TEMPORAL REGULATION OF CELL DIVISIONS IN THE EMBRYO OF *DROSOPHILA MELANOGASTER*

by

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Duke University

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Nicolas Buchler

Dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
in the Department of Cell Biology
in the Graduate School of
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2022

ABSTRACT

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Abstract

Cell proliferation is one of the elementary operations involved in building and maintaining the bodies of organisms, and animal development employs diverse regulatory strategies to ensure that it happens in the correct spatial and temporal arrangements. This dissertation is a study of some of the mechanisms involved in timing the early cell cycles of the embryo of *Drosophila melanogaster*. In chapter 1, we introduce many of the important concepts and provide the reader with background on developmental regulation of the cell cycle. In chapter 2, we turn our focus to the problems associated with the cell-cycle transitions that accompany the maternal-to-zygotic transition. Specifically, it had been shown that slowing of the cell cycle following the initial rapid cleavage divisions is linked to the downregulation of protein phosphatase $\mathrm{Cdc25}^{\mathrm{Twine}}$ activity. We pursue this problem with a structure-function analysis of $\mathrm{Cdc25}^{\mathrm{Twine}}$. In chapter 3, we turn our attention to the fourteenth round of cell divisions, which form exquisite spatio-temporal patterns called mitotic domains. Six heterochronic genes (*btd*, *ems*, *kni*, *slp1*, *h*, and *hkb*) had been identified that have dosage-sensitive effects on the timing of cell division in mitotic domain 2 (MD2). We tag two of these factors with GFPs using BAC trangenesis and measure their dynamics in MD2 and other head domains. We find that *btd* is expressed in a gradient that anticipates the mitotic schedule of MD2, and that *slp1* is a powerful repressor of mitosis in the head domains. We conclude that these two factors contribute to the timing of MD2 via a mixed

hourglass model that involves both activator-accumulation and repressor-depletion.

For my parents, Bryan and Jacque

"But our manner of knowing is so weak that no philosopher could perfectly investigate the nature of even one little fly." - St. Thomas Aquinas

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Chapter 1

Introduction

This project examines the mechanisms involved in timing cellular decisions during animal development. The specific cellular decision that I focus on in my experiments (presented in Chapters 2 and 3) is the cell division and the sense for doing so is the following: (1) it is an easy event to identify under a microscope; (2) certain embryonic cell divisions are extremely reproducible from embryo to embryo; (3) a great deal is known about embryonic cell cycle regulation; and (4) there are many tools available for studying the cell cycle. For those reasons, the cell division makes a great model for exploring the problem of developmental timing.

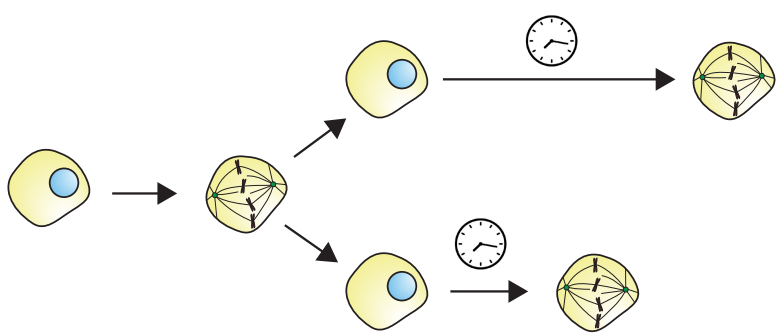

Figure 1.1: Developmental timing of cell divisions.

In this first chapter I will begin with a general discussion of the problems that relate to developmental time. I will review some of the experimental systems in the literature that exemplify the problems that I am interested in. Then I will provide

the reader with an overview of the eukaryotic cell cycle and the protein networks that regulate it. This will bring us to the model system that I have chosen to use for my experiments, namely the embryo of the common fruit fly *Drosophila melanogaster*. I will then guide the reader through what I consider to be the most important discoveries that either directly relate to my question or give the question its greatest inspiration.

1.1 Developmental time

Biology involves many different time scales [Ebisuya and Briscoe, 2018]. On one extreme there is natural history and Darwin's theory of evolution [Darwin, 1859], which take us all the way back to the first living organisms about 3.77 billion years ago [Dodd et al., 2017]. On the other extreme there are enzymatic reactions in our cells that have turnover times of microseconds [Shamir et al., 2016]. Here we are interested in development, which may be defined as the set of processes that take place over one life-cycle of an organism [Bonner, 1965]. This generally includes (among other things) the proliferation of cells and their progressive organization into tissues, as well as cellular differentiation and migration, and the morphogenetic events that lead to the formation of organs and organ systems [Gilbert, 2014]. These events must be coordinated in space and time and for that reason we suspect that there are molecular mechanisms of control. Here we are specifically interested in the mechanisms that ensure the regular progression of development through time.

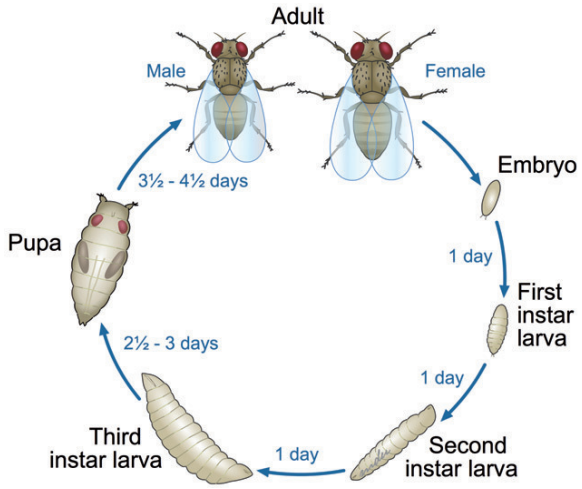

Figure 1.2: The life-cycle of *Drosophila melanogaster*.
Figure adopted from [Ong et al., 2015].

1.1.1 Sequence and duration

There are two frameworks for thinking about developmental time [Reiss, 1989]. On the one hand, there is the sense in which development has a sequential organization: $S_1 \rightarrow S_2 \rightarrow S_3 \rightarrow \rightarrow \rightarrow S_t$. As an example consider the life-cycle of the fly and the manner by which we represent this life-cycle in an arrow diagram (Figure 1.2). On the other hand, developmental time may refer to the species-specific amount of time—or duration—that it takes to complete the stages throughout one life-cycle of the organism. With these two ideas in mind it is possible to phrase sensible questions about the timing of animal development [Reiss, 1989]. We may ask (1) how the events of animal development get their sequential organization, and (2) what determines the duration of time between those events.

1.1.2 Clocks and hourglasses

In principle, there are two methods of keeping time in development. One involves a true clock and the other involves something that looks more like an hourglass or egg-timer [Pourquié, 1998, Johnson and Day, 2000, Negrete and Oates, 2021]. A true clock is a system that shares certain basic properties with the perfect harmonic oscillator, namely the property of cycling with a determinate period [Cooke and Smith, 1990]. In the context of animal development such a clock-type system might involve certain periodic oscillations in a gene network. Examples that satisfy this criterion include the somite segmentation clock [Oates et al., 2012] and the circadian rhythm [Patke et al., 2020]. Alternatively, hourglass-type models involve the rate-limiting production or depletion of a certain molecule until its concentration reaches a threshold [Pourquié, 1998]. Perhaps the best example of a mechanism like this is the timing of the mid-bastula transition [Ferree et al., 2016, Farrell and O'Farrell, 2014], but another commonly studied system is the sequential specification of neuroblasts in the fly [Averbukh et al., 2018]. We will consider some of these examples in detail below but first let us consider the concept of heterochrony.

1.2 Heterochrony

Heterochrony (which is Latin for *different time*) is a term that was originally coined in the context of evolutionary theory to describe a mechanism by which "ontogeny recapitulates phylogeny" [Haeckel, 1875]. The exact meaning of the term has undergone

some significant modifications [De Beer, 1951], but the core message remains one of rate-change in development [Smith, 2003]. It was the paleobiologist Stephen Jay Gould who brought the idea of heterochrony to prominence and ensured its place in our vocabulary [Smith, 2003]. In Gould's theory there are two main morphological contributions to the process of evolution [Gould, 1977, Gould, 1988]. One is the addition of certain novel unitary structures, and the other is heterochrony, which, according to Gould, involves perturbations to the relative timing of developmental events. Although mostly referenced in the literature on evolution [Smith, 2003], there has been a movement over the last few decades to understand the molecular and genetic basis for rate-change in the development of animals like flies [Kim et al., 2000] and nematodes [Ambros and Horvitz, 1984] as well as plants [Buendía-Monreal and Gillmor, 2018]. Let us consider briefly the discovery of heterochronic genes in nematodes.

1.2.1 Heterochronic genes in nematodes

Of the standard cohort of model systems, the tiny nematode called *Caenorhabditis elegans* is very well suited for careful studies of developmental timing [Moss, 2007]. This is in large part because of its simplicity: its life cycle is only 3 days at 25°C; the adult hermaphrodite has exactly 959 somatic cells in its body; and it is transparent under a light microscope [Corsi et al., 2015]. Most important though is the fact that the lineage of cell divisions throughout its development is near-perfectly invariant and

has been mapped in its entirety [Sulston and Horvitz, 1977]. This makes the system an extraordinary asset for those interested in how development achieves its temporal organization.

In a seminal paper on the subject, a collection of genes were identified in the *C. elegans* genome that when mutated (to a loss of function) lead to a disruption in the ordering of the cell division lineage [Horvitz and Sulston, 1980]. This was some of the earliest evidence at the level of molecular genetics for heterochronic genes reported in the literature. It was later revealed following more detailed studies that mutations in the heterochronic genes result in two basic phenotypic disparities [Ambros and Horvitz, 1984]. On one hand, loss of function mutations to the genes called *lin-14* and *lin-28* were found to accelerate *C. elegans* development in the sense that certain cells skip ahead to cell fates usually adopted much later [Ambros and Horvitz, 1984, Ambros and Horvitz, 1987]. On the other hand, loss of function mutations to the genes called *lin-4* and *lin-7* obstruct the normal cell-fate specification sequence, which leads to repetitive larval stages [Ambros and Horvitz, 1984].

Other heterochronic genes in *C. elegans* have since been discovered [Ambros, 2000]. Most are linked in some way or another to mechanisms of post-transcriptional regulation, including some small non-coding RNAs, such as *lin-4*, which is a 22 nucleotide micro RNA that acts as a translational repressor of *lin-14*. One feature of the heterochronic pathway—as it has come to be called—worth commenting on here is that it is implemented in a wide variety of developmental processes. Its

application is not unique to early cell-fate specification, which seems to suggest a certain level of generality in its function as a time-keeping system [Monsalve and Frand, 2012]. For instance, a recent study argues that *lin-4* is a central temporal regulator of post-mitotic neurons in *C. elegans* [Sun and Hobert, 2021]. It remains to be determined how exactly the heterochronic genes operate, but one popular model is an hourglass-type timer in which there is a progressive depletion of *lin-4* that encodes the timing of cellular decision making [Ebisuya and Briscoe, 2018].

1.2.2 Temperature and other environmental factors

The rate of animal development and growth is a function of temperature [Lillie and Knowlton, 1897], and in fact some of the earliest studies showing this were carried out in *Drosophila melanogaster* [Loeb and Northrop, 1916, Powsner, 1935]. At the most basic level this is because the rate constants for the biochemical reactions that contribute to growth are temperature-dependent. One interesting question that this raises is whether all of the component parts of development scale together uniformly with changes in temperature. It seems *a priori* they should not. After all, development is the product of many coordinating processes that each involve their own collection of biochemical reactions with their own rate constants. Nevertheless, morphogenesis in many *Drosophila* species has been shown to scale uniformly with temperature even with two-fold increases in the overall rate of embryogenesis [Kuntz and Eisen, 2014]. One way though to induce heterochrony in flies is to vary oxygen levels because organs

like the gut develop at different rates than the head under conditions of hypoxia [Kuntz and Eisen, 2015].

1.3 Segmentation: clocks and cascades

Animal development often involves metamerism in which the anterior-posterior axis of the body takes on a segmented structure [Diaz-Cuadros et al., 2021]. Two commonly studied examples of this include somitogenesis in vertebrate bodies like those of fish and mice [Maroto et al., 2012] and the segmented structure of the larval body of long germ-band insects like flies [Akam, 1987]. How these repetitive patterns emerge has been of interest to developmental biologists since the field's inception—for an early discussion of segmentation see for instance [Cope, 1871]—with wide-ranging hypotheses to explain the phenomena. What is interesting about these two instances of metamerism is that despite their obvious similarities in appearance, we now know that they diverge radically in mechanism [Diaz-Cuadros et al., 2021]. Whereas fly embryogenesis involves a mechanism of increased compartmentalization controlled by hierarchies of gene regulatory networks (GRNs) [Levine and Davidson, 2005], the somites are patterned by a wave-front of oscillating gene expression [Diaz-Cuadros et al., 2021]. Each of these systems invokes its own method of timing and so they are worth considering here in some detail. Moreover, the segmentation genes of the fly are directly relevant to the project presented here in chapter 3.

1.3.1 Segmentation network in anthropods

The genes that are responsible for the segmented structure of the fly larva were first discovered in a series of important mutagenesis screens [Nüsslein-Volhard, 1977, Nüsslein-Volhard et al., 1980, Nüsslein-Volhard and Wieschaus, 1980, Nüsslein-Volhard et al., 1984, Jürgens et al., 1984, Wieschaus et al., 1984, Wieschaus and Nüsslein-Volhard, 2016, Akam, 1987]. Over the course of the next few decades a picture emerged in which a cascade of genetic regulators progressively partitions the embryo. Furthest up-stream in the process is a small set of maternal-effect genes—including *bicoid* [Driever and Nüsslein-Volhard, 1988, Struhl et al., 1989]—that produce morphogen gradients along the anterior-posterior and dorsal-ventral axes. Cells detect concentration differentials in these gradients (in manners reminiscent of [Wolpert, 1969]) and respond accordingly by sequentially activating or inactivating the so-called gap genes, pair-rule genes, and segment polarity genes [Akam, 1987, Kornberg and Tabata, 1993]. Ultimately, the cascade initiates the Homeotic programs which specify the identities of the body compartments [Hajirnis and Mishra, 2021]. The Homeotic genes are themselves an interesting case of developmental timing in that, for reasons that remain unclear, the sequential ordering of their expression in time is exactly correlated with their spatial location in the genome [Montavon and Soshnikova, 2014].

The segmentation gene network has become a prominent model system for studying (among other things) gene regulation [Stanojevic et al., 1991, Boettiger and Levine, 2009, Fukaya et al., 2016]; patterning and positional information [Tomancak et al.,

2002, Gregor et al., 2007, Garcia et al., 2013, Gregor et al., 2014, Tikhonov et al., 2015]; biological precision, reproducibility, and the problem of molecular noise [Little et al., 2013, Dubuis et al., 2013a, Dubuis et al., 2013b, Petkova et al., 2014, Tkačik et al., 2015]; and more recently it has been proposed as a model for temporal regulation of development [Negrete and Oates, 2021]. As there are no oscillatory gene regulatory networks involved, the predominant understanding of time in this system is one of cascades and hourglasses.

1.3.2 Somite segmentation clock in vertebrates

The concept of positional information was instrumental in forming a wide variety of new hypotheses about the spatio-temporal organization of tissues [Wolpert, 1969, Cooke and Zeeman, 1976]. The French flag model illustrated in a simple way how a field of cells could compartmentalize. But certain features of the vertebrate segmentation process seemed to preclude this mechanism [Cooke and Zeeman, 1976]. Most important is the fact that whereas during anthropod development the segmentation process happens simultaneously, the procedure that patterns the vertebrae is sequential and truly rhythmic [Diaz-Cuadros et al., 2021]. The tissue to be patterned is called paraxial mesoderm and it runs along both sides of the notochord and neural tube. During somitogenesis the posterior region of the paraxial mesoderm is periodically partitioned into units of epithelial tissue called somites [Oates et al., 2012]. For our purposes, the important properties of the system include the regularity of size of

each somite, and the temporal frequency at which they are produced [Oates et al., 2012]. Although these dimensions vary widely from species to species (e.g, snakes versus frogs), within a given species somitogenesis is remarkably accurate at counting and sizing each segment. To explain the regularity, a clock and wave-front model was proposed based largely on theory [Cooke and Zeeman, 1976], but has since been bolstered by many genetic and physical experiments [Bessho et al., 2001, Dale et al., 2006, Giudicelli et al., 2007, Diaz-Cuadros et al., 2021]. The clock consists of oscillating gene expression in the pre-somitic mesoderm and the wave-front involves the progressive posterior movement of activity through the tissue that deactivates the clock [Oates et al., 2012].

1.4 Temporal regulation by hormones

Hormones are timing molecules commonly associated with large-scale body transitions during animal development [Kannangara et al., 2021]. For instance, bursts in production of the hormone called 20-hydroxy-ecdysone (see Figure 1.3) activate certain post-embryonic developmental transitions throughout the life cycle of the fly [Thummel, 1995, Kannangara et al., 2021]. Because of its broad implications as a developmental program the ecdysone system has been described as a mechanism of global temporal coordination [Jain et al., 2021].

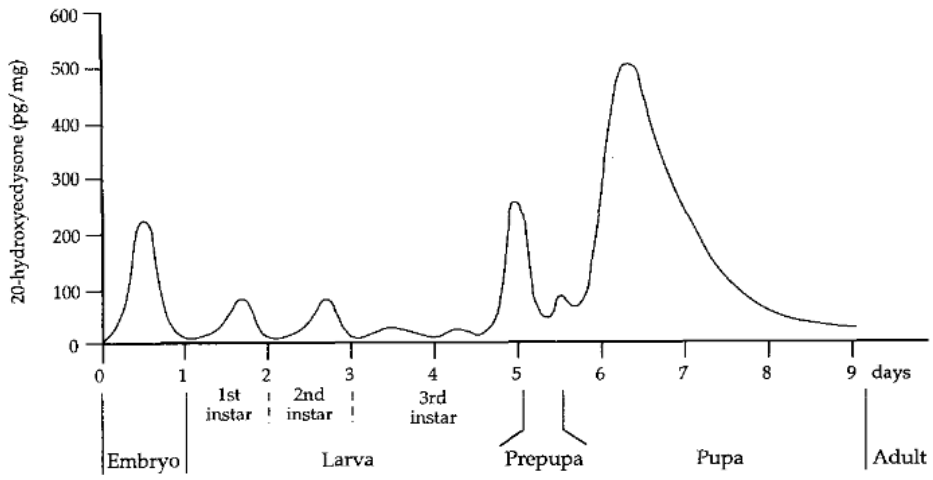

Figure 1.3: Ecdysone dynamics during *Drosophila* development. Figure adapted from [Thummel, 1995].

1.5 Circadian rhythm

The quintessential example of a time-keeping system in biology is the circadian rhythm, which involves the entrainment of certain biological features to the 24-hour day-night cycle of the earth [Patke et al., 2020]. To appreciate the relevance of this timer to development, consider that adult flies usually hatch from their pupal casings (an event called eclosion) in the morning. Taking advantage of this observation, a genetic screen was carried out in search of mutants that change the rhythmic pattern of eclosion [Konopka and Benzer, 1971]. This landmark paper reported the discovery of the first circadian gene called *period*, a transcriptional repressor, but others were to come including *timeless* [Sehgal et al., 1994], *cycle* [Rutila et al., 1998], and *clock* [Allada et al., 1998], also all transcription factors that together form a gene regulatory

network [Patke et al., 2020]. In the model that has emerged, Cycle and Clock proteins are positive regulators of *period* and *timeless*. Period and Timeless proteins peak in concentration in the evenings, which allows them to enter the nucleus and negatively regulate their own expression via inhibitory regulation of Cycle and Clock. But during the day Period and Timeless lose stability, which allows Cycle and Clock to again activate transcription of *period* and *timelesss* and the cycle starts all over [Patke et al., 2020]. When tested most of the tissues in the fly show time-dependent oscillations in expression of the circadian genes [Patke et al., 2020]. The circadian rhythm controls the timing of eclosion by feeding into the ecdysone hormone system [Myers, 2003].

1.6 Developmental control over the cell cycle

So far I have aimed to provide the reader with a broad introduction to the problems of developmental timing. I have explored some of the important systems that have contributed to our understanding of sequence, duration, clocks, hourglasses, and heterochrony. But the specific topic of this dissertation concerns the temporal regulation of cell division in embryonic development. In this section I will introduce the cell cycle and its regulatory proteins. Then I will turn our attention to the problem of cell cycle regulation in the early embryo and describe how this problem has been addressed in the literature. Although I will introduce other model systems along the way, indicating the contributions that they have made, I will ultimately focus on the embryo of *Drosophila melanogaster*, which is the experimental system I have chosen

to study in chapters 2 and 3.

1.6.1 Mechanisms of cell cycle regulation

The cell cycle is the path of operations involved in cell duplication [Nurse, 2000]. There are two broad categories of cells—eukaryotes and prokaryotes—but for our purposes we will consider only eukaryotes as those are the cells of animals. Most genes that contribute to the regulation of cell-cycle progression were isolated in screens carried out in budding and fission yeasts looking for temperature-sensitive mutants that fail to pass through various phases of the cell cycle [Hartwell et al., 1970, Hartwell et al., 1973, Nurse et al., 1976, Nasmyth and Nurse, 1981, Nurse, 2020]. These experiments turned up all kinds of *cdc* (cell division cycle) genes that are linked in some way or another to the cell cycle, but to identify the central regulators of mitosis would require a different strategy. With that in mind Paul Nurse took an interest in mutants that he termed *wee* because they were significantly smaller than their wild-type counterparts. This ultimately led to the discovery of *wee1* and *cdc2* as rate-limiting regulators of mitosis [Nurse and Thuriaux, 1980], and later another gene called *cdc25* was found to be a positive regulator of mitosis [Russell and Nurse, 1986]. There was parallel interest in purifying the maturation-promoting factor (MPF) from the oocytes of starfish, frogs, *et cetera* [Masui and Markert, 1971, Draetta et al., 1989]. MPF would later turn out to be Cdc2, which—following the discovery of the Cyclin/Cdc2 stoichiometric relationship—was later given the name Cdk1 for cyclin-dependent kinase 1 [Labbe

et al., 1989, Meijer et al., 1989]. Cdk1, Wee1, and Cdc25 are the core components involved in the regulation of mitosis, and although they were discovered in yeasts, they are conserved across all eukaryotes and so form the foundation on which cell division is regulated during animal development [Morgan, 1997].

The activity of Cdk1 is heavily regulated. Once it binds to a Cyclin partner it may be phosphorylated at threonine 161 (Thr161) by another kinase called Cdk1-activating kinase (CAK) [Morgan, 1997]. Because CAK is generally present at high levels throughout the cell cycle its covalent modification of Cdk1 is not rate-limiting. Instead, the activation state of Cyclin-Cdk1 is largely determined by two inhibitory phosphates, one at tyrosine 15 (Tyr15) and the other at threonine 14 (Thr14), both of which are near Cdk1's ATP-binding pocket [Morgan, 1997]. Wee1 and Myt1 are the kinases that catalyze the addition of phosphates to these two locations, and Cdc25-type proteins are the phosphatases that counter the operation by catalyzing their removal [Gautier et al., 1991, Strausfeld et al., 1991, Dunphy and Kumagai, 1991]. In this way, Cdc25 is a positive activator of mitosis. As a further level of regulation, Cyclin-Cdk1 then also modifies the enzymatic activity the Wee1/Myt1 and Cdc25 proteins [McGowan and Russell, 1995, Hoffmann et al., 1993]. This regulation is negative in the case of Wee1/Myt1, but positive in the case of Cdc25, leading to feedback loops that give the mitotic switch its all-or-nothing property [Morgan, 1997, Lindqvist et al., 2009].

1.6.2 Early embryonic cell cycles, in brief

There are many strategies of early embryonic development. In humans and mice the embryo is grown in the mother's womb and provided with nutritional support [Gilbert, 2014]. Most animals though, including flies and frogs, lay their eggs in the environment and leave them alone to fend for themselves [Farrell and O'Farrell, 2014]. One consequence of this behavior is that the eggs are relatively large as they must contain enough materials to complete embryonic development. Whereas the human zygote is only about 100 microns in diameter, the fly egg is five times that size, and the frog egg is about one millimeter in diameter [Gilbert, 2014]. The early embryonic cell cycles in these latter animals also tend to be very fast. Whereas a typical human cell spends about 24 hours traversing the cell-cycle landscape, some of the fastest embryonic cycles, like the ones in flies, are only about 10 minutes, which includes the whole of DNA replication. For these and other reasons the early embryonic cycles have contributed substantially to our knowledge of cell cycle regulation and its control by development. This is very-well illustrated by the maternal to zygotic transition, which we will turn our attention to now.

1.6.3 The maternal to zygotic transition

Early embryonic development in many animals involves a species-specific number of rapid cleavage divisions [O'Farrell et al., 2004]. For instance, in the frog (*Xenopus laevis*) there are twelve [Newport and Kirschner, 1982a, Newport and Kirschner,

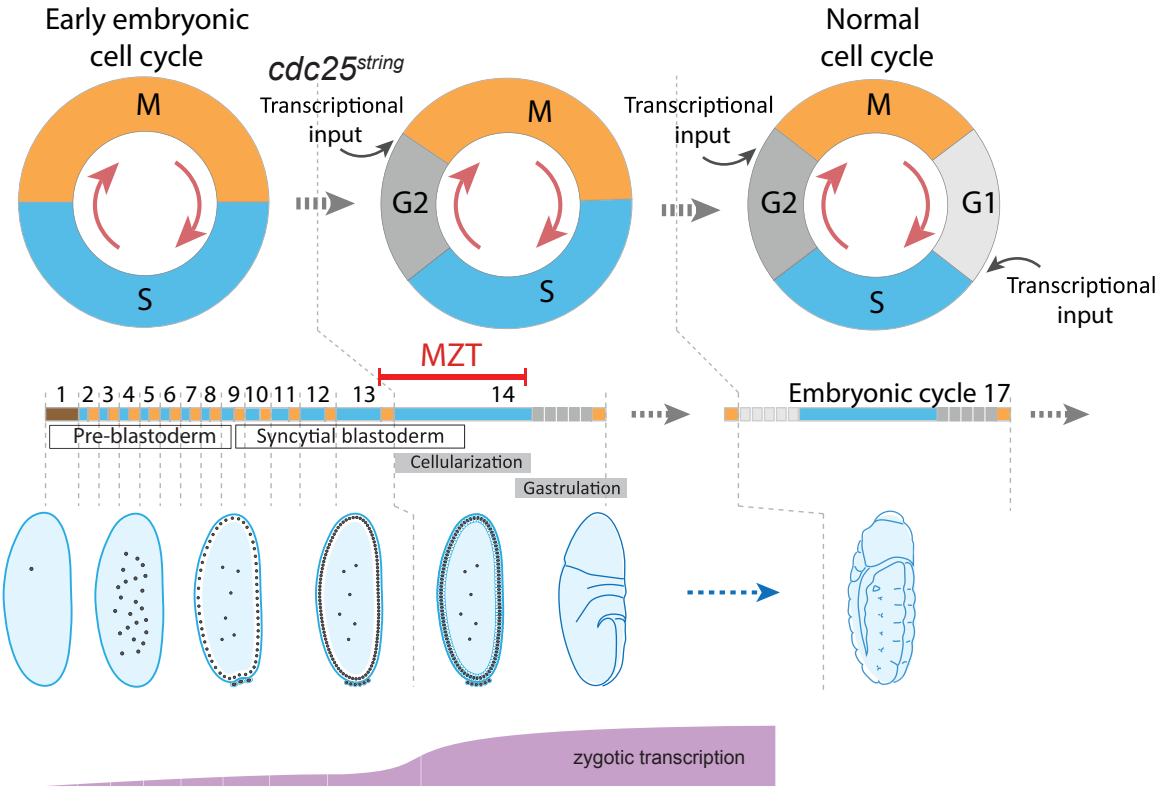

Figure 1.4: Early embryonic cell cycles in *D. melanogaster*.
This figure is modified from [Farrell and O'Farrell, 2014, Yuan et al., 2016].

Here is a schematic of the cell-cycle remodeling during early fly development. The initial thirteen cycles are abbreviated, consisting only of DNA synthesis (S-phase) and mitosis (M-phase). Cycle 14 coincides with gastrulation. Cycles 14-16 are regulated at the G2/M transition by transcription of *cdc25*^{string}. Embryonic cycles 17 onward are considered "normal" in that they include the four standard phases S-G2-M-G1.

1982b]; in the fly (*D. melanogaster*) there are thirteen [Foe and Alberts, 1983]; and in the starfish (*Asterina pectinifera*) there are ten [Mita and Obata, 1984]. These early divisions are generally synchronous (or very close to synchronous) and fueled only by the maternal contribution to the egg of mRNAs and proteins [Farrell and O'Farrell, 2014]. But in each example the initial free-running cycles are followed by slower, more somatic-like cell cycles, a great increase in the activity of the zygotic genome, and the onset of gastrulation [O'Farrell et al., 2004]. This transitional phase of development in the frog was initially called the mid-blastula transition (MBT) [Gerhart, 1980], and ever since its inception it has been a model for studying developmental time [Farrell and O'Farrell, 2014].

It is a topic for debate whether (and in what sense) the events of the MBT in the frog correspond to events in the fly—or the mouse for that matter [O'Farrell et al., 2004]. So as not to stir confusion it has become common to talk instead about the maternal to zygotic transition (MZT) as a much more general term. Figure 1.4 illustrates in more detail the many operations that accompany the MZT in *D. melanogaster*. The initial thirteen cleavage divisions are called nuclear cycles because they take place entirely in one shared cytoplasm called a syncytium [Foe and Alberts, 1983]. They are extremely fast (cycle 11 and 13 are 9 and 18 minutes, respectively) and composed only of S- and M-phases, with S-phase growing progressively longer between cycles 11 and 14 [Farrell and O'Farrell, 2014]. Similarly, the zygotic genome is essentially silent throughout the early cycles but becomes much more active between

cycles 11 and 14 [Yuan et al., 2016]. Consequentially, when we talk about the MZT in the fly, we generally have in mind the lengthening of the cell cycle (longer S-phases and the eventual addition of a G2-phase during cycle 14), cellularization (a special form of cytokinesis), activation of the zygotic genome, destruction of maternally deposited transcripts and proteins, and the onset of gastrulation (cell-fate specifications and tissue rearrangements) [Farrell and O'Farrell, 2014].

1.6.4 Nuclear-to-cytoplasm ratio

One long standing hypothesis in the field is that the timing of the events associated with the MZT involves the embryo measuring, in some sense, the ratio between the quantity of DNA and the quantity of cytoplasm, generally referred to as the nuclear-to-cytoplasm ratio (NC ratio). This is an hourglass-type mechanism in which, for instance, certain mitotic activators are present in the cytoplasm that progressively titrate out with each round of cleavage division. Once the NC ratio reaches some critical threshold and there is no longer enough mitotic activator, new cell-cycle programs take over. Evidence for this class of theories was provided by experiments on haploid embryos. Haploid fly embryos, for instance, proceed through an extra round of synchronized divisions [Edgar et al., 1986a]. But many of the arguments in favor of the NC hypothesis come from experiments carried out in the frog (*Xenopus laevis*). These include experimental perturbations to ploidy [Newport and Kirschner, 1982a, Kirschner et al., 1985], but also more substantial findings like the identification

of candidate molecules involved in the titration process, such as certain maternally deposited histones [Amodeo et al., 2015] and DNA replication factors [Collart et al., 2013].

1.6.5 Cell-cycle slowing at MZT is linked to Cdc25 activity

Further progress was made to understand the cell-cycle remodeling at the MZT in *D. melanogaster* when it was discovered that it requires the degradation of the maternally deposited mRNAs of two *cdc25* homologs called *cdc25*^{string} and *cdc25*^{twine} [Edgar and Datar, 1996]. It was known at the time that Cdc25-type proteins are activators of the mitotic kinase Cdk1 [Coleman and Dunphy, 1994]. Hence, the new results suggested an elegant model in which the post-transcriptional downregulation of Cdc25 activity in turn leads to Cdk1-deactivation initiated interphase. But a series of experiments later argued in favor of a model in which post-translational regulation of Cdc25, and in particular, Cdc25^{Twine}, controls cell-cycle remodeling at the MZT [Farrell and O'Farrell, 2013, Di Talia et al., 2013]. Not only does *cdc25*^{twine} mRNA outlive its corresponding protein, measurements of Cdc25^{Twine}'s stability showed that it sharply falls during cycle 13 suggesting its role as a switch [Farrell and O'Farrell, 2013, Di Talia et al., 2013]. In contrast, Cdc25^{String} proteins are targeted for degradation earlier and much more gradually, suggesting that the two homologs respond to distinct signals in the embryo [Di Talia et al., 2013]. The model that emerges is one in which zygotic genome activation is required for the abrupt destruction of Cdc25^{Twine} proteins, while

Cdc25^{String}'s stability is linked to activation of DNA replication checkpoints [Farrell and O'Farrell, 2013, Di Talia et al., 2013]. One of the questions this raises then concerns the identities of the zygotic genes that target Cdc25^{Twine} for degradation. The pseudo-kinase called *tribbles* was shown to be sufficient to destabilize Cdc25^{Twine} at the MZT but not required [Farrell and O'Farrell, 2013]. Overall these experiments suggested a model in which the onset of zygotic transcription triggers the production of certain molecules that destabilize Cdc25^{Twine}, leading to deactivation of Cdk1 and the slowing of the cell cycle during the MZT.

1.6.6 Victoria Foe's characterization of the mitotic domains

The fourteenth round of cell divisions in the fly embryo is spatially and temporally patterned into what Victoria Foe called mitotic domains (MDs) [Foe, 1989, Foe and Odell, 1989]. Although other examples of mitotic domains have been described, e.g., in zebrafish [Kane et al., 1992], the mitotic domains of *Drosophila melanogaster* have been very well characterized and provide an excellent model system for thinking about timing in development. Because her papers form the foundation for my project in chapter 3, I will now provide an overview of Foe's experiments, techniques, and hypotheses.

The general strategy that Foe followed was to fix and stain embryos and confirm her observations using video time-lapse recordings. She scored mitosis by staining for anti-β-tubulin, a common aid for visualizing microtubules, and therefore an indication

of the phase of the cell cycle. Some of her microtubule-stained micrographs are presented in the figure (Figure 1.5 A-D). From her images Foe was able to construct an atlas of the spatial pattern of the MDs and classify each MD with a number that signifies its location in the temporal order of development (Figure 1.5 E-H). Based on her assessment, there are 25 domains that are mitotic and synchronous; two domains that are mitotic but asynchronous and very late to divide; and two domains that are not mitotic (at least not mitotic during the time period she was looking).

The mitotic domains come in a variety of shapes and sizes and are remarkably invariant from embryo to embryo. This idea of "reproducibility" is an important part of Foe's project, and she goes to great lengths to provide the concept with a quantitative basis. She scores the timing of each MD based on the time that its first cells enter mitosis. She finds that cells begin to enter mitosis in the first mitotic domain called MD1 about 70 minutes after nuclear cycle 13, and cells begin to enter mitosis in the last mitotic domain called MD25 about 115 minutes after nuclear cycle 13. A complete list is provided in her paper. She calculates error based on her observations of many embryos and concludes that the timing of the MDs at 25°C is accurate to about $\pm$ 2 minutes.

Foe hypothesized that the segmentation genes [Akam, 1987] are what pre-pattern the mitotic domains [Foe, 1989, Foe and Odell, 1989]. One piece of evidence she provides in support of this idea is a micrograph of an embryo stained for *engrailed* (one of the segment polarity genes) which seems to show stripes aligned to the edges

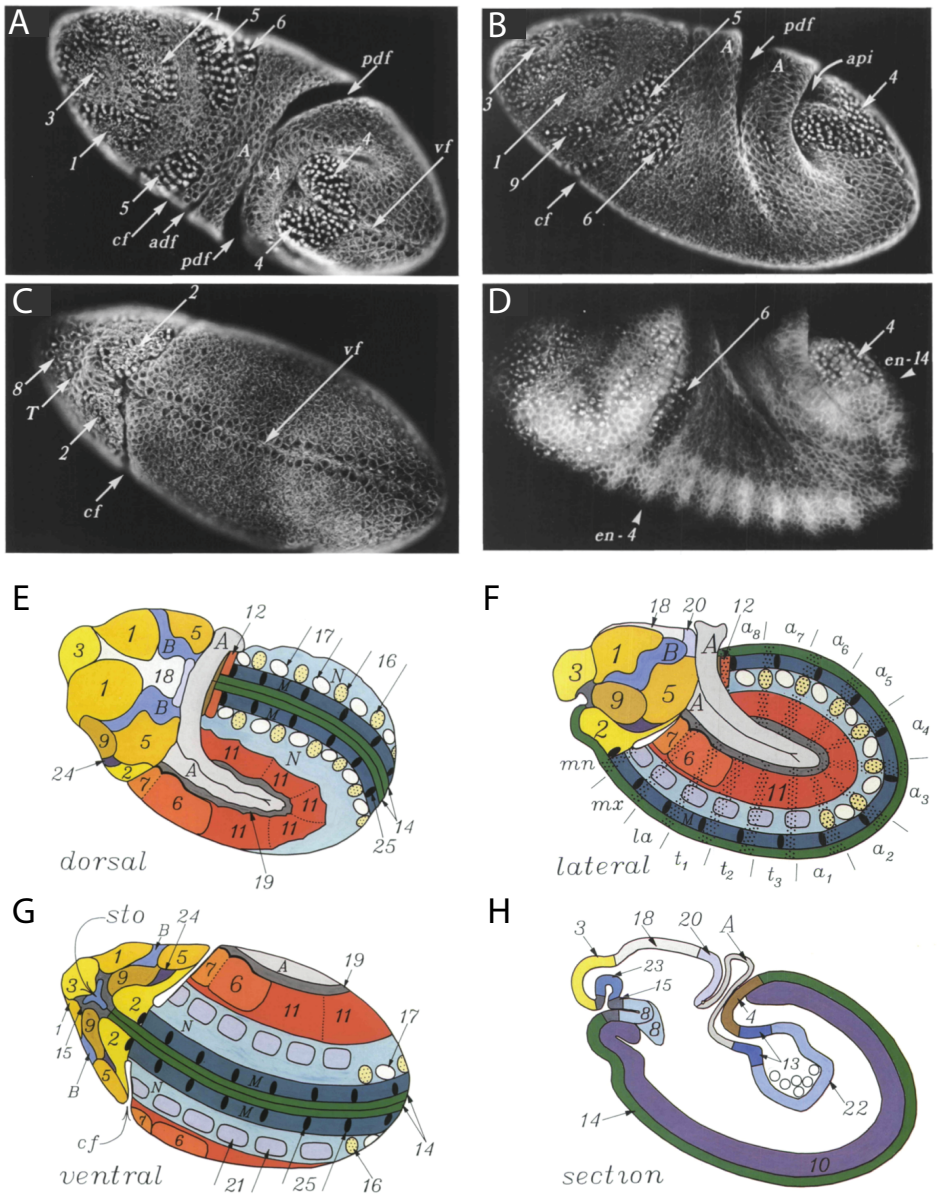

Figure 1.5: Victoria Foe's characterization of the mitotic domains.

(A) Dorsal side of cycle 14 embryo. (B) Lateral side of cycle 14 embryo. (C) Ventral side of cycle 14 embryo. (D) Lateral side of cycle 14 embryo stained for *engrailed*. (E-G) Cartoon illustrations of the images in (A-C). (H) Cartoon illustration of the sagittal plane of a cycle 14 embryo. [Foe, 1989].

of some mitotic domains (Figure 1.5D). Foe and others in the field were confident that the early patterning genes were acting in some combinatorial manner to direct cell fates and govern the massive morphogenetic movements that give rise to the larval body. In a moment of literary license Foe writes [Foe, 1989]:

> If the accumulating product of each early pattern gene contributes to the cells that expressed it a unique color, then, just prior to gastrulation, the blastoderm sheet of cells, each cell with a mix of colors, would appear clothed in an exceedingly complex plaid. By mechanisms not yet understood, this tapestry of gene products prefigures the larva's body.

She continues:

> It must be that the special combination of genes that cells in a particular group express impels them to act at a particular time in a particular way.

1.6.7 Patterning the mitotic domains involves *cdc25*^{string}

The mitotic domains require zygotic transcription [Edgar et al., 1986b]. When zygotic transcription is blocked embryos progress normally through the initial thirteen nuclear cycles but stall with cells in G2-phase. It was hypothesized that one of the cell cycle regulators was likely to be a product of maternal mRNA during the nuclear cycles and zygotic mRNA at the fourteenth cycle. This line of reasoning led to the discovery of *cdc25*^{string} [Edgar and O'Farrell, 1989].

The *string* alleles (as they were initially called) were first identified in the Heidelberg screens because they resulted in minor but recognizable disruptions in the appearance of the larval cuticle pattern [Jürgens et al., 1984]. Further investigation would later indicate that the problem was not a problem of patterning information but rather a problem of cell proliferation, and the reason for the diminished string-like appearance of the mutant embryo was its relative dearth of cells. Embryos homozygous for a completely amorphic *string* allele develop normally through the initial embryonic cycles but fail to pass through interphase 14, which satisfied exactly the desired phenotype [Edgar and O'Farrell, 1989]. The *string* locus was cloned and found to share 34% sequence homology with the C-terminal region of the fission yeast *cdc25* gene [Edgar and O'Farrell, 1989, Russell and Nurse, 1986]. Although there was not much yet known about the protein product of *cdc25*, it was known that the yeast *cdc25* mutants were not able to pass through the G2/M transition of the cell cycle [Russell and Nurse, 1986]. Consistent with this piece of information, the nuclei of mutant $cdc25^{string}$ arrested embryos have four copies of DNA, suggesting a normal transition through DNA synthesis in S-phase but an arrest in G2-phase [Edgar and O'Farrell, 1989].

Based on a time-series of images of mRNA *in situ* hybridization, it was found that the spatial and temporal arrangement of $cdc25^{string}$ transcription at cycle 14 mirrors or "anticipates" the mitotic pattern (visualized using a nuclear stain) [Edgar and O'Farrell, 1989]. This suggested that transcriptional activation of $cdc25^{string}$ may be

the rate-limiting step in the cellular decision to enter mitosis 14, and that perhaps studying its regulators would provide insights into the timing of developmental events. In fact, it would also turn out that the next two mitotic cycles (so 14, 15, and 16) are regulated at the G2/M transition and linked to the transcription of *cdc25*^{string} [Edgar and O'Farrell, 1990].

Three principal observations support the model in which *cdc25*^{string} transcription controls entry into mitosis 14 [Edgar et al., 1994b]

1. *cdc25*^{string} transcripts and proteins are absent during interphase 14 but reproduced just prior to mitosis;

2. the correct phospho-isoforms of Cdk1 (Cdc2) are detected during interphase 14;

3. as the level of *cdc25*^{string} transcripts increases, there is an increase in the level of dephosphorylated Cdk1 (Cdc2).

The first observation was tested and confirmed using immunoblots to measure the levels of the *cdc25*^{string} transcripts and proteins, and it was found that the transcripts disappear about 20 minutes into interphase 14, but are detected again before the entry into mitosis 14 [Edgar et al., 1994b]. The second observation was tested and confirmed using immunoblots to measure the levels of the four relevant phospho-isoforms of Cdk1 present in whole embryos at various stages of cell cycle 13 and 14 [Edgar et al., 1994b]. These experiments were newly possible because the protein product of the *cdc25* gene was further characterized and the target site on the Cdk1 protein was

identified [Dunphy and Kumagai, 1991, Gautier et al., 1991, Kumagai and Dunphy, 1992]. To make the last observation, a heat shock promoter was used to uniformly express *cdc25*^{string} in embryos. It was found that the ectopic *cdc25*^{string} not only drives cells into mitosis but also leads to the expected dephosphorylation of Cdk1 proteins [Edgar et al., 1994b].

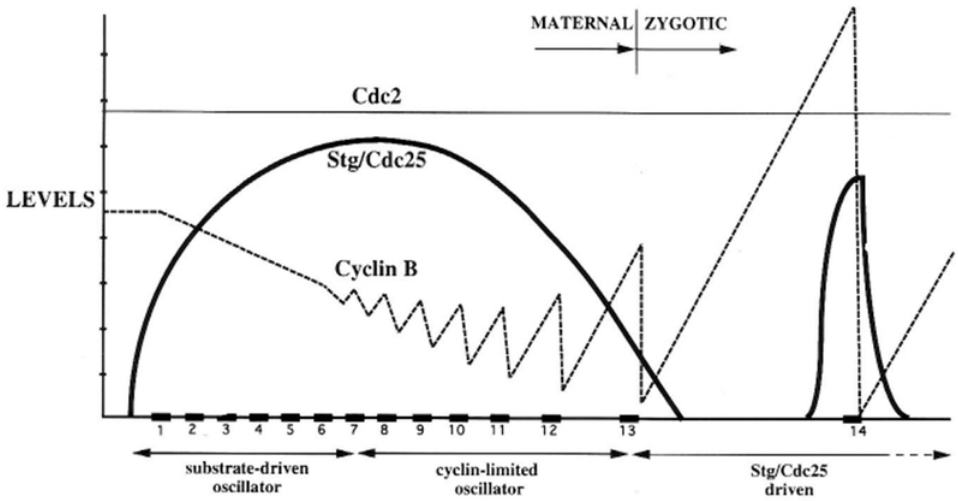

Figure 1.6: Protein levels of cell cycle regulators in the *D. melanogaster* embryo This figure was adapted from [Edgar et al., 1994b].

This particular paper is packed full of other information [Edgar et al., 1994b]. In addition to their experiments on *cdc25*^{string} and Cdk1, they also measure the levels of Cyclins A and B. Here too they present the interesting conundrum that Cdk1 activity is apparently constant throughout the early nuclear cycles, implying that there must be some (as of then) unexplained mechanism for the rapid S/M nuclear oscillations. They close the paper with a schematic that summarizes the suspected protein dynamics of each of the cell cycle regulators throughout the first 14 cell cycles.

I have included this diagram for the reader to see because it gets a lot of things right and gives us a good idea of what the next experiments should be (Figure 1.6). Two of the more interesting unknowns in the Edgar model concern the mechanisms of Cdc25 downregulation following mitosis 13 and Cdc25 upregulation during interphase 14. Those are the two problems that have interested me most throughout graduate school, and also the subject of the two projects I am presenting in my dissertation.

Now let us turn to another two papers, both of which investigate the problem from a genetics perspective, focusing less on the particular functions of the cell cycle regulators, and more on the nature of the DNA and its regulators that encode the precise expression pattern of *cdc25*^{string} transcription [Edgar et al., 1994a, Lehman et al., 1999]. At the time, many pattern-formation genes had been identified (described above in the section on segmentation genes), and the best model anyone could imagine suggested that those same types of genes were probably also acting on the enhancer elements of mitotic activators like *cdc25*^{string} to produce complex mitotic patterns such as those in the mitotic domains. Some evidence had already been provided for this hypothesis [Foe and Odell, 1989, Arora and Nusslein-Volhard, 1992]. To further explore the idea, *in situ* hybridization was used to visualize the pattern of *cdc25*^{string} expression (at gastrulation and other stages) in 36 pattern-formation mutants [Edgar et al., 1994a].

What was observed is that most of the pattern-gene mutants disrupt the pattern of *cdc25*^{string} expression [Edgar et al., 1994a]. Some of the mutants completely abolish

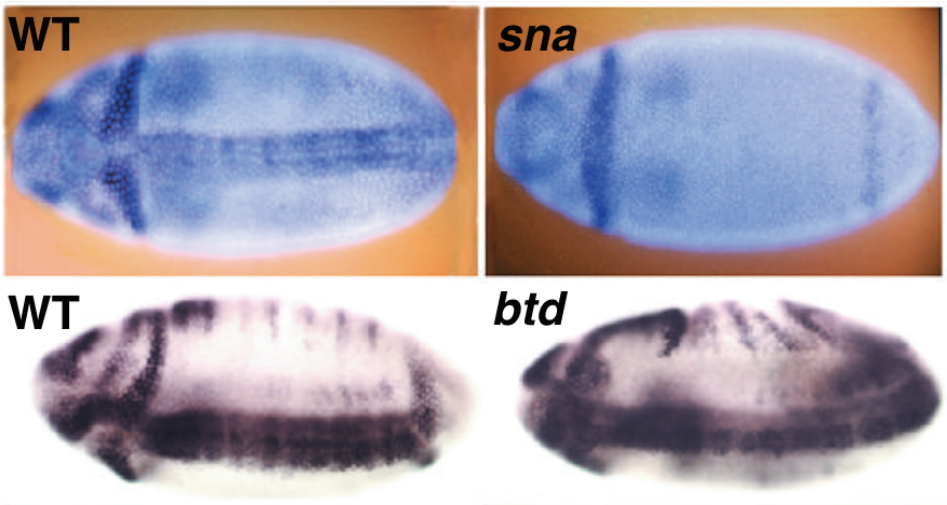

Figure 1.7: Altered expression of *cdc25*^{string} mRNA in pattern formation mutants. [Edgar et al., 1994a].

specific locations of *cdc25*^{string} expression. And other mutants only distort to various degrees what the *cdc25*^{string} pattern should be. I have provided some of the more relevant photographs from their paper for the reader to see (Figure 1.7). In *buttonhead* (*btd*) mutants, for instance, the stripe of *cdc25*^{string} that normally sits along the cephalic furrow (the specific location of MD2) is gone (Figure 1.7). And in *snail* mutants *cdc25*^{string} expression along the ventral furrow is completely abolished (usually the location of MD14) (Figure 1.7). The way these observations were interpreted was that complete deletion of *cdc25*^{string} expression means the pattern gene is a direct transcriptional activator, whereas distortions suggest indirect regulation [Edgar et al., 1994a]. By this logic, *buttonhead* is described as a direct activator of *cdc25*^{string} transcription in mitotic domain 2. These (and other) observations suggested an

impressive level of transcriptional regulation of *cdc25*^{string}. In a follow up study, the *cdc25*^{string} *cis*-regulatory DNA was dissected using *cdc25*^{string}-*lacz* reporter genes, and found to be large (>40 kb), complicated, but mostly modular [Lehman et al., 1999]. Small DNA fragments from the *cdc25*^{string} locus (as small as 500 bp) replicate the spatial and temporal expression pattern of *cdc25*^{string} in certain specific tissues. But none of the fragments rescue the *cdc25*^{string} mutant alleles [Lehman et al., 1999].

1.6.8 Cdc25^{String} accumulation times entry into mitosis 14

The molecular networks that contribute to the function and decision-making of cells are inherently open to random fluctuations, which in turn set limits on the possible precision of biological systems [Bialek and Setayeshgar, 2005]. Indeed, promoters that are experiencing the same concentration of activator have been shown to have differing levels of transcriptional activity [Elowitz et al., 2002]. This has led to the hypothesis that biological systems, and developmental systems in particular, have constructed mechanisms of molecular noise filtration. In recent years the fly embryo has proven to be an extraordinary system for asking questions about biological precision and noise [Gregor et al., 2014]. For instance, the timing of mitosis 14 involves two distinct steps that each must be carried out precisely [Di Talia and Wieschaus, 2012]. In the first step, *cdc25*^{string} transcription must be activated at precisely the right time; and in the second step, cells must respond accordingly to the rate of Cdc25^{String} production. An investigation of this second step has shown that the time between the initiation of

cdc25^{string} transcription and entry into mitosis is kept constant and controlled by the rate of Cdc25^{String} production [Di Talia and Wieschaus, 2012]. Moreover, arguments have been made that cells use covalent modification cycles to integrate Cdc25^{String} activity thereby ensuring timely entry into mitosis [Di Talia and Wieschaus, 2012, Di Talia and Wieschaus, 2014]. But the first step remains largely unexplored, namely the mechanisms that time *cdc25*^{string} transcription.

1.6.9 Transcriptional timers of *cdc25*^{string}

A whole-genome screen was carried out in the Wieschaus lab at Princeton for genes that have a dosage-sensitive effect on the timing of mitosis in mitotic domains 1 and 2 [Momen-Roknabadi et al., 2016]. The premise for the project was the idea that having half as many copies of a rate-limiting gene should lead to observable changes in the timing of its target event. Consequently, genes that affect the timing of mitosis could be identified by screening through embryos that are heterozygous for mutations. Embryos heterozygous for a mutation in a gene that activates mitosis would exhibit delayed entry, and embryos heterozygous for a mutation in a gene that inhibits mitosis would exhibit precocious entry. To initially screen through the whole genome, Momen-Roknabadi *et al.* used deficiency lines, which are flies that are heterozygous for large (well-annotated) DNA deletions. Their protocol involved fixing/staining embryos and counting the number of dividing cells in both MD1 and MD2. As a measure of absolute time, they used the extension of the posterior midgut.

They report eight genes in total that affect the timing of mitosis in MD2. Excluding Wee1 and Cdk1, which are general regulators of mitosis, the newly reported genes include three activators (*btd*, *ems*, and *kni*) and three repressors (*slp1*, *h*, and *hkb*) (Table 1.1).

All six genes code for transcription factors that are involved in various aspects of patterning the larval head and body, each with its own signature expression pattern during gastrulation (Figure 1.8). *btd* is a head gap gene that codes for a zinc-finger transcription factor and is expressed in a single stripe along the cephalic furrow [Wimmer et al., 1995, Wimmer et al., 1996, Wimmer et al., 1997, Schöck et al., 2000]. *ems* is a head gap gene that codes for a homeodomain transcription factor and is expressed (like *btd*) in a single stripe along the cephalic furrow [Walldorf and Gehring, 1992, Dalton et al., 1989]. *kni* is a gap gene that codes for a transcription factor of the steroid receptor super family [Nauber et al., 1988]. *slp1* is a pair-rule gene that codes for a fork-head domain transcription factor and is expressed in a characteristic set of stripes in the head and along the AP axis [Grossniklaus et al., 1992, Grossniklaus et al., 1994, Andrioli et al., 2004]. *hairy* is one of the classic pair-rule segmentation genes that codes for a basic helix-loop-helix transcription factor and is expressed in seven characteristic stripes along the AP axis [Carroll et al., 1988]. *hkb* is a gap gene that codes for a zinc-finger transcription factor and is expressed at the anterior and posterior poles of the embryo [Brönner et al., 1994]. Taken all together, we have a small set of genes that have been traditionally studied as spatial determinants of

gene	abbreviation	effect on MD2
buttonhead	*btd*	activator
empty spiracles	*ems*	activator
knirps	*kni*	activator
sloppy paired 1	*slp1*	repressor
hairy	*h*	repressor
huckebein	*hkb*	repressor

Table 1.1: Transcriptional timers of mitotic domain 2

pattern, but are now implemented as potential rate-limiting (heterochronic) regulators of cell division.

1.7 Subjects of this dissertation

Two distinct but related projects are presented in this dissertation. The first is presented in chapter 2 and is a study mainly of the mechanisms involved in timing the abrupt degradation of the protein phosphatase $\mathrm{Cdc25}^{\mathrm{Twine}}$ during the maternal-to-zygotic transition. The second project is located in chapter 3 and is an investigation of the mechanisms involved in timing $cdc25^{string}$ transcription and the resulting cell divisions that accompany gastrulation in *D. melanogaster*.

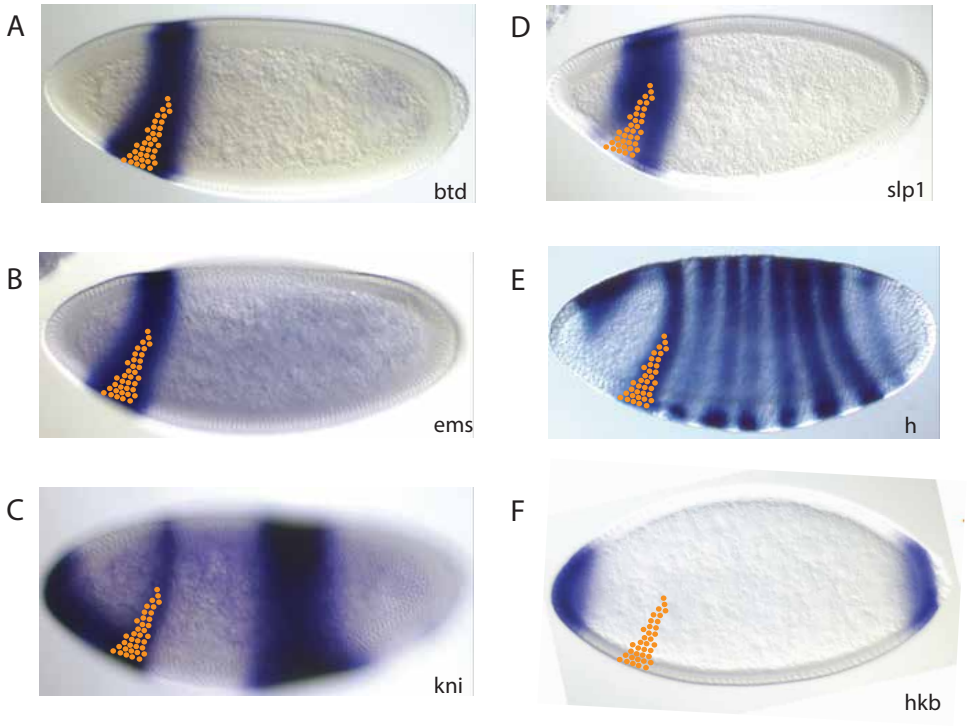

Figure 1.8: Expression patterns of transcriptional timers of MD2.

Images of mRNA *in situ* hybridization for each of the six transcription factors compiled from the Berkeley *Drosophila* Genome Project [Tomancak et al., 2002, Tomancak et al., 2007]. Included on each image is a cartoon illustration of the approximate location and relative size of mitotic domain 2.

Chapter 2

Structure-function analysis of Cdc25^{Twine} degradation at the *Drosophila* maternal to zygotic transition

This chapter was modified from a paper published under the same name along with Maggie Xing, Jenny Q. Zhang, and Stefano Di Talia [Ferree et al., 2022].

2.1 Abstract

Downregulation of protein phosphatase Cdc25^{Twine} activity is linked to remodeling of the cell cycle during the *Drosophila* maternal-to-zygotic transition (MZT). Here, we present a structure-function analysis of Cdc25^{Twine}. We use chimeras to show that the N-terminus regions of Cdc25^{Twine} and Cdc25^{String} control their differential degradation dynamics. Deletion of different regions of Cdc25^{Twine} reveals a putative domain involved in and required for its rapid degradation during the MZT. Notably, a very similar domain is present in Cdc25^{String} and deletion of the DNA replication checkpoint results in similar dynamics of degradation of both Cdc25^{String} and Cdc25^{Twine}. We also show that Cdc25^{Twine} degradation is delayed in embryos lacking the left arm of chromosome III. Thus, we propose a model for the differential regulation of Cdc25 at the *Drosophila* MZT.

2.2 Introduction

The earliest stage of embryonic development in most metazoans involves a series of extremely fast cell cycles punctuated by the onset of morphogenesis [O'Farrell et al., 2004]. For instance, the nucleus of the fertilized *Drosophila* egg reproduces itself, over thirteen nuclear cycles, into about six thousand nuclei in just two hours, at which point there is a major transition in the cell cycle. Whereas the initial thirteen nuclear cycles are meta-synchronous, syncytial, and largely abbreviated (composed only of S- and M-phases), the fourteenth round of cell divisions follow cellularization, a specialized form of cytokinesis, and include an extended gap phase (G2) following DNA synthesis [Farrell and O'Farrell, 2014].

Central to our current understanding of this cell-cycle transition is the much more global transition from maternal to zygotic (MZT) control of embryogenesis. At fertilization, many of the important molecules needed for driving the thirteen nuclear cycles have been synthesized and provided by the mother, including, for instance, mRNAs, proteins, and a significant portion of nucleotides [Farrell and O'Farrell, 2014, Liu et al., 2019b, Song et al., 2017]. But during the MZT many of these maternally deposited mRNAs and proteins are targeted for degradation and the expression of zygotic genes increases dramatically.

It was hypothesized early on that a candidate mechanism that could explain the lengthening of the cell cycle during the *Drosophila* MZT might involve zygotic downregulation of certain mitotic activators. Support for this idea came with the

discovery that the number of early nuclear cycles in the fly embryo is linked to the appropriately timed downregulation of mRNAs that code for Cdc25 tyrosine phosphatases [Edgar and Datar, 1996]. Cdc25 proteins are involved in a well described molecular circuit that regulates mitotic activity [Morgan, 1997]. At the center of this circuit is the master regulator of mitosis called cyclin dependent kinase 1 (Cdk1), which, when bound to a cyclin partner, may be activated or inactivated like a switch based on its phosphorylation state [Morgan, 1997]. Whereas Wee1 and Myt1 kinases place inhibitory phosphates at Cdk1's ATP-binding site, Cdc25 proteins exactly counter this operation by removing the phosphates [Dunphy and Kumagai, 1991, Gautier et al., 1991]. By this logic the downregulation of Cdc25 activity during the MZT allows for the accumulation of inhibitory phosphates on the cyclin-Cdk1 complexes [Morgan, 1997], which prevents further nuclear cycles and transitions cells instead into a G2 phase. Thus, the mechanisms that control the activity of Cdc25 are central in the timing of the cell-cycle transition at the MZT.

There are two Cdc25 proteins in Drosophila: $Cdc25^{String}$ and $Cdc25^{Twine}$ [Edgar and Datar, 1996]. Although both are expressed in the oocyte, $Cdc25^{Twine}$ alone is essential for driving the early nuclear cycles [Edgar and Datar, 1996], as well as instances of meiosis, but is not required for cell proliferation in later stages of development [Alphey et al., 1992, White-Cooper et al., 1993]. Both homologs are present as mRNA and protein in the early embryo and their dynamics have been characterized in detail. One important observation is that whereas $Cdc25^{String}$ proteins

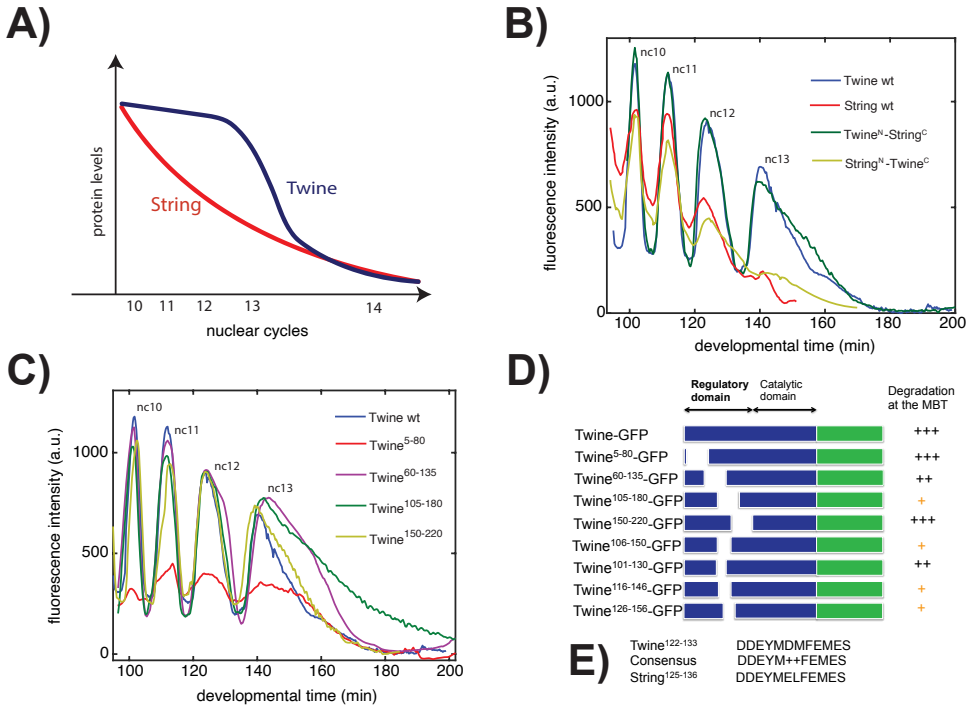

Figure 2.1: Domain analysis of Cdc25^{Twine}.

(A) Cartoon illustration of distinct protein dynamics of Cdc25^{Twine} and Cdc25^{String} prior to the MZT. (B) Quantification of dynamics of Cdc25^{Twine}-GFP, Cdc25^{String}-GFP, and two chimeras. (C) Quantification of dynamics of Cdc25^{Twine}-GFP constructs that are missing various sections of the N-terminus. (D) Cdc25^{Twine}-GFP constructs and their ability to degrade prior to the MZT. (E) Consensus sequence in Cdc25^{Twine} and Cdc25^{String}.

are gradually degraded leading into the MZT, the degradation pattern of Cdc25^{Twine} proteins is much more switch-like, which we have illustrated schematically in Figure 2.1A. Several experiments led to the hypothesis that—contrary to the earlier model based on the downregulation in the concentration of *cdc25* mRNA—the termination of the early nuclear cycles may be linked instead to the abrupt degradation of Cdc25^{Twine} proteins [Di Talia et al., 2013, Farrell and O'Farrell, 2013]. Further evidence that supports this idea is that expression of a Cdc25^{Twine} mutant with delayed degradation kinetics results in premature and unscheduled nuclear divisions in a large fraction of embryos [Di Talia et al., 2013]. Stabilized mRNA can also cause premature divisions prior to completion of cellularization and gastrulation [Farrell et al., 2012]. The central role of protein degradation is also supported by the observation that Cdc25^{Twine} mRNA outlives the switch-like degradation of Cdc25^{Twine} proteins [Di Talia et al., 2013, Farrell and O'Farrell, 2013]. While these data argue for a role of protein degradation, post-translational modifications that downregulate the enzymatic activity of Cdc25^{Twine} could also be important and in fact degradation might be a byproduct of such regulation. For instance, modifications from the protein phosphatase PpV have been recently implicated in the regulation of the initial levels of Cdc25^{Twine} present in the embryo [Liu et al., 2020]. However, these modifications do not seem to contribute to the dynamics of degradation, but rather the levels of protein loaded in the embryo maternally. In this paper, we specifically focus on the timing of degradation. The molecules involved in targeting Cdc25^{Twine} for degradation are not fully known. It

was shown that both the remodeling of the cell cycle at the *Drosophila* MZT and the abrupt degradation of Cdc25^{Twine} are linked to onset of zygotic gene transcription, particularly, the transcription of the pseudo-kinase *tribbles* [Farrell and O'Farrell, 2013]. The primary purpose of this paper is to identify regions of the Cdc25^{Twine} protein that mediate its timely degradation.

2.3 Materials and Methods

2.3.1 Reagents

Standard methods were used throughout. Here is a comprehensive list of the alleles and stocks that were used along with their Fly Base [Larkin et al., 2021] identification number: UASt-stgGFP (FBal026977), UASt-tweGFP (FBal0286379), UASt-stgDronpa (FBal0270090), UASt-tweDronpa (FBal0286381), His2Av-RFP (FBal0247236), matα4-GAL4-VP16 (FBal0086843), *grp*^{209} (FBal0197284), *grb*^{z5170} [LaRocque et al., 2007], *lok*^{30} (FBal0197286), and the deficiency line Df(3L)BSC839 (FBst0027917). Both the compound chromosome stock RM(3L); RM(3R) (=C(3)se) and the TM3.hb-GFP balancer stock were gifts from the Wieschaus lab at Princeton. Plasmids UASt-stg-tweGFP and UASt-twe-stgGFP (Cdc25^{String}/Cdc25^{Twine} chimera constructs) were generated using PCR-splice overlap. Plasmids of the form UASt-twe^{deletion}-GFP were generated using PCR-splice overlap. The *tweGFP* lines were generated using a genomic transgene inserted in attP40 (25C6) or attP2 (68A4) landing site. The *tweGFP* construct itself was synthesized by Genscript, and consists of a 3.7 kb *twine*

rescue fragment [Alphey et al., 1992] as well as a C-terminal fusion of *twine* with EGFP. This construct was then inserted into the vector pBabr by standard restriction digest and ligation, and the resulting pBabr-tweGFP constructs were injected into flies by Genscript.

2.3.2 Genetic Crosses

Embryos maternally expressing fluorescently tagged $Cdc25^{Twine}$ and $Cdc25^{String}$ type constructs (including chimeras and deletions) were obtained from the F1 generation of a cross between females carrying the UASt transgenes and males carrying Gal4 and His2Av-RFP (+/Y; His2Av-RFP matα4-GAL4-VP16 / His2Av-RFP matα4-GAL4-VP16; matα4-GAL4-VP16 / matα4-GAL4-VP16). DNA-checkpoint mutant embryos were trans-heterozygous for grapes (grp^{z5170}/grp^{209}) and homozygous for *loki* (lok^{30}) [LaRocque et al., 2007]. Those embryos were obtained from females with the following genotype: +/+; grp^{z5170} lok^{30} / grp^{209} lok^{30} ; UASt-tweGFP(Dronpa) (UASt-stgGFP(Dronpa)) / His2Av-RFP matα4-GAL4-VP16. Such females were generated by crossing +/+; grp^{z5170} lok^{30} / CyO; UASt-tweGFP (UASt-stgGFP) / TM3 females with +/Y; grp^{209} lok^{30} / CyO; His2Av-RFP matα4-GAL4-VP16 / TM3 males. Embryos lacking the left arm of chromosome III (3L- embryos) were obtained from a compound chromosome III stock RM(3L); RM(3R) (=C(3)se) also carrying the tweGFP rescue construct on the second chromosome. Embryos homozygous for a deficiency Df(3L)BSC839 covering the *tribbles* locus (maternally expressing fluores-

cently tagged Cdc25^{Twine}) were obtained from females with the following genotype: +/+; tweGFP, His2Av-RFP / CyO; Df(3L)BSC839 / TM3, hb-GFP, where the TM3 balancer is marked with hb-GFP. With this experimental set up, one quarter of the embryos were expected to be homozygous for the deficiency and they could be identified by the lack of the hb-GFP expression in the anterior half of the embryo by late cell cycle 14, when tweGFP has been fully degraded.

2.3.3 Microscopy

Quantitative live imaging experiments were performed using either an SP5 (Figure 2.1B and 2.1C) or SP8 (Figure 2.4) confocal microscope from LEICA. In the case of the SP5, the following specifications apply: a 20X/0.7 numerical aperture glycol-immersion objective, an argon ion laser, and a 405-nm and a 594-nm diode lasers. In the case of the SP8, the following specifications apply: a 20X/0.75 numerical aperture oil-immersion objective, an argon ion laser, and a 561-nm diode laser. For embryos expressing the fluorescently tagged Cdc25^{Twine} and Cdc25^{String} type constructs, images (1024x512 pixels) were acquired at a frame rate of about 1/20 seconds. A variable number of images (depending on the level of signal) were averaged together to reach a satisfactory signal-to-noise ratio. The 405-nm laser of the SP5 was used to covert the Dronpa from its dark to fluorescent state. The conversion of the Dronpa from fluorescence to dark was performed using the 488-nm and 496-nm light from the argon ion laser of the SP5. Dronpa fluorescent images were acquired with the 496-nm

excitation light along with sufficient averaging over images.

2.3.4 Data and Image Analysis

Custom MATLAB software was used to segment nuclei based on His2Av-RFP signal. Segmentation was used to generate a mask that was then used to estimate the nuclear fluorescence intensity of the tagged constructs.

2.3.5 Measurements of Cdc25^{String}-Dronpa and Cdc25^{Twine}-Dronpa

Dronpa is a photoswitchable fluorescent molecule that when irradiated with a 488-nm laser enters a dark state, but is restored to a light state when irradiated with 405-nm laser [Drocco et al., 2011]. In living tissue, one can convert the Dronpa molecules (generally fused to proteins of interest) in a particular region to the dark state, and then at a later time restore the light state. In doing so, one may calculate the converted/restored ratio, and determine a protein's effective rate of decay. Here, we use Dronpa to estimate the degradation rate of certain cell cycle regulators in the early nuclear cycles. Because the nuclear envelope breaks down during each nuclear cycle, the nuclear levels of Cdc25^{Twine} and Cdc25^{String} are very dynamic and complicate the measurement of the converted/recovered ratio. However, the peak in nuclear concentration during each cycle is stable for about 1-2 minutes and is consistently achieved about 4 minutes after Nuclear Envelope Formation (NEF) [Di

Talia et al., 2013]. Therefore, we determine protein's degradation rate per nuclear cycle, by converting to the dark state with illumination with the 488-nm laser at the peak of one cycle (4 minutes after NEF), and then restoring to the light state at the peak of the next cycle using the 405-nm laser (4 minutes after NEF).

2.4 Results and Discussion

We first set out to identify which general region of $Cdc25^{Twine}$ is responsible for targeting it for degradation. A general feature of Cdc25 phosphatases, shared by $Cdc25^{String}$ and $Cdc25^{Twine}$, is that they contain a well-conserved catalytic domain in the C-terminus and a divergent regulatory domain in the N-terminus [Rudolph, 2007]. Therefore, we suspected that the differential dynamics of $Cdc25^{Twine}$ and $Cdc25^{String}$ are most likely encoded by the N-terminus of the proteins. To test this hypothesis, we measured the dynamics of GFP tagged chimeras built from the N-terminus of $Cdc25^{Twine}$ and C-terminus of $Cdc25^{String}$ and vice versa (Figure 2.1B). The oscillatory nature of the dynamics reflects the fact that the reporters are dispersed at nuclear envelope breakdown (Figure 2.1 B). But the peak of fluorescence intensity during each cell cycle can be taken to represent the concentration of the reporter. As expected, we found that the dynamics were controlled by the N-terminus regions (Figure 2.1B).

Next, we set out to narrow down the region in the N-terminus responsible for targeting $Cdc25^{Twine}$ for degradation. We built a series of $Cdc25^{Twine}$-GFP fusion proteins with various sections of the N-terminus removed (Figure 2.1D). We measured

the nuclear levels over time for each fusion protein during the early embryonic cell cycles (Figure 2.1C). The nuclear levels of the Twine5-80 construct are likely decreased because the Cdc25^{Twine} protein has two NLS sequences, one of which is located at 36-41. However, its degradation dynamics is similar to the wild type protein. We found that the timing of the degradation was significantly delayed in two of the constructs: Twine60-135 and Twine105-180 (Figure 2.1C). Based on these observations and on similar ones using smaller deletions, we narrowed down the region of the N-terminus to about 20-30 amino acids.

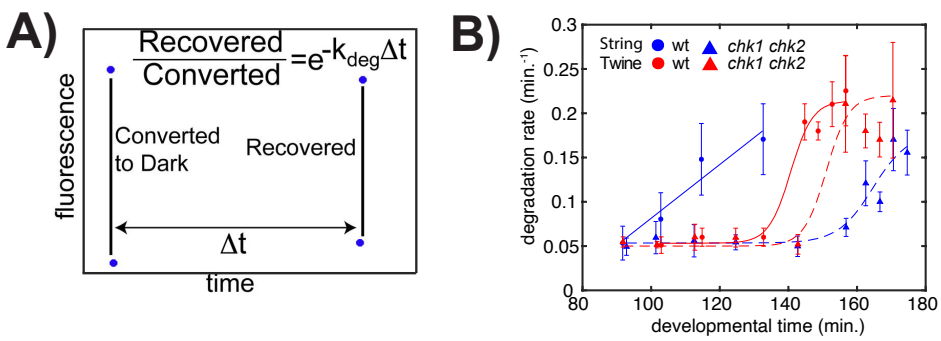

Figure 2.2: Measurement of protein degradation rates.

(A) Conceptual illustration of how protein degradation rate is measured using the Dronpa system [Di Talia et al., 2013]. (B) Degradation rates as a function of developmental time for Cdc25^{Twine} and Cdc25^{String} in wild-type and DNA-checkpoint mutant backgrounds.

While we were unable to identify any known domain or putative post-translational modifications in the region responsible for degradation—including, notably, the phosphorylation sites recently identified that control Cdc25^{Twine} levels [Liu et al., 2020]—we did identify a 12 amino acid sequence that is highly conserved in both

Cdc25^{String} and Cdc25^{Twine} (Figure 2.1E). This led us to speculate that perhaps both Cdc25s would be targeted for degradation similarly at the MZT and that such degradation is not observed in Cdc25^{String} because it is normally degraded prior to the MZT. To test this hypothesis, we used a photoinducible Dronpa system [Drocco et al., 2011, Habuchi et al., 2005] (Figure 2.2A) to measure the degradation rates for both Cdc25^{String} and Cdc25^{Twine} in embryos mutant for DNA checkpoint proteins (*chk1* and *chk2*) (Figure 2.2B). Consistent with previous work, the degradation dynamics for Cdc25^{Twine} is similar in both wild-type and DNA replication checkpoint mutants. But whereas in wild-type embryos Cdc25^{String} is slowly degraded in the cycles leading up to the MZT by a gradual increase in degradation rate, in DNA checkpoint mutants Cdc25^{String} is targeted for degradation only at the onset of the MZT (Figure 2.2B). This dynamic is similar to the degradation dynamic of Cdc25^{Twine}, although the increase in Cdc25^{String} degradation rate is less abrupt. Thus, in the absence of the DNA replication checkpoint, Cdc25^{String} still gets targeted for degradation, only much later, similar to Cdc25^{Twine}. We note that, while we had previously shown that a Cdc25^{String}-GFP protein is degraded in a DNA replication checkpoint-dependent manner [Di Talia et al., 2013], similar analysis argued that Cdc25^{String} is insensitive to the DNA replication checkpoint [Farrell and O'Farrell, 2013]. While it is possible that this difference stems from our use of transgenes lacking the endogenous 5'UTR and 3'UTR, our approach allows us to infer protein half-life regardless. Collectively, these observations argue that the differential protein stability of Cdc25^{String} and Cdc25^{Twine} is mediated by the

DNA replication checkpoint and that their MZT-specific degradation might involve the new domain we have identified.

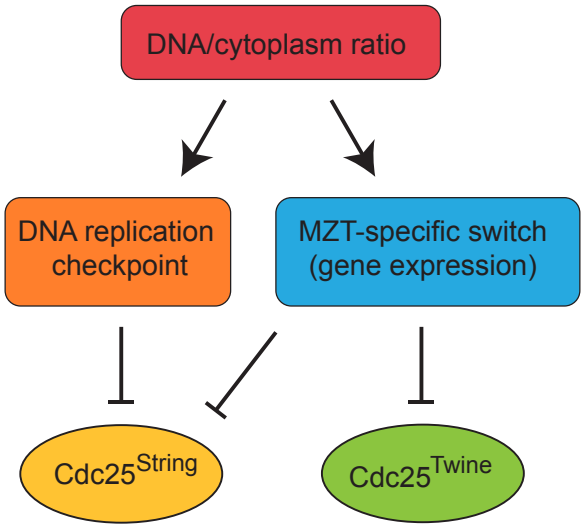

Figure 2.3: Proposed model for differential regulation of Cdc25^{Twine} and Cdc25^{String} at the MZT.

Finally, we set out to identify genomic regions responsible for targeting Cdc25^{Twine} for degradation. To this end, we generated novel lines expressing Cdc25^{Twine}-GFP from a genomic construct previously shown to rescue Cdc25^{Twine} mutants [Alphey et al., 1992]. We crossed flies carrying these constructs to compound chromosome flies to quickly scan through the genome for loci responsible for Cdc25^{Twine} degradation [Lu et al., 2009, Merrill et al., 1988]. The embryos which showed a clear delay in the degradation (Figure 2.4A) as well as a significant number of extra divisions (70%, N=10) were the ones lacking the left arm of chromosome III, referred to here as 3L- (notice that the number of extra divisions were computed in embryos which do not carry any Cdc25^{Twine} transgene, as the higher Cdc25^{Twine} expression might alter the

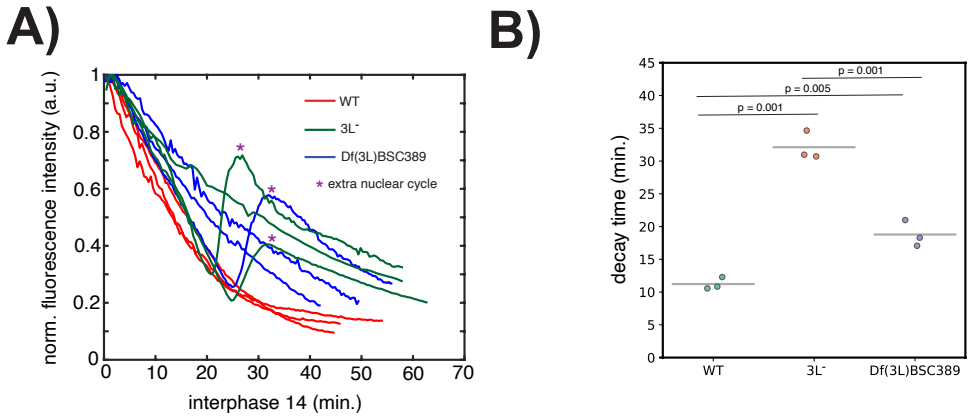

Figure 2.4: Search for genes that alter Cdc25^{Twine} degradation dynamics.

(A) Quantification of Cdc25^{Twine}-GFP dynamics in wild-type, flies having only the left arm of their chromosome III, and flies homozygous for a deficiency that covers the *tribbles* locus. (B) Cdc25^{Twine}-GFP decay times for each of the samples in (A) were calculated by fitting them with an exponential function and solving for the lifetime. Statistical significance was determined using a one-way ANOVA followed by a Tukey's test to compare the pairs.

frequency of extra divisions). Thus, our observations suggest that one or more genes on the left arm of chromosome III is responsible for Cdc25^{Twine} degradation. The gene *tribbles*, which is located on chromosome 3L, encodes a pseudo-kinase which has been shown to play an important role in targeting Cdc25^{Twine} for degradation [Farrell and O'Farrell, 2013]. Cdc25^{Twine} dynamics in embryos homozygous for a deficiency covering the *tribbles* locus were also significantly delayed (Figure 2.4B), in line with both previous findings [Farrell and O'Farrell, 2013] and more recent ones also quantifying Cdc25^{Twine} degradation kinetics [Liu et al., 2020]. Next, we calculated the decay time of Cdc25^{Twine} in these embryos and found that while it increases in both 3L- and *tribbles*- embryos, the increase in decay time is significantly more pronounced in the 3L- embryos (Figure 2.4B). Thus, we argue that there must be

at least one more gene on the left arm of chromosome III which plays an important role in the degradation of Cdc25^{Twine}. This idea is in line with previous experiments showing that the number of extra divisions observed in embryos lacking the left arm of chromosome III (70%, N=10) is significantly higher than observed in embryos mutant for *tribbles* (10%, N=27 as reported by Liu *et al.*) [Liu et al., 2019a]. One possibility is the other gene responsible for the extra divisions is *frühstart*, which is also located on chromosome 3L, and is a known mitotic inhibitor expressed following the last nuclear cycle [Großhans et al., 2003]. However, the mechanism by which Frühstart inhibits mitosis has been shown to involve inhibitory binding to the cyclin partners of Cdk1, and therefore should have no immediate effect on the degradation dynamics of Cdc25^{Twine} [Gawliński et al., 2007]. Hence, we propose there may be an additional, unknown, post-translational regulator Cdc25^{Twine}.

To conclude, our results, along with previous ones in the literature [Di Talia et al., 2013, Edgar and Datar, 1996, Edgar et al., 1994a, Farrell and O'Farrell, 2013, Farrell et al., 2012], suggest a model for the regulation of Cdc25^{Twine} and Cdc25^{String} involving both the DNA replication checkpoint and a MZT process (Figure 2.3). Our experiments also revealed a region of about 20-30 amino acids in Cdc25^{Twine}, which is required for its degradation. While we found no known protein domains within the region, we identified within it about 12 amino acids which are rather similar in Cdc25^{String} and Cdc25^{Twine}. Such domain is not present in Cdc25s of other species. At the moment the functional significance of this domain remains moot. Future

work will be required to elucidate whether (and how) this domain might mediate the degradation of Cdc25^{Twine} at the MZT.

2.5 Author Contributions

Patrick L. Ferree: Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration. **Maggie Xing:** Investigation. **Jenny Q. Zhang:** Conceptualization, Investigation, Resources. **Stefano Di Talia:** Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding Acquisition.

Chapter 3

Investigation of the mechanisms that time cell divisions during *Drosophila* gastrulation

This chapter is modified from a paper that is currently in preparation with Susanna Brantley, Tahj Starr, Aleksandra Walczak, Massimo Vergassola, Anna Chao, and Stefano Di Talia.

3.1 Abstract

The fourteenth round of cell divisions in the fly embryo is spatially and temporally patterned in what are termed mitotic domains [Foe, 1989, Foe and Odell, 1989]. Whereas the first thirteen nuclear cycles are meta-synchronous [Foe and Alberts, 1983] and maternally-driven [Farrell and O'Farrell, 2014], the fourteenth round is timed and localized via zygotic transcription of *cdc25*^{string} [Edgar and O'Farrell, 1990]. Due to their stereotypical temporal pattern, the mitotic domains are an excellent system for working out mechanisms of temporal specification and control. Previous work has shown that the enhancer element of *cdc25*^{string} is sensitive to activation and inactivation by pattern-encoding transcription factors [Edgar et al., 1994a, Lehman et al., 1999]. More recently, six genes (*btd*, *ems*, *kni*, *slp1*, *h*, and *hkb*) were identified

that have dosage-sensitive effects on the timing of cell division in mitotic domain 2 [Momen-Roknabadi et al., 2016]. In this chapter, we provide quantitative links between transcription factor dynamics and the timing of mitosis. In particular, we focus on *btd* and *slp1*. We built fluorescent reporters for each and measured their dynamics *in vivo* in single cells using confocal microscopy. We report that, in mitotic domain 2, our Btd-GFP construct is expressed in a dorsal-ventral gradient that mirrors a mitotic timing gradient. In addition, we report that our Slp1-GFP construct is expressed and degraded in at least four of the mitotic domains in the head region, and in each case, has significant dosage effects on the timing of mitosis (about 10 minutes per additional copy). We conclude that our findings support a model in which ratios of activators and repressors determine the timing of $cdc25^{string}$ transcriptional activation.

3.2 Introduction

Over the course of development, cells proliferate, migrate, or differentiate, based on the types and quantities of transcription factors they express. Gene promoters and their enhancer elements in the genome behave like molecular sensors that are sensitive to the presence and dynamics of transcriptional regulators [Levine, 2010]. In recent years our working model for gene regulation has become increasingly sophisticated [Tsai et al., 2020], with many distinct levels of analysis, including the thermodynamics of protein-DNA interactions, studies of open versus closed chromatin, sequence analysis

of *cis*-regulatory elements, and the tissue-level questions of gene regulatory networks that specify pattern. Although much is known about the spatial regulation of gene expression, we know relatively little about the mechanisms that keep track of time [Ebisuya and Briscoe, 2018, Negrete and Oates, 2021, Diaz-Cuadros et al., 2021]. Here, we use the precise timing of zygotic *cdc25*^{string} transcription in the *Drosophila* embryo as a model for studying temporal regulation of cellular decisions.

In the immediate aftermath of fertilization the fly embryo experiences a series of thirteen extremely fast (7-18 minutes) nuclear division cycles controlled by maternal doses of proteins and mRNAs [Farrell and O'Farrell, 2014]. This preliminary phase of proliferation proceeds in meta-synchronous waves of mitosis in one large shared cytoplasm [Foe and Alberts, 1983, Deneke et al., 2016, Deneke et al., 2019]. But during cycles 11 through 14 zygotic transcription expands, many maternal molecules are targeted for degradation, and there is progressive lengthening of S-phase [Farrell and O'Farrell, 2014]. During cell cycle 14, a special form of cytokinesis surrounds each nucleus with membrane and the newly minted cellular-blastoderm readies itself for gastrulation. From here on out cell proliferation in the animal is no longer perfectly synchronous. The fourteenth round of cell divisions are patterned spectacularly in twenty-five bilaterally symmetric, highly reproducible, patches that decorate the surface of the blastoderm (Figure 3.1 A) [Foe, 1989]. Genetic analysis showed that the "mitotic domains" get their spatial and temporal arrangement via the underlying pattern of *cdc25*^{string} expression [Edgar and O'Farrell, 1989, Edgar and

O'Farrell, 1990]. Cdc25^{String} proteins are tyrosine phosophatases that operate by removing inhibitory phosphates from the master regulator of mitosis Cdk1 [Dunphy and Kumagai, 1991, Gautier et al., 1991]. Entry into mitosis 14 is timed by transcriptional activation of *cdc25*^{string} [Di Talia and Wieschaus, 2012]. Furthermore, the *cdc25*^{string} enhancer is sensitive to many of the classic pattern-formation transcription factors [Edgar et al., 1994a, Lehman et al., 1999].

More recently, the mitotic domains were considered as a system to evaluate whether spatial and temporal information are separable in the fly embryo [Momen-Roknabadi et al., 2016]. Two general models were imagined. In one model, the spatial dimensions of each domain are specified by spatial-patterning genes, but their timing is specified by a global temporal regulator. The way this would work involves each domain having its own timing threshold. The timing molecule then accumulates in cells across the surface of the embryo and as it reaches each threshold in each domain, it triggers *cdc25*^{string} transcription and entry into mitosis. In the other model, the spatial-patterning genes simply *are* the temporal-patterning genes.

To distinguish between these models, and identify the genes involved, a genome-wide screen was carried out in the Wieschaus lab at Princeton for genes that have a dosage-sensitive effect on the relative timing of cell division in mitotic domains 1 and 2 [Momen-Roknabadi et al., 2016]. Preliminary motivation for the screen was provided by the observation that the timing of MD2 can be altered by changing the number of copies of *buttonhead* (*btd*), one of the head gap genes, in the genome. The timing of

MD2 relative to MD1 is significantly delayed in embryos that only have one functional copy of *btd*, but the timing of MD2 is advanced relative to MD1 in embryos that have four functional copies of *btd* [Momen-Roknabadi et al., 2016]. In fact, embryos without any functional copies of *btd* lack cell division and $cdc25^{string}$ expression in MD2 altogether [Edgar et al., 1994a]. Hence, *btd* is an essential activator of mitosis in MD2. To screen for other rate-limiting contributions to MD2, Momen-Roknabadi *et al.* took advantage of deficiency lines—flies that are heterozygous for large deletions of their genome. Ultimately, they were able to locate three activators of mitosis in MD2 (*btd*, *ems*, and *kni*) and three repressors of mitosis in MD2 (*slp1*, *h*, and *hkb*). One interesting conclusion they draw from their study is that there does not appear to be a single unifying molecular clock that times entry into mitosis across the embryo. Instead, it appears that the timing of mitosis is programmed into the very same molecules that specify spatial location.

The goal of the project presented here is to use the control of cell division during gastrulation to propose mechanisms of transcriptional regulation that can result in accurate temporal control of a cellular transition. What is remarkable about the mitotic domains is that cell cycles last at least 75 minutes and individual cells regulate their decisions to enter mitosis with an accuracy of 2 minutes and in a spatially-coordinated manner [Foe, 1989, Di Talia and Wieschaus, 2012]. We take a live-imaging approach to measure the dynamics of two of the rate-limiting factors. We use our results to argue in favor of a mixed hourglass model that involves both

Allele	Genotype	Source
His2Av-mRFP	w^*; P{His2Av-mRFP1}II.2	FBal0247236, FBst0023651
His2Av-mRFP	w^*;; P{His2Av-mRFP1}III.1	FBal0247236, FBst0023650
Btd-GFP	w; P{His2Av-mRFP1}II.2; EGFP-Btd-BAC/TM3	this study
Slp1-GFP	w; P{His2Av-mRFP1}II.2; EGFP-Slp1-BAC/TM3	this study
Slp1-GFP	w; P{His2Av-mRFP1}II.2; EGFP-Slp1-BAC	this study
Hairy-GFP	w; P{His2Av-mRFP1}II.2; EGFP-H-BAC	this study
UAS-Slp1-GFP	yw; P{His2Av-mRFP1}II.2; UAS-Slp1-GFP/TM3	this study
matα4-GAL4-VP16	+/Y; P{His2Av-mRFP1}II.2α4-GAL4-VP16; matα4-GAL4-VP16	FBal0086843
MS2	yw; cdc25^{string}-MS2;	Wieschaus lab
MCP-GFP	yw; P{His2Av-mRFP1}II.2/Cyo; MCP-GFP 4F	Garcia lab

Table 3.1: Stocks and Alleles

activator-accumulation and repressor-depletion.

3.3 Materials and Methods

3.3.1 Fly Stocks and Alleles

All *Drosophila melanogaster* lines were maintained under standard conditions. A list of stocks and alleles constructed or purchased for the purposes of this chapter are presented in Table 3.1. Details about the construction of the tagged transcription factors are provided in the next section.

3.3.2 Generation of transgenic lines

We used bacterial artificial chromosomes (BACs) purchased from P[acman] Resources (http://flypush.imgen.bcm.tmc.edu/pacmanfly/) [Venken et al., 2006, Venken et al.,

2009] to generate each of our transgenic lines that express fluorescently labeled transcription factors from *cis*-regulatory DNA. We followed a recombineering protocol developed by Shelby Blythe of Northwestern that allows one to insert a fluorescent tag at either the N- or C-terminus of the construct. Here is a list of the BACs that we purchased and used to build our fluorescent reporters: for *btd* we used CH322-47L15 (located on chromosome arm chrX from 9676471 to 9697982); for *slp1* we used CH322-184J09 (located on chromosome arm chr2L from 3809581 to 3829838); for *hairy* we used CH321-35J04 (located on chromosome arm chr3L from 8657822 to 8685687). Here is an outline of the steps we followed to transform the flies. (I) Once we acquired each BAC clone, we amplified it in EPI300 cells and collected DNA using a miniprep. (II) Then we designed and amplified our desired DNA inserts (in most cases EGFP). (III) We performed the "recombineering" of the BAC clone in SW102 cells. (IV) After the recombineering transformation we identified candidate recombinants and verified them using PCR. (V) Once we acquired a verified recombinant we transformed it into EPI300 cells, amplified it overnight, and isolated and prepared DNA for transformation into flies.

3.3.3 MS2/MCP system

The MS2/MCP system allows for *in vivo* visualization of the status of enhancers [Garcia et al., 2013, Bothma et al., 2014]. Here we use it to measure the activation timing of the $cdc25^{string}$ enhancer. A cartoon illustration of the system is provided

(Figure 3.2 A). DNA coding for MS2 hairpins are incorporated into a transgene under the control of an enhancer element (in our case, a *cdc25*^{string} minimal enhancer). MS2 hairpins are RNA structures specifically recognized by an RNA binding protein called MCP. In this approach, MCP is fused to a fluorescent protein such as GFP. When the MS2 transgene is transcribed, MCP-GFP molecules bind the MS2 hairpins, and the co-localized GFP signal provides an indirect readout for nascent transcription. The puncta of nascent transcription are readily observable by eye, and can be measured quantitatively to determine the onset and rate of transcription. MS2/MCP experiments are carried out in *Drosophila* by crossing females expressing MCP-GFP proteins under a maternal promoter to males carrying the *cdc25*^{string}-enhancer-MS2 transgene. This strategy avoids the problem of GFP maturation time, since mature MCP-GFP proteins loaded maternally in the embryo assemble rapidly at the site of MS2 transcription.

3.3.4 Microscopy

Quantitative live imaging experiments were carried out on a LEICA SP8 confocal microscope with the following specifications: a 20X/0.75 numerical aperture oil-immersion objective, an argon ion laser, and a 561-nm diode laser. Imaging the mitotic domains requires that the embryo lay in a certain orientation. Mitotic domain 2 sits along the cephalic furrow in the anterior region of the embryo. We generally collect a z-stack of 7 to 15 images (one micron spacing and 1024x1024 or 512x512 pixels^2) at a frame-rate of about one full z-stack every 30 seconds, though in the case

of the MS2 experiments this procedure requires 1 minute.

3.3.5 Image analysis

To analyze the sequences of image files we collected in our experiments, we wrote customized scripts in MATLAB with the Processing Toolbox installed. The general strategy that we followed was to segment nuclei based on their His2Av-mRFP signal, and then measure only the signal from the fluorescent reporter in that segmented region. We tracked each nucleus using standard methods. We defined the concentration of fluorescent reporter in a given nucleus at a given time as the signal in the brightest z-plane divided by the area of the nucleus. We assigned cells to their respective mitotic domains based on the atlas designed by Victoria Foe [Foe, 1989]. All software that was used is available upon request.

3.3.6 Spatial alignment of MD2 between embryos

To generate plots that refer to the the location of cells along the dorsal-ventral axis of MD2, we use the centroid of MD24 as an internal reference point to align embryos (Figure 3.1 B).

3.3.7 Calculation of protein lifetimes

Bleach-chase (FRAP) experiments are used to calculate protein lifetimes [Eden et al., 2011]. In these experiments a small region of interest is bleached, which destroys most of the existing fluorescent proteins, and the recovery is monitored.

3.4 Results

3.4.1 Timing of mitosis in MD2

Mitosis is not perfectly synchronous in any of the mitotic domains but nor is it random [Foe, 1989]. In fact, many of the domains are reported to exhibit wave-like patterns of mitosis [Foe, 1989] and such patterns have been confirmed in both MD1 and MD5 [Di Talia and Wieschaus, 2012]. To characterize the spatio-temporal patterning of mitosis in MD2 we imaged embryos expressing a His2Av-mRFP transgene. We developed algorithms to segment and track each nucleus in the field of view and determine its time of mitosis. Then we used Victoria Foe's atlas (Figure 3.1 A) to assign each nucleus to its appropriate mitotic domain (Figure 3.1 B). We scored mitosis times for four of the head domains (Figure 3.1 C-D). As expected [Foe, 1989] mitotic events in MD2 take place between 70 and 85 minutes after anaphase 13 (Figure 3.1 C-D). Further though when we plot the time of mitosis for each cell in MD2 against its location along the dorsal-ventral axis, we see that there is a timing gradient that starts in the most ventral region of the domain and traverses upwards (Figure 3.1 E). This is also evident from a heat map (Figure 3.1 F). In some embryos this gradient is more distinct than in others, but regardless we find that cells in the ventral compartment divide earlier than cells in the dorsal compartment.

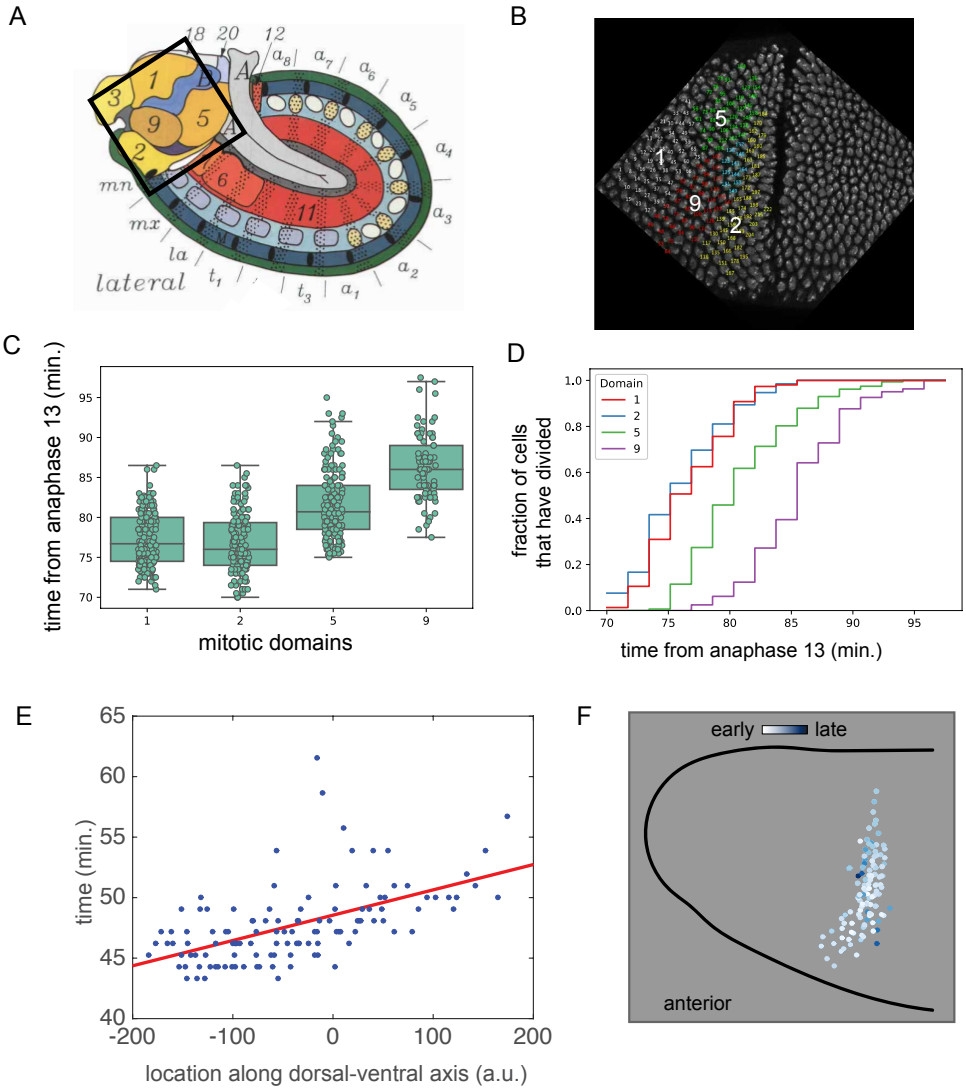

Figure 3.1: Timing of mitosis in domains 1, 2, 5, and 9.

Figure 3.1: (A) Victoria Foe's mitotic domain atlas for a gastrulating embryo on its lateral side [Foe, 1989]. The box indicates the location of the field of view in the image presented in (B). (B) An image of the anterior region of an embryo expressing His2Av-mRFP as a nuclear marker (shown here in white). The nuclei were tracked and assigned to their respective mitotic domains (the large white numbers): MD1 (white labels), MD2 (yellow labels), MD5 (green labels), MD9 (red labels), or MD24 (blue labels). The morphogenetic landmark that runs along MD2 is the cephalic furrow. (C) Mitosis times in MD1, MD2, MD5, and MD9, scored in four embryos. (D) Fraction of cells that have divided over time in the four domains. (E) Relative mitosis time is plotted against location along the dorsal-ventral axis. Here -200 is most ventral and 200 is most dorsal. (F) Heat map for mitosis times in MD2.

3.4.2 Timing of *cdc25*^{string} transcription in MD2

Next, we set out to determine how closely cell division in MD2 is linked to the timing of transcriptional activation of *cdc25*^{string}. Previous experiments in which a GFP was expressed from a *cdc25*^{string} enhancer argue in favor of a model in which *cdc25*^{string} transcription is the rate-limiting step involved in mitosis 14 [Di Talia and Wieschaus, 2012]. But the time between transcriptional activation of *cdc25*^{string} and entry into mitosis is on the order of minutes, which is not enough time for a GFP to fully mature. New methods have since been established, such as the MS2/MCP system (described above in the methods section), to measure the precise timing and dynamics of transcriptional activation *in vivo* [Garcia et al., 2013, Bothma et al., 2014] (Figure 3.2 A). To drive the MS2-stem-loops we use a ∼500 bp minimal enhancer element that was identified in the *cdc25*^{string} locus that is specifically activated in MD1 and MD2 [Momen-Roknabadi et al., 2016]. When we image embryos expressing an MS2-stem-loop construct from this minimal *cdc25*^{string} enhancer element and the MCP-GFP construct, we observed fluorescent puncta indicative of nascent transcription in the cells of MD2 (Figure 3.2 B). We measured the time between transcriptional activation and entry into mitosis (Figure 3.2 C). By using this approach we found that there is a close relationship between the timing of *cdc25*^{string} transcriptional activation and that of mitotic entry, with most cells activating *cdc25*^{string} transcription 24 ± 2 minutes before mitotic entry (Figure 3.2 C). This observation has two important implications: (1) the precision of cell division is encoded by the timing of activation

of *cdc25*^{string} expression; and (2) the timing of *cdc25*^{string} expression can be reliably estimated by knowing the timing of mitotic entry.

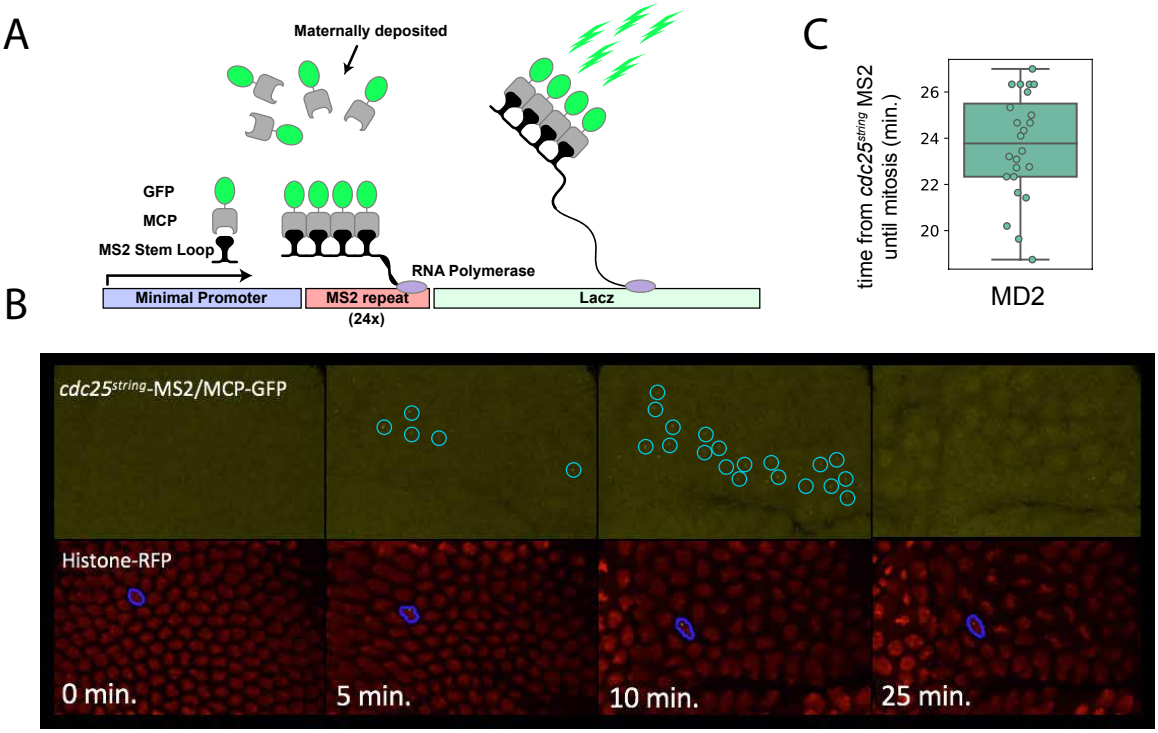

Figure 3.2: Transcriptional activation of *cdc25*^{string} in MD2.

(A) A cartoon illustration of the MS2/MCP system for measuring transcriptional dynamics *in vivo*. A minimal enhancer for *cdc25*^{string} drives transcription of MS2 hairpins, which bind maternally deposited MCP-GFP molecules, and generate fluorescent puncta at the site of transcription. (B) An embryo expressing His2Av-mRFP, MCP-GFP, and *cdc25*^{string}-MS2. In the top panel, fluorescent puncta are circled. In the bottom panel, a single nucleus is followed along with its corresponding MS2-MCP puncta. (C) The distribution of times between *cdc25*^{string} transcriptional activation and mitosis.

3.4.3 Btd dynamics

Embryos that lack a functional copy of *btd* fail to express *cdc25*^{string} mRNA in MD2 and in turn those cells fail to divide [Edgar et al., 1994a]. Moreover the timing of mitosis

in MD2 is affected by the *btd* copy number. MD2 cells in embryos that have additional copies of *btd* enter mitosis significantly earlier than in their wild-type counterparts [Momen-Roknabadi et al., 2016]. To capture this observation quantitatively, and better understand the behavior of Btd in MD2, we set out to measure its dynamics using a fluorescent reporter. Using BAC technology, we generated an N-terminally tagged Btd-GFP construct controlled by a large region of its *cis*-regulatory DNA. Our construct displays the same characteristic head-stripe visualized with *btd* mRNA *in situ* hybridzation (Figure 1.8 A). To measure its dynamics we imaged embryos expressing the Btd-GFP construct in addition to the His2Av-mRFP nuclear marker (Figure 3.3). We segmented, tracked, and assigned nuclei to their respective mitotic domains. We find that the nuclear concentration of Btd-GFP accumulates linearly in cells of MD2 but not in cells of the surrounding domains (Figure 3.4 A-B). Although there is a two-fold range in the Btd-GFP accumulation rate, the variability is not entirely random but rather reflects the spatial location of the cell (Figure 3.4 B-C). In fact, about 70% of the variability in the rate of accumulation is captured by the location along the dorsal-ventral axis (Figure 3.4 D). Similarly, the Btd-GFP "threshold" level at which time *cdc25*^{string} transcription activates is strongly associated with location along the dosal-ventral axis (Figure 3.4 E). So not only do cells in the most ventral region make Btd faster and enter mitosis earlier, and cells in the more dorsal region make Btd slower and enter mitosis later, these findings also suggest that cells respond to different levels of Btd. One explanation for this might be that there is

a systematic contribution (or balancing act) of other transcriptional regulators. That this might be the case is supported by the observation that other transcription factors contribute to the timing of mitosis in MD2 [Momen-Roknabadi et al., 2016]. Let us turn now to an analysis of one of those factors, namely the repressor Slp1.

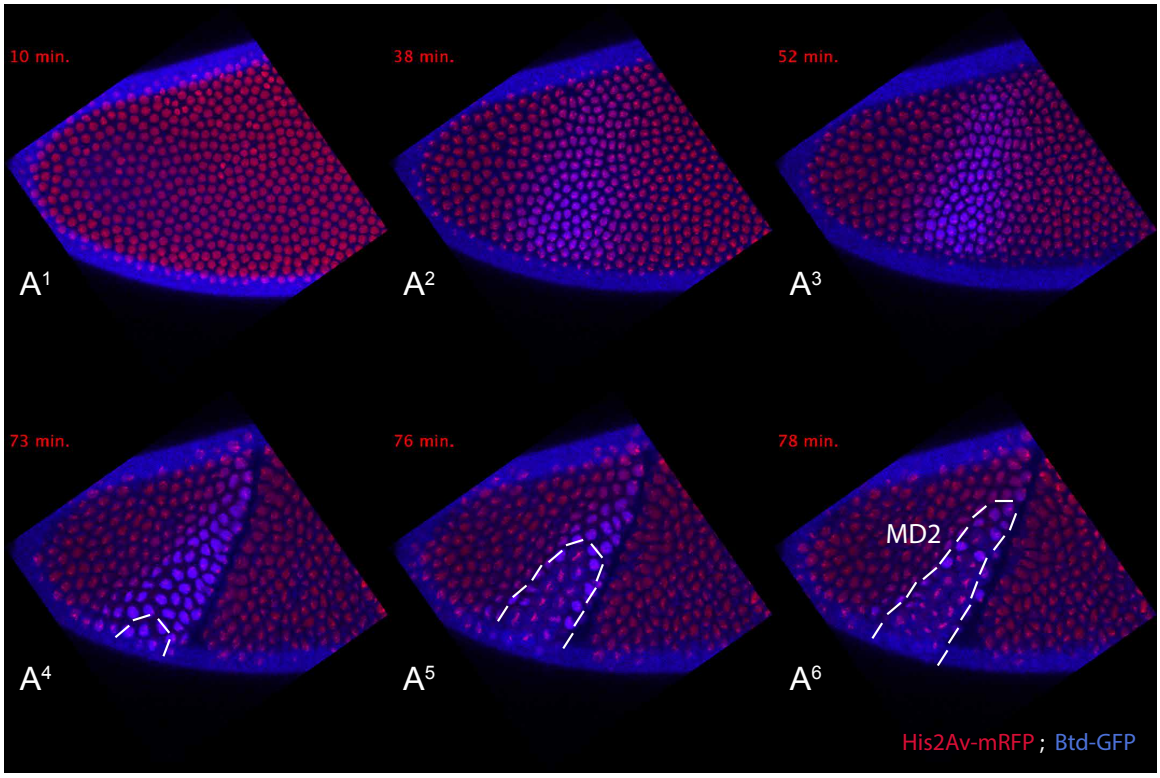

Figure 3.3: Btd-GFP expression pattern during cycle 14.

A strip of images from a movie of a cycle 14 embryo expressing His2Av-mRFP (in red) and Btd-GFP (in blue). A dashed white line indicates the location of MD2.

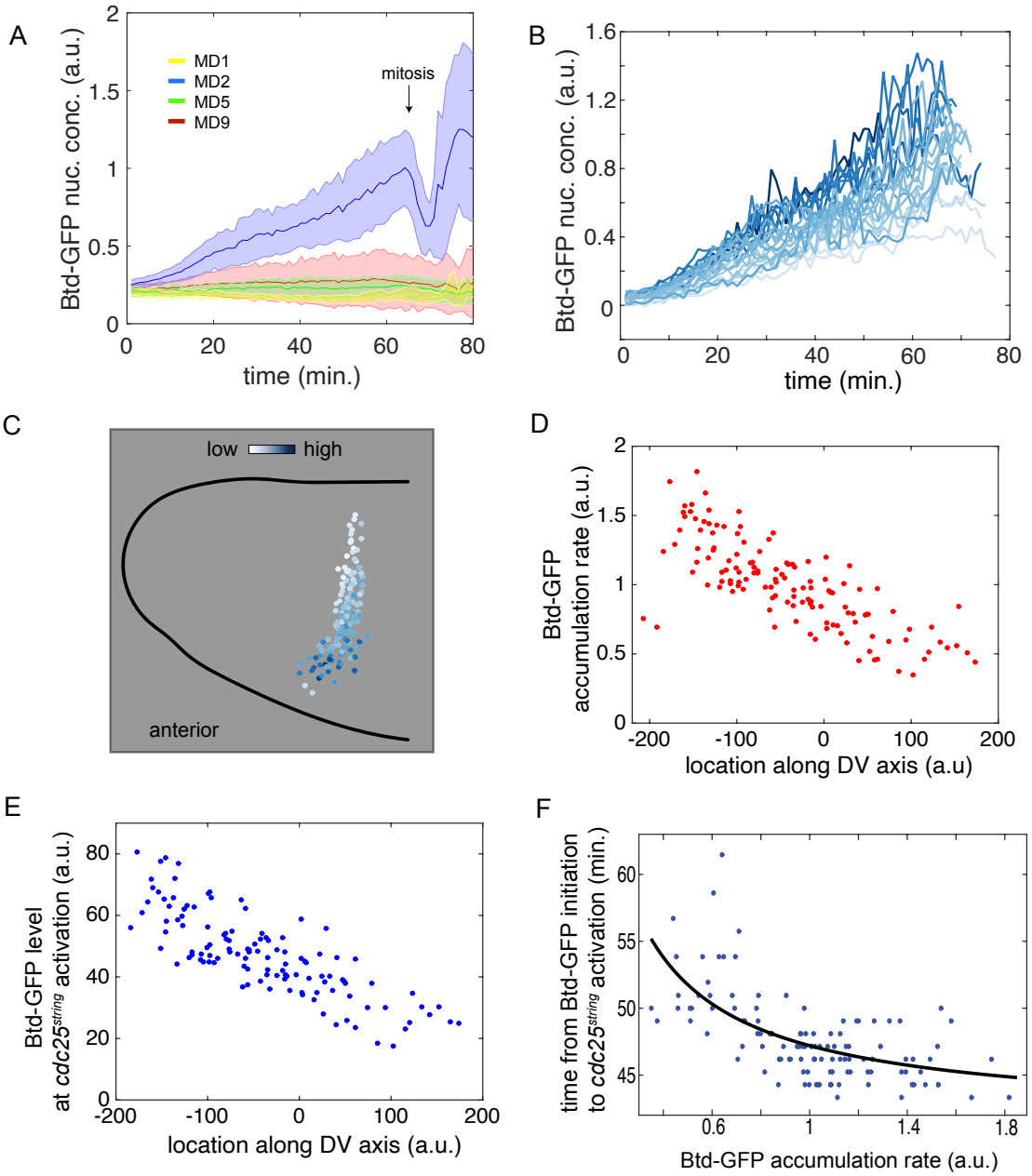

Figure 3.4: Btd-GFP is expressed in a dorsal-ventral gradient.

(A) Nuclear concentration of Btd-GFP as a function of time from cells in domains 1, 2, 5, and 9. (B) Nuclear concentration of Btd-GFP from single cells in MD2 with each trace color coded based on its rate of accumulation (darker blue is higher rate). (C) Here is a map with the relative locations of cells in MD2 color coded by their rate of Btd-GFP production (darker blue is higher rate). (D) Btd-GFP accumulation rate plotted against location along the dorsal-ventral axis. (E) Btd-GFP level at the time of $cdc25^{string}$ transcriptional activation. (F) Time from when Btd-GFP is visible to time of $cdc25^{string}$ transcriptional activation plotted against the rate of Btd-GFP accumulation.

3.4.4 Slp1 dynamics

Slp1 was identified as an inhibitor of mitosis in MD2 because *slp1* heterozygotes were found to exhibit precocious divisions in MD2 relative to MD1 [Momen-Roknabadi et al., 2016]. That *slp1* has interesting dynamics in the head region of the embryo had already been visualized using *in situ* hybridization [Grossniklaus et al., 1992, Grossniklaus et al., 1994, Fujioka and Jaynes, 2012, Andrioli et al., 2004]. We have reproduced some of those images here for the reader to see (Figure 3.5 A). Initially the expression of *slp1* mRNA covers the whole head region of the embryo before diminishing in the most anterior tip and leaving behind a single head stripe (Figure 3.5 A^2 and B^2). The head stripe then splits into two stripes, and six further stripes form sequentially along the length of the body (Figure 3.5 A^3 and B^3).

To better measure the dynamics of Slp1 *in vivo*, and relate those dynamics to mitotic properties, we built an N-terminally tagged Slp1-GFP construct using a similar protocol as the one used to generate the Btd-GFP construct. We imaged embryos from mitosis 13 through mitosis 14 expressing the Slp1-GFP construct in addition to the His2Av-mRFP nuclear marker (Figure 3.5 C). What we see in our movies is that the location of MD2 corresponds specifically to the location that results from the "splitting" of the head stripe (Figure 3.5 B^3 and C^6). This observation is confirmed in our measurements of nuclear Slp1-GFP concentration in MD2 (Figure 3.7). There we see that over the course of cycle 14, Slp1-GFP accumulates in the cells of MD2, but then slowly depletes leading up to mitosis 14 (Figure 3.7 A). To determine whether

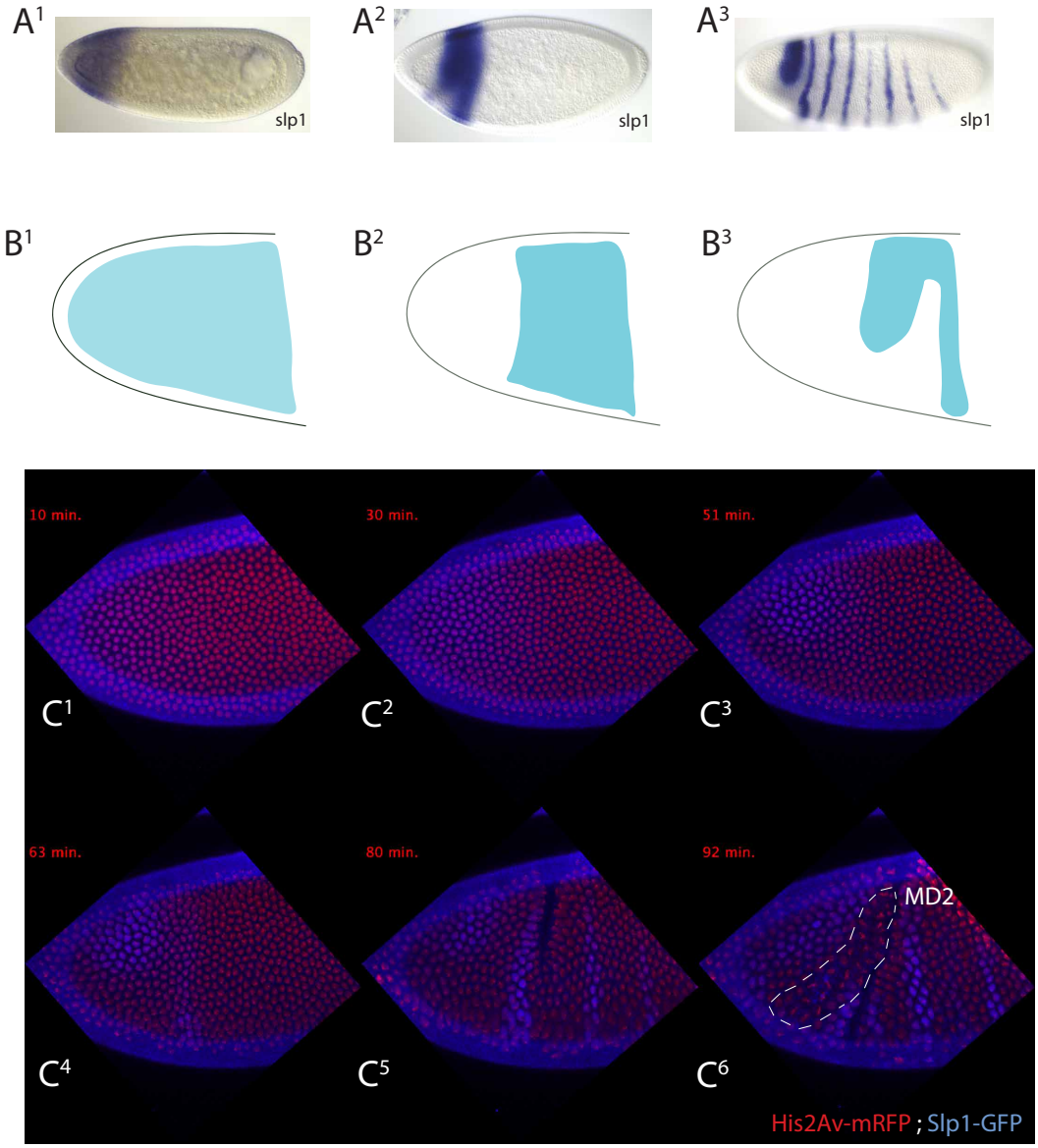

Figure 3.5: Slp1-GFP expression patterns during cell cycle 14.

(A) Images of *slp1* mRNA *in situ* hybridzation at three consecutive time points (borrowed from [Tomancak et al., 2002, Tomancak et al., 2007]): (A^1) late syncytial-blastoderm; (A^2) early cellular-blastoderm; (A^3) onset of gastrulation. (B) Cartoon schematic of *slp1* expression in the anterior most region of the embryo at the three consecutive time points: (B^1) broad expression duruing the syncytial-blastoderm; (B^2) loss of expression at the tip during early cellular-blastoderm; (B^3) narrowing of expression into two adjacent stripes leading up to the onset of gastrulation. (C) A series of still images from a movie of the lateral side of the anterior of an embryo expressing His2Av-mRFP as a nuclear marker (in red) and two copies of the Slp1-GFP construct (in blue). (C^6) Cells can be seen dividing in MD2 at this point. Note that the blue halo around the embryo is an imaging artifact.

these dynamics are due to *slp1* mRNA or protein regulation, we used a bleach-chase experiment [Eden et al., 2011]. We bleached the Slp1-GFP in a few nuclei and monitored its recovery (Figure 3.6A and B). From these experiments we calculate that the Slp1-GFP half-life is only about 5 minutes throughout cycle 14, suggesting that its dynamics are regulated transcriptionally.

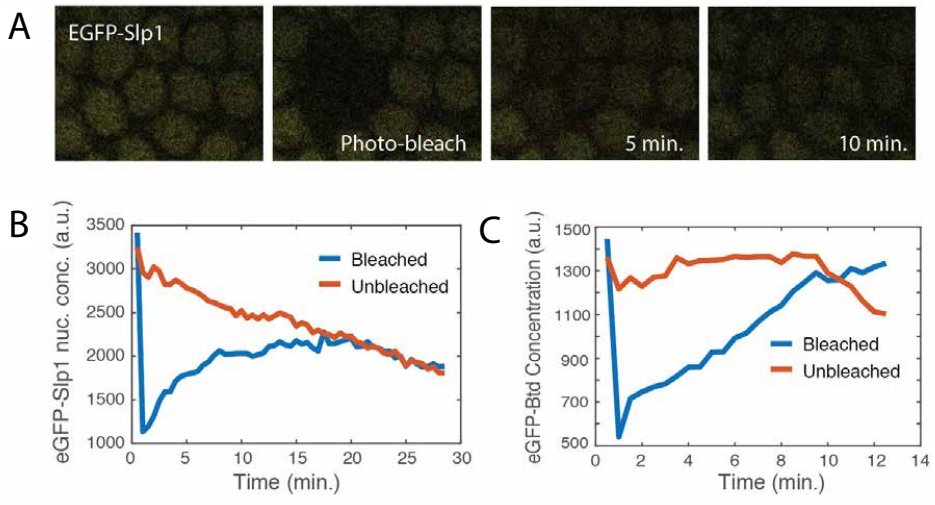

Figure 3.6: FRAP experiment to determine Slp1-GFP lifetime.

(A) Slp1-GFP during cycle 14. Three nuclei are bleached and allowed to recover. (B) Slp1-GFP nuclear concentration dynamics in bleached and unbleached nuclei. (C) Same experiment was performed on Btd-GFP during cycle 14. From these data the half-life of both Slp1-GFP and Btd-GFP were determined to be about 5 minutes.

3.4.5 Slp1 is a general repressor of mitosis in the head region

In support of the repressive role Slp1 was said to have on mitosis [Momen-Roknabadi et al., 2016], we find that the presence of extra copies of Slp1-GFP significantly delays the timing of mitosis in MD2 (Figure 3.7 B). In wild-type embryos, the mean time

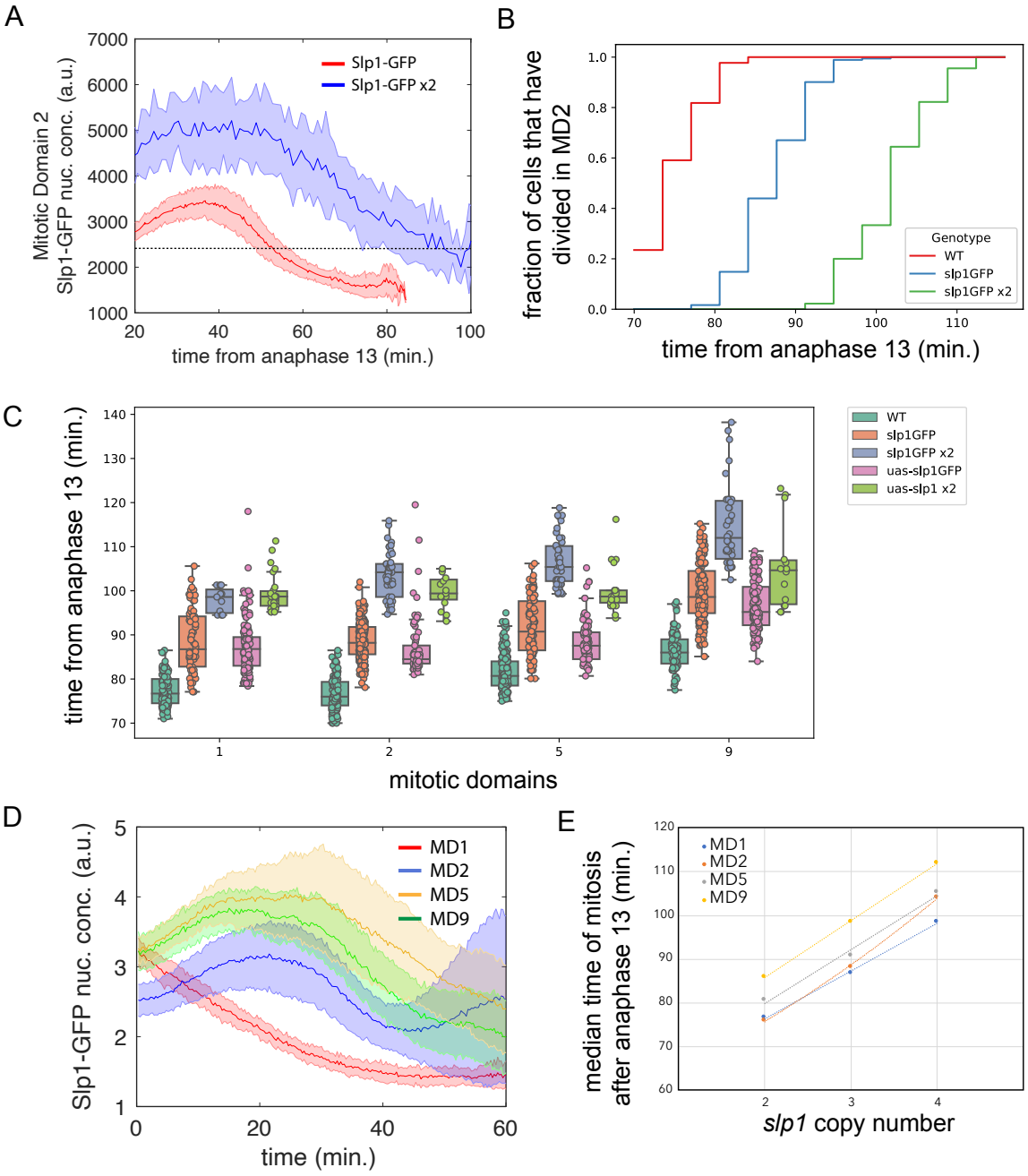

Figure 3.7: Slp1 dynamics and dosage effects.

(A) Average nuclear concentration of Slp1-GFP in cells of MD2 in embryos that have one or two copies of the Slp1-GFP BAC. The dotted line indicates the estimated time of *cdc25*^{string} transcriptional activation based on the MS2 experiments. (B) Fraction of cells in MD2 that have divided over time in wild-type embryos and embryos having one or two copies of the Slp1-GFP BAC. (C) Times of mitosis for five different genotypes. (D) Average nuclear concentrations of SLp1-GFP recorded from cells in domains 1, 2, 5, and 9. (E) Median time of mitosis for each of the four domains plotted against their *slp1* copy number (includes *slp1* and *slp1-GFP BAC* alleles).

of mitotic entry in MD2 (without corrections for spatial location) is 77 ± 4 minutes (std) following anaphase 13 (Figure 3.7 B). But in embryos expressing one or two extra copies of Slp1-GFP the mean time of mitotic entry in MD2 shifts to 88 ± 4 minutes and 104 ± 5 minutes, respectively (Figure 3.7).

Moreover, the fact that Slp1 is expressed widely across the anterior of the embryo suggested to us that we measure the timing of mitosis in other head domains. When we do so we find that, in addition to its effect on MD2, increased dosage of Slp1-GFP similarly delays mitotic timing in domains 1, 5, and 9 (Figure 3.7 C). It is evident from our measurements of the nuclear concentration of Slp1-GFP in the other domains, that they also involve a depletion-type process, though each with its own distinct dynamics (Figure 3.7 D). MD1 is interesting in that Slp1-GFP is removed from its cells much earlier than the other domains, and yet it experiences delays in mitosis timing to the same degree (Figure 3.7 C). In making these observations we were struck by the fact that Slp1 was initially identified partially because of its ability to shift the timing of mitosis in MD2 relative to MD1. One explanation for this might be that MD2 is more sensitive than MD1 to the dosage of Slp1. To get a sense for whether this is true we plotted the median mitosis time for each of the four domains in embryos that had genotypes consisting of 2x, 3x, or 4x copies of *slp1* (Figure 3.7 E). Whereas the median time of mitosis in domains 1, 5, and 9, scales linearly with the dosage of *slp1*, the dosage response of the median time of mitosis in MD2 is much sharper (Figure 3.7 E).

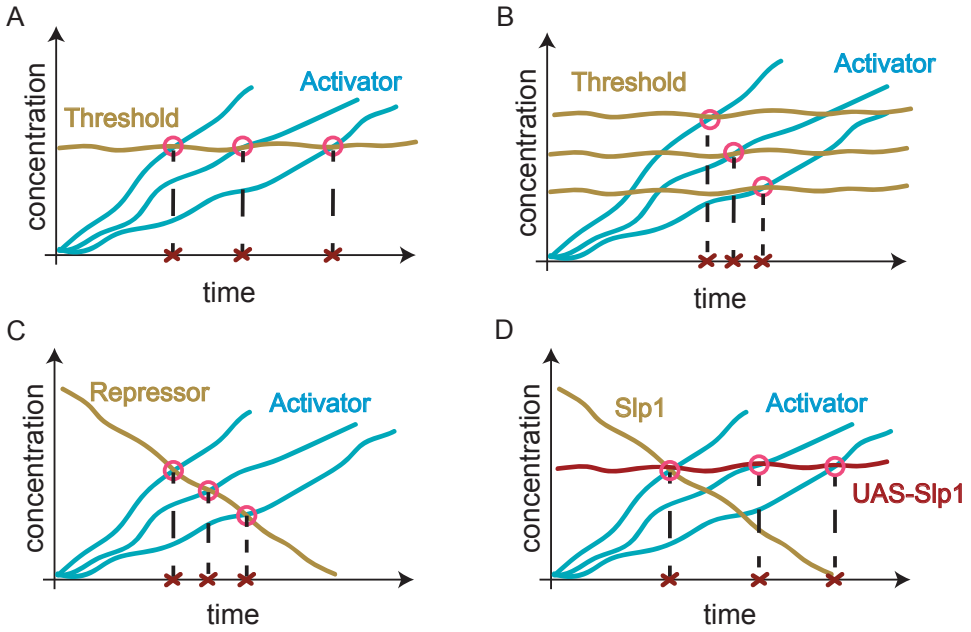

Figure 3.8: Threshold models for timing cellular events.

(A) An activator in three different cells (each blue line) accumulates to a constant threshold. (B) An activator in three different cells (each blue line) accumulates to a cell-specific threshold. (C) An activator in three different cells (each blue line) accumulates to a threshold that is determined by a diminishing repressor. (D) Illustration of an experiment in which the Slp1 threshold is perturbed by over-expressing UAS-Slp1.

3.4.6 Test the activator-repressor ratio hypothesis

Last, we set out to test the hypothesis that cells measure and respond to ratios in the concentration of activators (like Btd) and repressors (like Slp1). One implication of this hypothesis is that if we keep the threshold set by the repressor at a constant level, we should expect to see more variation in the timing of mitosis (Figure 3.8 D). To perform this experiment we used a maternal Gal4 driver to express a UAS-Slp1-GFP construct. Unlike the Slp1-GFP BAC, the UAS-Slp1-GFP construct remains transcriptionally active and so the proteins are present throughout cycle 14. When we do these experiments with a single copy of the UAS-Slp1-GFP, we observe two things: (1) just as in the Slp1-GFP BACs, the timing of mitosis in MD2 relative to anaphase 13 is significantly delayed; but (2) there is more variability in the timing of mitosis in MD2 (Figure 3.7 C). This second observation supports the idea that a ratio is at work. However, when we perform these experiments with two copies of the UAS-Slp1-GFP, we find comparable delays to the Slp1-GFP BAC x2, but less variability in timing of mitosis (Figure 3.7 C). We suspect that the reason for this has to do with the fact that embryos expressing more copies of UAS-Slp1-GFP also seem to have fewer cells in MD2, and so variability is more difficult to observe.

3.5 Discussion

Gastrulation is one of the more dramatic events in early development. Wide-spread coordination of cell division and migration begin to give the embryo its architecture

and organize the once singular layer of pluripotent cells into their three respective germ layers [Sweeton et al., 1991]. This orchestration is particularly pronounced in the *D. melanogaster* embryo because everything happens so fast [Farrell and O'Farrell, 2014]. Arguments have been made that cell division needs to be carefully timed so as not to interfere with the gross operations of morphogenesis [Johnston, 2000]. Consequently the system makes a great model for studying developmental timing of cell division.

The cell divisions that coincide with gastrulation are controlled by zygotic transcription of a mitotic phosphatase $cdc25^{string}$ [Edgar and O'Farrell, 1989, Edgar and O'Farrell, 1990]. In line with arguments elsewhere [Di Talia and Wieschaus, 2012] our MS2 experiments support the idea that $cdc25^{string}$ is the rate-limiting contribution to the timing of mitosis (Figure 3.2). Our main mission then throughout this chapter has been to link the timing of mitosis to some of its underlying regulatory molecules. Genetic analysis had indicated that $cdc25^{string}$ transcription is sensitive to the genes that pattern the larval body [Edgar et al., 1994a, Lehman et al., 1999]. Later six of these genes turned up in a screen for rate-limiters of mitotic domain 2 [Momen-Roknabadi et al., 2016]. Here we sought to understand how two of these factors contribute to the timing of mitosis in that domain.

An early observation we were presented with regarding MD2 is that there is a dorsal-ventral differential in the timing of mitosis (Figure 3.1 E-F). Cells divide earlier in the ventral compartment of the domain. We propose that a significant part of

the explanation for this timing gradient is that there is a mirrored gradient in the expression of the *cdc25*^{string} transcriptional activator Btd-GFP in MD2 (Figure 3.4 C). The simplest model that would account for this hypothesis is a classic threshold model in which Btd accumulates to some threshold, at which time it activates *cdc25*^{string} transcription (Figure 3.8 A). Interestingly though this model is wrong; cells in MD2 respond differently to the dynamics of Btd-GFP. Cells that produce Btd faster (the ventral cells) divide earlier but at significantly higher thresholds. We suspected that the presence of other factors identified in the screen [Momen-Roknabadi et al., 2016] might modify the thresholds in the MD2 cells respectively (Figure 3.8 B-C).

Of the set of repressors to choose from we chose to focus on Slp1. Although Slp1 was first identified as a MD2-specific timer of mitosis, our results suggest instead that the role of Slp1 is much more global, at least in terms of its effects on the timing of mitosis in the head domains (Figure 3.7 C). Whereas increasing the copy number of *btd* to four moves the timing of mitosis in MD2 to a few minutes earlier [Momen-Roknabadi et al., 2016], four copies of *slp1* shifts the timing of mitosis in MD2 to about 20 minutes later (Figure 3.7). Furthermore, based on our MS2 measurements (Figure 3.2), we estimate that *cdc25*^{string} transcription initiates midway the descent of Slp1 (Figure 3.7). This suggested to us that Slp1 may be a repressor-depletion timer of mitosis that works in parallel with the activator-accumulation mechanism of Btd (Figure 3.8 C). To probe the significance of the dynamics of depletion of Slp1, we perturbed the system in such a way that the concentration of Slp1 would remain high

and approximately flat (Figure 3.8 D). These experiments led to increased variability in the timing of mitosis in MD2 suggesting that perhaps the combined action or ratio of activators/repressors contributes to the temporal precision of the system.

In the future it will be interesting to study the dynamics of the other factors and build a more holistic model. For instance, *ems* and *hairy* may both have their own signature expression patterns in MD2 that contribute to the ratios that time transcriptional activation. Additionally, the tools and data we have generated open doors to more sophisticated studies that may help explain the speed and accuracy of the mitotic domains [Siggia and Vergassola, 2013].

3.6 Author Contributions

Patrick L. Ferree: Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project Administration. **Susanna Brantley:** Investigation. **Tahj Starr:** Software. **Aleksandra Walczak:** Formal Analysis. **Massimo Vergassola:** Formal Analysis. **Anna Chao:** Resources. **Stefano Di Talia:** Conceptualization, Methodology, Software, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding Acquisition.

Chapter 4

Conclusions

This dissertation began with a broad discussion of the study of developmental time. There I provided context for the questions that have interested me and motivated the projects presented in the two central chapters. Although the big question that ties the dissertation together concerns how cells make their decisions at precisely the right times, what we have specifically been occupied with amounts mostly to the idiosyncrasies of protein phosphatase Cdc25 regulation during the early cell cycles of the embryo of *Drosophila melanogaster*. In this last chapter I will summarize what was presented in the two empirical chapters on Cdc25, and offer up some suggestions about their limitations and future directions.

4.1 Cdc25^{Twine} degradation during the MZT

The backdrop for the research presented in chapter 2 is the remodeling of the cell cycle that accompanies the maternal to zygotic transition (MZT). At this stage of development in the fly embryo a procession of free-running S/M cycles transitions to slower more germane cycles with gap (G2) phases. One long standing hypothesis is that embryos in some sense measure their DNA to cytoplasm ratio (NC ratio) and use this information to count to the correct number of embryonic cycles. Some of the best

evidence for this idea is that embryos with more or less copies of their genome count to more or less cell cycles before pausing. But the molecular mechanism by which embryos might do such a calculation has remained largely elusive. In our current understanding of the fly embryo, the NC ratio (however it is measured) triggers (i) the activation of DNA replication checkpoints and (ii) a sharp increase in zygotic transcription [Farrell and O'Farrell, 2014, Yuan et al., 2016]. Involved in this process is the clearing of the maternal contribution to the egg, which was demonstrated to include the destruction of *cdc25* mRNAs and proteins [Edgar and Datar, 1996]. Given the function of Cdc25 as a positive regulator of mitosis, this was an intriguing discovery because it suggested a link between the NC ratio and the slowing of the cell cycle. It also raised a more specific question concerning the mechanism of its regulation. An early model suggested that the rate-limiting step was post-transcriptional, but later studies measured a sharp depletion of Cdc25 proteins, particularly $Cdc25^{Twine}$ [Farrell and O'Farrell, 2013, Di Talia et al., 2013]. Whereas $Cdc25^{String}$ is progressively depleted during the early cycles, $Cdc25^{Twine}$ undergoes a switch-like transition from being very stable to very unstable during cycle 13 [Di Talia et al., 2013].

In chapter 2 we sought to further study the stability of $Cdc25^{Twine}$ duing the MZT. Our approach was to measure the dynamics of $Cdc25^{Twine}$-GFP constructs that were missing certain sections of their protein sequence. Following this protocol we were able to narrow down the region required for timely $Cdc25^{Twine}$ degradation to a small peptide sequence. The presence of this peptide in both Cdc25 homologs led us to

speculate about their differential regulation. Earlier arguments favored a model in which Cdc25^{String}'s regulation is sensitive primarily to the DNA replication checkpoint, whereas Cdc25^{Twine} is sensitive to the activation of the zygote's genome [Di Talia et al., 2013]. But interestingly we found that when we removed the DNA replication checkpoint, Cdc25^{String} still loses its stability near the MZT, suggesting that it too is sensitive to some zygotic protein.

Meanwhile, we were also interested in locating novel zygotic genes involved in targeting Cdc25^{Twine} for degradation. We used compound chromosomes to screen through the fly genome in search of DNA regions that affect the timing of Cdc25^{Twine} degradation. In the process we found that embryos without the left arm of chromosome III experience significant delays in their Cdc25^{Twine} degradation dynamics. Two of the most commonly discussed zygotic genes thought to contribute to the remodeling of the cell cycle at the MZT are *tribbles* and *frühstart* [Farrell and O'Farrell, 2013, Farrell and O'Farrell, 2014], and both are on the third arm of chromosome III. Whereas *frühstart* has its effect through its regulation of Cdk1, *tribbles* is linked to Cdc25 regulation in the fly embryo [Mata et al., 2000]. We measured Cdc25^{Twine} dynamics in flies that lacked a functional copy of *tribbles* and found that although Cdc25^{Twine} degradation dynamics are stunted, the effect is smaller than in the complete absence of the larger chromosomal unit. We concluded then that there may be another gene on chromosome III that regulates Cdc25^{Twine} stability.

4.1.1 Future directions for Cdc25^{Twine}

Our results from chapter 2 suggest programs of research in two areas. From our first set of experiments it would be interesting to explore the small regulatory peptide sequence that is conserved between the two Cdc25 homologs. Recent studies have revealed new modes of post-translational regulation of Cdc25^{Twine} by some of the other protein phosphatases present in the early embryo, including, PpV [Liu et al., 2019a, Liu et al., 2020]. While none of the reported phosphorylation sites on Cdc25^{Twine} are situated in our peptide sequence, there are other sites we can mutate to dissect its mechanism. Another related experiment that we have yet to do is remove the particular putative sequence from a Cdc25^{String}-GFP construct and show that the construct remains stable during the MZT in the absence of DNA replication checkpoints. From our second set of experiments, namely the screens for regulators of Cdc25^{Twine} stability, it will be most natural to pursue the identity of the remaining gene on the left arm of chromosome III. We have yet to absolutely rule out *frühstart* and so the next experiment should measure the stability of Cdc25^{Twine} in null *frühstart* embryos. Otherwise the stability of Cdc25^{Twine} should be measured alongside smaller chromosome III deficiencies.

4.2 Temporal regulation of the mitotic domains

Ever since the mitotic domains were visualized and described in meticulous detail, they have been of interest as a model for studying developmental regulation of the cell cycle [Foe, 1989]. Victoria Foe hypothesized that it would turn out to be the underlying

segmentation and dorsal-ventral pattern genes that specify (along with information about the larval body plan) the timing and arrangement of cell proliferation [Foe, 1989, Foe and Odell, 1989]. Support for this idea was found when mutations in certain traditional patterning genes led to observable differences in the shapes of the mitotic domains [Foe and Odell, 1989, Arora and Nusslein-Volhard, 1992]. Then the domains were linked to the transcriptional regulation of *cdc25*^{string}, which suggested a model in which the *cdc25*^{string} enhancer is the direct connection between the pattern-formation genes and cell cycle control [Edgar and O'Farrell, 1989, Edgar and O'Farrell, 1990]. Further evidence for this model came with the discovery that many of the mitotic domains, and their co-localized *cdc25*^{string} transcripts, were abolished in embryos lacking functional copies of certain pattern genes [Edgar et al., 1994a]. These observations shifted research programs to the study of *cis*-regulatory DNA involved in patterning *cdc25*^{string} [Lehman et al., 1999]. The *cdc25*^{string} enhancer was shown to be >40 kb and remarkably complex, perhaps justifiably so, given that it must be regulated precisely in tissues throughout the life-cycle of the fly [Lehman et al., 1999]. But certain small DNA elements (0.5 to 7kb) from the enhancer recapitulate *cdc25*^{string} both spatial and temporal expression in specific tissues, and even individual mitotic domains, suggesting a level of modularity in its control system [Lehman et al., 1999]. Consequently, there has been a long-lasting interest in further dissecting the mechanisms that place and time the activation of the *cdc25*^{string} enhancer [Edgar et al., 2001, Di Talia and Wieschaus, 2012].

Our work presented in chapter 3 has its foundation in a screen that was carried out under the impression that there would be genes that affect the timing of the mitotic domains in a dosage-sensitive manner [Momen-Roknabadi et al., 2016]. That report provided the field with two interesting conclusions. First, based on absence of evidence, they argued against the idea that there is a unifying timer that coordinates the mitotic domain schedule. Second, they provided a list of putative transcriptional timers of mitotic domain 2 (*btd*, *ems*, *kni*, *slp1*, *h*, and *hkb*). The authors argue that this combination of activators and repressors may represent a novel mechanism to accurately time cellular events. To make their point they contrast this idea with the predominant view of gene expression in which repressors set boundaries and activators drive expression [Stanojevic et al., 1991].

In chapter 3, we sought to explore these ideas by measuring the dynamics of the transcriptional timers *in vivo* using BACs to tag them with GFPs. Preliminary investigations led us to focus on *btd* and *slp1*. Our work led to four principal discoveries: (1) we found that the transcriptional activator Btd is expressed in mitotic domain 2 in a dorsal-ventral gradient that mirrors a mitotic timing gradient; (2) the manner by which cells sense Btd dynamics varies along the dorsal-ventral axis; (3) we showed that the transcriptional repressor Slp1 has a much more global role in the timing of mitosis in the head region; and (4) Slp1 has a powerful dosage effect of about 10 minute delays in timing of mitosis per extra copy of *slp1*. Taken together, our findings suggest that mitosis in domain 2 is timed by both activator-accumulation and repressor-depletion,

which is significant because it is the first example of a developmental mechanism that uses both types of hourglasses to determine the timing of a single event.

4.2.1 Future directions for the mitotic domains

Our experiments on the mitotic domains raise many more questions than they answer. When we started this project we wished to use the system to build input-output models capable of making predictions about the behaviors of cells based on transcription factor concentrations and their dynamics. These studies were intended to contribute both to the study of developmental time, but also to our quantitative understanding of embryonic precision. One of the central debates in contemporary developmental biology that interests me, and which I wish to contribute to more fully, concerns whether embryonic precision is due to precision-all-the-way-down, or is instead a product of special corrective mechanisms that adjust for noise at each step along the way [Gregor et al., 2007, Little et al., 2013, Tkačik et al., 2015]. From other experiments in the fly embryo focused on the precise spatial arrangement of segmentation gene expression, we have come to understand the situation as one that involves transcriptional bursts that are prone to noise in their activation and rates of production. But the system manages these errant pathways admirably and so the suggestion in the field is that there must be corrective mechanisms [Gregor et al., 2014]. The mitotic domains lend themselves as interesting systems for studying this question because we know that *cdc25*^{string} transcription is itself timed accurately and so perhaps the corrective

mechanism exists at the level of *trans* and *cis* regulation. Beginning our experiments we had in mind two models. In one, the dynamics of the factors are highly regular and precise such that the timing of $cdc25^{string}$ transcription is also regular and precise (i.e., precision-all-the-way-down). In the other, the dynamics of the factors are noisy but their composite give the system its precision (i.e., a corrective mechanism). This dissertation does not conclusively differentiate between these two models, and it will be interesting in the future to do so. Although we provide measurements of the dynamics of Btd and Slp1, our conclusions about their relationship to the timing of mitosis are largely qualitative. They allow us to argue that the mitotic timing gradient in MD2 (and perhaps other domains too) is a direct result of underlying gradients in patterning genes—evidence for an old hypothesis [Foe, 1989].

In the future it will be interesting to use these measurements to make predictions about the timing of cell division. One theoretical framework for doing so might involve exploit-explore modeling [Siggia and Vergassola, 2013]. But invariably, successful modeling of this system will demand further and more careful experimental perturbations to the levels of the factors and their ratios. We have shown that over-expression of UAS-Slp1 leads to increased variability in the timing of mitosis in domain 2. What we would like to see though is how UAS-Slp1 over-expression affects the levels of Btd that cells divide with, and then incorporate this information into a quantitative model.

Moving forward it will also be important to measure the dynamics of the other

transcriptional timers. Though *hkb* is not expressed in MD2, *kni*, *ems*, and *hairy* each form stripes that overlay its location. Furthermore, while we were unable to generate a fluorescently tagged Ems construct, we did successfully generate one for Hairy and a Knirps-GFP BAC is available from Bloomington Drosophila Stock Center. This does raise the question though about the limitations of the BACs that we employ in our experiments. Neither our Btd-GFP nor our Slp1-GFP BACs rescue their respective null allele, which is perhaps reason enough to move to CRISPR-mediated genome editing in the future. One benefit of the BACs though is their ability to increase gene dosage in a systematic manner, as opposed to the use of Gal4/UAS over-expression systems which rely on promoters of variable strength. This proved useful in the context of the Slp1 experiments in which we inferred conclusions about the mechanism based on comparisons between BAC and Gal4/UAS over-expression. But nonetheless new molecular sensors and reagents, such as LlamaTags [Bothma et al., 2018] and light-sensitive LOVTRAPs [Wang et al., 2016], have been developed for studying protein dynamics *in vivo* and these might be promising steps forward.

One last remark on the future of the mitotic domains is a suggestion that the system be studied more fully using contemporary methods of whole embryo light-sheet microscopy [Stern et al., 2022, Abouakil et al., 2021, Amat et al., 2015]. One of the principal obstacles for studying MD2 has been its location on the cephalic furrow. Many of its component cells invaginate with the onset of gastrulation, which is part of the reason the system is interesting to begin with, but also contributes to the

difficulties of imaging it with a confocal microscope.

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Biography

Patrick Landon Ferree received a Bachelor of Science in chemistry and Bachelor of Arts in philosophy from the University of California in San Diego, where he graduated in 2012 *magna cum laude*. Afterwards he studied the neuroscience of drug addiction in Laura O'Dell's lab at the University of Texas, El Paso, where his work contributed to two publications [Torres et al., 2014, Carcoba et al., 2014]. In 2014, he joined Duke University's Program for Cell and Molecular Biology (CMB), where he rotated through Danny Lew's lab studying mechanisms of symmetry breaking in budding yeast and Chris Nicchitta's lab studying mechanisms of mRNA localization in *HeLa* cells. He ultimately joined Stefano Di Talia's lab for quantitative developmental biology in the Department of Cell Biology where he completed the research presented in this document, published one first author research paper [Ferree et al., 2022], two reviews [Ferree et al., 2016, Ferree and Di Talia, 2018], and two previews [Ferree and Di Talia, 2017, Ferree and Di Talia, 2019]. Patrick has also presented his research at many schools and conferences, notably: the Winter School in Trieste, Italy, which focused on the physics-biology intersection (2017); Challenging the Systems in Heidelberg, Germany (2018); and Quantitative Biology in Oahu, Hawaii (2019).