these metrics, we measured E2F1 temporal dynamics in ~100 individual cells for each level of serum activation and calculated their values for each cell. Values of the four metrics (as shown for $Amp$, $t_1$, $t_2$ and $t_3$) were highly variable among individual cells (Fig. 2.4a-d) because of stochastic gene expression. Notably, $Amp$ exhibited bimodal distribution (ON/OFF) at an intermediate serum concentration (Fig. 2.4a), consistent with the bistability of the Rb/E2F switch. Moreover, decreasing serum concentration led to significant change in the distribution of $Amp$ but moderate increase of average values of $t_1$, $t_2$ and $t_3$ (Fig. 2.4b-d). Pairwise correlation analyses revealed a weak correlation between $Amp$ and $t_2$, and no significant correlations between other pairs, suggesting little dependence among these metrics (Fig. 2.5).
FIGURE 2.4: Distribution of different metrics. (a) Histogram shows the distribution of Amp at different serum levels. (b-d) Histograms show the distribution of each metrics value among \( t_1 \), \( t_2 \) and \( t_3 \) (in divided cells) at different serum levels, respectively. The mean values were compared among different serum concentrations and plotted as insets.

FIGURE 2.5: Pairwise correlation between E2F1 dynamics metrics. Data points under different serum levels are mixed in plotting and labeled with different colors.
2.3 Discussion

Here we provide the first measurement of E2F transcriptional dynamics at the single-cell level. Our results indicate that E2F1 shows a pulse-like expression pattern within the first cell cycle. This pattern is consistent with the previous finding that endogenous E2F1 protein level is periodic, peaking at the beginning of S phase and then decaying rapidly as S/G2 progress (Marti et al., 1999). The overall consistent pattern among all cells examined also underscores the fact that E2F1 expression is tightly regulated during cell cycle progression.

Our data clearly indicate the bimodal distribution of the metric \( Amp \) among a population of cells, consistent with previous findings based on flow cytometry analysis (Yao et al., 2008). Remarkably, temporal characteristics of E2F1 dynamics define three distinct phases: an initial delay (\( t_1 \)), an activation window (\( t_2 \)) and a post-activation window (\( t_3 \)). These observations strongly argue that regulation of endogenous E2F1 transcription during the first cell cycle is itself occurring in three different stages. During the first stage (lasting about 8~10 hours), E2F1 is transcriptionally repressed. Interestingly, it was reported that cultured mouse fibroblast NIH 3T3 cells experience an extra 8 hour delay when released from quiescence into the cell cycle by serum stimulation (Coller, 2007). It will be interesting to investigate at the single-cell level whether this extra delay could be explained by the initial delay of E2F1 transcriptional dynamics. In contrast, E2F1 transcription is stably induced during the activation
window that spans the late G1 and S phases. This finding is not surprising because S phase progression requires the activation of G1/S transcriptional program that is initialized by E2F1 activation (Bertoli et al., 2013). However, it will be interesting to investigate how this activation trend responds to perturbation of core components in the Rb/E2F network. Lastly, we observed that E2F1 signal decreases from late S phase to mitosis. The timing for this repression wave temporally matches activation of E2F7/8, cyclin A and Skp2, which are known to mediate E2F1 negative auto-regulation for the shutdown of E2F1 transcriptional program (Krek et al., 1995; Liu et al., 2013; Marti et al., 1999). These consistent findings further confirm the reliability of using our reporter system to monitor endogenous E2F1 transcriptional regulation.

The experimental platform that I developed provides a convenient tool that not only increases measurement resolution and quantitation of E2F dynamics at the single level, but can also be easily integrated with additional experimental manipulations and functional assays. In Chapter 3, we will combine the monitoring of E2F dynamics with measurements of cell fate decisions, in order to explore the quantitative relationship between the two phenomena.

2.4 Methods

2.4.1 E2F1 transcriptional reporter system

A DNA fragment encoding d4Venus (destabilized Venus, ~ 4 h half-life) was isolated from pd4Venus-N1 and fused in frame at its 5’ end to four repeats of SV40
nuclear localization sequence (NLS) to generate a cassette encoding the 4NLS-d4Venus-
fusion protein. This DNA cassette was then sub-cloned downstream of the human E2F1 
promoter (-784 to +32) and the resultant fragment was inserted into the pQCXIP 
retroviral vector (Clontech), to generate the pQCXIP-hE2F1p::4NLS-d4Venus reporter 
plasmid. This plasmid was transfected into an ecotropic packaging cell line, Plat-E. 
Forty-eight hours after transfection, the culture medium containing retrovirus particles 
was filtered and applied to REF52 cells. Clonal pools of cells transduced with the 
reporter system were selected in medium containing puromycin (2 μg/ml) for 
approximately 10 days. Single clones (REF52-hE2F1p::4NLS-d4Venus) were then picked 
up from the clonal pool population by limiting dilution. The hE2F1p::4NLS-d4Venus 
reporter system leads to two significant improvements that are critical for single-cell 
analysis. First, the addition of four NLSs to the reporter protein restricts its localization 
into the nucleus, thereby improving sensitivity of detection by increasing the intensity of 
the fluorescent signal. Second, restricted localization to the nucleus facilitates 
segmentation and signal extraction in individual cells, as nuclei in different cells are well 
separated. To further confirm that expression of the improved reporter construct 
recapitulated faithfully E2F1 transcriptional expression, a mouse E2F1 promoter (-1,165 
to +123) was substituted for the human E2F1 promoter in hE2F1p::4NLS-d4Venus to 
derive the similar reporter, pQCXIP-mE2F1p::4NLS-d4Venus. This construct was 
introduced into mouse embryonic fibroblast NIH3T3 cells to derive the NIH3T3-
mE2F1p::4NLS-d4Venus reporter cells. Identical findings were observed with this second reporter.

### 2.4.2 Cell culture

REF52 (an immortal line of post-crisis Fischer rat embryo cells) and NIH3T3 mouse fibroblasts (CRL-1658, ATCC) were routinely grown in Minimum Essential Medium Alpha Medium (Gibco/Invitrogen) supplemented with 10% bovine growth medium (BGS, Hyclone/Thermo Scientific).

### 2.4.3 Live cell imaging

For E2F dynamics measurements using time-lapse microscopy, cells were first trypsinized, resuspended at a density of $1 \times 10^5$ per ml and then seeded in μ-Slide I (tissue culture treated, ibidi) channel slides by adding 1 ml volume of the cell suspension. After overnight incubation, cells were synchronized in quiescence by shifting into Minimum Essential Medium Alpha medium with 0.02% BGS (starvation medium) for 36 h. At that point, quiescent cells growing in μ-Slide I slides were released from starvation by shifting to serum containing medium and placed under Leica DMI 6000 B inverted fluorescence microscope (Leica). Images were taken using Leica N PLAN L 20×0.4 objective lens with phase contractor a Semrock BrightLine YFP filter set (500/542 nm, excitation/emission) and Hamamatsu ORCA AG digital camera (Hamamatsu) with uniform parameter setting: binning = 4, offset = 0, gain = 255 and exposure time = 0.01 s (phase channel) or 0.15 s (YFP channel). The microscope was enclosed with an
environmental chamber with 37 °C temperature, atmosphere (5% CO₂) and humidity. Images were acquired every 30 or 60 min for 24–48 h. Time-series image acquisition was controlled by SimplePCI6 Software (Hamamatsu).

2.4.4 Image analysis

The time-lapse microscopy involved taking two series of raw images of the cells in 60-min increments for 36 h, one series for the phase channel and the second for the YFP channel. E2F signals were extracted from these images using ImageJ (NIH) software. The first time-point of each series of images was loaded side-by-side into the software. Using the ROI Manager Tool, a circular selective mask with a fixed area was placed around each cell nucleus in the phase channel image. The location of each mask was then copied to the YFP channel image and the integrated grey value of the selected pixels was measured. In the case of cell division, the selective mask was applied to one of the daughter cells. This value was normalized to the background by subtracting the integrated grey value of the same area of pixels in an empty part of the image. This process of measuring normalized grey value of the nuclei was repeated for each time point by adjusting the location of the selective mask to account for the movement of cells, thus giving a time-series measurement of the fluorescence reporter on the E2F sequence. Throughout this process, data from cells that left the field of view at any time were discarded; and the time of cell division was also noted.
2.4.5 qRT-PCR analysis

RNA extracts were prepared from REF52-hE2F1p::4NLS-d4Venus reporter cells using the RNeasy Protect Cell Mini Kit (Qiagen) according to the manufacturer’s protocol. E2F1 RNA expression was interrogated by real-time PCR using the Power SYBR Green RNA-to-CT 1-step Kit (Applied Biosystems). Gene-specific primers used: 5′-TTG ACCCCTCTGGATTTCTG-3′ and 5′-CCCTTTGGTCTGCTCAATGT-3′ for rat E2F1; 5′-GTCGTACCACACTGGCATTGTG-3′ and 5′-CTCTCAGCTGTGGTGTAAGG-3′ for rat β-actin.

2.4.6 Western blot analysis

Antibodies against E2F1 (1:1,000, Cell Signaling), GFP (XP Rabbit mAb, 1:1,000, Cell Signaling), and Actin (C-2, 1:1,000; Santa Cruz) were selected for the quantification of protein expression at the population level. REF52-E2F1p::4NLS-d4Venus cells were harvested and a protein extract was obtained by lysis at different time points after release from quiescence. Total protein amount was quantified with the bicinchoninic acid (BCA) assay (Pierce). Equal protein amounts were separated by 4–20% Mini PROTEAN TGX gradient gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes by electro-blotting. Membranes were then blocked with 5% nonfat dried milk, incubated overnight at 4 °C with primary antibody, washed in TBS with 0.2% Tween, and incubated again with secondary antibody coupled to peroxidase. Protein
levels were detected by using LumiGLO Peroxidase Chemiluminescent Substrate Kit (Cell Signaling) after additional washing steps.
Amplitude of E2F accumulation as the predictor of commitment and division

3.1 Introduction

Previous studies performed on a cell population indicated that E2F accumulation is associated with S phase entry and cell proliferation. Remarkably, Johnson et al. showed that expression of E2F1 transcriptional factor alone is able to induce S phase entry in quiescent rat fibroblast cells (Johnson et al., 1993; Kowalik et al., 1995). This phenomenological observation was later explained in subsequent studies when a large set of E2F target genes were identified to participate in S phase progression and initialize DNA replication. Interestingly, overexpression of E2F1 protein was reported to induce apoptosis, suggesting a fail-safe mechanism that cells evolve to respond to excessive amount of proliferative signals (Polager and Ginsberg, 2008; Shan and Lee, 1994). These seemingly contradictory findings suggest that E2F expression must be tightly regulated in individual cells but also raise a very interesting possibility: Does E2F expression dictate the fate of a particular cell?

The experimental platform described in Chapter 2 provides a convenient practical means to address that question. With the use of that platform, we documented for the first time how E2F transcriptional dynamics looked like at the single cell level. In
addition, we were able to quantify this activation accurately. It allowed us to define a set of metrics encompassing the properties of the E2F dynamics trajectories, metrics that evaluated in our attempts to link a particular cell fate to the observed dynamics. Specifically, we sought to identify a single metric that could predict cell fate (commitment or division) (Fig. 3.1).

FIGURE 3.1: A schematic diagram links E2F dynamics to phenotypes at the single-cell level.

3.2 Results

3.2.1 Amp as the predictor of commitment

To examine the extent by which the metrics introduced in Chapter 2 could predict cell-cycle commitment, we combined measurements of single-cell E2F dynamics with measurement of 5-ethynyl-20-deoxyuridine (EdU) incorporation into newly synthesized DNA, a surrogate for determining cell cycle entry (Fig. 3.2a-b). We found that Amp is a reliable predictor of commitment. Specifically, plotting EdU signals against Amp values for ~100 cells revealed two distinct groups of cells: cells with high EdU
incorporation had an $Amp$ value above a threshold ($Amp_{th}$), whereas cells with no EdU labelling showed a value of $Amp$ below it (Fig. 3.2c). This relationship was also confirmed in NIH3T3 cells with the mouse E2F1 reporter (Fig. 3.2d). Two $Amp$-related metrics—$k$ and $S$—were also informative, albeit slightly less accurate at predicting cell cycle entry (Fig. 3.3a-b). The other metrics ($t_1$ and $t_2$) were much less reliable predictors (Fig. 3.3c-d).
FIGURE 3.2: *Amp* as the predictor of commitment. (a) The diagram indicates the experimental design to correlate E2F dynamics with commitment into cell cycle entry. Serum-starved cells were released by adding 1% BGS together with EdU and then incubated under time-lapse microscope for E2F1 dynamics measurement. After 44 hours incubation, cells were fixed and subjected to EdU staining. (b) EdU labeling after E2F dynamics measurement and cell fixation. Circles mark the nuclei and white arrow indicates the cell with negative EdU signal. Scale bar, 50 μm. (c) Scatter plot based on cell fate (circle, uncommitted; dot, committed) as determined by *Amp* in REF52-hE2F1p::4NLS-d4Venus cells. Dotted line indicates *Amp*<sub>th</sub> (at which an individual cell has 50% probability to commit into cell cycle). Solid line indicates the boundary of signal between EdU-positive and -negative cells. (d) Scatter plot based on cell fate as determined by *Amp* in NIH3T3-mE2F1p::4NLS-d4Venus cells.
3.2.2 Amp as the predictor of division

As we showed that the Amp and $k$ parameters correlate with the commitment into S phase, it stood to reason that they would also predict cell division. To confirm this prediction, we followed cell division over time in single cells and linked it to E2F dynamics. Data points from individual cells stimulated with different serum levels were aggregated and split into two groups depending on whether the cells had divided. A small proportion of cells (~ 5%) within the undivided group displayed high Amp. These cells were likely within the $t_2$ interval, and would have been observed to divide if we had kept monitoring them longer (Fig. 3.4). In subsequent analyses, we excluded these cells as we lacked definitive information about their ultimate behavior. The aggregate data were used to plot the distribution of cells as a function of the values of the corresponding E2F1 dynamic metrics measured in single cells. When plotted against
Amp or k, we observed a clear-cut boundary between the group of cells that had divided and the one in which cells had not (Fig. 3.5a and Fig. 3.6a). A similar trend was observed in the NIH3T3 reporter cells (Fig. 3.5b). This distinction became less apparent when the same data were plotted against S and was lost for t₁ or t₂ (Fig. 3.6b-d).

**FIGURE 3.4: Representative E2F trajectories in different sub-groups.** Divided cells (green curves) show significant E2F increase while undivided cells (red curves) show little increase in E2F Amp. In addition, a small proportion of cells (< 5%, yellow curves) show a fluorescence signal that is rising at the end of the observation window but have not divided during the observation window. This sub-group of cells was omitted from the entire population subjected to statistical analysis of the relationship between Amp and cell fate.
FIGURE 3.5: *Amp as the predictor of division.* (a) Histogram based on cell fate (red, undivided; green divided) as determined by *Amp* in REF52 cells. A total amount of 250 cells were analyzed. (b) Histogram based on cell fate as determined by *Amp* in NIH3T3 cells.

FIGURE 3.6: Division as determined by relevant metrics. (a-d) Histogram based on cell fate (red, undivided; green divided) as determined by *k, S, t₁* or *t₂* in REF52 cells.

### 3.2.3 Correctness as predicted by different E2F1 dynamics metrics

We performed logistic regression to estimate the probability of cell commitment or division as a function of different metrics. Our results demonstrated that *Amp* serves as the most accurate predictor, with a confidence value of 95% ± 1% for commitment (96% ± 1% for division; Table 3.1). Importantly, we observed an ultrasensitive
dependence between the probability of cell commitment/division and the $Amp$ value, reflected in Hill coefficients of $>7$ for logistic regression curves (Fig. 3.7). A slight increase of $Amp$ from 2.2 to 2.8 a.u. (12% in the scale of mean $Amp$ in E2F1-activated cells) within the ultra-sensitive region can lead to a 50% relative increase in the proportion of divided cells (Fig. 3.7). This observed ultrasensitive dependence further defines a threshold value $Amp_{th}$. If $Amp$ of an individual cell reaches this threshold, it has at least a 50% probability to commit into the cell cycle.

Table 3.1 Accuracy of prediction for cell commitment or division as assessed by different E2F1 dynamics metrics

<table>
<thead>
<tr>
<th>Metric</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Commitment</td>
</tr>
<tr>
<td>$Amp$</td>
<td>95</td>
</tr>
<tr>
<td>$k$</td>
<td>90</td>
</tr>
<tr>
<td>$S$</td>
<td>85</td>
</tr>
<tr>
<td>$t_1$</td>
<td>81</td>
</tr>
<tr>
<td>$t_2$</td>
<td>75</td>
</tr>
<tr>
<td>Random$^a$</td>
<td>50</td>
</tr>
</tbody>
</table>

$^a$ As a control, a random guess predicts 50% correctly
FIGURE 3.7: Probability of cell commitment or division as function of \( Amp \). Logistic regression was performed for cell cycle commitment (dashed line, data from Fig 3.2c) and for division (solid line, data from Fig 3.5a). The shaded area around each logistic regression curve indicates a 95% confidence interval for 1,000 bootstrapping iterations.

3.3 Discussion

In this Chapter, we established for the first time the precise relationship between gene expression dynamics and cell fate at the single-cell level. Specifically, we demonstrated that a single metric, \( Amp \), serves as an accurate predictor of both cell cycle commitment and division. Moreover, this quantitative relationship was shown to hold across different cell lines. These findings suggest that gene expression is tightly regulated in individual cells and its amount has definitive effect on the cell phenotypic output.

An important question is whether this relationship still holds in different tissues and species, given that the Rb/E2F network is evolutionally conserved across a variety of organisms for the control of cell proliferation (van den Heuvel and Dyson, 2008). In
addition, it will be interesting to explore whether this principle is altered in cancerous cells and, if so, how it is altered (Sherr, 1996).

The existence of a quantitative relationship between $Amp$ and cell fate provides an analytical tool for the study of cell proliferation at the single-cell level in various contexts. This reporter can be used to probe the function of any gene or signaling pathway that is known to affect cell proliferation simply through measuring how $Amp$ is influenced when that gene or pathway is perturbed. In Chapter 5, we will apply this strategy for determining the roles of Myc and G1 cyclin/CDKs in the control of cell cycle entry.

### 3.4 Methods

The methods used in this Chapter for cell culture, live cell imaging and image analysis are the same as those described in Chapter 2 (refer to 2.4.2, 2.4.3 and 2.4.4 for details).

#### 3.4.1 EdU staining

EdU staining was performed after E2F dynamics measurement in ibidi μ-Slide I slides by using Click-iT EdU Imaging Kits (Invitrogen) with Alexa Fluro 594 azide according the manufacturer’s instructions. The only modification of the protocol was to wash cells with PBS with 0.2% Tween three times after all the staining steps. Images were taken using Leica N PLAN L 20×0.4 objective lens with phase contrast or a Semrock BrightLine Texas Red filter set (562/624 nm, excitation/emission) and
Hamamatsu ORCA AG digital camera with uniform parameter setting: binning = 4, offset = 0, gain = 255 and exposure time = 0.01 s (phase channel) or 0.8 s (Red channel).

3.4.2 Analysis of E2F dynamics trajectories

This time-series E2F dynamics trajectories were analyzed in Matlab (MathWorks) to define various E2F metrics. We developed a smoothing algorithm for the trajectories after we removed the large fluorescence spike that occurs during mitosis, possibly as a result of either the increased concentration of endogenous E2F that accompanies cell mass shrinkage or the change from a “flat” to a “globular” cell shape that immediately precedes division. The processed trajectories without spikes were then smoothened by using a 3-window Gaussian averaging algorithm. Each smoothed and processed trajectory was then fitted to the following two-phase regression model (Equation (1)) to automatically derive optimal values for $t_1$ and $t_2$:

$$y = y_0, \text{ if } 0 \leq t < t_1; \quad y = (y_{\text{max}} - y_0) \cdot \frac{(t - t_1)}{t_2}, \text{ if } t_1 \leq t \leq t_2.$$  \hspace{1cm} (1)

Thus, the problem equals to search for $\arg\{t_1, t_2\}$ that gives

$$\min \left\{ \sum_{t \in [t_1,t_2]} \left[ y_{E2F}(t) - y(t) \right]^2 \right\}.$$

Relevant parameters represent:

$t_1$: initial delay
$t_2$: activation time
$y_0$: E2F basal level (the average of fluorescence values of the initial four time points)
$y_{\text{max}}$: E2F peak level (maximum fluorescence value of each trajectory)
$y_{E2F}(t)$: E2F signal in dynamic trajectory at the moment $t$
Distinct roles of Myc and G1 cyclin/CDKs revealed by modeling analysis

4.1 Introduction

Mammalian G1 cyclins and CDKs have been generally regarded as the critical upstream regulators of the Rb/E2F network, which translate proliferative signals to commit to cell cycle entry (Massague, 2004; Musgrove et al., 2011). In this canonical view, mitogenic stimuli increase the transcription of D-type cyclins, which combine with either CDK4 or CDK6 to phosphorylate and inactivate Rb (Morgan, 1997; Murray, 2004). Phosphorylation of Rb causes a conformational change resulting in the dissociation of E2F, which when freed from Rb, can initialize the E2F-dependent G1/S transcriptional program (Bertoli et al., 2013). Moreover, the increase in cyclin E level (a direct target of E2F transcriptional activity) further phosphorylates Rb in a quantum level, resulting in a positive feedback loop to commit cells into cell cycle entry.

However, the assumption that cyclin D/CDK4/6, Rb, E2F and cyclin E/CDK2 act in such a linear fashion might be somewhat simplistic. Indeed, knock-in replacement of the cyclin D gene by a cyclin E coding sequence in mice was able to rescue many of the phenotypic defects in cyclin D-deficient mice (Geng et al., 1999). Moreover, recent studies using elegant knockout mouse models has provided increasing evidence that
challenge the prevailing view that G1 cyclin/CDKs are essential for cell cycle entry. Mice with genetic deletion of all three D-type cyclins develop until mid/late gestation and die due to heart and hematopoietic abnormalities (Kozar et al., 2004). However embryonic fibroblasts established from these mutant mice can proliferate (albeit slightly less efficiently as wild-type fibroblasts) largely due to a lengthened cell cycle re-entry (Kozar et al., 2004). A similar finding was reported for mice lacking CDK4 and CDK6 (Malumbres et al., 2004). In addition, genetic deletion of cyclin E and CDK2 genes in mice showed this complex is largely dispensable in mouse development (Berthet et al., 2003; Geng et al., 2003). Mouse embryonic fibroblasts with CDK2 deletion proliferate fine although they show a delay in entering S phase. In contrast, deletion of both E-type cyclins (E1 and E2) completely abolished cell cycle re-entry, but this phenotype was attributed to a kinase-independent mechanism – that is the failure of incorporating MCM proteins into DNA replication origins during G1/S progression (Geng et al., 2007).

In contrast, the role of Myc in the control of Rb/E2F network had remained elusive. Myc is a well-known proto-oncogene and has dramatic effect on the control of cell proliferation. Myc can integrate growth signals from a number of upstream signaling pathways (Dang, 2012). Myc overexpression is frequently linked to cell over-proliferation, while inactivation of Myc results in very slow growth in fibroblasts and can cause dramatic regression of various tumor types in mouse models (Adhikary and Eilers, 2005; Jain et al., 2002; Schorl and Sedivy, 2003; Soucek et al., 2008). The
mechanism by which Myc transduces these signals to the core Rb/E2F network had been postulated to result from its ability to activate cyclin D transcription (Obaya et al., 1999). However, rescue of Myc-deficient cells with ectopic expression of cyclin D failed to rescue the slow-growth phenotype in these cells despite the fact that Rb phosphorylation had been restored to normal (Mateyak et al., 1999; Obaya et al., 1999). This observation suggested that the accepted description for the role of Myc in the cell cycle control might be either incomplete or incorrect.

Previous efforts relying on molecular biology approaches aided little to address this pressing question. In this Chapter, we employed mathematic modeling to explore the system-level emergent property of the Rb/E2F network. We used our mathematical model to predict the effects on E2F dynamics when G1 cyclin/CDKs or Myc were inhibited. The model predictions were biologically validated through chemical or genetic interference of the above nodes in single cells.

4.2 Results

4.2.1 Modularity revealed by sensitivity analysis

We revised an established kinetic model of Rb/E2F network and employed it to perform time-course simulations, which can be checked with the use of our experimental platform (Tables 4.1-4.4) (Lee et al., 2010; Wong et al., 2011b; Yao et al., 2008; Yao et al., 2011). We applied the full mathematical model (see Table 4.4) to generate dynamics trajectories for all 9 variables (Fig. 4.1). Among them, E2F
transcriptional dynamics show a consistent relationship to our biological observations (Fig. 4.1).

**FIGURE 4.1: Simulated temporal trajectories of all the factors in Rb/E2F network.** The initial molecule concentration for the nine variables (Table 4.1) was set as ([Myc], [E2Fm], [E2Fp], [CD], [CE], [RB], [RE], [RP], [R]) = (0, 0, 0, 0, 0.4, 0.25, 0, 0) μM.

We also performed sensitivity analysis to investigate how the perturbation of each parameter value affects the pattern of E2F dynamics. The characteristic E2F pulsatile dynamics are maintained even when each parameter is varied by 100-fold around its base value. We further performed sensitivity analysis of each metric (Amp, t_s, k and S) with respect to changes in 33 parameters in the full model. Our analysis indicates that only parameters associated with Myc-dependent E2F autoregulation can
lead to dramatic change in $Amp$ (Fig. 4.2a). Parameters associated with Myc-dependent E2F autoregulation, Rb-E2F interaction, or cyclin D and cyclin E accumulation can significantly affect $t_2$, $k$ and $S$ (Fig. 4.2b-d). In comparison, parameters associated with the E2F negative feedback loop have little effect on either $Amp$, $t_2$ and $k$ (Fig. 4.2a-c). These results suggest that different modules within the full system influence different aspects of E2F dynamics.
FIGURE 4.2: Sensitivity analysis of $Amp$, $t$, $k$ and $S$ to different model parameters. (a-d) Results were plotted in for each metric ($Amp$, $t$, $k$ and $S$). According to their definitions in the full model, 33 parameters (see Table 4.4) were divided into five functional groups corresponding to five modules: Myc-dependent E2F autoregulation, RB-E2F, cyclin D/CDK4/6 (CycD), cyclin E/CDK2 (CycE) and negative feedback loop (NFL).
4.2.2 Distinct roles of Myc and G1 cyclin/CDKs revealed by simulation analysis

We next examined the modulation of Amp after perturbations of three main regulators – cyclin D/CDK4/6 (CycD), cyclin E/CDK2 (CycE), and Myc (Wong et al., 2012; Wong et al., 2011b; Yao et al., 2008). As mentioned, CycD and CycE nodes play a critical role in controlling the function of Rb through phosphorylation, facilitating activation of E2F. Accordingly, they have been proposed to constitute the rate-limiting step in cell cycle entry (Blagosklonny and Pardee, 2002; Malumbres and Barbacid, 2001). Myc, on the other hand, has been shown to be critical for modulating the strength of E2F auto-regulation, and to contribute to the expression of cyclin D (Johnson et al., 1994; Leung et al., 2008). We simulated the inhibition of each node by reducing their synthesis rate constant, while maintaining the other model parameters constant. Using our full ODE model, we ran simulations under a wide range of either CycD or CycE inhibition. Under these conditions, normalized Amp exhibits only a slight decrease (Fig. 4.3a, b and g). In contrast to the robust behavior of Amp, t2 shows a significant lengthening as inhibition of either CycD or CycE is increased (Fig. 4.3d-e). Moreover, sensitivity analysis suggests that CycD inhibition has much stronger influence on t2 extension than CycE inhibition (Fig. 4.3h). This is likely because CycD triggers Rb phosphorylation before CycE can perform quantum phosphorylation of Rb. We next assessed the consequence of perturbing Myc, as it is known to exert a positive feedback loop on the Rb/E2F network and contribute to the bistable property of E2F signaling (Johnson et al.,
1994; Leung et al., 2008). Somewhat to our surprise, our simulation analysis indicated that increased inhibition of Myc activity induced a significant decline in normalized $Amp$ (Fig. 4.3c and g). Moreover, $t_2$ was predicted to moderately increase within the range of Myc inhibition that fails to prevent the ability of cells to commit (Fig. 4.3f and h).

**FIGURE 4.3:** Distinct roles of Myc and G1 cyclin/CDKs revealed by simulation analysis. (a-c) Normalized $Amp$ (to the $Amp$ for base parameter values) within 120 hours under the inhibition of CDK4/6, CDK2 or Myc as a function of inhibition rate. Horizontal dashed lines mark that the expected threshold value for normalized $Amp_{th}$ is 0.5, given that the measured $Amp_{th}$ is around half of the average $Amp$ of cells. (d-f) Normalized $t_2$ to its initial level as a function of inhibition rate under each inhibition case. Yellow color highlights the region where cells are expected to commit into cell cycle. (g) Log sensitivity analysis of $Amp$ to inhibition rate. (h) Log sensitivity analysis of $t_2$ to inhibition rate. For $k_{inhM}$, log sensitivity was calculated within the committed region.
4.2.3 Distinct roles of Myc and G1 cyclin/CDKs interpreted by system analysis

As the G1 cyclin/CDKs and Myc are both positive regulators of E2F, their qualitatively different impact on E2F Amp appears counter-intuitive. Nevertheless, close inspection indicates that this is an intrinsic dynamic property of the underlying network. To help illustrate the control logic, we simplified the network to a motif comprising an E2F positive feedback and a negative regulation through Rb sequestration (Fig. 4.4a). Other regulatory inputs impinge on this motif by affecting the positive feedback loop strength or affecting Rb phosphorylation. This motif can be described by a single equation (2):

\[
\frac{d[E2F]}{dt} = k_{\text{syn}} \cdot \frac{[E2F]_e}{K + [E2F]_e} - d \cdot [E2F]
\]

\[ [E2F]_e = 0, \text{if} \ [E2F] < [Rb]; \ [E2F]_e = [E2F] - [Rb], \text{if} \ [E2F] \geq [Rb]; \]

where \([E2F]\) represents E2F concentration, \([Rb]\) the concentration of active Rb (unphosphorylated) that can bind to and titrate E2F, \([E2F]_e\) the concentration of free E2F, \(k_{\text{syn}}\) the E2F synthesis rate constant, \(K\) the half-maximal constant for E2F auto-regulation, and \(d\) the degradation rate constant. This equation describes the dynamics of the motif in the limiting case of extremely tight binding between Rb and E2F.

As illustrated by our steady-state analysis, inhibition of Myc drastically reduces the E2F amplitude by decreasing E2F synthesis rate, suggesting that Myc is largely responsible for setting maximum levels of E2F (Fig. 4.4c). In contrast, inhibition of CycD...
or CycE decreases the phosphorylation rate of Rb, which acts as a “sink” against the initial E2F activation. Despite the drastic slowdown in phosphorylation, and as long as Rb gets completely phosphorylated by cyclin/CDK complexes, E2F will reach its maximum level set by Myc (Fig. 4.4b). As a result, inhibition of the cyclin/CDK nodes primarily modulates the time it takes to overcome the Rb-sink but has little impact on the eventual E2F amplitude.

FIGURE 4.4: Distinct roles of Myc and G1 cyclin/CDKs interpreted by system analysis. (a) The central motif of Myc/Rb/E2F network includes E2F auto-regulation (module for level control) and its titration by Rb (module for timing control). (b) Steady-state analysis of the central motif under the inhibition of Rb phosphorylation through reduction of G1 cyclin complexes activity. The decrease in Rb phosphorylation rate leads to only a slight reduction (ΔAmp) in steady-state E2F level. Black and gray lines are E2F synthesis curves under low (black) and high (gray) cellular unphosphorylated Rb levels, respectively. Red line indicates the degradation curve. Blue line indicates the threshold Amp_th. Green dots, stable steady states; red dots, unstable steady states. (c) Steady-state analysis of the central motif. Black and gray lines are E2F synthesis curves given high (black) and low (gray) synthesis rate, respectively.
4.3 Discussion

Using sensitivity analysis for all the parameters within the full ODE model, we have illustrated the modularity of the system structure. The Rb/E2F network can be divided into two fundamental modules: a Myc-dependent E2F auto-regulatory module and a Rb-E2F interaction module. These modules exert their influences on different aspects of E2F dynamics. Specifically, Amp is only sensitive to perturbations directly affecting E2F transcription. Using a similar analysis, we specifically addressed how the inhibition of G1 cyclin/CDKs or Myc affected E2F dynamics. We showed that Amp responds to the inhibition of Myc but not (or very little) to that of the G1 cyclin/CDKs. Interestingly, our results also indicate that $t_2$ remains stable to a level of Myc inhibition that still allows Amp to be above the Amp$_{th}$ level, suggesting fairly separate effects from the perturbation of these two nodes. Furthermore, we reached similar conclusions when we simplified the full network down to a two-node central motif. System analysis revealed that this motif is sufficient to generate non-linear dynamics that account for effects on E2F dynamics when G1 cyclin/CDKs or Myc is inhibited.

Collectively, these findings suggest an unexpected design principle of the Rb/E2F network. In contrast to the canonical view that argues for Myc and G1 cyclin/CDKs to work in a consecutive manner, we found that these two nodes transduce signals and regulate different aspects of E2F dynamics in a parallel mode. In Chapter 5, we will
follow and analyze E2F dynamics by using small molecule inhibitors or RNAi reagents to validate this novel prediction.

4.4 Methods

4.4.1 Mathematic modeling

A modified version of a previously developed ODE model of Rb/E2F network was applied for simulation analysis (Wong et al., 2012; Yao et al., 2008). In contrast to models applied in a previous analysis, we neglected the hypothetical feed-forward loop from Myc to E2F mediated by microRNAs, making the model more concise but still retaining all the essential regulatory characteristics. In addition, we include in our model a representative factor R, which represents regulators in E2F-related negative feedback loops, such as E2F7/8, cyclin A and Skp2 (Fig. 1.2b) (Krek et al., 1994; Krek et al., 1995; Marti et al., 1999). The introduction of R does not influence steady-state analysis but shapes the curves with slight decrease after Amp reaching the maximum level, which is consistent with experimental observation. The revised model includes 10 variables, 9 equations and 33 parameters, as illustrated in Tables 4.1-4.4.
Table 4.1 Description of variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
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<tbody>
<tr>
<td>S</td>
<td>Serum and mitogenic stimulation</td>
</tr>
<tr>
<td>MYC</td>
<td>Myc</td>
</tr>
<tr>
<td>E2Fm</td>
<td>E2F mRNA</td>
</tr>
<tr>
<td>E2Fp</td>
<td>Free E2F protein (not binding to Rb)</td>
</tr>
<tr>
<td>CD</td>
<td>Cyclin D</td>
</tr>
<tr>
<td>CE</td>
<td>Cyclin E</td>
</tr>
<tr>
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<td>Rb pocket protein</td>
</tr>
<tr>
<td>RE</td>
<td>Rb-E2F complex</td>
</tr>
<tr>
<td>RP</td>
<td>Phosphorylated RB</td>
</tr>
<tr>
<td>R</td>
<td>Repressor mediating E2F negative feedback loop</td>
</tr>
</tbody>
</table>

Table 4.2 Description of reaction terms

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>φ → S → MYC</td>
<td>$k_{MC} \cdot \frac{[S]}{K_s + [S]}$</td>
<td>Serum-dependent Myc production</td>
</tr>
<tr>
<td>MYC → φ</td>
<td>$d_{MC} \cdot [MYC]$</td>
<td>Myc decay</td>
</tr>
<tr>
<td>φ → MYC → E2Fm</td>
<td>$k_b \cdot \frac{[MYC]}{K_{MC} + [MYC]}$</td>
<td>E2Fm synthesis regulated by Myc alone</td>
</tr>
<tr>
<td>φ → MYC&amp;E2F → E2Fm</td>
<td>$k_{E2Fm} \cdot \frac{[MYC]}{K_{MC} + [MYC]} \cdot \frac{[E2Fp]}{K_{EF} + [E2Fp]}$</td>
<td>E2Fm synthesis regulated by Myc/E2F cooperation</td>
</tr>
<tr>
<td>E2Fm → φ</td>
<td>$d_{E2Fm} \cdot [E2Fm]$</td>
<td>E2Fm decay</td>
</tr>
<tr>
<td>E2Fm → E2Fp</td>
<td>$k_{E2Fp} \cdot [E2Fm]$</td>
<td>E2Fp production through translation</td>
</tr>
<tr>
<td>Reaction</td>
<td>Kinetic Expression</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td>--------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>E2Fp + RB → RE</td>
<td>( k_{RE} \cdot [RB] \cdot [E2Fp] )</td>
<td>Rb-E2F complex formation</td>
</tr>
<tr>
<td>E2Fp --- ( \rightarrow \phi ) ( R )</td>
<td>( \frac{K_R + [R]}{K_R} \cdot d_{E2Fp} \cdot [E2Fp] )</td>
<td>R-regulated E2Fp decay</td>
</tr>
<tr>
<td>( \phi \rightarrow \text{MYC} \rightarrow \text{CD} )</td>
<td>( k_{CD} \cdot \frac{[\text{MYC}]}{K_{MCD} + [\text{MYC}]} )</td>
<td>Myc-dependent cyclin D production</td>
</tr>
<tr>
<td>( \phi \rightarrow \text{S} \rightarrow \text{CD} )</td>
<td>( k_{CDS} \cdot \frac{[S]}{K_s + [S]} )</td>
<td>Serum-dependent cyclin D production</td>
</tr>
<tr>
<td>CD ( \rightarrow \phi )</td>
<td>( d_{CD} \cdot [CD] )</td>
<td>Cyclin D decay</td>
</tr>
<tr>
<td>( \phi \rightarrow \text{E2F} \rightarrow \text{CE} )</td>
<td>( k_{CE} \cdot \frac{[E2Fp]}{K_{EF} + [E2Fp]} )</td>
<td>E2F-dependent cyclin E production</td>
</tr>
<tr>
<td>CE ( \rightarrow \phi )</td>
<td>( d_{CE} \cdot [CE] )</td>
<td>Cyclin E decay</td>
</tr>
<tr>
<td>( \phi \rightarrow \text{RB} )</td>
<td>( k_{RB} )</td>
<td>Constitutive Rb synthesis</td>
</tr>
<tr>
<td>RP ( \rightarrow \text{RB} )</td>
<td>( k_{RBDP} \cdot \frac{[RP]}{K_{RP} + [RP]} )</td>
<td>Rb production through dephosphorylation of RP</td>
</tr>
<tr>
<td>( \text{RB} \rightarrow \text{CD} \rightarrow \text{RP} )</td>
<td>( k_{RBP1} \cdot \frac{[CD] \cdot [RB]}{K_{CD} + [RB]} )</td>
<td>Cyclin D-dependent phosphorylation of Rb</td>
</tr>
<tr>
<td>( \text{RB} \rightarrow \text{CE} \rightarrow \text{RP} )</td>
<td>( k_{RBP2} \cdot \frac{[CE] \cdot [RB]}{K_{CE} + [RB]} )</td>
<td>Cyclin E-dependent phosphorylation of Rb</td>
</tr>
<tr>
<td>RE ( \rightarrow \text{CD} \rightarrow \text{E2Fp} )</td>
<td>( k_{RBP1} \cdot \frac{[CD] \cdot [RE]}{K_{CD} + [RE]} )</td>
<td>E2Fp release due to cyclin D-dependent phosphorylation of RE</td>
</tr>
<tr>
<td>RE ( \rightarrow \text{CE} \rightarrow \text{E2Fp} )</td>
<td>( k_{RBP2} \cdot \frac{[CE] \cdot [RE]}{K_{CE} + [RE]} )</td>
<td>E2Fp release due to cyclin E-dependent phosphorylation of RE</td>
</tr>
<tr>
<td>( \text{RB} \rightarrow \phi )</td>
<td>( d_{RB} \cdot [RB] )</td>
<td>Rb decay</td>
</tr>
<tr>
<td>( \text{RP} \rightarrow \phi )</td>
<td>( d_{RP} \cdot [RP] )</td>
<td>RP decay</td>
</tr>
</tbody>
</table>
\[
\begin{align*}
\text{RE} & \rightarrow \phi & d_{\text{RE}} \cdot [\text{RE}] & d_{\text{RE}} \cdot [\text{RE}] \\
\phi & \rightarrow \text{E2F} \rightarrow \text{R} & k_{\text{R}} \cdot [\text{E2F}] & K_{\text{R}} + [\text{E2F}] \\
\text{R} & \rightarrow \phi & d_{\text{R}} \cdot [\text{R}] & d_{\text{R}} \cdot [\text{R}] \\
\end{align*}
\]

RE decay

E2F-dependent R transcription

R decay

Table 4.3 Equations for the full ODE model that describes Rb/E2F network

\[
\begin{align*}
\frac{d [\text{MYC}]}{dt} &= k_{\text{MC}} \cdot \frac{[S]}{K_{S} + [S]} - d_{\text{MC}} \cdot [\text{MYC}] \\
\frac{d [\text{E2Fm}]}{dt} &= \frac{k_{\text{E}}}{K_{\text{MC}} + [\text{MYC}]} \cdot [\text{MYC}] + k_{\text{E2Fm}} \cdot \frac{[\text{MYC}]}{K_{\text{MC}} + [\text{MYC}] K_{\text{EF}} + [\text{E2F}] - d_{\text{E2Fm}} \cdot [\text{E2Fm}]} \\
\frac{d [\text{E2Fp}]}{dt} &= \frac{k_{\text{E2Fp}}}{K_{\text{R}} + [\text{R}]} \cdot [\text{E2F}] + k_{\text{RBP1}} \cdot \frac{[\text{CD}] [\text{RE}]}{K_{\text{CD}} + [\text{RE}]} + k_{\text{RBP2}} \cdot \frac{[\text{CE}] [\text{RE}]}{K_{\text{CE}} + [\text{RE}]} + k_{\text{RE}} \cdot [\text{RB}] \cdot [\text{E2Fp}] \\
\frac{d [\text{CD}]}{dt} &= \frac{k_{\text{CD}}}{K_{\text{MCCD}} + [\text{MYC}]} \cdot [\text{MYC}] + k_{\text{CDS}} \cdot \frac{[S]}{K_{S} + [S]} - d_{\text{CD}} \cdot [\text{CD}] \\
\frac{d [\text{CD}]}{dt} &= \frac{k_{\text{CE}}}{K_{\text{EF}} + [\text{E2Fp}]} \cdot [\text{E2F}] - d_{\text{CE}} \cdot [\text{CE}] \\
\frac{d [\text{RB}]}{dt} &= \frac{k_{\text{RB}}}{K_{\text{RP}} + [\text{RP}]} \cdot [\text{RP}] + k_{\text{RE}} \cdot [\text{RB}] \cdot [\text{E2Fp}] + k_{\text{RBP1}} \cdot \frac{[\text{CD}] [\text{RB}]}{K_{\text{CD}} + [\text{RB}]} + k_{\text{RBP2}} \cdot \frac{[\text{CE}] [\text{RB}]}{K_{\text{CE}} + [\text{RB}]} \\
-k_{\text{RB}} \cdot [\text{RB}] \\
\frac{d [\text{RP}]}{dt} &= \frac{k_{\text{RBP1}}}{K_{\text{CD}} + [\text{RB}]} \cdot [\text{CD}] [\text{RB}] + k_{\text{RBP2}} \cdot \frac{[\text{CE}] [\text{RB}]}{K_{\text{CE}} + [\text{RB}]} + k_{\text{RBP1}} \cdot \frac{[\text{CD}] [\text{RE}]}{K_{\text{CD}} + [\text{RE}]} + k_{\text{RBP2}} \cdot \frac{[\text{CE}] [\text{RE}]}{K_{\text{CE}} + [\text{RE}]} \\
-k_{\text{RBDP}} \cdot \frac{[\text{RP}]}{K_{\text{RP}} + [\text{RP}]} - d_{\text{RP}} \cdot [\text{RP}] \\
\frac{d [\text{RE}]}{dt} &= k_{\text{RE}} \cdot [\text{RB}] \cdot [\text{E2Fp}] - k_{\text{RBP1}} \cdot \frac{[\text{CD}] [\text{RE}]}{K_{\text{CD}} + [\text{RE}]} - k_{\text{RBP2}} \cdot \frac{[\text{CE}] [\text{RE}]}{K_{\text{CE}} + [\text{RE}]} - d_{\text{RE}} \cdot [\text{RE}] \\
\frac{d [\text{R}]}{dt} &= \frac{k_{\text{R}}}{K_{\text{RS}} + [\text{E2F}]} \cdot [\text{E2F}] - d_{\text{R}} \cdot [\text{R}] \\
\end{align*}
\]
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Base Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{MC} )</td>
<td>1 ( \mu M/h )</td>
<td>Myc synthesis rate (by serum)</td>
</tr>
<tr>
<td>( d_{MC} )</td>
<td>0.7 /h</td>
<td>Myc decay constant</td>
</tr>
<tr>
<td>( k_b )</td>
<td>0.15 ( \mu M/h )</td>
<td>E2Fm synthesis rate (by Myc alone)</td>
</tr>
<tr>
<td>( k_{CD} )</td>
<td>0.03 ( \mu M/h )</td>
<td>Cyclin D synthesis rate (by Myc)</td>
</tr>
<tr>
<td>( k_{CDS} )</td>
<td>0.45 ( \mu M/h )</td>
<td>Cyclin D synthesis rate (by serum)</td>
</tr>
<tr>
<td>( d_{CD} )</td>
<td>1.5 /h</td>
<td>Cyclin D decay constant</td>
</tr>
<tr>
<td>( k_{RBP1} )</td>
<td>18 /h</td>
<td>RB phosphorylation rate (by cyclin D/CDK4/6)</td>
</tr>
<tr>
<td>( k_{E2Fm} )</td>
<td>0.40 ( \mu M/h )</td>
<td>E2Fm synthesis rate (Myc/E2F co-regulation)</td>
</tr>
<tr>
<td>( d_{E2Fm} )</td>
<td>0.25 /h</td>
<td>E2Fm decay constant</td>
</tr>
<tr>
<td>( k_{E2FP} )</td>
<td>0.40 /h</td>
<td>E2Fp translation rate</td>
</tr>
<tr>
<td>( d_{E2FP} )</td>
<td>0.35 /h</td>
<td>E2Fp decay constant</td>
</tr>
<tr>
<td>( k_{RBP2} )</td>
<td>18 /h</td>
<td>Rb phosphorylation rate (by cyclin E/CDK2)</td>
</tr>
<tr>
<td>( k_{CE} )</td>
<td>0.35 ( \mu M/h )</td>
<td>Cyclin E synthesis rate (by E2Fp)</td>
</tr>
<tr>
<td>( d_{CE} )</td>
<td>1.5 /h</td>
<td>Cyclin E decay constant</td>
</tr>
<tr>
<td>( k_r )</td>
<td>0.10 ( \mu M/h )</td>
<td>Repressor synthesis rate</td>
</tr>
<tr>
<td>( d_r )</td>
<td>0.10 /h</td>
<td>Repressor decay constant</td>
</tr>
<tr>
<td>( k_{RB} )</td>
<td>0.18 ( \mu M/h )</td>
<td>Rb synthesis rate</td>
</tr>
<tr>
<td>( d_{RB} )</td>
<td>0.06 /h</td>
<td>Rb decay constant</td>
</tr>
</tbody>
</table>
4.4.2 Simulation analysis

Given the fact that Myc and cyclin D levels usually start to rise 6–8 hours after serum addition during cell cycle entry (Jones and Kazlauskas, 2001), we reasoned that the initial delay ($t_i$) is mainly due to the belated activation of these upstream factors. Therefore, we performed simulation with an initial constraint that Myc and cyclin D are forced to be zero until the simulation reaches the 8-hour time point in the temporal scale.
Simulations based on these assumptions were able to generate E2F dynamics trajectories including a significant length of time. On the other hand, we noted the fact that a time lag exists between cyclin E activation and that of cyclin A (Coverley et al., 2002; Kalasyczynska et al., 2009). Moreover, population dynamics of Skp2 show a peak after that of E2F1 (Marti et al., 1999). Therefore, we reasoned that the activation of negative feedback loop through R is a delayed event after E2Fp increases. We introduced a time lag between these two events by setting a threshold for E2Fp above which E2F starts to transcribe R. The introduction of this delayed negative feedback loop was able to generate slight decrease in E2Fm dynamics after it reaches the peak level, consistent with our experimental observations (Fig. 2.3b).

Time-course simulation was performed based on the model mentioned (Tables 4.1-4.4) by using a stepwise updating algorithm. As mentioned, Myc and cyclin D were constrained to be zero during the first 8 hours whereas R was constrained to be zero until E2Fp concentration reached a threshold of 0.4 μM. The interval for variable value update is 6 seconds. Simulation was performed with a relatively high serum input for 48 hours.

To mimic the perturbation of CycD, CycE or Myc node, we multiplied the degradation term with a coefficient \( k_{inh} \), inhibition rate) as shown in the following equation (3):

\[
\frac{d[MYC]}{dt} = k_{MC} \cdot \frac{[S]}{K_s + [S]} - k_{inh} \cdot d_{MC} \cdot [MYC]
\]
Through varying the inhibition rate $k_{in}$ within a wide range (1~100 fold), we performed simulations and derived a series of E2F temporal trajectories. The normalized peak values of E2F (corresponding to $Amp$) for each trajectory were calculated and plotted against the inhibitor concentration. $t_2$ was calculated from each trajectory by using the similar method as described above in 3.4.2.

4.4.3 Log-sensitivity analysis

Sensitivity analysis was performed using the full model after varying each parameter value within 0.1 and 10-fold. Log sensitivity of the four metrics to each parameter was calculated by the following equation (4):

$$\text{Sensitivity}_{\log} (\text{metric}) = \left| \frac{d(\log(\text{metric}))}{d(\log(\text{parameter}))} \right|$$

(4)
Roles of Myc and G1 Cyclin/CDKs confirmed by analysis of E2F dynamics

5.1 Introduction

In Chapter 4, we used mathematic modeling to explore the design principle of the Rb/E2F network. Results from both simulation and system analysis revealed unexpected control logic inherent to the network structure. In particular, simulation analysis based on the full model predicted that Myc and G1 cyclin/CDK2 affect different aspects of E2F transcriptional dynamics, possibly related to distinct functional outputs. Myc is critical for regulating the maximum E2F level ($Amp$) while G1 cyclin/CDKs have little effect on $Amp$ but do contribute to the modulation of E2F activation duration, $t_2$.

In order to validate these predictions, we took advantage of the reporter system to measure E2F temporal dynamics under the conditions of G1 cyclin/CDKs or Myc inhibition by using small molecule inhibitors or RNAi. Here, we present the characterization of single-cell E2F dynamics under perturbations aimed at directly validate the theoretical predictions derived from mathematically modeling the Rb/E2F network.
5.2 Results

5.2.1 Control of timing but not commitment by G1 cyclin/CDKs

To experimentally monitor the behavior of the network under CycD/E inhibition, we used the CDK4/6 and CDK2-specific inhibitors – PD0332991 and CVT-313 – respectively in a wide range of concentrations (Brooks et al., 1997; Campaner et al., 2010; Choi et al., 2012; Fry et al., 2004). We confirmed the inhibitory effect at each concentration by monitoring phosphorylation of Rb at various serine or threonine residues (Fig. 5.1a). To exclude the possibility of off-target effect from CDK2 inhibition, we generated short hairpin RNAs (shRNA) to knockdown endogenous CDK2 mRNA levels (and consequently of CDK2 protein) (Fig. 5.1b and Table 5.1). Single-cell analysis of E2F1 dynamics after serum stimulation was combined with EdU incorporation or visual inspection of cell division in the presence of the CDK4 and CDK2-specific inhibitors or CDK2 shRNA. Consistent with our modeling analysis, inhibition of CycD or CycE complexes had no effect on the distribution of Amp (<5% of cells found in the OFF mode) (Fig. 5.1c-e). Concomitant inhibition of both complexes moderately increased the proportion of cells showing a low-mode Amp (~25% of cells in the OFF mode) given a 48-hour observation window (Fig. 5.1c and e). When cells were monitored for cell division, we observed that inhibition of CycD or of CycE complexes (or both) led to a decrease in the fraction of cells that completed division during the time window of our assay (Fig. 5.1f and g). However, it had little effect on the fraction of cells that incorporated EdU (and presumably had committed to the cell cycle) (Fig. 5.1f and g).
FIGURE 5.1: G1 cyclin/CDKs have little effect on commitment decision. (a) Rb phosphorylation (at Ser-780 or Thr-821/826 sites) variation detected by western blot in
different cases of cyclins perturbation. Actin was used as a loading control. (b) Efficacy of CDK2 knockdown by two different shRNAs as determined by western blot. Sorted cells with shCDK2-inducible system were cultured in medium with 10% FBS and doxycycline at indicated doses for 48 hours and then collected for western blot. (c) *Amp* distribution in different cases of cyclins perturbation. Mean ± std. is shown on top left of each panel. (d) Scatter plot based on commitment as determined by *Amp* given the inhibition of CDKs (PD0332991 for CDK4/6 and CVT-313 for CDK2). Committed cells were measured by EdU labeling after 48 hours incubation, and divided cells were counted 48 hours after addition of 10% BGS. (e) Histogram based on cell division as determined by *Amp* under inhibition of CDKs (48 hours observation). (f) Proportion of committed/divided cells under different CDK inhibitions during a 48- and 92-hour (the latter only for the combined inhibition) observation window. The exact number of total cells followed and that of cells committed/divided are indicated on top of each error bar. (g) Proportion of committed/divided cells under different conditions of CDK2 knockdown during a 48-hour observation window. Fate of REF52-hE2F1p::4NLS-d4Venus cells transduced with shCDK2#1 was followed in single-cell analysis.

In contrast, and consistent with our simulation results, inhibition of CycD, CycE or both led to significantly increases in $t_2$ and its variance (Fig. 5.2a, b and f). The prolonged $t_2$ results in a significant extension of the entire cell cycle length (Fig. 5.2c and g). As $t_1$ and $t_3$ show moderate changes under inhibition of CycD/E (Fig. 5.2d and e), the increase in $t_2$ is the primary contributor to cell cycle elongation. For this reason, the observed reduction in the proportion of cells that undergo cell division under dual inhibition is likely an overestimation. Indeed, we found a significant increase in the proportion of divided cells and the recovery of *Amp* distribution in the case of combined inhibition if we extended the time of observation to 92 hours (Fig. 5.1c and f). Our experimental data therefore fully validate the predictions from the model and suggest
that cells under CycD/E inhibition can commit to cell cycle entry but progress more slowly on the way to cell division.
5.2.2 Control of commitment into cell cycle entry by Myc

To experimentally test the functional consequences induced by Myc inhibition, quiescent cells were stimulated with full serum in the presence of EdU and of different concentrations of either Myc-specific inhibitor 10058-F4 (which efficiently interferes with Myc/Max hetero-dimerization) or the bromodomain inhibitor (+)-JQ1 (which substantially suppresses c-Myc transcription) (Delmore et al., 2011; Mertz et al., 2011; Nie et al., 2012; Rahl et al., 2010; Wang et al., 2007; Yin et al., 2003) (Fig. 5.3e). Consistent with our modeling analysis, single-cell analysis indicates that Myc inhibition dramatically prevents Amp to switch from an OFF mode to an ON mode, resulting in a sharp decrease in the proportion of both committed and divided cells (Fig. 5.3a-c, h). Fitting the curves to a Hill function yielded a sharp sigmoid pattern for both division and commitment (Fig. 5.3c and h). Moreover, we note a bimodal response at an
intermediate inhibitor level (90 μM for 10058-F4 and 0.8 μM for (+)-JQ1) (Fig. 5.3a, b, f and g), which is around the half-inhibition threshold. At this dose, a fraction of the cells exhibiting a strong E2F response was able to commit (Fig. 5.3d). In contrast, the other cells that remained quiescent exhibited weak E2F responses (Fig. 5.3d). We also performed shRNA knockdown of endogenous c-Myc level to rule out the potential for a non-specific effect of the inhibitors (Fig. 5.3i, Table 5.1). A large proportion of the cells remained in the OFF mode after serum stimulation, fully supporting our findings obtained with two independent small molecule inhibitors of Myc (Fig. 5.3j). All these results are consistent with the bistable nature of E2F activation in response to upstream signals and suggest a critical role for Myc in this regulation (Yao et al., 2008). Importantly, they further underscore the predictable power of the parameter Amp in E2F dynamics for cell-cycle commitment.
FIGURE 5.3: Myc controls the commitment into cell cycle entry. (a) Scatter plot of commitment as determined by Amp under different c-Myc inhibitor (10058-F4) concentrations (80, 90 and 100 μM). 101, 100 and 93 cells were analyzed for each condition. (b) Histogram of cell division as determined by Amp under the conditions in (a). (c) Dose-response curves (fitted to data points as Hill functions) indicate the proportion of divided/committed cells under different c-Myc inhibitor concentrations. (d) Representative E2F1 dynamics trajectories (green for committed cells; red for uncommitted cells) at 90 μM 10058-F4 inhibitor concentration. (e) Validation of (+)-JQ1 effect on endogenous c-Myc and E2F1 level under different concentrations. REF52-hE2F1p::4NLS-d4Venus reporter cells were released from serum-starvation by adding 10% BGS and different concentrations of (+)-JQ1. Samples were collected at 14 hours after release. (f) Scatter plot of commitment as determined by Amp at 0.8 μM (+)-JQ1. (g) Histogram of cell division as determined by Amp at 0.8 μM (+)-JQ1. (h) Dose-response
curves indicate the proportion of divided/committed cells under different (+)-JQ1 concentrations. (i) Confirmation of shRNA knockdown effect on c-Myc in REF52-hE2F1p::4NLS-d4Venus cells with tet-inducible shc-Myc system at different doxycycline concentrations. (j) E2F dynamics trajectories at doxycycline concentration of 0 or 1000 ng/ml. Committed/divided cells were plotted in green color while uncommitted/undivided ones were plotted in red.

5.3 Discussion

In this Chapter, we provide experimental evidence that fully validate the predictions derived from modeling analysis. By using CDK4/6- and CDK2-specific inhibitors, we show that the inhibition of CycD, CycE or both results in a prolonged E2F activation time but has little effect on Amp when a long enough observation window is allowed. Interestingly, the effect of CycD inhibition on \( t_2 \) is more dramatic than that from the inhibition of CycE, consistent with the prediction obtained from simulation analysis. This difference can be explained by two mechanisms. First, CycE is induced after the induction of CycD at the temporal scale, implying that CycD may play a dominant role in determining the timing. Second, it was recently reported that CycE phosphorylates Rb in a quantum manner while CycD can only mono-phosphorylate Rb, suggesting that these two types of cyclin/CDKs may have different catalytic abilities (Narasimha et al., 2014). In addition, we find that inhibition of G1 cyclin/CDKs has little effect on the other two stages, \( t_1 \) and \( t_3 \). Given that the entire cell cycle length is a sum of all three stages, we conclude that G1 cyclin/CDKs are primarily responsible for the control of cell cycle progression through the modulation of E2F activation duration.
In contrast, it is remarkable that both the inhibition of Myc activity or the reduction of Myc level can abolish cell cycle entry. Interestingly, under these circumstances, failure of cell cycle entry correlates with a severely blunted E2F dynamics trajectory (with maximum Amp values less than $Amp_{th}$) in individual cells. These findings nicely establish a critical role for Myc in controlling of commitment into the cell cycle through by modulating Amp.

Our results challenge the prevailing view about how cell cycle entry is controlled and provide a revised paradigm, which reconciles previous controversies or inconsistencies in the model. It is no longer surprising that the knockout of D- or E-type cyclins or their corresponding CDKs only leads to delayed cell cycle entry. Moreover, as Myc and G1 cyclins have distinct functions, it becomes evident that overexpression of cyclin D cannot rescue the slow-growth phenotypes in Myc-deficient cells.

### 5.4 Methods

The methods and procedures used in this Chapter for cell culture, live cell imaging, image analysis and western blot, unless specified, are the same as those described in Chapter 2 (refer to 2.4.2, 2.4.3, 2.4.4, 2.4.6 for details).

#### 5.4.1 Cell culture

Cells were plated according to procedures described in 2.4.2. For perturbation experiments, PD0332991 (CDK4/6 inhibitor, ChemieTek), CVT-313 (CDK2 inhibitor, Enzo Life Science), 10058-F4 (c-Myc inhibitor, Sigma Aldrich) and (+)-JQ1
(bromodomain and extra terminal domain inhibitor, Cayman Chemical, MI) were added into cells immediately after cells were released from serum-starvation (by adding 10% BGS) in either single or combined way. For 92-hour perturbation experiment with both PD0332991 and CVT-313, cells were growing with replaced fresh medium with fresh inhibitors after the initial 48 hours.

**5.4.2 Western blot analysis**

Antibodies against E2F1 (1:1000, Cell signaling), phosphorylated Rb Ser780 (C-15, 1:1000, Santa Cruz), phosphorylated Rb Thr821/826 (sc-16669-R, 1:1000, Santa Cruz), c-Myc ((D84C12) XP® Rabbit mAb, 1:1000, Cell signaling), CDK2 (78B2 Rabbit mAb, 1:1000, Cell signaling) and Actin (C-2, 1:1000; Santa Cruz) were selected for the quantification of protein expression at the population level. REF52-hE2F1p::4NLS-d4Venus cells were treated with different concentrations of inhibitors and then harvested. The same procedures as described in 2.4.6 were used in the following steps.

**5.4.3 RNAi interference**

shRNAs for targeting CDK2 or c-Myc were generated by cloning shRNA sequences (Table 5.1) into Tet-pLKO-2A-mCherry vector (He et al., 2012). Lentiviral packaging reactions were performed in the 293T cell line in the presence of packaging plasmids psPAX and pMD2.G using Lipofectamine Transfection Reagent (Invitrogen). Viral supernatants were collected 48 h after transfection, filtered through disposable 0.45 μm acrodisc syringe filters (Pall Corporation, NY). For infection cells were plated in 10
cm tissue culture dishes and allowed to achieve 20–30% confluence before adding viral supernatant in the presence of 7 μg/ml polybrene for 12 hours (EMD Millipore, MA). Cells were collected and sorted by flow cytometry for 5% of the population with highest mCherry signals. Sorted cells were grown up and induced to express shRNA at different concentration of doxycycline (Sigma Aldrich) for 48 hours. After induction, cells were lysed and knockdown efficiency was determined by western blot. Meanwhile, sorted cells with confirmed knockdown effect were subjected to the measurement of E2F dynamics in the presence of doxycycline at different doses.

<table>
<thead>
<tr>
<th>shRNA</th>
<th>Hairpin Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>shCDK2#1</td>
<td>5’-CCGG-TACTTCTATGCGCTGATTATAA-CTCGAG-TTATAATCAGGCATAGAAGTA-TTTTTG-3’</td>
</tr>
<tr>
<td>shCDK2#2</td>
<td>5’-CCGG-TTCTTCCAGGATGTGACTAAA-CTCGAG-TTTAGTAGCACATCCTGGGAAGAA-TTTTTG-3’</td>
</tr>
<tr>
<td>shc-Myc</td>
<td>5’-CCGG-TCTACTCACCAGCAACAATTAT-CTCGAG-ATAATTGTGCTGGTGAGATAGATAAGTGA-TTTTTG-3’</td>
</tr>
</tbody>
</table>
6 Measurement of E2F dynamics in cycling cells

6.1 Introduction

Cell cycle decisions are flexibly controlled by mechanisms that enable either transient division, followed by quiescence, differentiation or apoptosis, or continuous proliferation. For example, many adult stem cells or differentiated cells, rarely enter the cell cycle and proliferate infrequently or exhibit a prolonged G1 phase (Orford and Scadden, 2008). In contrast, the fast cycling crypt cells in the intestinal epithelium take as short as 9–10 hours to complete a cell cycle. In all these events, the same Rb/E2F network plays a key regulatory role in controlling how a cell is choosing to respond; however, the mechanistic details involved in eliciting a response might be different given the fact that cells start from very different initial states. Unfortunately, conventional approaches based on population analysis are ill suited for mechanistic studies of cycling cells, because cells usually reside in distinct states that are unsynchronized with each other. In contrast, single-cell analysis provides a viable tool for addressing this particular question. Indeed, in a recent publication using single-cell analysis, Spencer et al. showed cells to either continue immediately into the next cycle or enter a G0-like state, depending on the residual CDK2 activity found within the cell, following the initial
division. This activity was controlled by either p21 (a member of the Cip/Kip family of negative regulators of the G1-phase cell cycle progression) or mitogen signals (Spencer et al., 2013).

In this Chapter, we applied the similar strategy used in previous chapters to investigate E2F transcriptional dynamics at the single-cell resolution but in cycling cells. We characterized the measured trajectories and quantified them by a different set of metrics. The analysis of metrics revealed novel mechanisms and their contribution to the control of cell cycle progression in cycling cells.

6.2 Results

6.2.1 Measurement of E2F dynamics in cycling cells

I optimized the Kozak sequence of the previously used E2F transcriptional reporter and derived a new version with greatly increased fluorescence signal intensity and better nuclear localization (Fig. 6.1a). The updated reporter was introduced into REF52 cells through viral infection and genomic integration. Single clones were screened for the analysis of E2F transcriptional dynamics in cycling cells.

E2F dynamics was followed over time in individual cells after cells they had been released from serum starvation. The entire observation time window was 72 hours, allowing cells to finish the first cell cycle as well as one or two additional cycles. Our measurement revealed robust oscillatory E2F dynamics over a number of cells during this time window (Fig. 6.1c). The rise and fall of E2F signal within a single period
corresponds precisely to a complete round of cell cycle (Fig. 6.1b). Similar to the pattern within the first cell cycle, the E2F signal in subsequent cell cycles exhibits an initial delay, before it robustly increases to a peak level and then slightly decreases. Around the time of cell division, we observed a quick drop in the signal, owing to the dilution of signal split between the two daughter cells (Fig. 6.1b). On the basis of these observations, we defined a new set of metrics to quantify the observed E2F dynamics in cycling cells (Fig. 6.1b). These include initial delay ($t_{c-1}$), activation time ($t_{c-2}$), post-activation time ($t_{c-3}$) and the cell cycle length ($T_c$). Despite similarities, there are two significant differences in E2F dynamics between cells going through the first cell cycle and cells going through the later cell cycles. First, the initial E2F signal levels are quite different. In cells just released into the cell cycle after serum starvation, there is a very low basal level of E2F activity, which increases after an initial delay (confirming our observations in previous chapters), whereas in cycling cells (cycle 2 and 3) the E2F signal increases from an intermediate level that is much higher than the basal E2F activity. This difference in starting E2F activity levels suggests distinct initial conditions for the initial and subsequent divisions (Fig. 6.1c). Second, the initial delay is relatively constant within the first cell cycle but varies dramatically in the second and third round of cell division (Fig. 6.1c). Interestingly, the E2F signal slowly decreases within the initial delay in cycling cells, suggesting a degradation of E2F transcripts without new synthesis during this stage.
FIGURE 6.1: Measurement of E2F transcriptional dynamics in cycling cells. (a) Time-lapse microscopy images of cycling REF52 rat fibroblasts expressing hE2F1p::4NLS-d4Venus reporter (with the Kozak sequence optimized in the promoter region) for a complete cell cycle. Cells were cultured in medium with 10% bovine growth serum (BGS). Upper panel, phase channel; low panel, Venus channel. Scale bar, 100 μm. (b) Characterization of E2F1 dynamics trajectory in cycling cells with defined metrics: $t_{c-1}$, initial delay; $t_{c-2}$, activation time; $t_{c-3}$, post-activation time; $T_c$, the entire cell cycle length. Blue triangles indicate cell division time points. (c) Representative trajectories of E2F dynamics in single cells (#1-6) for continuous cycles. Cells were released from serum
starvation and monitored for 72 hours. If a cell divided, two daughter cells were separately monitored. Each trajectory reflects a dynamics curve over a cell lineage within 72 hours.

**6.2.2 \( t_{c-1} \) primarily contributes to the variation of cell cycle length**

Next, we analyzed the distributions of the values of different temporal metrics in cycling E2F dynamics trajectories. The average cell cycle length \( (T_c) \) is about 21 hours with a standard deviation (std.) of \( \sim 7 \) hours (Fig. 6.2a). Remarkably, the cell cycle length can be as short as 12 hours for cycling REF52 cells, although the most frequent time takes about 17 hours. Of the three sequential stages, \( t_{c-1} \) takes 9 hours on average with a high standard deviation (std.) value of \( \sim 7 \) hours (Fig. 6.2b). In contrast, \( t_{c-2} \) and \( t_{c-3} \) average around 11 and 2 hours respectively, with std. values less than 2 hours, suggesting a stable regulation of E2F dynamics within these two phases (Fig. 6.2c-d). Moreover, the scatter plot between the values of \( T_c \) and \( t_{c-1} \) for individual cells displays a highly linear relationship between these two metrics. These findings altogether indicate that the initial delay, \( t_{c-1} \), rather than the other two phases, is primarily responsible for the variation in the whole cell cycle length.
FIGURE 6.2: $t_{c-1}$ primarily contributes to the variation of cell cycle length. (a-d) Distribution of $T_c$, $t_{c-1}$, $t_{c-2}$, and $t_{c-3}$ over ~100 cycling cells. The mean ± std. was calculated and plotted at the right top corner for each panel. (e) Scatter plot of $T_c$ and $t_{c-1}$. R-squared value was calculated and shown in plot.
6.2.3 Correlation of E2F dynamics between sibling cells

To explore whether the E2F dynamics of sibling cells are correlated, we classified the E2F dynamics trajectories into two pairwise sibling groups. After we determined the values for the different metrics from each trajectory, the cell cycle length ($T_c$) was plotted in a pairwise manner between sibling cells. Our results indicated that $T_c$ is not correlated between two sibling cells (Fig. 6.3a). We next made pairwise correlations in each of the three stages and, in all cases examined, found poor correlations between sibling cells (Fig. 6.3b and c). Based on these E2F temporal dynamics, we conclude that cell cycle commitment and duration are fairly uncorrelated between sibling cells.

FIGURE 6.3: Correlation of E2F dynamics between sibling cells. (a) Scatter plot of $T_c$ between sibling cells. (b) Scatter plot of $t_{c-1}$ between sibling cells. R-squared value was calculated and shown in both plots. (c) R-squared values for the correlation analysis of all three metrics were calculated and shown in bar plot.
6.3 Discussion

In this Chapter, we measured E2F transcriptional dynamics in cycling cells. Our results reveal that E2F transcriptional dynamics show robust oscillations, corresponding to periodic cell cycles. These results demonstrate periodic E2F activation at the single-cell level in continuous cell cycles, suggesting that E2F expression remains tightly regulated, during cell cycle progression.

Second, we find that E2F dynamics in cycling cells show similar consistent patterns as in the case of the first cell cycle after release from quiescence, but the pattern can vary significantly at the temporal scale. Using a new set of metrics to quantitatively describe each trajectory, we show that the initial delay ($t_{c-1}$) is the phase with the highest variance, and the largest contributor to the variation of the whole cell cycle length ($T_c$). These data suggest that each cycling cell displays flexible control around the cell cycle timing. Moreover, we show that cell cycle progression through all three stages is largely uncorrelated between sibling cells, suggesting that genetics or epigenetics properties inherited from the same ancestor cell have little effect on cell cycle control in progeny cells.

Detailed analyses remain be performed in the future to address the following questions about E2F dynamics in cycling cells. First of all, it will be interesting to explore how different stages ($t_{c-1}$, $t_{c-2}$ and $t_{c-3}$) are regulated at the molecular level. $t_{c-1}$ is of particular interest since it is the most variable stage within the entire cell cycle. By using
a CDK2 activity reporter, Spencer et al. recently showed that cells are able to enter a G0-like state after mitosis (Spencer et al., 2013). Moreover, the length of this G0-like state is variable among different cells. It will be necessary to combine these two different reporters and map \( t_{c-1} \) with the G0-like state. Second, it will be interesting to investigate which differences, at the molecular level, affect the length of the initial delay (\( t_1 \) and \( t_{c-1} \)) between the first cell cycle and the continuous cycles.

**6.4 Methods**

Unless specified, the methods and procedures used in this Chapter for cell culture, live cell imaging and image analysis, are the same as those described in Chapter 2.

**6.4.1 Analysis of E2F dynamics trajectories in cycling cells**

To analyze E2F dynamics trajectories in cycling cells, we first divided the entire trajectory of several continuous cycles into time intervals that span individual cell cycles. For each trajectory of an individual cell cycle, the signal has a minimum level and a maximum level. The minimum level marks the boundary between \( t_{c-1} \) and \( t_{c-2} \), while the maximum level marks the boundary between \( t_{c-2} \) and \( t_{c-3} \). Through mapping the time points at which cells arrive at these boundaries, we calculated the values for \( t_{c-1} \), \( t_{c-2} \) and \( t_{c-3} \).
7 Conclusion

7.1 Summary and discussion

For my thesis work, I developed an integrated platform to perform measurements of single-cell E2F dynamics in real time and proposed a quantitative approach to interpret how E2F dynamics might inform on whether a cell chooses to remain in quiescence or proliferate. The outlined methodology not only advanced the study of cell cycle entry to an unprecedented quantitative level at the single-cell resolution, but also revealed the emergent property of the Rb/E2F network that underlies the control of cell cycle entry and pace.

Measurements of real-time dynamics of E2F1 transcription in single mammalian cells revealed pulse-like E2F dynamics during cell cycle progression. The availability of quantitative measurements performed on a large number of single cells allowed us to define a set of metrics to precisely capture the nature of E2F transcriptional regulation during cell cycle progression for each cell under observation. Details of the inherent E2F dynamics that had been masked in previous studies based on population analysis were clearly revealed through single-cell analysis. Moreover, the single-cell approach that I developed can be adapted to study the system-level emergent properties of virtually any other cell signaling pathways, providing that appropriate reporters can be generated. To
successfully apply this strategy to my own work, I decided to develop a reporter for E2F1, because it is a hub node located at the core of the Rb/E2F signaling network. Like most signaling pathways, the Rb/E2F pathway shows a hierarchical structure where a hub node integrates multiple signals from other effector nodes in the pathway. As such, measurements of the hub node activity are particularly instructive and, upon specific perturbations of the network, allow one to dissect the various contributions made by other effectors in the pathway, whether they are directly or indirectly connected to the hub node.

Quantitative analysis of E2F dynamics trajectories together with cell fate decision has allowed us to identify the key metrics that accurately predict cell commitment and proliferation. We find that the values of $Amp$ and $t_2$ respond to two intimately linked but differentially controlled modes of regulation by the Rb/E2F network. $Amp$ is critically dependent on Myc activity, whereas $t_2$ is highly sensitive to the activity of G1 cyclin/CDK complexes. Interestingly, $Amp$ serves as the single most reliable predictor of cell cycle commitment in single cells. In particular, we show an ultrasensitive dependence on $Amp$ for the probability of each individual cell to commit into the cell cycle. Importantly, this predictive power is probabilistic in nature. If $Amp$ is at $Amp_{th}$ (2.5), a cell has a 50% chance to commit. However, a slight increase in $Amp$ (from 2.2 to 2.8) greatly boosts the probability (from 25% to 75%). However in such cases, there is still a small probability by which a cell can stay in quiescence due to the stochastic
nature of single-cell behavior. At the temporal scale, transition across the $Amp_{th}$ defines a critical time point after which a cell becomes likely to commit into division. This time point is reminiscent of the classical restriction point (Blagosklonny and Pardee, 2002; Pardee, 1974), but has the advantage that it can be precisely determined in single cells.

Remarkably, $Amp$ has remained a reliable predictor of cell cycle commitment even under drastic perturbations of the Rb/E2F network in NIH3T3 and REF52 fibroblasts. As this network is highly conserved among different tissues and species (van den Heuvel and Dyson, 2008), it will be interesting to test the generality of this principle among different cell types, including normal and cancerous cells. $t_2$, another metrics that we defined, seems to account on the other hand for the main variability in cell cycle duration, and its variable value correlates with that of the cell cycle length. At the temporal scale, $t_2$ covers the late G1 phase and most (if not all) of S phase. This time window is most variable during the cell cycle, perhaps allowing cells to optimize conditions for the ordered completion of the cell cycle in response to internal stress signals such as DNA damage response (Zetterberg and Larsson, 1985).

Although the role of the Rb/E2F pathway in regulating cell cycle entry has been well documented, the precise contribution of how its core constituents mediate temporal steps in its execution had not been fully established. Our analysis has generated new mechanistic insights into this issue. First, we find that Myc is critical for cell cycle entry. Myc has long been recognized as a potent proto-oncogene, and its effect on cell
proliferation were largely attributed to its regulation of G1 cyclins expression and CDK activities (Dang, 2012; Obaya et al., 1999; Pelengaris et al., 2002). However, our results show that Myc’s contribution – when cells re-enter the cell cycle after a prolonged quiescence – is primarily due to a direct modulation of E2F $\text{Amp}$. They also suggest a revised role for the G1 cyclins and their associated kinases. In our simulations and biological experiments, the primary role of G1 cyclins was found to tune the timing and duration of the cell cycle. These observations challenge the canonical view that G1 cyclins are predominantly responsible for controlling the commitment to cell cycle entry. Taken together, our results reveal that the Rb/E2F network coordinates two independent control modes of cell cycle progression: the decision of “whether” by Myc and that of “when” by G1 cyclins.

Although our revised view challenges a salient aspect of the canonical paradigm that postulates that Myc, G1 cyclin/CDKs, Rb and E2F are working in a linear manner, it remains fully consistent with results that were used to support the original model. For example, it was reported that the microinjection of anti-cyclin D1 antibody into normal diploid human fibroblasts prevented cells from proceeding to S phase. This observation had been held as strong evidence that the cyclin D complex was responsible for initializing S phase entry (Baldin et al., 1993). However, our single-cell analysis suggests an alternative explanation. The observed “prevention” of S phase entry was not a true abrogation of cell cycle entry, but rather the result of a postponed process. Moreover,
our model reconciles a number of previous findings that were at odds with the canonical view. First, it explains previous observations made with knockout mouse models where G1 cyclins and CDKs were shown to be largely dispensable for cell cycle entry although they affected efficacy or timing of commitment (Berthet et al., 2003; Geng et al., 2003; Kozar et al., 2004; Malumbres and Barbacid, 2009; Malumbres et al., 2004). Second, unlike the original model, our model is completely consistent with the observation that constitutive overexpression of cyclin D cannot overcome the G1 arrest induced by deprivation of epidermal growth factor signals (Chou et al., 1999). Third, our model easily explains why restoration of cyclin D level in c-Myc-deficient cells fails to rescue their slow-growth phenotypes despite succeeding in restoring wild-type kinetics of Rb phosphorylation (Mateyak et al., 1999; Obaya et al., 1999). Therefore, our model (henceforth referred to as “parallel” model), based on a parallel mode of control between the G1 cyclin complexes and Myc, resolves all the observations regarding how control of cell cycle entry is achieved and is more inclusive than the canonical “linear” model.

Importantly, the “parallel” model provides an alternative rationale to interpret a number of biological phenomena. Indeed, the phenotype of “cell cycle arrest” caused by the inhibition of G1 cyclin/CDKs can now be viewed as a tunable “cell cycle delay” depending on the intrinsic cyclin/CDK complex activity. Likewise, the quiescence state can be ascribed to the fact that transcription of E2F by Myc or other factors is repressed, rather than invoking inhibition of G1 cyclin/CDK activities as the main trigger. In fact, a
body of evidence adds support to our interpretation. Indeed, signaling pathways that are critical for the maintenance of quiescence have been shown irrevocably to have a direct effect on the regulation of Myc activity (Artavanis-Tsakonas et al., 1999; Fleming et al., 2008; Palomero et al., 2006; Sears et al., 1999). Interestingly, our model may also cast new light on cellular senescence, the so-called irreversible state characterized by the permanent inability of a cell to proliferate. Cellular senescence has been long regarded as one special case of quiescence, but one that differs in two key aspects: a characteristic morphology of cells undergoing senescence and their inability to reinitiate cell proliferation (Dimri, 2005; Kuilman et al., 2010). The molecular mechanism underlying senescence had been attributed to the activation of two tumor suppressors – Rb and p53 – and a potent inhibition of G1 cyclin/CDK activities by CKIs. According to the canonical “linear” model, the same logic is invoked in mediating the two different states (quiescence and senescence) despite the fact that the two cellular states are functionally different. However, the “parallel” model nicely resolves this inconsistency. The role ascribed to the G1 cyclin complexes in our model implies that senescence is akin to a state in which “the cell has entered but is trapped within an infinitely long and futile cell cycle” (Collado et al., 2007; van Deursen, 2014). Finally, the mechanism(s) underlying the control of terminal differentiation can also be re-interpreted according to our proposed model. It is well known that induction of p21 drives this permanent cell cycle exit. According to the “linear” model, p21 induction results in the inhibition of G1
cyclin/CDK activities, which then promotes differentiation. However, it is interesting to note that p21 can also interfere with Myc transcriptional activity by blocking Myc/Max complex formation or directly participate in the repression of E2F transcriptional program (Kitaura et al., 2000; Vigneron et al., 2006). With this in mind, the transcription-repressive function of p21 may actually be the relevant activity in the initiation of terminal differentiation.

Since the loss of cell cycle control is a hallmark of cancer development, the “parallel” model also provides fresh insights in understanding the process of oncogenesis (Hanahan and Weinberg, 2000). Central to our argument, we point out that the driving force of cell proliferation resides in the regulation of E2F transcription, rather than the induction of G1 cyclin/CDK activities. This novel interpretation highlights the importance of Myc during the process of transformation. Indeed, a brief inactivation of Myc by either a tetracycline-inducible system or the expression of the dominant interfering Myc bHLHZip dimerization domain mutant Omomyc are able to induce fast tumor regression in mouse models, suggesting a universal strategy by targeting Myc for curbing the growth of various types of cancer (Jain et al., 2002; Soucek et al., 2008). Moreover, the “parallel” model is particularly relevant to the case of colorectal carcinogenesis. The loss of APC gene and activation of β-catenin is a hallmark event that promotes the initial neoplastic growth (Clevers and Nusse, 2012). This oncogenic event was shown to be accounted for through an increase in Myc level rather than cyclin
D/CDK4/6 activation, which was difficult to be explained within the “linear” model but perfectly consistent with the logic of the “parallel” model (Sansom et al., 2007; Sansom et al., 2005).

Furthermore, if the “parallel” model also holds for stem cells, it would provide a simple interpretation for the differences in cell cycle regulation among stem cells at the different stage of life. Embryonic stem cells usually have very short cell cycle duration because constitutive cyclin E/CDK2 activity maintains hyper-phosphorylation of Rb (He et al., 2009). However, over time, stem cells (from the initial embryonic stem cells to fetal stem cells, young adult stem cells and eventually old adult stem cells) display increasing cell cycle length. This lengthening of the cell cycle during development remains an unresolved question in the field of stem cell research and self-renewal. As G1 cyclin/CDKs were believed to mediate the decision to enter the cell cycle but not the duration, a speculative explanation based on a dynamic interplay between developmental changes in G1 cyclin/CDKs and CKIs expression and a switch from mitogen independent to mitogen-dependent signaling was postulated to account for the lengthening of the cell cycle in later stem cells (He et al., 2009). However under the “parallel” model, the prolonged cell cycle can be simply explained by the gradual increase of CKI levels and the loss of G1 cyclin/CDKs activities as cells age.

In sum, the measurement of single-cell E2F dynamics provides a quantitative and sophisticated framework for understanding the control logic of cell cycle entry that can
be adapted to different settings. Furthermore, a clear delineation of the precise function of each effector in the Rb/E2F network implicated in cell cycle control has direct implications for developing effective strategies for cancer therapy.

7.2 Future directions

There are many interesting directions to explore that are directly relevant to or suggested by our current research.

First of all, it will be necessary to examine if the “parallel” model still holds in different biological settings. We have demonstrated that Amp is an accurate predictor of cell cycle entry in rat and mouse fibroblast cells. To broaden the significance of this observation, we plan to integrate the E2F transcriptional dynamics reporter into hTert-immortalized normal human epithelial HME1 cells. These cells have been used as an ideal model for the study of cell proliferation, mammary sphere formation (differentiation), and cancer stem cells (after introduction of oncogenes such as Ras) (Mani et al., 2008). These cells provide a much richer biological platform to investigate how E2F dynamics influence specific cell state, and how external perturbations modulate E2F kinetics and the ensuing phenotype or adopted cell fate.

The recent advancement in CRISPR/Cas9 technology provided an opportunity to apply genome-editing tools for generating E2F reporter cell lines in a more specific way through integration of the reporter in the endogenous locus (Hsu et al., 2014). Because E2F1 heterozygous deletion has little effect on S phase entry in primary embryo
fibroblasts (Tsai et al., 2008), integration of the fluorescent protein coding sequence (4NLS-d4Venus) into the endogenous E2F1 locus will allow us to measure single-cell E2F dynamics under normal conditions, which truly reflect the locus-specific transcriptional regulation.

An ideal strategy will be to build a locus-specific E2F reporter in embryonic stem cells through homologous recombination and generate a mouse model with this reporter construct expressed in all cells. This mouse model could serve as a powerful and convenient source, from which we can derive all types of cells for the study of cell cycle control at the single-cell resolution. Moreover, this reporter mouse line can be crossed with mouse lines that carry genetic mutations of interests, including those involved in cell cycle progression or cancer susceptibility and/or progression, to study how the cell cycle is regulated in a variety of genetic contexts.

Second, we have established in Chapter 6 a phenomenological foundation to study how E2F dynamics is controlled in cycling cells. Our preliminary data show that E2F dynamics robustly oscillate in continuous cell cycles. Importantly, although the dynamics tracings in these cells show a similar pattern to that observed in the first cell cycle, they can be broadly classified into two groups on the temporal scale. Accordingly, we have defined a new metrics to quantify these properties. However, we do not know of this variance for E2F dynamics at the temporal scale is due to. It will be of particular interest to explore how relevant signaling pathways reset the initial conditions after the
first mitosis. Live-cell imaging technique will be of great value in that regard because it will capture the entire cell signaling history over the period of observation. This record may provide meaningful information that could not have been gleaned from population analysis. Another key question will be to understand how different pathways control the different phases defined by the new E2F dynamics metrics applied to continuous cell cycles.

Third, it will be informative to combine measurements of E2F transcriptional dynamics with measurement of dynamics of other cell cycle effectors to explore their temporal correlation and crosslinks at the single-cell level. By using a CDK2 activity reporter, Spencer et al. showed that cycling cells can enter a transient G0-like state after mitosis (Spencer et al., 2013). As described in Chapter 6, we also observe cells characterized by different values in E2F amplitude after the first mitosis. One obvious direction will be to combine our E2F reporter with the CDK2 activity reporter in the same cells but with distinguishable fluorescence (e.g. use Venus for E2F reporter but use mCherry for CDK2 activity reporter) to explore how this transient G0-like state is reflected in E2F dynamics.

Previous studies suggested that the three E2F activators (E2F1, E2F2 and E2F3) have redundant functions in the control of cell proliferation, yet in some contexts, they have been observed to be associated with unique function(s) that are essential and non-redundant (Chong et al., 2009). One way to address this issue will be to generate either
E2F2 or E2F3 reporters and combine them with the E2F1 reporter so that we can examine whether their dynamics show temporal correlation at the single-cell level during cell cycle progression. Furthermore, it will be interesting to examine their dynamics and the extent of correlation with each other under circumstances where these activator E2Fs have shown to have a unique function.

Fourth, it will be valuable to develop other types of reporters for probing E2F regulation at different levels. For this purpose, a fusion reporter of E2F1 and fluorescent protein can be developed for probing the regulation of E2F degradation by specific ubiquitin ligases (Marti et al., 1999). Binding sites for several known microRNAs that target E2F1 mRNA may also be inserted within the 3’ end of the coding region of a fluorescent reporter to evaluate their contribution in the post-transcriptional regulation of E2F1 (O’Donnell et al., 2005). Moreover, our simulation analysis has indicated that E2F activity (free E2F dissociated from Rb) increases after a time delay when compared to the E2F transcriptional dynamics. This is because Rb immediately sequesters the initially synthesized E2F molecules. Once this sequestration becomes saturated, the level of free E2F starts to increase. These simulation results are interesting and argue for the development of a reporter that could reflect E2F activity dynamics. One approach to construct such as reporter is to take a truncated E2F promoter that only includes canonical E2F binding sites. The fluorescence signal driven by this promoter is likely to mimic the work from free E2F molecules alone, but not of other factors. Using this
strategy, I have built up such a reporter, which was introduced into rat fibroblasts. Single cell measurements of this reporter indicated that the activity dynamics show a tight correlation at the temporal level with S phase onset and progression (data not shown). In the future, we plan to combine this activity reporter with the transcriptional reporter to explore how E2F is regulated at different levels during cell cycle progression and how the different aspects of regulation are related to sequential cellular events.

Fifth, it will be important to upgrade the current ODE model of the Rb/E2F network to a sophisticated stochastic model. The ODE model has advantages; it can represent non-linear dynamics and is computationally efficient, which are two important requirements for model single-cell analysis. However, it lacks the ability to capture the stochastic nature of biochemical reactions. When applied to complex biological networks, stochasticity becomes critical, because under some circumstances, it can lead to the so-called “deviant effect”, which is the discrepancy between the manifestations of behaviors and the predictions from continuous deterministic classical chemical kinetics (Samoilov and Arkin, 2006). This effect can become crippling for systems with complex dynamics and strong non-linearity and/or in a small reaction volume with limited number of molecules. Therefore, if future use of the ODE model is marred by an increased occurrence in discrepancies between the measurements and the simulation results, the “deviant effect” might be the likely explanation, and a switch to a stochastic model would be warranted.
Finally, to more fully capture the complex regulation that occurs at the biological level, we can expand the current model of the core Rb/E2F module by including other regulatory factors. This would allow us to explore how different signals are processed within a broader network structure. One interesting direction will be to include different CKIs. A body of evidence has shown that CKI proteins, such as p27 and p21, can buffer the effect between cyclin D/CDK4/6 and cyclin E/CDK2 complexes. However, it remains unclear how this buffering mechanism works within the G1/S progression to regulate E2F activation. Extending the model to include the functions of CKIs may generate meaningful predictions that warrant further experimental validation.

At yet a higher level, it would be very interesting to combine modeling of the Rb/E2F network with that of the p53 pathway. A lot of evidence point to a strong crosstalk exists between these two pathways in the context of their ability to control multiple cell fates, such as proliferation, cell cycle arrest, senescence and apoptosis (Polager and Ginsberg, 2009). G1 cyclin/CDK activities are readily repressed by p21 expression that is primarily controlled by p53 signaling. Moreover, high dose of E2F1 has been reported to induce p53-dependent cellular senescence or apoptosis. Interestingly, p53 protein dynamics were reported to exhibit spontaneous pulses under normal conditions, robust oscillations under condition of ionizing radiation and maintenance of high level of expression when cells were exposed to UV radiation (Loewer et al., 2010; Purvis et al., 2012). The development of a full modeling framework
incorporating these two modules may assist our understanding of how different cellular phenotypic outputs are determined through a balanced and dynamic gene expression response. As for all models, this approach would require the development of a dual reporter system that can simultaneous reflect E2F and p53 dynamics to validate those theoretical predictions.
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Biography

Peng Dong was born January 1st, 1983 in the county of Xinyi, Jiangsu Province, People’s Republic of China. He grew up in the nearby town of Mohe before he moved to the city of Xuzhou with parents. He then attended the primary school of Tongshan Road, the No. 4 Middle School and No. 1 High School in Xuzhou.

He matriculated at Tsinghua University in 2001 in Beijing, and earned a Bachelor Degree in Engineering from the Department of Automation. He also pursued a M.S. degree in School of Medicine in Tsinghua University from 2005. During that time, he worked with Dr. Jing Cheng, who was a professor in Medical Systems Biology Research Center in School of Medicine.

In 2009, he joined the Duke University Computational Biology and Bioinformatics Ph.D. program. In 2010 he began to work with Dr. Bernard Mathey-Prevot in Department of Pharmacology and Cancer Biology and Dr. Lingchong You in Department of Biomedical Engineering.

Publications (* denotes co-first author)