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Developmental Cis-Regulatory Analysis of the Cyclin D Gene in the Sea Urchin Strongylocentrotus purpuratus

Christopher Michael McCarty

DEVELOPMENTAL *CIS*-REGULATORY ANALYSIS OF THE CYCLIN D GENE IN THE SEA URCHIN, *STRONGYLOCENTROTUS PURPURATUS*

By

Christopher Michael McCarty

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Advisory Committee:

James Coffman, Associate Professor, Mount Desert Island Biological Laboratory, Advisor

Carol Bult, Professor, The Jackson Laboratory

Thomas Gridley, Senior Scientist, Maine Medical Center Research Institute

Robert Gundersen, Associate Professor, The University of Maine

Antonio Planchart, Assistant Professor, North Carolina State University

DISSERTATION ACCEPTANCE STATEMENT

On behalf of the Graduate Committee for Christopher M. McCarty I affirm that this manuscript is the final and accepted dissertation. Signatures of all committee members are on file with the Graduate School at the University of Maine, 42 Stodder Hall, Orono, Maine.

James A. Coffman 8/15/14

Dr. James A. Coffman, Associate Professor <Date>

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An Abstract of the Dissertation Presented
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Proper execution of animal development requires that it be integrated with cell division. In part, this is made possible due to cell cycle regulatory genes becoming dependent upon developmental signaling pathways that regulate their transcription. Cyclin D genes are important bridges linking the regulation of the cell cycle to development because these genes regulate the cell cycle, growth and differentiation in response to intercellular signaling. In this dissertation, a *cis*-regulatory analysis of a cyclin D gene, *Sp-CycD*, in the sea urchin, *Strongylocentrotus purpuratus*, is presented. While the promoters of vertebrate cyclin D genes have been analyzed, the *cis*-regulatory sequences across an entire cyclin D locus that regulate its expression pattern have not.

From conducting the *cis*-regulatory analysis of *Sp-CycD*, regulatory regions located within six defined regions were identified. Two of these regions were found upstream of the start of transcription, but the remaining regions were found within introns. Regarding their activity patterns, two intronic regions were most strongly active at the time of induction of *Sp-CycD* expression, implying they contributed to this induction. The activity patterns of other regions indicated that each could have distinct

roles, including controlling and maintaining *Sp-CycD* expression as it becomes spatially restricted during and after gastrulation.

The sequences of the regulatory regions were analyzed. In three regions subregions containing the *cis*-regulatory modules responsible for activity were found, and in two other regions, sequences that lacked activating regulatory activity were found, allowing the identities of active regulatory sequences to be inferred. The sequences of each region were further analyzed for bearing significantly represented potential binding sites for transcription factors expressed in developmental lineages of the embryo where *Sp-CycD* is expressed. The transcription factors included those that act downstream of Wnt-beta catenin and Delta-Notch signaling pathways that induce the development of the endoderm and mesoderm; and those expressed within the Gene Regulatory Networks that contribute to the development of these lineages. From this, testable linkages between these binding sites and transcription factors that could regulate the expression of *Sp-CycD* as development progresses were identified, providing the foundation for future work.

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CHAPTER 1:

THE CELL CYCLE AND DEVELOPMENT, AND THE ROLE OF CYCLIN D GENES IN REGULATING THOSE PROCESSES

1.1 Overview and rationale

This dissertation describes a *cis*-regulatory analysis of the cyclin D gene, *Sp-CycD*, in the sea urchin, *Strongylocentrotus purpuratus*. Genes of the cyclin D family, which are primarily regulated at the level of transcription [1], are important contributing regulators of both the cell cycle and development. Despite this, to date, no cyclin D gene has been subjected to a comprehensive *cis*-regulatory analysis to identify the regulatory sequences within its locus that allow the gene to transcriptionally respond to developmental signals. As a result of the *cis*-regulatory analysis of *Sp-CycD*, *cis*-regulatory regions were identified in discreet regions found both upstream of the start of transcription, but also, intronically. Because, as will become apparent below, cyclin D family genes function within the context of both the cell cycle and development, before describing the results of the *cis*-regulatory analysis in more detail, an overview of the cell cycle, its link to development, and the role of cyclin D family genes in these processes is given.

Please note: A number of genes are introduced in this dissertation. Generally, within the main text, the most common names are given. For official names and Gene Identification numbers, provided by NCBI Gene [2] for all genes except for those derived from the sea urchin, *Strongylocentrotus purpuratus*; or by SpBase [3] for genes described in *S. purpuratus*, see Appendix A, Table A.1.

1.2 Overview of the cell cycle, and the discovery of cyclins and their partners

In animal development, cells become integrated into a cooperative community. To do this, cells must successfully reproduce themselves, and they must do so in relationship to their neighbors. At the heart of this process is the cell cycle – the means by which cells reproduce themselves. The cell cycle involves a large number of molecular players. The first group consists of the group of proteins, such as DNA helicases, polymerases, topoisomerases and associated factors that replicate the cell's DNA, along with the histone proteins, acetylases and deacetylases, that regulate the disassembly and assembly of DNA into chromatin and chromosomes, which must be mitotically segregated into daughter cells following replication of the DNA. However, this multitude of proteins must be set into motion in a coordinated manner, and groups of them must also silenced after cells have been replicated and further replication is either permanently, or temporarily not needed. The involved players were discovered over many years [4], and will be introduced as this Introduction proceeds.

Important regulatory drivers of the cell cycle are a family of proteins known as cyclins. The first cyclins were discovered in the sea urchin, *Lytechinus pictus* by the Hunt group, working at the Marine Biological Laboratory, who labeled proteins from fertilized eggs with [^{35}S]methionine, ran the proteins on an SDS gel, and discovered a protein in early cleaving embryos that abruptly was destroyed before each cleavage, then appeared again, in a cyclical manner [5, 6]. Proteins showing this periodic behavior were likewise discovered in clam [5, 6]. Due to its cyclical synthesis and destruction coinciding with the beginning and end of each cell cycle, this protein was called "cyclin"[5, 6] This cyclin, later termed cyclin B, is a member of a larger family of cyclin proteins [1]. The

Hunt group hypothesized but did not prove that the cyclin protein they had discovered played a role in regulating the cell cycle; their evidence was purely correlative. Ruderman and colleagues [7] provided direct evidence that a cyclin protein in clams, cyclin A, when injected into G2/M arrested oocytes, could induce M phase. Since that time, other cyclins were discovered, found to be expressed in all eukaryotes, from yeast to mammals, and together with a network of other proteins with which they interact, found to be fundamental players in the eukaryotic cell cycle [1, 8]. How could cyclins regulate the cell cycle? In part, cyclins were found to accomplish this by interacting with and activating cyclin-dependent kinases (CDKs), the first characterized of which, cyclin-dependent kinase 2 was discovered in yeast [9]. In each case, the interaction between each cyclin protein and its CDK partner is mediated by a 100 amino acid "cyclin box" within each cyclin protein. This interaction requires the presence on the CDK of the amino acid motif PSTAIR [1]. The CDKs are serine/threonine protein kinases. There are a number of different CDKs, each of which is involved in phosphorylating specific substrate proteins to allow specific stages of the cell cycle to proceed. For example, CDK4 and 6 phosphorylate the retinoblastoma (RB) protein, which acts as a cell cycle inhibitor in the absence of such phosphorylation. In the presence of such phosphorylation, RB releases E2F transcription factors needed for the progression of S phase [1].

1.3 The protein players involved in controlling the cell cycle

A transition is now made to listing and giving some of the functions of the network of proteins that drive the cell cycle, focusing first on members of the cyclin family, the proteins with which they directly interact, and the stages of the cell cycle that are set in motion by those interactions. As will become evident below, it has been shown that specific stages of the cell cycle are associated with the activities of specific members of the cyclin and CDK families. However, it should be noted that recent work by Coudreuse and Nurse [10] showed that in fusion yeast, it is possible to engineer a single CDK to drive the entire cell cycle in this organism, without the need for the input from any cyclins, despite the fact that this organism possesses at least 4 different cyclins. This relates to the fact that the seemingly unique roles of specific cyclin-CDK complexes may in part be due not to intrinsic properties of the complexes themselves, but due to where they are localized within a cell [1].

Herein, a simplified overview of how the cell cycle is set in motion by extracellular signals [1, 8, 11, 12] is presented. An important caveat is that many of the experimental findings upon which this overview is based are derived from work on cultured cells, especially mammalian cells [12] rather than from developing organisms. As this Introduction proceeds, how the cell cycle is linked to the gene regulatory networks within a whole developing organism will be described, but first, the discussion of the cell cycle overview begun above will be finished. In a cell cycle permissive signaling environment, combinations of developmental signaling pathways converge to activate transcription of cyclin D gene(s). Cyclin D family genes are indeed important integrators of multiple developmental signaling pathways and their associated

downstream activated transcription factors [13]. Due to this, cyclin D family genes have been called "signal sensors" that couple signals received by cells to progression from G1 to S phase of the cell cycle [14], and this characterization relates to findings pertaining to their discovery. Cyclin D genes were first characterized by the Sherr group [15], although the newly identified cyclins were not yet given the designation "cyclin D" at the time of this characterization. The newly identified cyclins, originally named $p36^{CYL}$, based on their size of 36 kd, were required for mouse macrophages to overcome G1 and enter S phase in response to the growth factor Colony-Stimulating Factor 1, but, after this, were no longer required for the cells to complete the cell cycle, their protein levels falling during S phase to a low after mitosis. In the absence of such stimulation, the cells never entered S phase, and died. Subsequent work provided support for the role of cyclin D genes as the "signal sensors" that couple signals received by cells to progression from G1 to S phase of the cell cycle [14].

Cyclin D family genes may also actively prevent the cell cycle from proceeding forward under appropriate conditions. This is based on work by Kozar et al [16]. These authors obtained fibroblasts from day 13.5 C57BL/6 mouse embryos in which all three mammalian cyclin D genes, *Ccnd1*, *Ccnd2* and *Ccnd3*, had been knocked out. As a control, fibroblasts from littermate controls were used. When both groups of fibroblasts were transfected with retrovirus encoding the cell cycle inhibitor *P16ink4a*, the proliferation of control cells was inhibited, as expected. However, the inhibition of proliferation by this cell cycle inhibitor was almost completely prevented in the cyclin D-null fibroblasts.

An explanation of how the cell cycle is driven forward will now be presented. Cyclin D mRNA levels are low in the absence of inducing signals, and, in addition, cyclin D proteins are unstable, exhibiting half lives of about 20 minutes [1]. The instability of cyclin D proteins is due in part to the presence of C-terminal PEST sequences, which signal for these proteins to be destroyed by ubiquitination [1]. Once transcribed and translated, cyclin D proteins bind to and activate serine/threonine protein kinases, termed cyclin-dependent kinases (CDKs), such as CDKs 4 and 6. CDK4 and CDK6 phosphorylate proteins of the RB family. The path to discovery of the first described gene of this family, *RB,* was begun in 1971 by Knudson [17], who discussed how retinoblastoma tumors of the eye were brought about in patients who had inherited a mutated version of a gene. This one mutant copy could not by itself elicit cancer, but if the second copy became mutated somatically, retinoblastoma tumors would result. Ultimately, the *RB* gene was cloned by Friend et al. in 1986 [18].

Proteins of the RB family are termed "pocket proteins" [19, 20], because they share a conserved "pocket domain" which binds to target proteins that bear the motif LXCXE [21]. Besides RB, the family also contains the proteins P107 and P130 [22] . All three of these proteins play primarily inhibitory functions at the gene promoters that are regulated by the E2F transcription factor family, with P107 and P130 acting as a complex at such promoters [22]. There is also evidence that RB and P107 + P130 differ in terms of the E2F target genes they regulate. This was shown in 1997 by Hurford et al [23]. These authors demonstrated that deletion of either *Rb*, or both *P107 + P130* (but not either of the latter singly) in mice led to either the upregulation or downregulation of different cell cycle regulatory genes in cell cultures derived from these mice. For

example, the cell cycle regulators B-MYB, CDK2, and E2F1, and cyclin A2 were de-repressed by deletion of *P107 + P130*, whereas cyclin E was derepressed by deletion of *Rb*. Another way that proteins of the RB family carry out their regulatory function is by, in their hypo-phosphorylated state, recruiting transcriptional repressors, such as histone deacetylases to the promoters of *E2F*-regulated genes [21, 24, 25].

As introduced above, the activity of cyclin-CDK and RB family proteins regulates the transcription of genes in part by regulating the interaction of proteins of the E2F family with these genes' promoters. The *E2F* genes have multiple family members, which regulate the transcription of different genes. They carry out their transcriptional regulation through forming heterodimers with proteins termed DP proteins. By carrying out this transcriptional regulation, *E2F* family genes can affect cell proliferation, and also developmental fate (reviewed in [26]). The target genes of *E2F* family genes have been queried by genome wide analysis of binding sites [27, 28] . This has shown that *E2F* family genes regulate a variety of genes, including those involved in the regulation of chromatin, DNA replication, DNA repair, the cell cycle, and development. The fact that *E2F* family genes undertake such diverse processes is of relation to cyclin D family genes, which, as described later in this Chapter, regulate developmental processes as well as the cell cycle.

Among the genes that are transcribed by activated E2F transcription factors is a second group of cyclins, of which focus is made on cyclins of the cyclin E family [12]. Cyclin E proteins interact with CDK2 family proteins, leading to their activation. This has at least two consequences. First, the cyclin E-CDK2 complexes further phosphorylate RB family proteins, which have already been phosphorylated by cyclin D-

CDK4. Therefore, the actions of signal-sensing cyclin D-CDK4 ultimately set in motion a positive feedback loop that contributes to making a single cell cycle irreversible. Because of this, the state through which cells pass to reach this irreversible status is known as the "restriction point." However, because each subsequent cell cycle includes another G1 stage, these subsequent cell cycles depend on the continued presence of induction signals, in the absence of which, these cycles will cease [12, 14, 29]. Continuing with the discussion of the activation of cyclin E-CDK2 complexes and its relationship to cell cycle progression, the second consequence of the activation of cyclin E-CDK2 complexes is the activation by phosphorylation of various transcription factors, which ultimately leads to the transcription of genes critical for progression through the cell cycle. These include genes necessary for DNA synthesis, along with those needed for mitosis [4, 12].

It is in part through the above mechanisms that cells progress from the first gap phase, G1, to the DNA synthesis stage, S, of the cell cycle. After this, if conditions are favorable, cells will then prepare for and undergo mitosis, as described herein [8, 12]. The commencement of mitosis is brought about through passage through another restriction point, the G2-M phase. Key players involved in this progression include the A type cyclins, which associate with CDK1 and CDK2, and are active first, followed by the B type cyclins, which become active as the A type cyclins are ubiquitinated and degraded. At least 70 proteins involved in mitosis are phosphorylated through cyclin B-induced CDK activity. Another of several important players includes CDC25 phosphatase proteins. The role of these proteins only becomes clear in light of the fact that not all phosphorylation events that occur during the cell cycle are activating; some

are inhibitory, and these inhibitory phosphorylations relate to the negative regulation of the cell cycle, discussed further below. These inhibitory phosphorylations are carried out by kinases of the WEE and MYT families [8, 12]. These inhibitory phosphorylations, which act as another safeguard gate to prevent the cell cycle from proceeding inappropriately, occur on cyclin-dependent kinases involved in both the G1 to S phase and G2 to M phase of the cell cycle. Proteins of the CDC25 phosphatase family act as positive regulators of the cell cycle by removing these inhibitory phosphates, thus allowing the cell cycle to proceed. After the completion of mitosis, cells face another decision, to either continue cycling or to enter a resting stage termed G0 [8, 12]. Cycling cells may enter G0 for a number of reasons, of which focus is given to developmental ones. Cells may find themselves at a stage of development where they must differentiate, a process often referred to as terminal differentiation. An important theme arises with respect to this fact: development and the cell cycle must somehow be linked in order for cells to behave in a manner that relates to their temporal and spatial position within a developing organism. As signal-responsive cyclins that play a role in the decision of cells to cycle or not to cycle in response to extracellular signals, cyclin D genes play important contributory roles in this process. Further expansion on the relationship of the cell cycle to development is described in the section of this Introduction, "How regulation of the cell cycle relates to development."

1.4 Regulation of the cell cycle by the availability of nutrients

Besides being regulated by developmental signaling pathways, the cell cycle is also regulated by the availability of nutrients. An important pathway that cells use to couple the availability or lack of nutrients, along with the presence of growth factors to

the decision about whether to proceed with the cell cycle is the mTOR pathway [30]. It has been shown that this pathway exerts its effect, at least in part, by regulation of the cyclin D1 gene (in a human cell line), both at the level of transcription [31], and also by controlling the levels of both cyclin D1 mRNA and cyclin D1 protein (in a 3T3 mouse cell line). It should be noted that animal cells are not unique in becoming dependent on extrinsic cues for their cell cycles to proceed. For example, in the plant *Arabidopsis,* evidence suggests that cyclin D type genes couple development from juvenile to adult plant by the availability of sugar [32]. Polymenis and Schmidt showed that in the unicellular yeasts, the cyclin protein involved in the G1 to S phase transition, CLN3, is translationally regulated by a 5' sequence in its mRNA that senses the level of translation in the yeast [33]. The theme that arises from these observations is that the eukaryotic cell cycle is not solely autonomous – its passage is coupled to the availability of nutrients and/or developmental signals, depending on the the identity of the organism in which the cell cycle is taking place. The next section explores this theme further – by describing how the the cell cycle and development are related

1.5 How regulation of the cell cycle relates to development

Up until now, most of the discussion has focused on how the cell cycle is driven forward. However, in order to better understand how the cell cycle is linked to development, it is critical to understand how the cell cycle can be negatively regulated [8, 34, 35]. Both driving the cell cycle forward and inhibition of the cell cycle must be properly coordinated with an organism's developmental status. This importance will become evident as some of the mechanisms for inhibiting the cell cycle are discussed.

In acting as cell cycle inhibitors, proteins of the RB family play important roles in allowing cells to differentiate [21]. For example, RB contributes to the differentiation of adipocytes by at least two mechanisms. First, in line with its aforementioned role, RB, inhibits cell cycle in adipocytes in part by inhibiting cell cycle promoting transcription factors, such as those of the E2F family. In concert with this, RB family proteins induce differentiation in this system by activating the differentiation promoting transcription factor C/EBPα, thus exhibiting a transcriptional activation as well as inhibitory role.

Results from work in knockout strains of mice demonstrate that members of the *Rb* family are needed for normal development, due in part to the necessity for their cell cycle inhibitory and differentiation-inducing properties. This is shown by the fact that knockout of these genes in mice is embryonic lethal, due to defects in the erythrocyte lineage and over-cell proliferation in the liver [20]. Of interest, cyclin D triple knockout C57BL/6 mice likewise die in utero, but due to under-production of hematopoietic cells rather than due to over-production [16]. This is not surprising given that, as explained above, RB family proteins function downstream of signal-activated cyclin D proteins [14].

The relationship between cyclin D, cyclin E and E2F is likely not simply linear. This was shown through work in *Drosophila* by Buttitta et al [36]. Given that E2F acts downstream of cyclin D-CDK4 and cyclin E-CDK2, a reasonable hypothesis would be that simply activating E2F, irrespective of either cyclin D or cyclin E, could prevent cells from exiting the cell cycle. However, these authors showed that, at least in *Drosophila*, it is necessary to activate both E2F, plus either cyclin D or cyclin E to prevent cells from exiting the cell cycle before completing differentiation.

Given that cells exit the cell cycle and enter G0 when they differentiate, it might be hypothesized that the states of cycling through the cell cycle and differentiation are mutually exclusive. Is this a developmental rule? Related to this question, Korzelius et al. showed that in *C. elegans*, artificially activating cyclin D-CDK4 or cyclin E-CDK2 could cause differentiated muscle cells enter S phase or mitosis, respectively [37]. In a related study, Sage et al. [38] showed that targeted deletion of *Rb* genes in mammalian hair cells of the ear causes those cells to undergo the cell cycle but still maintain functions such as the abilities to respond to mechanosensation and express at least some markers of differentiation. Similarly, Ajioka et al.[39] characterized, in vivo, differentiated interneurons in mice (strain not provided) lacking two of the *Rb* family members, *Rb* and *P13*0, but not *P107*. These authors found that after several weeks, differentiated interneurons bearing this genotype would re-enter the cell cycle. However, these cells maintained various phenotypes of differentiation, such as the ability to form neurites and synapses. Whether these interneurons were fully differentiated was not clear, because the authors did not compare the gene expression pattern of these interneurons to differentiated interneurons in wildtype mice.

These findings relate to another aspect of the cell cycle– that it can be modulated during development, as the two processes are linked [40]. During the earliest cleavage stages in vertebrates and sea urchins, the fertilized egg divides a number of times in preparation for subsequent rearrangements that begin with gastrulation. These earliest cell divisions are driven by maternal factors that are stored in the egg cytoplasm [41, 42]. During these earliest divisions, the cell cycle is essentially intrinsic, moving forward

without the cues of extracellular signals. At this stage, the cell cycle consists of just two phases, S, where the DNA is synthesized, followed rapidly by M, mitosis.

However, even during these earliest divisions in animals, cells are not found within a developmental void: their position within the developing embryo will dictate their eventual developmental fate. For example, in the sea urchin, cells that will become various developmental lineages are formed in distinct parts of the cleaving embryo [42, 43]. This is due to exposure of the cells in different embryonic territories, initially, to maternally stored factors that will subsequently set in motion specific developmental programs for each uniquely located group of cells [41, 43]. Maternal factors also include mRNAs that encode cyclins A and B, which can play a role in the transition from S to M phase by activating cyclin A and B dependent kinases [41].

There then arrives an important transition termed the maternal to zygotic transition [44]. At this stage, two critical events occur to set the developing embryo on its independent trajectory. First, maternal regulators of the cell cycle are degraded. Second, transcription of the embryo's own genes that regulate the cell cycle and development is commenced. Degradation of maternal RNAs is triggered by the presence of sequences within the maternal RNAs that signal for the binding of factors, such as enzymes that remove the polyA tails. Maternal RNAs with different functions are degraded at different rates, with those that code for factors that regulate the cell cycle among the first to be eliminated [44]. This allows the cell cycle to begin to be regulated by external rather than maternal cues.

As maternal transcripts become degraded, activation of transcription of the zygotic genome begins. A combination of factors may induce transcription of zygotic

genes. These factors include changes in the nuclear to cytoplasmic ratio with successive cell divisions, during which cells become successively smaller during cleavage; presence of a molecular clock, for which the molecular components are being elucidated; and changes to chromatin within the embryo's nuclei [44]. The timing of the onset of transcription from the zygotic genome varies between animals [44]. In sea urchin, transcripts synthesized by the embryo itself are detected at the zygote stage [44]. These include transcripts of genes that comprise the Gene Regulatory Networks (GRNs), introduced more fully below, that control sea urchin embryogenesis [45]. However, these development-regulating GRNs are activated by maternal factors that are stored in the egg cytoplasm. For example, the GRN that controls the development of the lineage comprising the endoderm and mesoderm, that is, the endomesoderm, requires maternal Wnt6 transcripts in order to be activated [46].

An important event for which the timing coincides with the maternal to zygotic transition is the introduction of gap phases in the cell cycle. The introduction of these gap phases, G1 and G2 [41] is important for a number of reasons. First, as noted, their terminal boundaries serve as cell cycle checkpoints whereby cells will not commit to replicating their DNA or undergoing mitosis if errors are present. Second, and related to the theme being developed for this dissertation, the checkpoints are important from a developmental perspective: after completion of M, there exists another gap phase G0, during which cells can decide to exit the cell cycle and differentiate. Cells make this decision based in part on the developmental context in which they find themselves. In short, cells sense and respond to developmental signaling factors. The maternal factors that cells encounter differ upon their position in the embryo [42, 43]. Cells respond to

these factors by activating the transcription of a specific subset of genes [45]. Some of these genes code for other transcription factors, and others code for specific terminal differentiation factors that do not themselves activate other genes, but impart on a cell a specific phenotype related to its temporal and spatial position within the developing embryo [43]. Ultimately, what is set in motion within a specified cell type is a network of transcriptional-regulatory interactions between specific genes within the organism's developmental program [45]. This relates to gene regulatory networks (GRNs), which are explained more below.

1.6 *Strongylocentrotus purpuratus* – a useful system for studying development

The purple sea urchin, *Strongylocentrotus purpuratus* is an ideal system for studying questions relating to development and the cell cycle, due to a number of recent developments. These include the fact that the genome of this organism has been sequenced, and its genes have been annotated [47], revealing that most of the gene families found in vertebrates are also found in *S. purpuratus*. These include, for example, most transcription factor family members, developmental signaling pathways, genes involved in the immune and complement systems, ABC transporters, genes involved in adhesion, such as integrins and cadherins, and genes expressed in the nervous and sensory systems [47]. With respect to transcription factor families, the members of various families have been well annotated, including, for example, Fox genes [48], Ets genes [49], Zinc finger genes [50], and Homeobox genes [51]. In addition, the transcriptome of the sea urchin embryo was studied by Samanta et al. [52]. These investigators identified thousands of genes across many functional classes that were transcribed during embryogenesis. Of interest, the Samanta et al. study described

transcription from intergenic regions. Although the function of these latter transcripts was not determined by Samanta et al.[52], this study has not been the only one to identify such entities. For example, Kim et al. [53] identified RNA species they termed enhancer RNAs that were transcribed from neuronal enhancers. Likewise, the functions of these species remained unknown, but it was speculated that they might play a role in gene regulation. The existence of these newly characterized RNAs is of interest, because it relates somewhat to the project described in this dissertation, which identifies and characterizes conserved non-exon regions within a cyclin D gene that regulate its expression, although it does not address whether any RNAs are transcribed from these regions. An update on the status of the transcriptome of *S. purpuratus* was published in 2012 [48]. Although that study focused on protein coding genes, the knowledge obtained in that project allowed gene models postulated in the previous work of Sodergren et al. [47] to be revised based on the identity and pattern of transcription of genes that are expressed from early embryo through juvenile stages.

Of relevance to this project, in *S. purpuratus*, the genes involved in regulating the cell cycle in this organism have been annotated [54]. This annotation showed that with the exception of the INK4 and ARF tumor repressor families, all family members involved in both positive and negative control of the cell cycle were present, although often with fewer representative members than found in vertebrates.

As noted earlier, the cell cycle is linked to development [40, 41]. In this Introduction, an attempt has also been made to show specific examples of how the cell cycle and developmental signaling and environmental factors related to nutrition are linked. To date, the role of cell cycle regulatory genes in controlling developmentally

important transcriptional networks has been largely neglected in the field of animal development. For example, in *S. purpuratus*, cell cycle regulatory genes have not yet been linked to the developmental GRNs in this organism [55]. The relationship of cell cycle regulatory genes to the transcriptional regulatory networks of which they are part has been studied in systems such as yeast [56, 57] but not so much in the development of animals, except as pertains to the study of cancer, and in such studies, the techniques used are largely computational methods that make predictions that have yet to be experimentally verified [58]. As alluded to above and will become further evident below, genes of the cyclin D family, could play an important role in linking the cell cycle to the GRN. With this in mind, this project focuses on a *cis*-regulatory analysis of the cyclin D gene, *Sp-CycD*, of *S. purpuratus*. Cyclin D genes are now described in more detail.

1.7 Cyclin D genes -- overview of roles in the cell cycle and development

As described above, the eukaryotic cell cycle is regulated by the cyclins [59]. As described earlier, cyclins were first identified in sea urchin embryos as proteins that accumulated and then were destroyed with different phases of the cell cycle [5]. While the cyclins expressed during early development before the maternal to zygotic transition are byproducts primarily of maternal mRNAs, as noted, the D-type cyclins become active at the maternal to zygotic transition. Linked to this fact, analysis of cyclin D promoters, generally in vitro, and primarily with the vertebrate cyclin D1 gene, has shown the existence of binding sites for dozens of transcription factors that act downstream from most of the developmentally important signaling pathways, giving further evidence for roles of cyclin D genes as developmental sensors that contribute to the regulation of development by linking receipt of extracellular signals to downstream developmental

responses [13]. This is related to the fact that the well characterized role of cyclin D genes in bringing about the G1 to S transition in the cell cycle is triggered by receipt by the cell of mitogenic signals, stemming from virtually all the developmental signaling pathways [59].

Driving the G1 to S phase of the cell cycle may be one of many roles for cyclin D genes, and in fact, in certain developmental contexts, cyclin D genes may not be needed for the G1 to S phase transition. For example, work carried out by the Sicinski lab has shown that knockout mice lacking all three of the mammalian cyclin D genes are viable throughout much of embryogenesis, before dying due to deficits in the hematopoietic lineages [16]. It is possible that these findings could be due to functional redundancy with other cyclin genes. For example, in 1999, Geng et al. [60] showed that in a mouse strain where the cyclin D coding sequence had been replaced with that of cyclin E, cyclin E rescued the phenotypes caused by cyclin D loss. Further support of this came from Keenan et al. in 2004 [61]. These authors showed that if cyclin D1 synthesis was blocked in Chinese hamster embryonic fibroblasts, progression through G1 to S phase of the cell cycle was blocked. However, this block was overcome by expression of cyclin E-CDK2. Moreover, cyclin E-CDK2 carried out this rescue through inactivation of RB via phosphorylation, and concomitant activation of E2F. Moore et al.[62] showed that depletion of cyclin D in developing sea urchin embryos did not affect total cell number in late gastrula stage embryos. However, Robertson et al. [63] examining the effect of cyclin D knockdown on cell numbers in blastula stage embryos, showed that depletion of cyclin D did reduce cell numbers at that stage of development.

In addition to their important role in regulating the cell cycle in response to developmental signals, genes of the cyclin D family also play other developmental roles. For example, Datar et al.[64] showed that in *Drosophila*, cyclin D and its partner CDK4 induce cellular growth (increase in cell size) but not cell proliferation. Related to its role in regulating cell growth, cyclin D genes have also been shown to down-regulate catabolic genes [37]. Moore et al. [62] showed that cyclin D in the developing sea urchin embryo is not expressed until blastula stage, and that this expression is required for development of normal larval morphology. Inducing cyclin D expression during cleavage caused death. Similar findings were reported by Tanaka et al. [65] who, working in a different developmental system, *Xenopus laevis*, showed that cyclin D1 RNA in that organism was not detected until the midblastula stage. Both Moore et al. and Tanaka et al. showed that cyclin D expression became successively restricted as development proceeded, to dividing cells of the gut and ectoderm in the sea urchin, and to neural plate and eye vesicles in *Xenopus* [62, 65].

A point of contention has been the role of cyclin D genes in differentiation. The most common view has been that cyclin D cells are cell cycle regulators, and that it is their down-regulation that allows cells to exit the cell cycle and differentiate [66]. This view is supported by studies, such as that of Adachi et al. [67] who demonstrate that degradation of cyclin D1 and D2 caused by switching growth factor medium is associated with ceasing of the cell cycle in immature myeloid cells and their differentiation into neutrophils. In developing mouse spermatogonia, cyclins D1 and D3 appear to regulate the cell cycle, whereas the expression cyclin D2 appears to be required for differentiation into A1 spermatogonia [68]. The complexity of this situation is further revealed by the

fact that cyclin D3's role may be context dependent, regulating the G1 to S transition in spermatogonia, but perhaps regulating differentiation in Sertoli and Leydig cells [68]. In skeletal muscle, cyclin D3 and its associated CDK4 has been shown to repress differentiation by directly inhibiting the association of the transcriptional regulators MEF2C and GRIP-1 required for the muscle cell differentiation program to be activated [69].

Understanding the mechanisms through which the expression of cyclin D family genes is regulated is also medically pertinent, with cyclin D genes, particularly cyclin D1, being commonly mis-regulated in various cancers, with the cyclin D1 gene being the second most amplified gene in human cancers [70, 71], and its mis-regulation being associated with the development of a variety of these diseases [72-74]. Moreover, this gene could be an important chemotherapeutic target, based on a recent finding that expression of this gene may be required for the viability of certain cancers, but may not be needed in adult tissues that have completed development [75]. Also of medical relevance, cyclin D and its partners have been shown to regulate the activity of telomerase [76-78], findings which are pertinent to better understanding both cancer and aging [79].

Clearly genes of the cyclin D family play important roles in development, and in both normal and disease-compromised biological processes. Of interest, recent work has provided evidence that cyclin D proteins may carry out some of their functions by pathways distinct from the best characterized activation of CDKs. In particular, recent work has shown that cyclin D proteins may act directly as transcription factors, perhaps in concert with other transcription factors. For example, the Sicinski group [80] showed

that during mouse embryogenesis, the cyclin D1 protein was found associated with promoters of developmentally active genes, and, in particular, was shown to recruit CREB binding protein histone acetyltransferase to the *Notch1* gene. Moreover, if the cyclin D1 gene was ablated in retinas, NOTCH1 activation was lessened, leading to decreased cell proliferation in that organ, an effect that could be rescued by introduction of an artificially activated *Notch1* gene. In related work, Lukaszewicz and Anderson [81] showed that the cyclin D1 protein promotes neurogenesis in the developing mouse spinal cord by inducing expression of the transcription factor Hes6. As described near the end of Chapter 3, the weight of the evidence indicates that cyclin D genes carry out their transcriptional roles indirectly, via protein-protein interactions with sequence-specific DNA binding transcription factors.

How are levels in the cell of the developmentally important cyclin D genes regulated? Due to its instability as a protein, cyclin D is primarily regulated at the level of transcription [1]. Work from numerous groups has provided evidence in support of this by describing how developmentally important signaling pathways and their associated transcription factors regulate the transcription of cyclin D genes. For example, transcription factors of the TCF family that are the effectors of the Wnt-β-catenin pathway regulate the expression of cyclin D genes. Shtutman et al. [82] and Tetsu and McCormick [83] showed that activation of β-catenin, working through the TCF homologue LEF1, increased transcription of cyclin D1 via LEF1 binding sites in the promoter. Pradeep et al. demonstrated that cyclin D1 activation depended primarily on activation in its promoter of a CRE responsive element, but that a TCF4 site contributed to a lesser extent [84]. Baek et al. [85], working on a mouse cell line, showed that LEF1,

along with histone deacetylase 1 and a complex of E2F4 and P130, repress the cyclin D1 promoter until repression is lifted by activation of the Wnt-β catenin pathway.

The regulation of cyclin D expression has also been linked to Runx transcription factors. For example, Bernardin-Fried et al. [86] found that levels of the Runx protein AML1 varied during the cell cycle in a pattern similar to that displayed by cyclin D3. Inhibition of AML1 lead to loss of cyclin D3 expression, and AML1 was shown to interact with and activate the cyclin D3 promoter. Knockdown of the sea urchin Runx gene *Runt1* caused a decrease in cyclin D RNA expression, as well as decrease in expression of several Wnt genes, such as *Wnt4, Wnt7, Wnt8, Wnt6, Wnt7* and *Wnt9* [63]. Further, Robertson et al. [63] showed that blocking *Runt1*, *Wnt8*, or cyclin D expression caused a decrease in cell numbers in blastula stage embryos, and that Runt1 bound the 5' flanking regions of *CycD*, *Wnt6* and *Wnt8*.

The regulation of cyclin D genes by other developmentally important signaling pathways and associated transcription factors has also been examined. Examples include the MAPK cascade [87]; heat shock proteins [88]; E2F (of interest since E2F transcription factors are themselves regulated by cyclin D genes during the G1 to S phase transition of the cell cycle) [89]; G proteins, steroid hormones and nuclear receptors [90]; Sp1 [91]; STAT5 [92]; STAT3 [93]; and TGFα [94].

Transcription factors mediate their effects, in part, by binding to gene promoters. Related to this, the cyclin D1 promoter has been extensively analyzed, although the work involved has focused mostly on in vitro systems [13]. Examples of specific papers analyzing cyclin D promoters include Kitazawa et al. [95] and Matsumura et al [92]. To date, cyclin D promoters have not been subjected to a great deal of analysis in an in vivo

context. An exception concerns work done by Tanaka et al. working with *Xenopus* [65]. After examining the in vivo expression profile of endogenous cyclin D1, these authors created reporter constructs with specified deletions of the cyclin D1 promoter, and analyzed the effect on reporter gene activity. These authors found that the regulatory elements identified in the promoter were not sufficient to explain the full expression profile of cyclin D1, so they suggested that other sequence elements might be involved. This finding also provides an impetus for undertaking the project described in this dissertation – a comprehensive *cis*-regulatory analysis of a cyclin D gene.

1.8 The rationale for performing a *cis*-regulatory analysis on a cyclin D gene

Focus is now made on the main subject of this dissertation – a *cis*-regulatory analysis of the *Sp-CycD* gene in the sea urchin *Strongylocentrotus purpuratus*. To understand how the expression of a gene is regulated during development requires a *cis*-regulatory analysis of that gene. Typically, developmentally regulated genes contain multiple DNA sequence regions, up to several hundred basepairs in length, that bind groups of transcription factors that play a role in regulating a gene's pattern of expression [45]. These regulatory regions are termed *cis*-regulatory modules (CRMs). Some of these regions play stimulatory roles in specific cells, others have inhibitory roles, and still others act as boosters or inhibitors of other *cis*-regulatory modules [45]. The function of *cis*-regulatory modules can be examined by incorporating them into reporter constructs, injecting the latter into developing embryos, and observing the spatial and temporal expression pattern of the reporter genes. Such *cis*-regulatory analyses have been successfully applied in *S. purpuratus* to numerous genes, such as *CyIIIa* [96], *SM50* [97], *Endo16* [98, 99], *CyIIa* [100], *Wnt8* [101], *Nodal* [102], and *Delta* [103].

The efficiency with which potential CRM-containing regions of a gene are identified can be increased using a number of computational approaches. One such method is to identify regions of sequence conservation. This method, termed "phylogenetic footprinting," is based on the premise that sequences within the same gene that are evolutionarily conserved between different species of sufficient evolutionary distance may exhibit this conservation because they are functional [104, 105]. With respect to this, sequence comparisons between the genes of *S. purpuratus* and the sea urchin *L. variegatus* have been shown to reliably predict CRMs [106, 107]. A comprehensive program for identifying conserved and potentially functional regulatory sequences is FamilyRelationsII [106]. This program has been demonstrated to accurately predict *cis*-regulatory regions ([106] and references therein). The identification of regions containing potential *cis*-regulatory modules can also be facilitated by identifying sequence regions that have clusters of binding sites for known transcription factors, as such regions have been shown to often be regulatory in nature [108].

Performing a *cis*-regulatory analysis of a gene is the only way to definitively, by experiment, link that gene to the gene regulatory network (GRN) of which it is a part, because such an analysis is required to identify the transcription factors of a gene regulatory network that directly regulate the expression of the gene being studied [45].

1.9 Overview of developmental GRNs

Gene regulatory networks (GRNs) are important "drivers" of development [45, 55, 109]. Gene regulatory networks prescribe how the information encoded in the genome is to be used during development of an organism. Visualized in diagrammatic form [55] GRNs consist of networks of all regulatory genes known to be active in

development. Among the best worked-out lineages in developing embryonic *S. purpuratus* are the endomesoderm lineages, and, to a lesser extent, the lineage specifying the ectoderm [55]. GRNs show not only the genes involved in specifying a developmental lineage or structure, but, more importantly, the regulatory interactions between those genes. These interactions can range from simple, as for example, when a transcription factor activates a gene that produces an end product, such as a skeletal protein that is expressed in and characteristic of a particular cell type, or complex, as in circuits where transcription factors can successively activate or inhibit other transcription factors through negative and/or positive feedback loops [45].

Development is best described as a system property that results from the interactions between genes. Developmental GRNs present these interactions, and explain how they lead to specific phenotypes at specific times and specific places within a developing embryo [45, 109-112]. Developmental GRNs are modular, being composed of individual subcircuits of interacting genes. These subcircuits, which can be classified based on their function, have been described as the "building blocks" of developmental GRNs. The genes within these subcircuits can be classified based on whether they only receive signals from other genes, but do not themselves communicate with other genes; or both receive input from other genes, but respond with an output that regulates the transcription of other genes. An example of the former would be a gene that encodes a structural product but does not transcriptionally regulate any other genes [111]. Examples of the latter would be transcription factors, and signaling genes that lead to the transcriptional expression of such transcription factors [45].

The subcircuits within developmental GRNs can be classified into a number of different types [45]. Among developmental questions that can be answered by study of subcircuits are: what causes a particular transcription factor to be expressed in a particular spatial domain but not in others; what causes a particular gene to be activated at a particular time and place, and then have its expression become extinguished; is a particular gene activated by binding of one transcription factor, or does it require binding of more than one specific transcription factor to become activated; how is "community effect" signaling, in which all cells within a given spatial territory express the same assortment of genes, maintained? Developmental GRNs ultimately consist of all the subcircuits that are active in all regions of an embryo, and how they change over time to bring about developmental phenotypes. A goal of researchers who decipher GRNs is to eventually construct global GRNs that encompass all regulatory genes expressed during development. Progress toward this goal is being made by analyzing the entire transcriptome during sea urchin embryonic development [113].

Despite the fact that their structures are still being deciphered, the developmental GRNs of *S. purpuratus* that regulate the development of specific tissue lineages within embryos are complete enough to allow them to be used to explain how certain regulatory genes that are active in specific developmental lineages communicate and cooperate with each other to bring about specific phenotypes in terms of expressed genes and resultant developmental morphology and behavior, within those lineages. This knowledge was gained by either individually perturbing expression, generally by knockdown using morpholino antisense oligonucleotides but sometimes by over-expression, of each regulatory gene in the regulatory network, followed by cataloging the

effect on expression of every other gene in the network. From this analysis, it can be determined which genes are regulated by each gene whose expression was experimentally perturbed. To determine whether each gene whose expression is affected by the experimental perturbation of the each regulatory gene is direct or indirect, *cis*-regulatory analyses of genes whose expression profiles were affected by perturbation of each regulatory gene were, and are being conducted. Therefore, direct transcriptional regulatory interactions between genes in the network can be deduced, verified by direct experimental evidence [45].

1.10 Gaps in our understanding of the developmental role of cyclin D family genes

At least two gaps in understanding exist with respect to cyclin D family genes. First, to date, the cyclin D gene of *S. purpuratus* (*Sp-CycD*) has not been linked to sea urchin developmental GRNs. GRNs of strongest interest include that specifying the endomesoderm, the precursor to the endoderm and mesoderm lineages; and that specifying the ectoderm. This is because *Sp-CycD* becomes confined to the endomesoderm and oral ectoderm as development proceeds [62], and this pattern of expression is likely controlled by the genes expressed in those territories, which is in turn controlled by the respective GRNs. Second, as noted above, Wnt signaling has been shown to regulate expression of cyclin D genes, and *Wnt8* is a key gene in the endomesoderm GRN, showing multiple linkages [55]. *Runt1*, which is required for both *Wnt8* and cyclin D expression in the blastula [63], is also ultimately expressed in the endomesoderm, as well as in oral and ciliated band ectoderm, in an overall pattern that is similar to *Sp-CycD*'s pattern of expression [114].

A second gap in understanding with respect to cyclin D family genes is that none has been subjected to a comprehensive *cis*-regulatory analysis, the experimental method needed to verify linkages between a gene and the developmental GRNs of which it is a part. Evidence has also been provided in this Introduction that cyclin D genes, due to their transcriptional regulation by numerous developmentally important pathways, and due to their ability to in turn regulate aspects of both the cell cycle and development, play important developmental roles. Due to the above noted gaps in understanding, a *cis*-regulatory analysis of the entire *Sp-CycD* gene has been undertaken, as described in the following chapters, based on the premise that genes of the cyclin D family are an important bridge linking the cell cycle to development [40]. A *cis*-regulatory analysis of *Sp-CycD* in *S. purpuratus* would identify the DNA sequence modules that control its expression pattern. Since *cis*-regulatory elements control expression by interacting with transcription factors from developmental pathways, they can link a gene to a GRN of which it is a part. Indeed, a gene is confirmed to be part of a GRN by just such an analysis [45]. Therefore, as described in Chapter 2, a developmental *cis*-regulatory analysis of *Sp-CycD* of *S. purpuratus* was conducted.

CHAPTER 2

DEVELOPMENTAL *CIS*-REGULATORY ANALYSIS OF THE CYCLIN D GENE IN THE SEA URCHIN *STRONGYLOCENTROTUS PURPURATUS*

Herein, a developmental *cis*-regulatory analysis of the cyclin D gene, *Sp-CycD*, in *S. purpuratus* is presented. As explained in Chapter 1, it is proposed that this work can serve as the basis for incorporation of this developmentally important gene into the GRNs that regulate embryonic development in *S. purpuratus*. The methods used to carry out this work are first described. Subsequently, the results, and the interpretation of those results are presented. It should be noted that the material presented in this Chapter is taken, essentially in whole, with only slight modifications, from a recently published paper [115].

2.1 Materials and methods

2.1.1 Rearing and maintenance of *Strongylocentrotus purpuratus*, and obtaining gametes

Strongylocentrotus purpuratus adults were obtained from the Pt. Loma Marine Invertebrate Lab (Lakeside, CA), and kept in a seawater aquarium at ~12^{o}C. Sperm and eggs were obtained by shaking, or by injection with 0.55 M KCl using established methods [116]. Embryos were cultured in artificial sea water.

2.1.2 Sequence comparisons between *Sp-CycD* and *Lv-CycD*

The cyclin D sequence from *Lytechinus variegatus* (*Lv-CycD*) used for comparison to *Sp-CycD* sequence was obtained from two sources, a BAC containing 17 kb of sequence upstream of exon 1, and as a series of isotigs from an *Lv-CycD* draft sequence available at SpBase [3]. Sequence comparisons were made using Family

Relations II [106, 117]. FamilyRelationsII compares sequences using a "sliding window," so that conserved sequences found in the genes being tested will be identified irrespective of their location or orientation in each gene. Sequences in *Sp-CycD* of at least 20 bp that shared at least 90% similarity with *Lv-CycD* were selected for further analysis.

2.1.3 Generation of reporter constructs

To generate EpGFPII-linked reporter constructs [118], regions of interest were amplified by PCR using high fidelity DNA polymerases purchased from Roche or New England BioLabs. For template, either BAC DNA bearing the *Sp-CycD* locus, or if PCR from that template was unsuccessful, sea urchin genomic DNA, was used. Primers were modified on their 5' and 3' ends to have KpnI and SmaI sites, plus 15 bp homology with the multiple cloning site of EpGFPII cut with those enzymes. The primer modifications were 5'-CTATCGATAGGTACC and 5'-ACAGTTTAACCCGGG, for the forward and reverse primers, respectively. Primers were designed using Primer 3, available online [119]. For regions to be incorporated into 13-tag vectors rather than EpGFPII, the forward primer was not modified, while the reverse primer was modified by the addition of 5'-TTGAAGTAGCTGGCAGTGACGT at its 5' end to enable linkage by fusion PCR to 13 tag-bearing reporters as described below. The sequences of primers used to amplify all regions used for analysis are shown in Appendix B Table B.1.

Amplified regions of interest were ligated to EpGFPII reporter vectors using conventional methods. Reporter constructs were then linearized with KpnI followed by purification with a PCR purification kit (Nucleospin Gel and PCR Cleanup, Clontech) before being used for injecting embryos.

13-tag-linked reporter constructs were made as follows. Bacterial cultures bearing each 13 tag reporter were grown up from stab cultures (provided by J. Nam, Davidson lab, California Institute of Techology) as follows. First, derivatives of each stab culture were individually streaked onto LB agar plates containing chloramphenicol (12.5 μg/ml). Colonies from each plate were then placed into 5 ml LB + chloramphenicol (12.5 μg/ml) and grown overnight at 37^{o}C, with shaking. 200 μl of each overnight culture was then used to inoculate 1 ml LB + chloramphenicol (12.5 μg/ml) + 1 μl Copy Control Induction Solution (epicentre). These cultures were then incubated at 37^{o}C, shaking at 290 rpm for 5 hours before being subjected to miniprepping (Spin Miniprep Kit, Qiagen). The resultant minipreps were then used as templates for PCR that would be used to modify their structure somewhat from that presented in the original Nam et al. paper [120] (J. Nam, personal communication). These modifications involved replacing, on each 13 tag reporter, the *Sp-gatae* basal promoter given in the Nam and colleagues paper [120] with an *Sp-nodal* basal promoter. For this modification, a forward primer, new_mNBP,

(5'-

ACGTCACTGCCAGCTACTTCAACTTGGAAGGTAAGGTCTCAAGTATTTAAGATTGAGGGCTCACGGGCACCTTCtcatcttacaagtgaatcacaa), bearing the *Sp-nodal* basal promoter annealed just 3' to the *Sp-gatae* promoter on each original 13 tag vector. In this primer, the non-underlined nucleotides in red font on the 5' end were for subsequent linking by fusion PCR to the 3' end of a regulatory region to be tested bearing the complementary sequence, 5'-TTGAAGTAGCTGGCAGTGACGT; the underlined sequence corresponded to a disarmed *nodal* basal promoter; and the lowercase part

annealed to the 5' end of each 13 tag vector being amplified (J. Nam, personal communication). The reverse primer, end_core-polyA, (5'-CACAAACCACAACTAGAATGCA) annealed ~23 nucleotides downstream of the 13 tag basic unit unique on each reporter (J. Nam, personal communication, May, 2011). Minipreps of each of the 13 tag vectors were then used as templates in PCR reactions containing the two above primers. For these reactions, Phusion DNA polymerase (New England BioLabs) and the following cycling conditions were used: 98^{o}C x 30 sec; 35 cycles of 98^{o}C x 7 sec, 60.8^{o}C x 20 sec, 72^{o}C x 20 sec; 72^{o}C x 10 min. PCR products of the 13 tag reporters, which now bore the *Sp-nodal* basal promoter instead of the *Sp-gatae* promoter, were subjected to PCR purification (Nucleospin Gel and PCR Cleanup, Clontech). At this point, these PCR products could be used for subsequent linking by fusion PCR to amplified potential regulatory regions of interest from *Sp-CycD*.

Potential regulatory regions in *Sp-CycD* were amplified with either Expand High Fidelity DNA polymerase (Roche) or Expand Long Template PCR System (Roche) and purified as described in Nam et al [120]. Amplified regions were linked by fusion PCR to 13-tag reporter constructs using Expand High Fidelity DNA polymerase (Roche) as described in Nam et al [120]. If fusion PCR products could not be generated using Expand High Fidelity DNA polymerase (Roche), then Expand Long Template PCR System (Roche) was used. Fusion PCR products were run on a gel and subjected to gel purification (Nucleospin Gel and PCR Cleanup, Clontech). PCR products run on the gel were visualized by blue light from a Safe Imager (Invitrogen) rather than ultraviolet illumination to limit damage to the DNA. By comparing the activity of reporter constructs bearing known active regions that had been purified by either gel purification

with the aid of blue light or by PCR purification, it was determined that gel purification with the aid of blue light did not prevent the detection of active regulatory regions (data not shown). All PCR products were sequenced to ensure generation of desired products. From analysis of these sequences, it was determined that gel purification was successful in removing the majority of contaminating PCR side products for all 13 tag-linked regions except for 13 tag-linked region 3, for which sequencing showed a roughly 1:1 mixture of 13-tag linked region 3 and non-specific amplification products (data not shown). Despite multiple attempts at optimization, it was not possible to remove these non-specific amplification products from 13-tag linked region 3.

The sequences for upstream regions 2 and 4 presented in this dissertation are from the full sequencing of clones bearing these regions used in this study. The sequences of all of the other regions, for which the correct identity in each case was confirmed by partial sequencing and by running 13 tag-linked reporters of each on a gel to check sizes, are taken directly from *Sp-CycD* sequence accessed using GBrowse V3.1, located at the SpBase website [3, 121].

Each region was attached to a specific 13 tag reporter, X-13Y, where X denotes the region and Y denotes the tag, as indicated in Appendix C, Table C.1.

2.1.4 Microinjection of fertilized eggs

For reporter constructs containing region(s) linked to the reporter vector EpGFPII [118], a 10 μl injection solution contained ~10 nmols of reporter construct along with 165 to 200 ng of HindIII digested then purified genomic DNA; and 0.12 M KCl. Injection solutions comprising potential CRM-containing regions linked to 13-tag vectors were made based on Nam and colleagues' paper [120], but with some modifications. First, a

Master Pool containing ~10-12 13-tag linked reporter constructs was made as directed [120]. However, for the final injection solution of 10 µl, the volume of Master Pool mix used was increased form 0.5 µl to 1 µl. The final mix also contained ~200-270 ng HindIII digested then purified genomic DNA, plus 0.12 M KCl. Microinjection was done using established methods [122], with ~100-150 embryos being injected with injection solution containing EpGFPII-linked reporters and $\geq$ 200 embryos being injected with injection solution containing 13-tag-linked reporters. For this study, a BAC (BAC 4013 F-18 mCherry, prepared by the Sp Genome Research Resource at Caltech) bearing the *Sp-CycD* gene plus ~90 kb upstream and ~13 kb downstream sequence was also utilized. BAC DNA was prepared using a BACMAX DNA Purification Kit (epicentre) from bacterial stab cultures that were grown up under selection from chloramphenicol (12.5 µg/ml). BAC DNA was dialyzed and microinjected based on previous methods [123] . Injection needles were pulled from capillary tubing (FHC, catalog number 30-30-0) using a Flaming/Brown Micropipette Puller (Sutter Instrument Co, Model P-97).

2.1.5 Procurement of RNA, and cDNA synthesis

For assays of endogenous *Sp-CycD* expression, embryos were cultured at a concentration of ~1200 embryos per 4 ml at 15^{o}C in 4 ml each in 6 well plates. At specified time points, embryos were harvested by centrifugation and RNA was obtained using an Rneasy Plus mini kit (Qiagen). Lysates were first passed through a QIAshredder (Qiagen) before processing to obtain RNA. DNA was removed from lysates as described in the kit's instructions. For each time point, RNA equivalent to 30 ng per 20 µl reaction was converted to cDNA using random hexamers and the FirstStrand cDNA Synthesis kit (Invitrogen Life Technologies). For embryos injected with

EpGFPII-based reporter vectors, RNAs and DNAs were obtained with a DNA/RNA ALL Prep kit (Qiagen). cDNA synthesis was carried out using random hexamers as directed by the manufacturer, with 3 µl RNA used for each 20 µl reaction. For embryos injected with 13-tag-linked reporter vectors, RNAs and DNAs were extracted for each time point using the DNA/RNA ALL Prep kit (Qiagen). Before cDNA synthesis, RNAs were treated with DNAse as directed by the DNA/RNA ALL Prep kit instructions. cDNA synthesis was conducted using the FirstStrand Synthesis kit on RNA equivalent to 3 µl per 20 µl reaction using a gene specific primer, that is, one specific for the 13 tag vectors, 5'-ATGCTTTATTTGTTC [120]. The exception for this was the experiment for biological replicate #5 (Fig. 2.4), for which random hexamers were used.

2.1.6 Real-Time PCR procedure and analysis

Real-Time PCR experiments were conducted using Perfecta SYBR Green Fast Mix (Quanta BioSciences) and a LightCycler 480 II instrument (Roche). cDNA and DNA equivalent to 1.3 µl and 1.6 µl per 12 µl reaction were used. Unless indicated otherwise, all reactions were done in duplicate. The reaction profile used was 95^{o}C for 10 minutes, followed by 40 cycles of 95^{o}C for 30 seconds, 60^{o}C for 1 minute. The relative quantification setting was used. All reactions were subjected to melt curve analysis as well.

To determine endogenous *Sp-CycD* expression, primers specific for exon 1 of cyclin D were used (5'-TTTGTTGTGCTTTGAGCAAGA and 5'-CGAACATCCAATCCACGACT). Ct values were obtained for each time point and compared to those derived from expression of ubiquitin in the same samples. *Sp-CycD* expression levels for each time point were determined by finding the difference in Ct

values between the Real-Time PCR reactions conducted for *Sp-CycD* expression and ubiquitin expression. The primers used to detect ubiquitin expression were: 5'-CACAGGCAAGACCATCACAC and 5'-GAGAGAGTGCGACCATCCTC. Next, the Ct value difference between *Sp-CycD* and ubiquitin from each time point was compared to this difference at the first time point, generally 10 hours post-fertilization (hpf), yielding a ΔΔCt value for each time point. Relative expression values at each time point were then computed using the formula Expression = $1/2^{\Delta\Delta Ct}$. These Ct values were derived from cDNA samples subjected to Real-Time PCR.

To calculate expression of GFP derived from injection of embryos with EpGFPII-region of interest-linked reporter vectors, Ct values derived from expression of GFP were determined using GFP specific primers (5'-AGGGCTATGTGCAGGAGAGA and 5'-CTTGTGGCCGAGAATGTTTC). Ct values derived from GFP expression were then normalized to Ct values derived from expression of ubiquitin by finding the difference between Ct values of GFP and ubiquitin at each time point. These Ct values were derived from cDNA samples subjected to Real-Time PCR. To account for how much GFP-linked construct was injected for each time point, Ct values were likewise obtained using the same GFP specific primers on DNA samples derived from each time point. The difference between each ubiquitin normalized Ct value and the corresponding value derived from Real-Time PCR with GFP primers on the corresponding DNA sample for that time point was determined for each time point. All such ubiquitin- and amount-injected-normalized values were then further normalized to that of the first time point by finding the difference between the former and each of the latter. The resultant ΔΔCt values were used to calculate the relative expression of GFP at each time point as above.

Activity levels of microinjected mcherry-bearing BAC (BAC 4013 F-18 mCherry) were determined as for microinjected GFP-bearing constructs, except that primers specific for mcherry (5'-AAGGGCGAGGAGGATAACAT + 5'-ACATGAACTGAGGGGCAGG) replaced those specific for GFP.

To determine the activity of each 13-tag-linked reporter derived from embryos co-injected with these, each linked to a potential regulatory region of *Sp-CycD*, a primer pair unique for each 13 tag reporter being assayed was used to obtain a Ct value for that reporter. Primers used to detect 13 tag reporters are provided in Nam and colleagues' Supplemental Data [120]. Ct values were derived from both the cDNA samples, to determine how much reporter was expressed, and for the corresponding DNA samples, to determine how much of each was injected. For each 13-tag reporter linked to a specific potential regulatory region, activity was first determined in the same manner as for GFP from EpGFPII-based reporter. However, for each time point, Ct data for co-injected empty 13 tag reporter 1302 were also collected, enabling relative expression of both empty reporter and reporters linked to regions of interest to be determined at each time point. As a final step, the relative activity value determined for each region-linked reporter was divided by that of empty 1302 for each time point. These calculations led to the relative expression values for each region reported in the Results and Discussion.

Some deviations from these procedures were made for some of the experiments presented in Fig. 2.4, as follows. 1. The graph for Experiment #8 is a composite of three individual biological replicates, for which Real-Time PCRs were conducted one time each. This graph also contains one region, 13_orig, for which the final boundaries had not been finalized to account for conservation with *Lv-CycD*, because this latter sequence

was unavailable when Experiment #8 was done. 2. In Experiment #7, region 18, not discussed, showed significant activity. This region was considered to be of interest before the boundaries of regions 5 and 6, which were also shown to be active, as discussed in the Results, had been finalized. Since the termini of region 18 overlap with regions 5 and 6 (see Fig. 2.3A), and since regions 5 and 6 contain all of the conserved sequence found in region 18 (Fig. 2.3A), region 18 was not further studied.

2.1.7 Examination of injected embryos by fluorescence microscopy

Eggs were arrayed on 50 mm glass bottom dishes (MatTek), and fertilized and injected as described above. At time points of interest, injected embryos were visualized with an Axiovert 200 fluorescence microscope (Zeiss).

2.2 Results

2.2.1 Temporal expression of *Sp-CycD*

The temporal profile of embryonic *Sp-CycD* expression was assayed by quantitative RT-PCR. As reported previously by others [62], expression commenced ~10-12 hpf (early blastula), then increased at least up to pluteus stage (72 hpf) (Fig. 2.1). Interestingly, there was substantial variation between biological replicates.

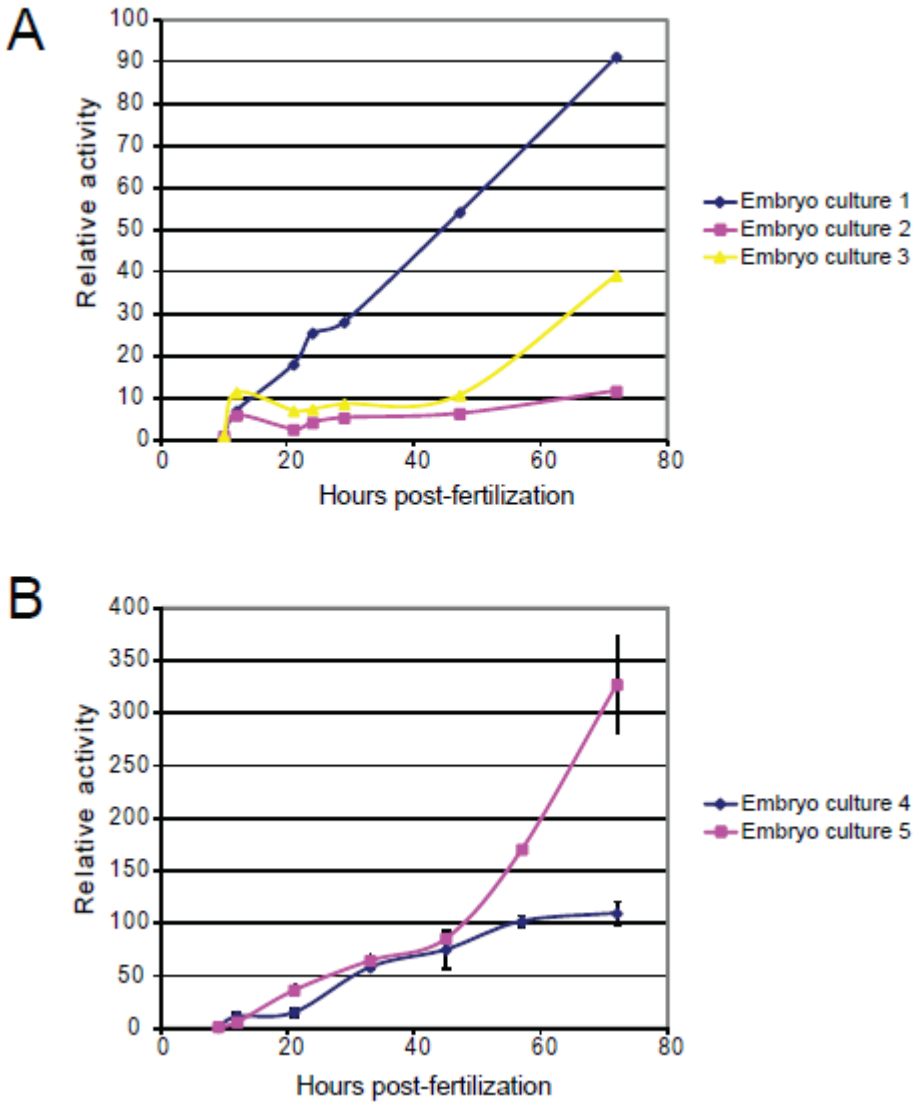


Fig. 2.1 Endogenous *Sp-CycD* expression from different embryo cultures, as determined by quantitative RT-PCR. Expression values are of relative expression with respect to that at the first time point. **A.** Temporal expression patterns of *Sp-CycD* in experiments derived from embryo cultures 1-3. Each experiment shown in panel A consisted of one technical replicate on a unique embryo culture. **B.** Graph of experiments derived from embryo cultures 4 and 5. In this case, each graph represents the mean of two technical replicates done on one embryo culture each.

The temporal activities of endogenous *Sp-CycD* and a bacterial artificial chromosome (BAC) bearing *Sp-CycD* with mCherry knocked into exon 1 were co-assayed. This BAC encompassed sequence from ~90 kb upstream of the gene to ~13 kb

downstream. Both endogenous *Sp-CycD* and the injected BAC exhibited similar temporal activities (Fig. 2.2, panel A), suggesting the information needed to regulate embryonic *Sp-CycD* expression is within this BAC. It should also be noted that the expression profiles of endogenous *Sp-CycD* and the *Sp-CycD*-mcherry BAC were similar to that of *Sp-CycD* derived from the transcriptome analysis of *S. purpuratus*, worked out by the Davidson lab (Fig. 2.2, panel B, [3]).

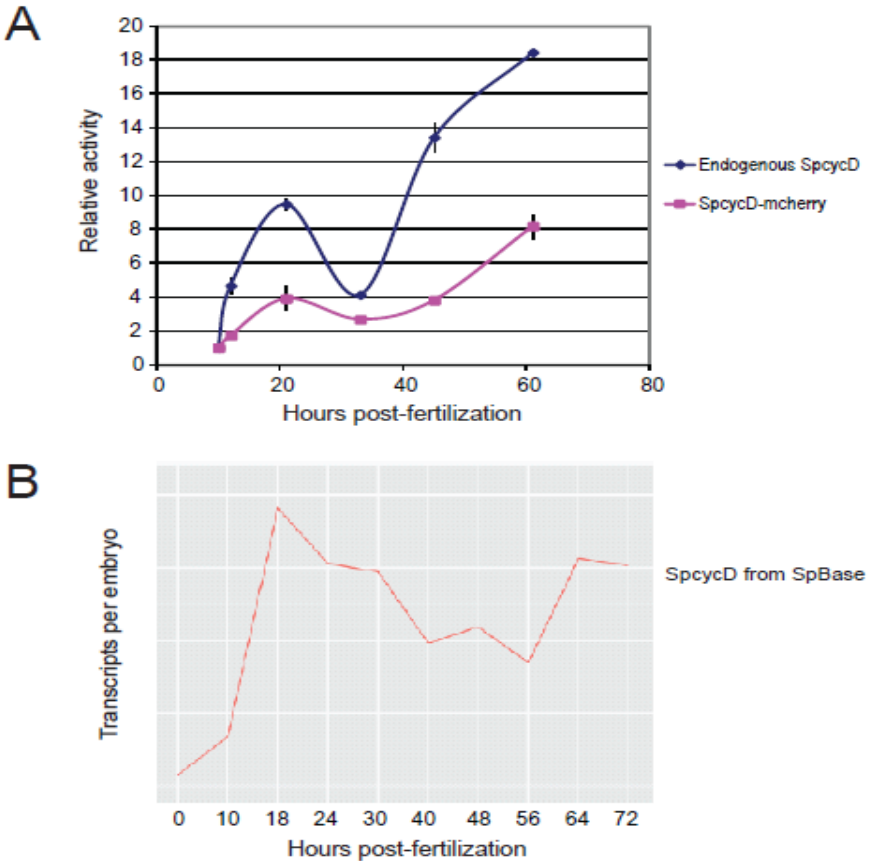


Fig. 2.2. A. Expression of endogenous *Sp-CycD* and microinjected mcherry-linked BAC bearing *Sp-CycD* plus 90 kb and 13 kb of up and downstream sequence. Relative levels of *Sp-CycD* mRNA were measured at each indicated time point by qRT-PCR as described in the text. Each graph represents two technical replicates done on one biological replicate. B. Transcription profile of *Sp-CycD* as taken from SpBase [3]. The original data are from Tu et al [124].

The *cis*-regulatory analysis conducted for this project encompassed from ~13 kb upstream of exon 1 to ~7 kb downstream from the end of exon 5 (Fig. 2.3A).

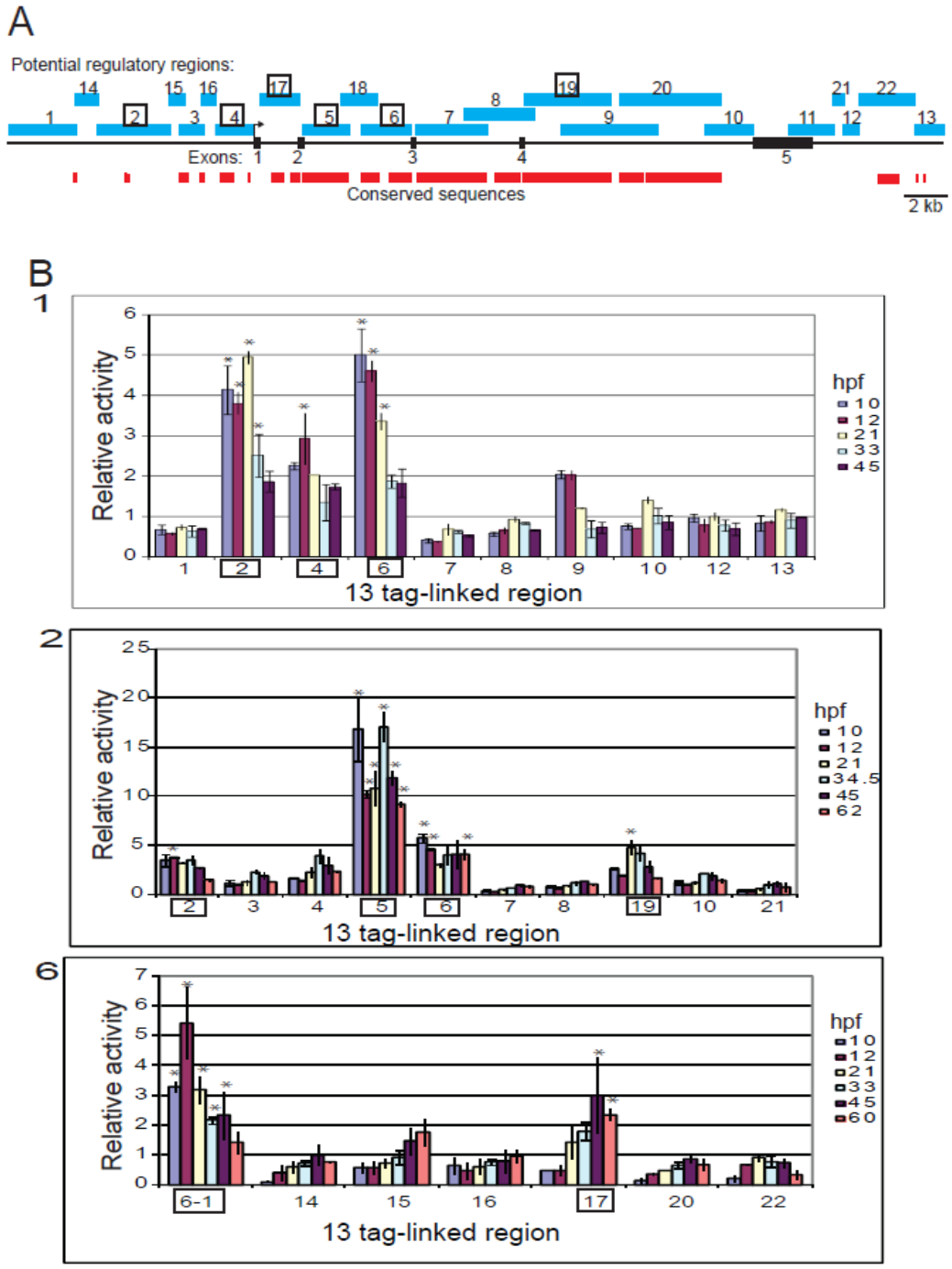


Fig. 2.3. Identifying *cis*-regulatory sequences. A. Regions tested for CRM-containing activity. *Sp-CycD*, plus 13 kb upstream and 7 kb downstream sequence is shown. Exons: black; potential CRM-containing regions: blue; sequences with > 90% similarity to *Lv-CycD*: red; active regions: boxed. **B. Representative activity profiles.** Each panel is from the indicated experiment 1, 2 or 6. Asterisks denote significant activity. See Fig. 2.4 for additional activity profiles.

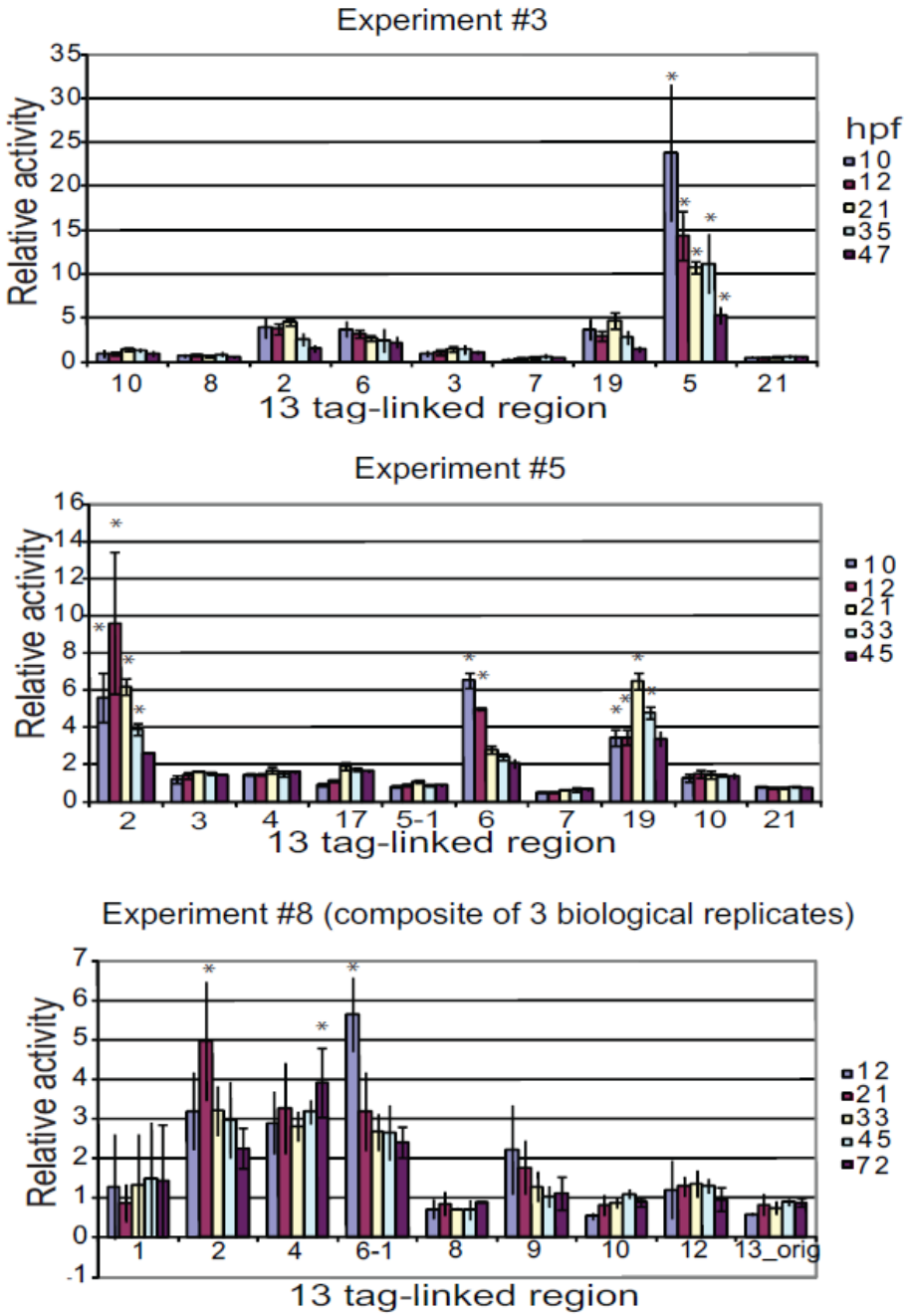


Fig. 2.4 Results of additional experiments showing the activities of tested regions.
Notes: 1. The fact that region 21 showed significant activity at 10 hpf in Experiment #7 was attributed to the low background expression level in that experiment. Region 21 did not show significant activity in other experiments. 2. In at least two additional experiments assaying each, regions 12 and 13 showed only background activity; and in one additional experiment, region 22 showed only background activity (data not shown)
Figure continues on next page.

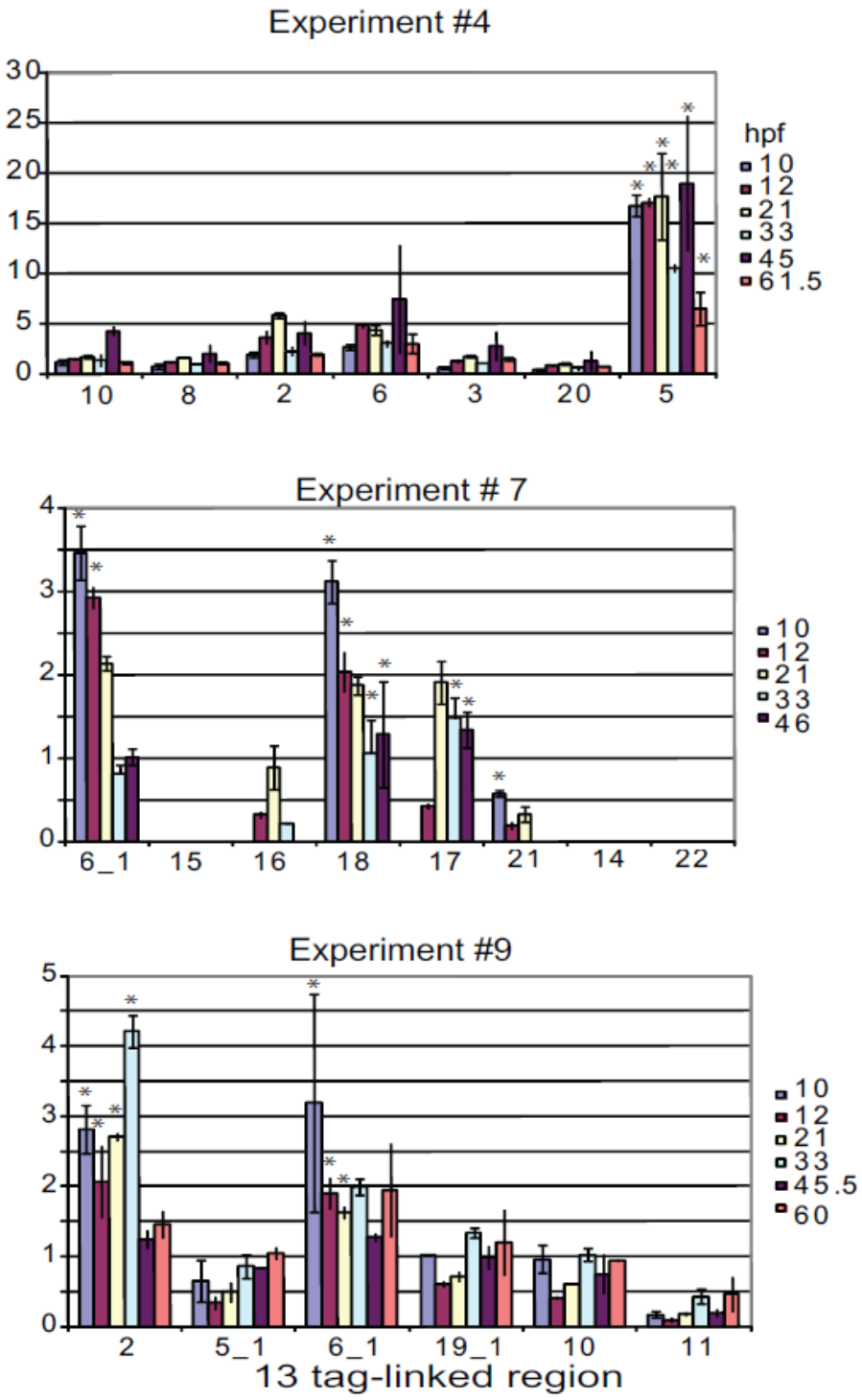


Fig 2.4 continued.

2.2.2 Identification of *cis*-regulatory regions

Twenty-two regions spanning upstream and intronic sequence of *Sp-CycD* were selected to assay for regulatory activity (Fig. 2.3A). The boundaries of most were chosen based on the presence of sequences of $\geq$ 20 bp with $\geq$ 90% similarity to *Lv-CycD* from *L. variegatus* (Fig. 2.3A) [3]. This criterion was based on the fact that sequence comparisons between genes in *S. purpuratus* and *L. variegatus* reliably predict *S. purpuratus* CRMs [106, 107]. This analysis was comprehensive: all non-exonic sequence except 1 bp between the 3' end of region 10 and the 5' end of exon 5, and 2 bp between the 3' end of region 11 and the 5' end of region 21 was tested.

Candidate *cis*-regulatory regions were assayed for activity using the '13-tag' reporters developed by Nam and colleagues [120]. Representative results are in Fig. 2.3B and Fig. 2.4. In each experiment, a region was classified as significantly active if activity at one or more time points was $\geq$ 2.5 times that of the mean activity of regions in the middle 40% of the distribution [120].

Several active regions were identified. Region 5, (2.4 kb) in the first half of intron 2 (Fig. 2.3A) showed the strongest activity, with significant activity at all tested time points from ~10-60 hpf. This activity was ~15 times greater than that of empty reporter at its peak, and at least 2 times higher than those of the next most active regions. The next most active regions were region 2 (~3.6 kb), located ~4.6 kb upstream from the beginning of exon 1; region 6 (2.7 kb), comprising the 3' half of intron 2; region 19 (4.6 kb), in intron 4; followed by region 4 (2.1 kb), which abuts exon 1; and region 17 (2.1 kb) in intron 1 (Fig. 2.3 and 2.4). Regions 2 and 6 always showed significant activity for at

least one time point when injected without region 5-linked reporter, but not always in its presence (Fig. 2.4).

2.2.3 Temporal activity profiles of *cis*-regulatory regions

To gain further insight into the roles of each active region, temporal activity profiles were extracted from experiments in Fig. 2.3B and Fig. 2.4, and are presented in Fig. 2.5. This analysis reveals substantial inter-experimental variation in the temporal activity profiles of each region. An exception concerned region 19, as discussed below. Possible sources of this variation include biological variability, the fact that injection solutions contained different mixtures of 13-tag-linked regions, and the fact that each time point was from a separate injection plate because it was technically not possible to inject more than ~200 embryos per plate.

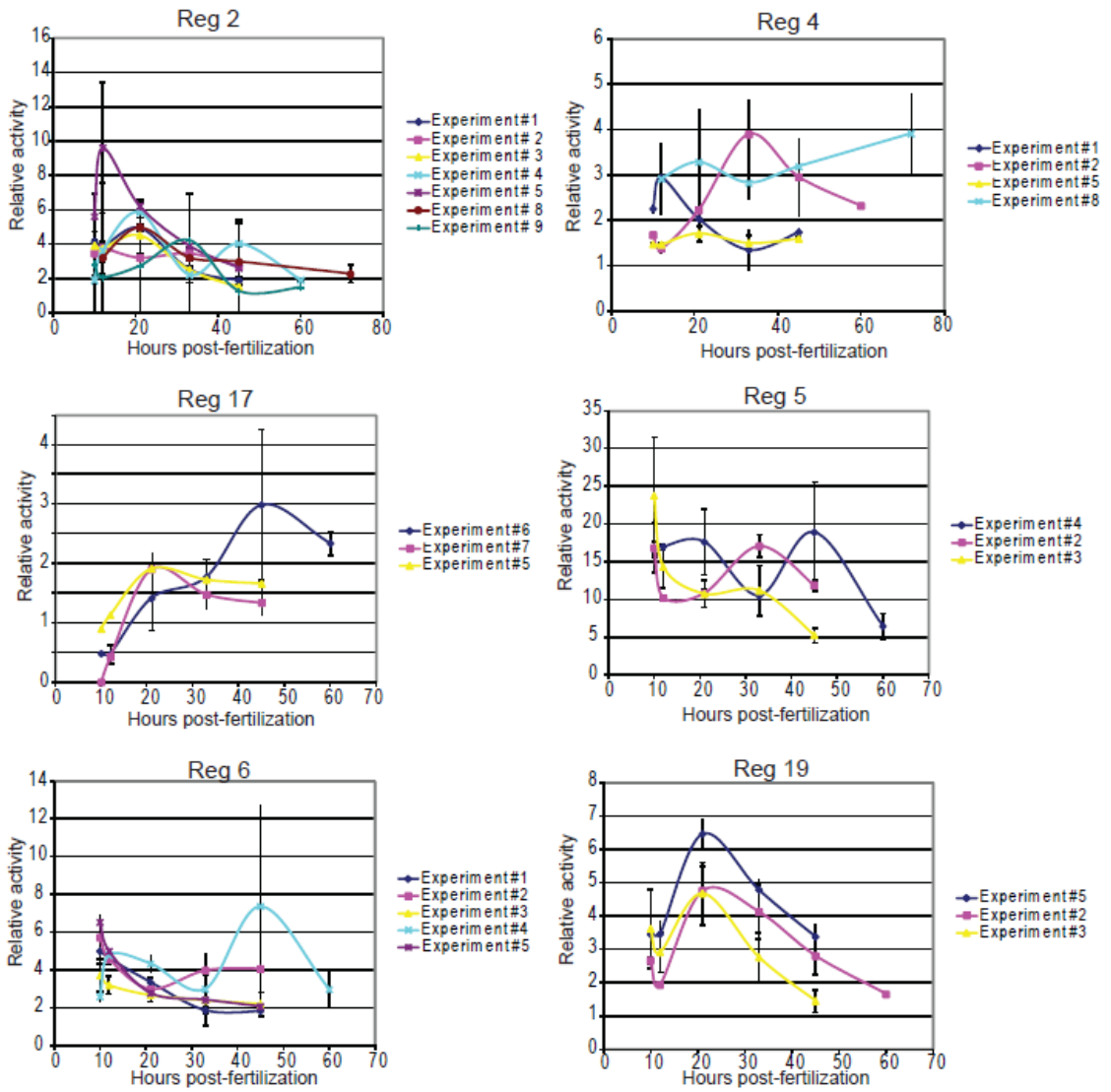


Fig. 2.5. Comparison of the temporal activities of regulatory regions of *Sp-CycD*, with the results of individual experiments for the temporal activity of each region shown. Temporal activity profiles are derived from embryos injected with regions linked to 13-tag reporters. Experiments shown in the key for each graph each correspond to a unique experiment corresponding to a unique embryo culture. Experiment "X" in a given panel utilized the same embryo culture as Experiment "X" in a different panel. For example, Experiment 1 in the graphs for regions 2, 4 and 6 corresponds to the same experiment. Note also that Experiments #1, #2 and #6 are extracted from panels 1, 2 and 6, respectively, in Fig. 2.3B. The other labeled time course graphs are extracted from the graphs bearing the same labels in Fig. 2.4. In all cases, activity at each time point is with respect to that of 1302 empty reporter at the corresponding time point.

To more clearly discern canonical aspects of the temporal activity patterns, the activity values across experiments were averaged (Fig. 2.6).

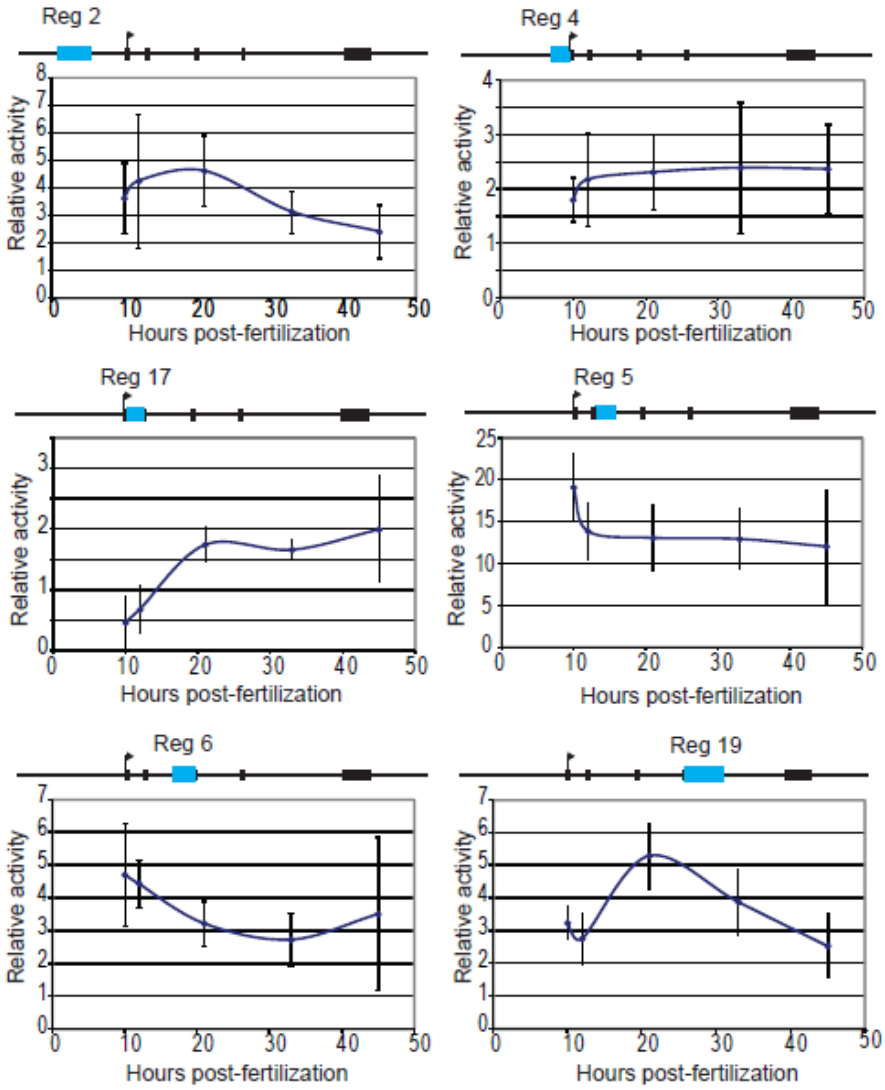


Fig. 2.6. Averaged temporal activity profiles. Grand means and standard deviations were calculated from the means of all experiments in Fig. 2.5. Small differences between time points in different experiments (for example, 45 and 47 hpf) were ignored.

From this analysis, the following patterns were found. (Please see Figs. 2.5 and 2.6, plus other figures when indicated). Region 5's activity was highest at 10-12 hpf, when *Sp-CycD* is initially activated. As other regions became active, region 5's activity

declined somewhat, but remained significant (Fig. 2.3B). Region 6 likewise showed the strongest activity at ~10 hpf. During the first ~33 hours, activities of regions 5 and 6 paralleled each other, then region 6's stabilized, suggesting that region 6 contributes to maintaining *Sp-CycD* expression after ~33 hpf, corresponding to gastrulation and later stages.

On average, region 2's activity peaked at ~21 hpf (Fig. 2.6), although peak activity varied from ~12-33 hpf (Fig. 2.5). Region 2's activity peak occurred after that of regions 5 and 6. Therefore, region 2's primary role may be to activate transcription during late blastula stage.

Region 4's activity varied considerably (Fig. 2.5), but on average (Fig. 2.6) increased to low but stable levels by ~21-33 hpf. Thus, region 4 may contribute to maintaining *Sp-CycD* expression.

Region 17's activity slowly increased to stability by ~21-33 hpf (Figs. 2.5 and 2.6), indicating that this region may contribute to maintenance or lineage-specific activation of *Sp-CycD* during and after gastrulation.

Region 19's activity peaked at ~21 hpf, the mesenchyme blastula stage (Figs. 2.5 and 2.6), suggesting that this region may act as a switch that regulates *Sp-CycD* at the onset of gastrulation. As noted, region 19's activity showed much less variation than those of other active regions (Fig. 2.5; compare Experiments #5, 2 and 3). Therefore, region 19 may be under especially strong control.

As a control, activities of region 2-linked 13-tag vectors at 12 hpf (Fig. 2.7A), and 13-tag vectors linked to unique regions (Fig. 2.7B) were compared. There was significantly less variation between activities of 13-tag reporters linked only to region 2

than between those linked to different regions, indicating that differences in activity among regions could mostly be attributed to region-specific differences rather than 13-tag reporter-specific differences.

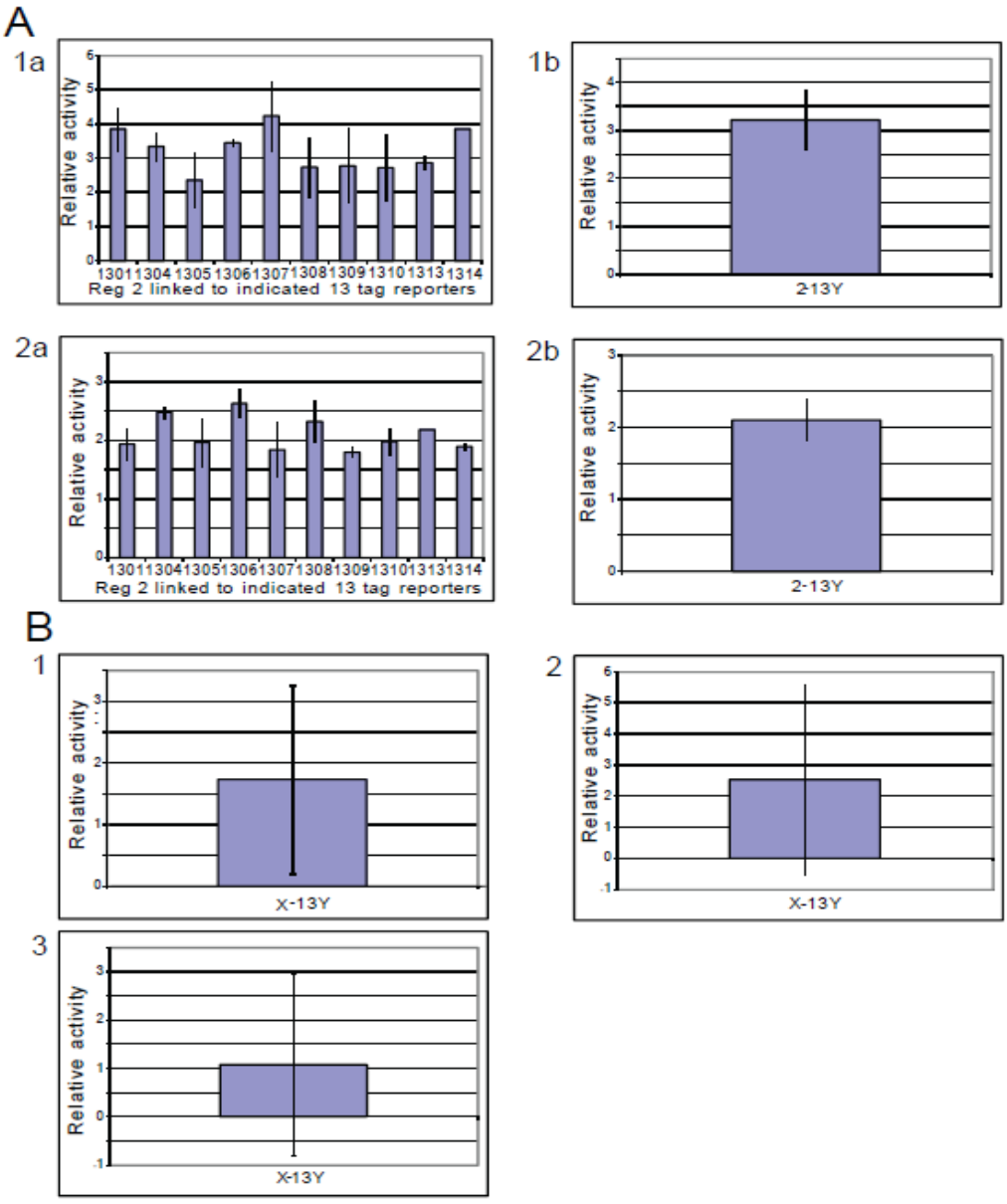


Fig 2.7. Testing for variations in activity attributed to differences between 13-tag reporters at 12 hpf. A. Testing for variation in expression between activities of the same region (region 2) when linked to different 13-tag reporters. Two biological replicates, 1 and 2, each broken down into two graphs, a and b, are presented. In each case, "a" shows the activity of each individual region 2-linked reporter, whereas "b" shows the grand mean of the activities of all region 2-linked reporters, along with the standard deviation of those means. B. The grand means and standard deviations resulting from averaging the activities of multiple regions (not just region 2) when linked to 13-tag reporters. To construct these graphs, the average activity level and standard deviation for all regions at 12 hpf was determined for each experiment in Fig. 2.3. Note that the standard deviations are much less when all 13-tag reporters are linked to the same region (region 2) than when these reporters are all linked to different regions.

2.2.4 Identification of candidate *cis*-regulatory modules

Since the sizes of the identified regulatory regions ranged from ~2-5 kb (Fig. 2.3A), additional analysis was needed to identify CRMs, which are generally only up to several hundred bp [45]. By using a combination of computational approaches to analyze each region (Fig. 2.8; Appendix D, Fig. D.1; Appendix E, Fig. E.1), candidate CRMs were identified within each. The activities of several of these were verified experimentally. (Please note: Several transcription factor binding sites highlighted in Appendices D and E may only be briefly introduced in this Chapter, or not mentioned at all. Further discussion is provided in Chapter 3).

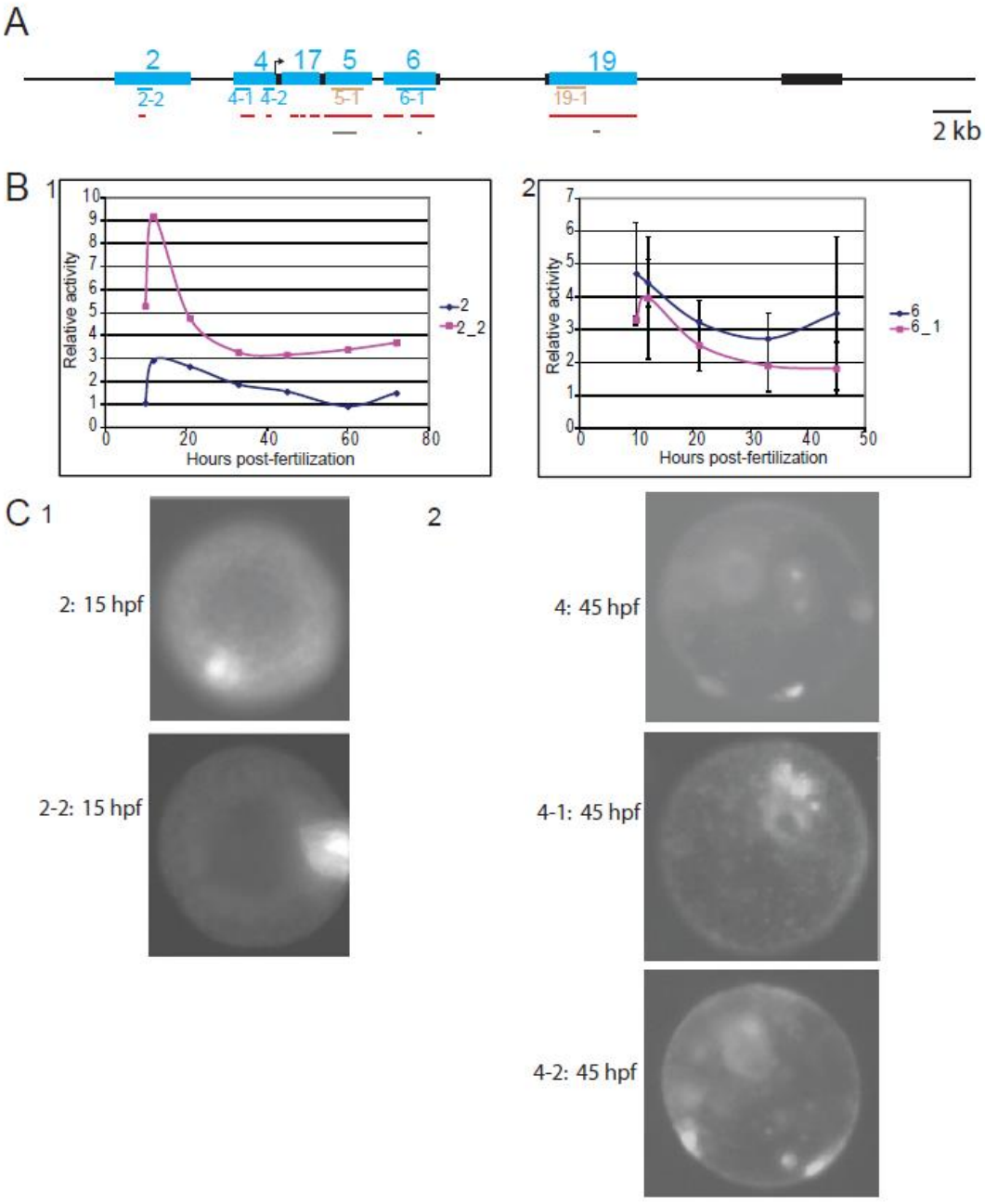


Fig. 2.8. Identification of *cis*-regulatory modules. A. *Sp-CycD* showing active *cis*-regulatory regions. Exons: black rectangles; active regions: blue rectangles; active and inactive subregions: blue and tan lines, respectively; conserved sequences: red; Cluster-Buster-identified sequences: gray. **B. Activities of 13-tag-linked regions 2, 2-2, 6 and 6-1.** Panel 1 shows the activities of region 2 and subregion 2-2 in co-injected embryos (one experiment). Panel 2 shows the averaged temporal activities and standard deviations of region 6 and subregion 6-1 from all presented experiments where either region was assayed. **C. Fluorescence micrographs from injection with EpGFPII-linked region 2, 2-2, 4, 4-1 or 4-2.** Brightness and contrast were adjusted equally in all images.

Region 2 contains a 0.5 kb subregion, 2-2, encompassing sequence conserved at ≥ 90% with *Lv-CycD* (Fig. 2.8A; Appendix D, Fig. D.1). Experimental analysis using both 13-tag and EpGFPII-linked versions of region 2 and subregion 2-2 showed that subregion 2-2's temporal activity mirrored region 2's (Fig. 2.8B, panel 1; Fig. 2.9). Further analysis showed that the activities of each were detected at blastula stage by fluorescence microscopy (Figs. 2.8A and 2.8C, panel 1). Together, these findings indicate that subregion 2-2 contains a CRM.

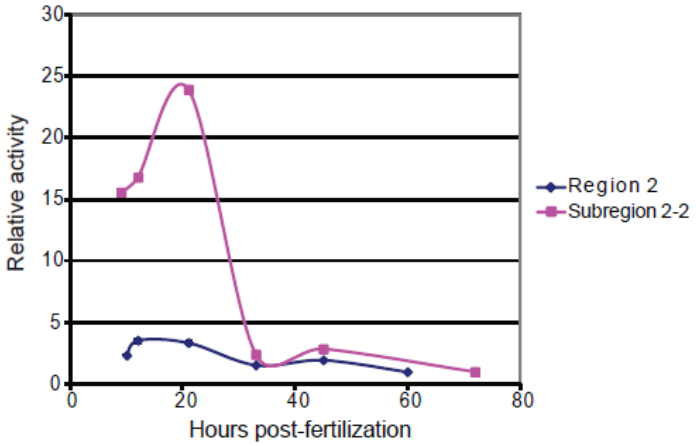


Fig. 2.9. Comparison of the temporal activities of region 2 and subregion 2-2 when linked to the reporter vector EpGFPII. The plots are from separate experiments derived from different embryo cultures, in each of which EpGFPII-linked region 2 or subregion 2-2 were separately injected. Activity in each case is with respect to that at the time point with the lowest activity. Error bars for region 2 (error bars are small) are standard deviations of two technical replicates done on a representative biological replicate. Note that error bars are not shown for subregion 2-2, for which one technical replicate of one biological replicate is shown.

Region 4 contains two active subregions (4-1 and 4-2; Fig. 2.8A). Subregion 4-1 overlaps partly with conserved sequence (Fig. 2.8A; Appendix D, Fig. D.1), and bears a potential Runx site (Appendix D, Fig. D.1). Sequence within subregion 4-1 was previously found by chromatin immunoprecipitation to bind the Runx protein SpRunt-1, which was shown to regulate *Sp-CycD* [63]. Subregion 4-2 contains a 22 bp conserved

sequence (Fig. 2.8A; Appendix D, Fig. D.1), and a potential Runx site [125] (Appendix D, Fig. D.1). When tested for activity by fluorescence microscopy, subregions 4-1 and 4-2 were both shown to be active at gastrula stage (Fig. 2.8C, panel 2), suggesting that both encompass CRMs.

Analysis of the intronic regulatory regions, which contain longer stretches of sequence conservation than the upstream regions (Fig. 2.8A, red lines), was chiefly computational. In this analysis, a number of sequence elements of interest were identified. Among these, were potential binding sites for TCF and Runx. Wnt-TCF signaling is known to regulate cyclin D expression in a variety of other systems [82, 83, 87, 126]; and, as noted above, the Sp-Runt-1 protein is known to regulate *Sp-CycD*. In addition, a search was done for sequences with clustered binding sites for transcription factors identified by the program Cluster-Buster, of interest because sequences where transcription factor bindings sites cluster are hypothesized to be regulatory [108, 127, 128]. These areas are highlighted on the sequence for each region in Appendix D, Fig. D.1. Identities of transcription factors identified by Cluster-Buster are in Appendix E, Fig. E.1. In Chapter 3, further analysis of the sequence of each regulatory region is presented. The sequence of each identified regulatory region was also studied to identify possible CRMs within each. One candidate CRM in region 5 was subregion 5-1, found 6 bp upstream of a potential transcription factor cluster site to 14 bp downstream from a potential TCF binding site (Fig. 2.8A, Appendix D, Fig. D.1). However, subregion 5-1 showed only background activity (Fig. 2.4, Experiments #5 and 9). This was surprising because within its boundaries, which overlapped with conserved sequence, subregion 5-1 contains 6 potential TCF and Runx sites, respectively, most of which overlap with the

transcription factor cluster site. Therefore, 5-1 may be necessary but not sufficient for region 5's activity. Further analysis (presented in Chapter 3) uncovers the possible reasons why subregion 5-1 is inactive.

Within region 6, it was reasoned that the 3' two-thirds of this region could contain a CRM, as most of the potential regulatory elements of interest (discussed further in Chapter 3) were found in that portion (Fig. 2.8; Appendices D and E). This subregion, 6-1, was verified to be active (Fig. 2.3B, panel 6; Fig. 2.4, Experiments #7, 8 and 9), and its temporal activity closely resembled region 6's (Fig. 2.8B, panel 2).

Within region 19, a sequence termed subregion 19-1, which bears few of the potential regulatory elements of interest highlighted in Appendix D, showed only background activity (Fig. 2.4, Experiment #9), indirectly supporting the hypothesis that the highlighted sequence elements shown for region 19 likely mark one or more CRMs. The hypothesized roles of specific potential transcription factor binding sites in regulating the activity of this and all regions are discussed in greater detail in Chapter 3.

2.2.5 Conclusions

The entire *Sp-CycD* locus was analyzed to identify *cis*-regulatory regions and modules (CRMs) within those regions that mediate expression. Intronic and upstream regions that impart distinct activity patterns were identified, and likely CRMs were found in two upstream regions, 2 and 4; and within intronic region 6. A future aim is to determine the specific roles of each regulatory region and candidate CRM by individual deletion of each from a BAC bearing *Sp-CycD*. Finally, to link *Sp-CycD* to GRNs that control early embryogenesis, the spatial activity of each CRM should be studied and compared to that of both endogenous *Sp-CycD*, *Sp-CycD*-bearing BAC, and *Sp-CycD*-

bearing BAC in which each of the regions in question has been individually deleted. In Chapter 3, further analysis of the sequence of each regulatory region is presented in order to gain better insight into how the expression of *Sp-CycD* could be regulated by endomesoderm and ectoderm-specifying transcription factors expressed during embryogenesis.

CHAPTER 3

POSSIBLE LINKAGES OF THE REGULATORY REGIONS OF *SP-CYCD* TO DEVELOPMENTAL SIGNALING PATHWAYS AND LINEAGE SPECIFYING TRANSCRIPTION FACTORS

3.1 Overview

During development, *cis*-regulatory modules (CRMs) carry out their tasks by binding to transcription factors that are expressed within the cells as development proceeds. In *S. purpuratus*, the set of transcription factors that is expressed during embryogenesis is well worked out [129]. As presented in Chapter 1, transcription factors that regulate development do so via Gene Regulatory Networks (GRNs).

In Chapter 2, a *cis*-regulatory analysis of *Sp-CycD* during development was described. In addition, the sequence of each active regulatory region was analyzed to identify candidate transcription factors that could potentially regulate each region's activity (Appendices D and E). In Chapter 2, only a preliminary discussion of the results of this analysis was provided. The purpose of this Chapter is to provide a more in depth analysis. In addition, at the end of the chapter, how *Sp-CycD* itself could regulate the expression of developmental regulatory genes will be discussed.

In addressing how *Sp-CycD*, through its regulatory regions, could be regulated by specific, developmentally-expressed transcription factors, this Chapter discusses a number of different groups of transcription factors. The first group comprises transcription factors expressed within the endomesoderm, the lineage that gives rise to the endoderm and mesoderm lineages. This lineage is one of two major lineages in the embryo where expression of *Sp-CycD* becomes confined during and after gastrulation

[62]. Insight into how this localized expression is controlled can be gained by identifying transcription factors active within that lineage that could bind to the regulatory regions of *Sp-CycD*. From the large set of transcription factors expressed within the endomesoderm GRN [55], focus will be made on a subset of transcription factors that are expressed within a conserved subcircuit that plays a central role in the specification of endoderm and mesoderm from that lineage [130, 131]. Since the transcription of the genes expressed within the endomesoderm is largely induced by two signaling pathways, the Wnt-beta catenin and Delta-Notch pathways [111], available evidence that transcription factors activated directly downstream from these two signaling pathways regulate the expression of *Sp-CycD* is given. This Chapter also presents evidence that Runx transcription factors could regulate the transcription of *Sp-CycD*. As discussed in Chapter 1, Runx transcription factors act in a context-dependent manner to regulate the transcription of genes, in part, by inducing the recruitment of other transcription factors [132]. Finally, since, along with the endoderm, *Sp-CycD* becomes confined to the oral ectoderm after gastrulation [62], the evidence that the transcription of *Sp-CycD* could be regulated by transcription factors expressed within the GRN that regulates the development of the oral ectoderm is discussed. While this Chapter is essentially conjecture, it provides the basis for future work.

3.2 Comparing the expected and actual number of binding sites for transcription factors of interest

As described in section 3.1 above, the regulatory regions of *Sp-CycD* identified in Chapter 2 were analyzed for binding sites for transcription factors present in GRNs active in developmental lineages where *Sp-CycD* is expressed during embryogenesis.

This current section first describes the statistical calculations done to determine whether the actual number of potential binding sites for each transcription factor of interest compared to the predicted numbers of each such site was significantly significant, then presents the results as a graph. This graph is then referred to in subsequent sections of this Chapter, which discuss which transcription factors of interest could regulate the expression of *Sp-CycD* during embryogenesis.

This statistical analysis was performed as follows. First, the GC and AT content of each region was determined using an online GC percent calculator [133], so that the probability of finding each nucleotide in the consensus binding site for each transcription factor of interest within the regulatory region being examined could be determined. For example, if the GC content was 38.19C%, then the proportion of G or C would be 19.095% or 0.19095, and the proportion of A or T would be (100 - 38.19C)/2/100 = 0.30905. The probability, P, of finding each consensus sequence and its reverse complement in a region of length N was then found using the generalized formula:

$$\mathbf{2N(P\ of\ G\ or\ C)^{(\#\ of\ G\ and\ C\ in\ sequence)}\ (P\ of\ A\ or\ T)^{(\#\ of\ A\ and\ T\ in\ sequence)}}$$

The purpose of multiplying by 2 was to account for both the forward version and reverse complement version of each consensus sequence. The above formula, as noted, is a generalized version. In cases where it was possible for a nucleotide within a consensus sequence to have more than one identity, the formula was modified. In Table 3.1 below, the consensus binding site sequences of most transcription factors discussed in subsequent sections are provided, along with the modified versions of the above formula

used to calculate the predicted number of forward and reverse complement binding sites for each transcription factor in a regulatory region of sequence length N.

Table 3.1 Formulas used to determine the expected number of binding sites for the given consensus sequences in regulatory regions of length N.

Note: Lowercase "n" within a sequence denotes any nucleotide; capital "N" in a formula denotes sequence length; and "P" in a formula denotes probability. The consensus sequences were determined by examining the references cited below. These sequences are composites of the sequences provided in the references cited in this table. The figure legend of Appendix D, Fig. D.1 shows the original sequences that were used to determine the consensus sequences shown in this table.

Bra
Consensus sequence: (A/G)(A/T)(A/T)nTn(A/G)CAC(C/T)T
Formula: 2N(PA+PG)^2(PA+PT)^2(PT or PA)^3(PC)^2(PC+PT)^1
Reference for consensus sequence: [134]

FoxA
Consensus sequence: (A/G)(A/C)(A/C)T(G/A)TT(A/T/G)(A/T)TT(T/C)
Formula: 2N(PA+PG)^2(PA+PC)^2(PA or PT)^5(1-PC)^1(PA+PT)^1(PT+PC)^1
Reference for consensus sequence: Reverse complement of sequences identified by Cluster-Buster [127]

GataC
Consensus sequence: (T/G/A)(T/A)(G/C)AGACT(T/A)AGC(T/G)
Formula: 2N(1-PC)^1(PT+PA)^2(PC+PG)^1(PA or PT)^4(PC or PG)^4(PT+PG)^1
References for consensus sequence: Gata-1 binding sites identified by Transfac [135] were stated to be GataC sites, because GataC is a homolog of Gata-1 [136].

Su(H)
Consensus sequence: (C/G)(G/A)TG(A/G)GA(A/T/G)
Formula: 2N(PC+PG)^1(PG+PA)^2(PA or PT)^2(PG)^2(1-PC)^1
Reference for consensus sequence: [137]

Runx
Consensus sequence: (C/T)G(C/T)GGTn
Formula: 2N(PC+PT)^2(PG)^3(PT)^1
References for consensus sequence: [63, 125]

TCF
Consensus sequence: ACAAAG
Formula: 2N(PA)^4(PA or PG)^2
References for consensus sequence: Cited in [63].

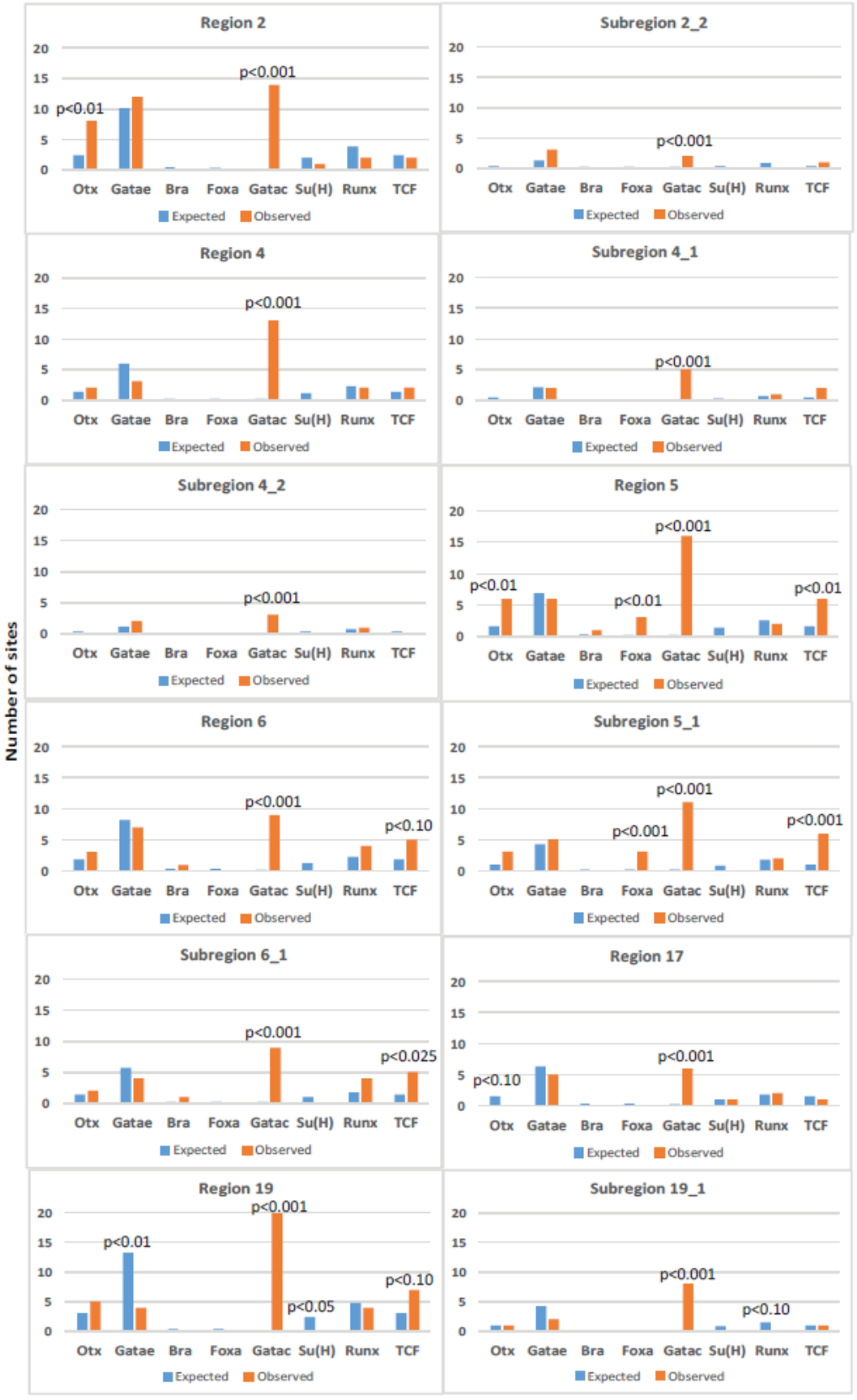
Fig. 3.1 on page 64 presents the predicted and actual numbers of potential binding sites in each regulatory region for the transcription factors presented in Table 3.1, and indicates whether the difference between predicted and actual values are statistically significant, as determined by Goodness of Fit Tests (G Tests) [138], by providing the p values in each case of a statistically significant difference. The calculations used to perform these tests are shown in Appendix F, Fig. F.1 (see separate Excel file provided). As described in Robin et al., the Goodness of Fit Test, can be used to determine whether a sequence motif is significantly more or less represented in one sequence than another [139]. Although Robin et al. were comparing counts of motifs in two different sequences, the Goodness of Fit Test was appropriate in the individual analysis of each regulatory region of *Sp-CycD* because the distributions of the predicted numbers of each binding site are not normally distributed. Rather, each starts at zero, rises to a mean that is the predicted number of binding sites, then decreases to successively smaller values. Each of these distributions is therefore skewed to the left. As shown in Appendix F (in separate Excel file), each G test examined sufficient numbers of binding sites to be reliable, because, for each binding site, the G score was calculated by using the predicted and actual numbers of not only the binding site in question, but also, its non-version. For example, region 2 had 2.4 expected Otx binding sites and about 598.1 expected non-Otx binding sites. These non-Otx binding sites would be motifs of the same length as the Otx binding site, but with different sequences. Therefore, information encompassed in the whole sequence was taken into account when undertaking the statistical calculation. In the current example, the sequence would be considered a population of Otx binding sites and non-Otx binding sites, ultimately summing up to all sites of the same length in

that sequence. The degrees of freedom for each G test, where N = the number of sequence categories being tested (with N designating, in the above example, Otx binding sites and non-Otx binding sites) was N -1 = 2-1 = 1. The statistical analysis was similar to that which would be performed to compare the predicted number of offspring bearing each phenotype to the observed number in a genetic cross. In that case, also, one desires to know whether the numbers of each phenotype, which ultimately sum up to all the phenotypes in the entire population of offspring, are statistically significant [138].

In terms of statistical significance, a p value cutoff of 0.10 was considered to be statistical significant. Although this was greater than the customary value of 0.05 [138], using a higher cutoff would provide greater assurance that no binding sites of interest, whose function could be confirmed or refuted by future experimental analysis, would be over-looked. As shown in Fig. 3.1, Appendix F and in the text below, the actual p values for all significantly represented transcription factor binding sites are provided in all cases.

The locations of potential binding sites for transcription factors of interest within the sequence of each active region are shown in Appendix D, which highlights each consensus sequence and also cites references from which these consensus sequences were taken.

Fig. 3.1. Number of potential binding sites in regions and subregions of *Sp-CycD* for selected transcription factors discussed in the text. For each transcription factor binding site in each regulatory region, both the predicted number and actual number of potential binding sites in each region are provided. Whether the difference between the predicted and actual number of binding sites for each transcription factor in each regulatory region was significant, as determined by a Goodness of Fit Test, is indicated in each graph by the p values appearing above different comparisons. If a p value is not shown, this indicates that the difference between actual and predicted number of a given binding site was not statistically significant. Statistical calculations were done as described in the current section (3.2) and associated Table 3.1.


Region 2
p<0.01
p<0.001
Subregion 2_2
p<0.001
Region 4
p<0.001
Subregion 4_1
p<0.001
Subregion 4_2
p<0.001
Region 5
p<0.01
p<0.01
p<0.001
p<0.01
Region 6
p<0.001
p<0.10
Subregion 5_1
p<0.001
p<0.001
p<0.001
Subregion 6_1
p<0.001
p<0.025
Region 17
p<0.10
p<0.001
Region 19
p<0.01
p<0.001
p<0.05
p<0.10
Subregion 19_1
p<0.001
p<0.10
Otx Gatae Bra Foxa Gatac Su(H) Runx TCF
Expected Observed
Number of sites

The expression profiles of transcription factors that could regulate the expression of *Sp-CycD* have been worked out [124]. The expression profiles of some of these transcription factors, taken directly from SpBase [3] are reproduced in Fig. 3.2.

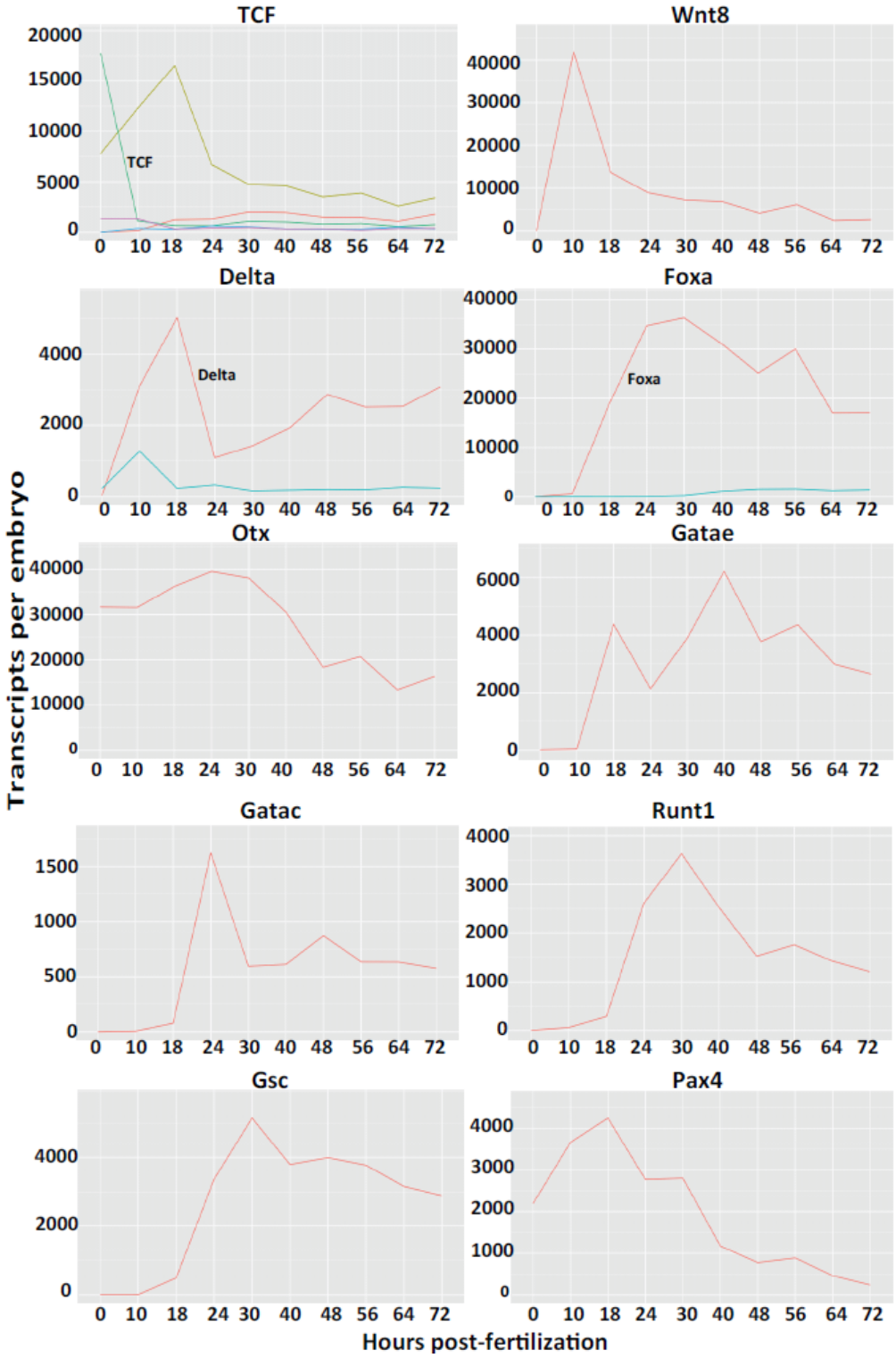


Fig. 3.2. Expression profiles of selected transcription factors discussed in the text. These expression profile graphs were taken directly from SpBase [3], and the original data are from Tu et al [124]. If multiple graphs are shown in a panel, the graph corresponding to the gene of interest is labeled.

3.3 Are transcription factors directly downstream of Wnt-beta catenin and Delta-Notch signaling regulators of *Sp-CycD* expression during embryogenesis?

In *S. purpuratus*, the developmental divergence of the endodermal and mesodermal lineages from endomesoderm (one of the two major areas, the other being oral ectoderm, where *Sp-CycD* expression becomes confined as embryogenesis proceeds [62]) is primarily directed by the Delta-Notch and Wnt-beta catenin signaling pathways [111, 140, 141]. Endodermal and mesodermal fates are attained by gradual activation of solely Wnt-beta catenin signaling in presumptive endoderm and Delta-Notch signaling in presumptive mesoderm [141]. Within presumptive mesoderm, Delta-Notch signaling inhibits expression of Hox 11/13B, which is a key transcription factor in an endoderm-specific gene regulatory subcircuit that contains the transcription factors *Bra, Foxa,* and *Blimp1b*. When allowed to be active, this regulatory subcircuit also leads to the maintenance of expression of the Wnt ligand. Furthermore, in presumptive mesoderm, Delta-Notch signaling triggers export of TCF transcription factors from cell nuclei. This makes these cells resistant to Wnt signaling, prevents them from becoming induced to become endoderm, and sets them on a developmental trajectory to become mesoderm [141]. Therefore, one role for Delta-Notch signaling within presumptive mesoderm is an inhibitory one: inhibiting the expression of genes involved in the specification of endoderm.

The above description would suggest that mesoderm formation induced through Delta-Notch signaling takes place solely through a passive process – the inhibition of Wnt signaling. However, Su(H), the transcription factor induced by Delta-Notch signaling, directly activates expression of the transcription factors HesC, Gcm and Gatae

in presumptive non-skeletogenic mesoderm [55]. Regarding presumptive endoderm, since Hox 11/13B is not inhibited by Delta-Notch signaling in this lineage, expression of the Wnt ligand is able to be maintained there. This activates beta-catenin, which interacts with the TCF transcription factor, converting it from an inhibitor to an activator of transcription of endodermal-specific genes. This further sets this region on a trajectory to become endoderm [141].

To gain insight into how the expression of *Sp-CycD* might be regulated during the specification of endoderm and mesoderm, the active regulatory regions within it were queried for possible binding sites for the above described transcription factors whose expression is regulated by Wnt-beta catenin and Delta-Notch signaling (Fig 3.1; Appendix D, Fig. D.1).

There is evidence, based on sequence analysis of active regions for potential TCF binding sites, that *Sp-CycD* expression is regulated by the Wnt-beta catenin-TCF pathway (Fig. 3.1; Appendix D, Fig. D.1; Appendix F, Fig. F.1). Of the active regulatory regions, regions 5 ($p<0.01$), 6 ($p<0.10$) and 19 ($p<0.10$) all have significantly more potential TCF binding sites than would be predicted by chance (Fig. 3.1; G-test results in Appendix F, Fig. F.1). Potential binding sites for TCF within region 5 all fall within subregion 5-1 (Appendix D), which, as described in Chapter 2 (Fig. 2.4), is an inactive subregion. This does not mean that these TCF sites are non-functional. The fact that there are 6 such potential sites within a relatively short sequence argues against that idea, as does the fact that this number of TCF binding sites in subregion 5-1 compared to the number predicted is clearly statistically significant (p value <0.001) (Fig. 3.1; Appendix F). Rather, it is hypothesized based on these findings that TCF is necessary but not sufficient to induce

the activity of region 5. Regarding region 6, all of the potential TCF binding sites fall within subregion 6-1, (Appendix D, Fig. D.1). In addition, like region 6, the number of TCF binding sites compared to the number predicted in subregion 6-1 is statistically significant ($p<0.025$; Fig. 3.1 and Appendix F, Fig. F.1). This finding supports the proposition that TCF may regulate the activity of region 6, and that of subregion 6-1 within it.

Region 19 has the greatest number of potential TCF binding sites of all the active regions (Fig. 3.1; Appendix D, Fig. D.1). In addition, the number of such sites is significantly more than would be predicted (p value < 0.10; see Appendix F, Fig. F.1; and Fig. 3.1). Therefore, TCF likely plays a role in regulating the activity of region 19. This hypothesis is further supported based on the locations of the potential TCF binding sites within active region 19 and inactive subregion 19-1. All but one of the 7 potential TCF binding sites fall outside of subregion 19-1 (Appendix D, Fig. D.1). Since region 19 as a whole is active, this finding further strengthens the hypothesis that TCF regulates the activity of region 19. As discussed below, region 19 contains binding sites for other potentially regulatory transcription factors as well.

To determine which regulatory regions might be regulated by Delta-Notch signaling, potential Su(H) binding sites in the regulatory regions of *Sp-CycD* were searched for based on the sequences of Su(H) binding sites given in a 2006 paper by Ransick and Davidson [137]. The only potential Su(H) binding sites were found within regions 2 and 17, which each bore one such site. However, this number was not statistically significant for either of these regions, as determined by a G test (Fig. 3.1;

Appendix F, Fig. F.1). Related to this, Region 19, which was predicted, based on its length to have ~2 Su(H) binding sites, bore none, significantly less than expected ($p < 0.05$). None of the active regions had any identified binding sites for the transcription factors HesC or Gcm, whose transcription within presumptive non-skeletogenic mesoderm is directly activated by Su(H) [55]. However, Su(H) also activates the expression of *Gatae* in non-skeletogenic mesoderm [55]. Region 19, which, as discussed later in this Chapter, could play an important role during gastrulation, when mesodermal cells, such as blastocoelar cells, delaminate from the archenteron [142], has significantly over-represented binding sites for Gatae ($p < 0.01$; see Fig. 3.1 and Appendix F). Therefore, Delta-Notch signaling could indirectly regulate the expression of *Sp-CycD* through region 19 by activating expression of Gatae.

There is additional evidence that Delta-Notch signaling could indirectly regulate the temporal transcription of *Sp-CycD*. As described near the end of section 3.4, the regulatory regions of *Sp-CycD* all contain many potential binding sites for Gatac at levels much greater than would be predicted by chance (see Fig. 3.1; in all cases, $p < 0.001$). Because this transcription factor is activated downstream from Delta-Notch signaling [143], Delta-Notch signaling could regulate the expression of *Sp-CycD* indirectly via this transcription factor.

In addition, Delta-Notch signaling could act in another capacity – an inhibitory one. As described above, Delta-Notch signaling during embryogenesis in sea urchin leads, within presumptive mesoderm, to the inhibition of a subcircuit containing the transcription factors Bra, Foxa, and Blimp1b that are involved in the specification of

endoderm. Of these, as discussed again below, Foxa is the transcription factor whose change in expression mediated by Delta-Notch signaling would most likely affect the expression of *Sp-CycD*, through region 5. This is because region 5 bears three potential Foxa binding sites, a statistically significant number ($p < 0.01$), since this region was not predicted to bear any such sites (Fig. 3.1). In contrast, Blimp1b binding sites are not found within any of the regulatory regions of *Sp-CycD* discovered in this analysis, and Bra is not statistically over or under-represented in any region.

The explanation for why cyclin D can be expressed in mesoderm may lie partly in the fact that, while TCF can act as a transcriptional activator, as it does when beta-catenin is triggered by Wnt signaling to translocate to the nucleus, in the absence of such signaling, TCF, by complexing with Groucho, acts as a transcriptional repressor [144]. Delta-Notch signaling can trigger export of TCF from the cell nuclei [141]. It is possible that Delta-Notch signaling, by triggering the export of inhibitory TCF from cell nuclei in mesoderm, removes this repressive barrier and allows *Sp-CycD* to be expressed in this lineage.

One way to test if Delta-Notch signaling regulates the expression of *Sp-CycD* would be to compare the transcript levels of cyclin D in control embryos to those in which Notch signaling was blocked. Notch signaling occurs when the binding of Delta ligand on one cell binds to the Notch receptor on an adjacent cell, triggering the enzyme gamma secretase to cleave an intracellular portion of the Notch receptor [145] . Since this signaling can be blocked by administering inhibitors of gamma secretase [145], it is

proposed that such inhibitors could be used to test the effect of inhibiting Notch signaling on the expression of *Sp-CycD* during embryogenesis.

3.4 Does a conserved subcircuit that regulates the specification of endoderm and mesoderm contribute to the regulation of *Sp-CycD* expression during embryogenesis in *S. purpuratus*?

In section 3.3, the roles of Delta-Notch and Wnt-beta catenin-TCF signaling in possibly regulating the expression of *Sp-CycD* was discussed. As noted, these pathways are important in inducing the formation of mesoderm and endoderm, respectively. Based on this theme – the relationship between regulation of expression of *Sp-CycD* and the formation of mesodermal and endodermal lineages, this section explores whether a conserved subcircuit within the GRN controlling the development of mesoderm and endoderm could regulate the expression of *Sp-CycD*. The conservation of this subcircuit was uncovered through a comparative study of the endomesoderm GRNs of the sea urchin *S. purpuratus* and the sea star *A. miniata* [130, 131]. This study revealed transcription factors of which both their identities and pattern of linkages to other transcription factors is conserved. These transcription factors included Blimp1, Otx, Bra, Foxa, Gatae, Gatac, and Bra [130]. The lineage specifying functions of these transcription factors were also conserved. That is, in both sea urchin and sea star, Blimp1, Bra and Foxa contribute to the specification of endoderm; Gatac contributes to the specification of mesoderm; and Gatae and Otx contribute to the specification of both endoderm and mesoderm [130]. An important purpose of the conserved subcircuit between sea urchin and sea star is to ultimately allow the expression of Gatae [131].

While, as just noted, this transcription factor is expressed in both mesoderm and endoderm, its expression is essential for the expression of regulatory genes expressed in the endoderm [130, 131]. A direct reproduction of a figure from the 2007 paper by this group is given in Fig. 3.3. Both the transcription factor genes and many of the linkages between them by which they regulate each other's expression are conserved in both sea urchin and sea star.

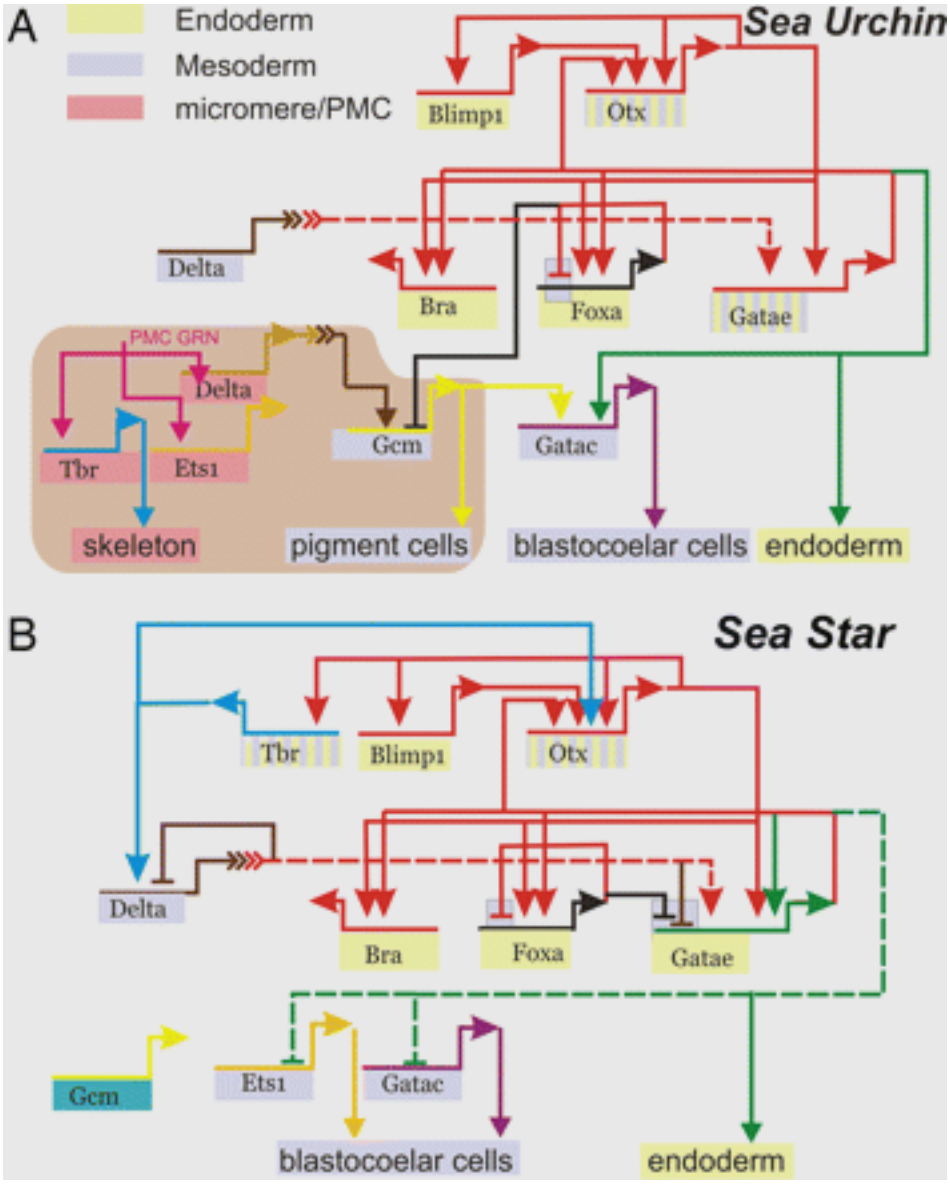


Fig. 3.3. The GRN subcircuit specifying endomesoderm in sea urchin and sea star. Taken from [130].

Since the sea urchin and sea star last shared a common ancestor ~500 million years ago [130], this conservation in terms of identity, linkages and functions of each of these transcription factors was considered to be remarkable [130]. Regarding the analysis presented in this Chapter, each regulatory region of *Sp-CycD* was queried for potential binding sites for transcription factors expressed in this conserved subcircuit (Fig. 3.1;

Appendix F, Fig. F.1). Within this section, each region is discussed separately for potential binding sites for all transcription factors expressed within this conserved subcircuit except for Gatac. Since all the regulatory regions bore significantly more potential binding sites for this transcription factor than would be predicted based on their lengths (Fig. 3.1; Appendix F, Fig. F.1), and since the number of binding sites were statistically significant in all cases (Fig. 3.1; Appendix F, Fig. F.1) the possible roles of this transcription factor in regulating the expression of *Sp-CycD* are discussed primarily at the end of this section.

Region 2 is notable for bearing 8 potential binding sites for Otx, which is expressed in the gut [130] ($p < 0.01$; Fig. 3.1; Appendix F, Fig. F.1), whereas it would be predicted to bear only 2 of these binding sites. As shown in Moore et al. [62], one of the lineages where *Sp-CycD* becomes confined as development proceeds is the gut. It is hypothesized that one of the regulatory regions responsible for this expression pattern is region 2, and that region 2, in part, mediates this through its Otx binding sites.

As described in Chapter 2, region 2 also bears within it an active subregion, 2-2, whose expression profile is similar in shape to that of region 2 (Chapter 2, Figs. 2.8 and 2.9). None of the potential Otx binding sites in region 2 are within the boundaries of subregion 2-2. These potential Otx binding sites in region 2 are likely to be important due to their statistical over-representation (Fig. 3.1; Appendix F, Fig. F.1; $p < 0.01$). Binding sites for Otx can also serve as binding sites for the transcriptional repressor Gsc [146]. It could be argued that lack of binding sites for a repressor, such as Gsc, may explain why subregion 2-2 has a higher activity profile than region 2. In terms of activating the activity of subregion 2-2, Gatac could play an important role, as potential

binding sites for this transcription factor are over-represented in region 2 ($p < 0.001$; Fig. 3.1; Appendix F, Fig. F.1). Of note, of the discussed transcription factor binding sites, only Gatac binding sites are significantly over-represented in subregion 2-2. Therefore, Gatac may be the only one of the discussed transcription factors that could be activating subregion 2-2.

Region 4 is most notable for containing an excess of potential Gatac binding sites ($p < 0.001$; Appendix F, Fig. F.1; Fig. 3.1). Region 4 does not bear an excess of actual to predicted binding sites for any other transcription factors conserved within the conserved endomesoderm-specifying subcircuit. This could indicate that the expression of this region is controlled primarily by Gatac. Alternatively, the fact that a regulatory region does not bear a statistically significant number of binding sites for a transcription factor of interest does not mean that the binding sites it does possess are non-functional. Indeed, the number of potential Runx binding sites in region 4 (2 actual vs. ~ 2 predicted; see Fig. 3.1; Appendix F, Fig. F.1) was not significant. However, as described in both Chapter 2 and section 3.5, one of these Runx binding sites has been confirmed previously to be functional. As described in Chapter 2, region 4 bears two subregions, 4-1 and 4-2, which were active (Fig. 2.8), although their temporal activity profiles were not compared quantitatively to that of region 4. It is of interest that two subregions separated by intervening sequence, as is the case for subregions 4-1 and 4-2 in region 4 (Appendix D, Fig. D.1) could both be functional, indicating that both could be separate CRMs.

Region 5 was of strong interest due to it having by far the most robust activity of all the active regulatory regions identified in *Sp-CycD*, showing statistically significant activity at all developmental time points examined from when *Sp-CycD* becomes induced

at ~10-12 hours post-fertilization (hpf) through mid-gastrula stage (Chapter 2, Fig. 2.3). These results would indicate that region 5 would have many linkages to transcription factors expressed in the endomesoderm GRN. The analysis of region 5's sequence for binding sites for such transcription factors indicates that, indeed, this may be the case. Region 5 bears six potential binding sites for Gatae (Fig. 3.1; Appendix D, Fig. D.1), although this number was not significantly more than the ~ 7 such sites predicted (Fig. 3.1; Appendix F, Fig. F.1). Region 5 also contains three potential binding sites for Foxa (Fig. 3.1) compared to none predicted (p value <0.01; Fig. 3.1; Appendix F, Fig. F.1). What is especially interesting regarding the potential Foxa binding sites is that region 5 is the only region with binding sites for this endoderm-specifying transcription factor (Fig. 3.1). The expression of this transcription factor commences at ~10 hpf (as shown at SpBase [3]), which would support the hypothesis that it could contribute to the induction of region 5's activity. The potential binding sites of Foxa are all within subregion 5-1 (Appendix D, Fig. D.1). The fact that this subregion is inactive does not mean that these Foxa sites are non-functional. Given their over-representation within this subregion, three sites compared to the zero predicted by chance ($p < 0.001$) (Fig. 3.1; Appendix F, Fig. F.1), that hypothesis is unlikely. Rather, it is proposed that the Foxa sites are necessary but not sufficient for the activity of region 5.

In a related finding, region 5 bears a potential binding site for the endoderm-expressed factor Bra. Although the possession of one such site was not statistically significant (Appendix F, Fig. F.1; Fig. 3.1), it could still be of interest. Along with region 6 (where the possession of a single potential binding site for Bra is likewise not statistically significant as shown in Appendix F, Fig. F.1; and Fig. 3.1), region 5 is one of

only two of the six regulatory regions that has a binding site for Bra. In support of a functional role of Bra in regulating the expression of regions 5 and 6, subregion 5-1, which is inactive (Chapter 2, Fig. 2.4) lacks a potential binding site for Bra, while subregion 6-1, which, like region 6, is active (Fig. 2.8) contains region 6's potential Bra binding site.

Region 5 also bears six potential binding site for Otx (Fig. 3.1), a significant number ($p < 0.01$; Fig. 3.1; Appendix F, Fig. F.1). Of interest, the potential binding sites for Bra and Otx fall in the regions located 5' and 3' to inactive subregion 5-1 (Appendix D, Fig. D.1). The majority of the other potential transcription factor binding sites in region 5 fall within subregion 5-1. From these findings, it is hypothesized that the transcription factor binding sites within region 5 that are within the boundaries of subregion 5-1 are necessary but not sufficient to allow the activity of region 5, and, by extension, of *Sp-CycD*. For region 5 to be activated, the above noted Bra and Otx sites, which are outside the boundaries of subregion 5-1, may be critical.

It would be informative to compare the spatial expression of region 5 to that of the other regions, and to test the effect of mutating the above noted transcription factor binding sites on that activity pattern. It would be predicted, based on its possession of binding sites for both Bra and Foxa, both of which are endoderm-specifying transcription factors [130], that region 5 would be more strongly expressed in endoderm than the other regions, but, due to also containing binding sites of transcription factors Gatac and Otx, (Fig. 3.1), that are expressed in mesoderm; and in both mesoderm and endoderm, respectively, would also be expressed in mesoderm. Indeed, region 5 may play an especially important role in allowing *Sp-CycD* to be expressed in both of these lineages.

As has already been partly discussed, region 6 has nearly the same contingent of transcription factor binding sites as region 5, with most of these sites falling within subregion 6-1, which shows a similar temporal expression profile to the whole of region 6. However, unlike region 5, region 6 does not include any site for Foxa. In addition, unlike region 5, which has significantly more than predicted potential binding sites for Otx, region 6 does not possess sufficient Otx sites compared to the number predicted to reach statistical significance (Fig. 3.1; Appendix F, Fig. F.1). These observations may explain why its expression is much lower, in absolute levels, than that of region 5. It would also be predicted that region 6, along with other regions that lack Foxa, might have less of a role in mediating the expression of *Sp-CycD* in endoderm than would region 5. This would be tested by examining spatial activity profiles of region-linked reporters.

Region 17, the region with the lowest activity level of all the regulatory regions (Chapter 2), is also notable for bearing five potential binding sites for a transcription factor from the conserved endoderm-mesoderm specifying subcircuit, Gatae. However, approximately 6 such sites were predicted, and the possession of five such sites was not statistically significant (Fig. 3.1; Appendix F, Fig. F.1). However, other than Gatac, Gatae provides the best candidate for functional analysis, simply because binding sites for several other candidate regulators were either missing, or were under-represented (Fig. 3.1; Appendix F, Fig. F.1). Otherwise, compared to the other regulatory regions of *Sp-CycD*, region 17 has the least number of potential binding sites for the above discussed transcription factors. This sparseness of binding sites for regulatory transcription factors may account for region 17 having the lowest activity of all discovered regulatory regions of *Sp-CycD*. This does not mean that this region has an

unimportant regulatory role. The fact that its activity continuously rises argues against this. The fact that its activity is low could in fact argue that this region plays an important role in mediating the spatial activity of *Sp-CycD* as this gene's spatial activity becomes increasingly restricted after gastrulation. This finding may relate to that of a *cis*-regulatory analysis done by Arone and Davidson from 1998 [147], where they showed that a *cis*-regulatory module required for expression of the *CyIIa* gene, which is expressed after most cell types have already been specified, is much simpler in structure than that of the *cis*-regulatory modules controlling the expression of genes that are expressed earlier in development, when territories are still being specified (as reviewed in a 1997 paper by the same authors) [148]. Region 17 becomes most active (by ~21 hpf, as shown in Chapter 2, Fig. 2.6), as *Sp-CycD* is becoming restricted to cells in well established territories, such as the gut and oral ectoderm [62]. Based on the work of Arone and Davidson just described, a relatively simple regulatory structure might therefore be expected of region 17.

Region 19 was most notable for having a distinctive temporal activity profile that reproducibly peaked at ~21 hpf, a time point that occurs shortly before gastrulation begins (Chapter 2, Fig. 2.6). As described in Chapter 2, region 19 contains a subregion, 19-1, which, by itself, is not functional. Located 3' with respect to subregion 19-1 is sequence that is rich in potential binding sites for various transcription factors of interest (Appendix D, Fig. D.1). Four of these transcription factors have numbers of potential binding sites that occur significantly more often than would be predicted by chance within the whole of region 19 (Fig. 3.1; Appendix F, Fig. F.1). It should be noted here that although region 19 possesses fewer potential Runx binding sites that would be

predicted based on its length (4 actual vs. ~ 5 predicted, a non-significant difference; Fig. 3.1), one of the potential Runx binding sites overlaps with a potential TCF binding site (Appendix D, Fig. D.1, toward 3' end of region 19). This finding is of interest because region 19 is the only one of the identified regulatory regions of *Sp-CycD* that shows this overlap between a potential Runx and TCF binding site. This overlap could indicate that this potential TCF site is functional, for reasons described in the next section. As described in section 3.3, TCF acts directly downstream of Wnt-beta catenin signaling that is involved in the specification of endoderm. Given that the activity of region 19 peaks at ~21 hpf, which just shortly precedes the beginning of gastrulation [43, 111], the overlapping potential Runx and TCF site in region 19 could contribute significantly to this temporal activity pattern.

A general observation is that none of the regulatory regions of *Sp-CycD* had any potential binding sites for Blimp1 (Fig. 3.1). However, this does not preclude the regulation of *Sp-CycD* transcription by this transcription factor. This is because within the endomesoderm specifying subcircuit conserved between sea urchin and starfish, the *Otx* and *Blimp1* genes regulate each others' expression through a positive feedback loop, in which each gene activates transcription of the other [130]. Blimp1 could thus regulate the expression of *Sp-CycD* indirectly by regulating the transcription of Otx, for which, as noted, regions 2, 5 and 17 have significantly over-represented potential binding sites (Fig. 3.1).

Of potential binding sites for transcription factors in the conserved GRN subcircuit, the most prevalent are those for TRANSFAC 4.0 flagged binding sites for Gata1 (Appendix D, Fig. D.1; Fig. 3.1). All the identified regulatory regions of *Sp-CycD*

possess statistically significant numbers of potential binding sites for this transcription factor ($p < 0.001$ in all cases; Fig. 3.1; Appendix F, Fig. F.1). These sites were hypothesized to mark potential binding sites for Gatac, since Gatac is a homolog of vertebrate Gata1/2/3 [136]. Gatac is expressed strongly in blastocoelar cells, which act as immune cells, as shown in unpublished work by Rast, and described in [130] and [142]. In addition, the transcription of Gatac is regulated by the Delta-Notch-induced transcription factor Gcm, and also, by another transcription factor within the conserved endomesoderm-specifying subcircuit, Gatae [130]. Delta-Notch activated Gatac has also been shown to be expressed in the non-skeletogenic mesoderm [143] from which the blastocoelar cells are derived [142]. Delta-Notch signaling could therefore contribute to the regulation of *Sp-CycD* expression in non-skeletogenic mesodermal-derived cells, such as blastocoelar cells, through activation of Gatac.

Also of interest, in several instances (Appendix D, Fig. D.1), the potential Gatac binding sites overlap with the binding sites for other transcription factors, including TCF, Gatae, Otx and Runx, indicating potential cooperative interactions. Since the marked potential Gatac sites are TRANSFAC-identified binding sites for Gata1, they may not all correspond to Gatac sites. However, any region that possesses such sites would have the potential to be expressed in blastocoelar cells. This could be readily tested.

3.5 Do Runx transcription factors regulate the expression of *Sp-CycD* during embryogenesis in *S. purpuratus*?

Runx transcription factors are developmentally important proteins that regulate transcription by interacting with other developmentally expressed transcription factors [132]. Moreover, Runx transcription factors interact with the two signaling pathways –

Wnt-beta catenin and Delta-Notch [132] – that, as described in section 3.3, are involved in the specification of endoderm and mesoderm. It was shown by Robertson et al. [63] that SpRunt1 binds to and regulates the expression of the *Wnt8* gene, which functions upstream of TCF. There is also evidence that Runx transcription factors regulate the expression of cyclin D genes. The embryonically expressed Runx gene *SpRunt1* shows an expression profile similar to that of *Sp-CycD*, being globally expressed at mesenchyme blastula stage, then becoming restricted mainly to gut and oral ectoderm [114]. In addition, as described by Robertson et al. [63], knockdown of *SpRunt1* leads to under-expression of *Sp-CycD*. Also, as described in Chapter 2, chromatin immunoprecipitation experiments indicate that SpRunt1 binds to one of the predicted Runx binding sites in region 4, within sequence corresponding to subregion 4-1. Along with this, potential Runx binding sites are distributed among several of the regulatory regions of *Sp-CycD*.

Since Runx transcription factors carry out their functions by interacting with other transcription factors, the binding sites of strongest interest included those that were adjacent to or overlapped for binding sites for other transcription factors discussed in this Chapter (see Appendix D). This is true for region 2, where, toward the 3' end, a potential Runx binding site overlaps with a potential Gatae site; and, as first introduced in the previous section, for region 19, where a potential Runx binding site overlaps with a potential binding site for TCF. Regarding the sequence site in region 19 where a potential Runx binding site overlaps with a potential TCF binding site (Appendix D, Fig D.1), there is reason to propose that this overlap could be functional, based on the findings and discussion presented by Robertson et al. [63]. In that study, the transcription

of the *Wnt8* gene was shown to be regulated by a *cis*-regulatory element in which a TCF binding site overlapped on its 3' end with a Blimp1 binding site. Since it was known that binding of TCF to can induce looping of that DNA, which in turn can cause nearby transcription factors that bind to sites in that loop to functionally interact with each other, it had been predicted by Minokawa et al. that just upstream of the TCF binding site, there existed the binding site for another transcription factor [63, 101]. Robertson et al. showed that this was a Runx binding site, and demonstrated that it was functional using site-directed mutagenesis.

The 3' end of the overlapping potential TCF and Runx binding sites in region 19 ends at position 4186 (Appendix D, Fig. D.1). Of interest, a potential binding site for Gatac was found about 50 bp from the 3' end of the overlapping potential Runx and TCF binding sites. There were also several other instances of Runx and Gatac binding sites being in close proximity, sometimes adjacent or overlapping (Appendix D, Fig. D.1). In addition, analysis of the region 19 sequence with TRANSFAC 4.0 revealed a potential binding site for C/EBPalpha from position 4182 to 4191 (data not shown), a position that overlapped with this potential Runx binding site. This latter finding was of interest because Puig-Kroger et al. (2003) [149] found that Runx and C/EBP transcription factors regulated the *CD11a* integrin gene in myeloid cells by binding to overlapping binding sites within the regulatory region of this gene. In *S. purpuratus*, blastocoelar cells, which, which, like myeloid cells, are immunocytes [142], delaminate from the tip of the ingressing gut [142]. Region 19, its activity peaking at ~21 hpf, could, in addition to perhaps acting as a switch to contribute to expression during gastrulation, also help activate expression during the differentiation of future blastocoelar cells.

It should be noted that the existence of a Runx binding site without any nearby binding sites for other transcription factors discussed in this Chapter does not diminish the potential importance of these sites. One example of such a site would be the earlier mentioned potential binding site for SpRunt1 in subregion 4-1, which does not overlap with or fall adjacent to any binding sites for the transcription factors discussed in this Chapter. There could be other, non-discussed transcription factors with which SpRunt1 could interact. In the case of the Runx binding site in region 4, this site extends from position 725-731 within this region. Analysis of the region 4 sequence for TRANSFAC 4.0 identified transcription factors revealed binding sites for several nearby transcription factors, including Sp1 and USF (data not shown). That the Sp1 and Runx binding sites could function together is based on the finding that an enhancer active in osteoblasts was bound by both of these transcription factors, although the binding sites were separated by about 25 bp [150]. From this discussion, it is argued that, although the regulatory regions of *Sp-CycD* each bear less than the predicted number of potential Runx binding sites (Fig. 3.1), at least some of these sites, including at least one in region 4, and perhaps those that may mediate the interaction of Runx with other transcription factors, either are, or could be functional.

3.6 Is *Sp-CycD* transcription during embryogenesis regulated by transcription factors involved in the specification of oral ectoderm?

During and after gastrulation, as noted, the expression of the cyclin D gene in the sea urchin becomes confined to the endomesoderm, oral ectoderm and ciliary band. In the previous sections of this Chapter, discussion focused on the transcriptional inputs

that might regulate the expression of *Sp-CycD* in the endomesoderm. The purpose of this section is to identify transcriptional inputs that could regulate the expression of *Sp-CycD* in another region where it becomes confined during and after gastrulation: the oral ectoderm. The structure of the GRN that contributes to the development of the ectoderm in *S. purpuratus* [55, 151] was more recently deciphered than that of the endomesoderm GRN [152]. The expression patterns of the transcription factors comprising this GRN are regulated by Nodal signaling, the distribution of which along the oral-aboral axis is regulated by Lefty and a mitochondrial redox gradient [153-155]. Among the transcription factors expressed within this GRN [151] that could regulate the expression of *Sp-CycD,* focus is made on *Pax41* and *Gsc*. These two transcription factors may play roles in regulating the expression of *Sp-CycD* by directly binding to its regulatory regions. With respect to Gsc, this transcription factor acts as a transcriptional repressor in the oral ectoderm [151], restricting the expression of a number of genes. In 2001, the Angerer lab showed if translation of *Gsc* was blocked, then both gastrulation and the separation of the ectoderm into oral and aboral lineages were blocked or inhibited [146]. Related to this finding, this transcription factor was shown to be expressed in some cells of the vegetal plate that later ingressed during gastrulation, and to be strongly expressed in lineages that became the oral ectoderm [146]. Further study showed that Gsc competed for the same binding sites as Otx, a transcription factor expressed throughout the ectoderm (along with endomesoderm, as described in section 3.4). By doing so, Gsc interfered with the function of Otx in presumptive oral ectoderm, and contributed to the development of this lineage. Since Otx and Gsc bind to the same sequence, at least some of the potential Otx binding sites in regulatory regions can also be hypothesized to be

potential Gsc binding sites. Regions that bear significantly greater than the predicted number of binding sites for Otx, and therefore, for Gsc, include regions 2 and 5 ($p < 0.01$ in both cases; Fig. 3.1; Appendix F, Fig. F.1). One observation that requires further analysis is that oral ectoderm is one of the areas where *Sp-CycD* expression becomes confined as development proceeds [62]. Given that Otx is a transcriptional activator and Gsc is a repressor, further work is needed to determine how each cooperate to regulate the expression of *Sp-CycD*.

As noted, another transcription factor involved in the specification of the oral ectoderm GRN, Pax4, is likewise a possible candidate for regulating the expression of *Sp-CycD* within the oral ectoderm. This transcription factor is expressed relatively early during development, with it showing its second highest expression level at 10 hpf, before peaking at 18 hpf (Fig. 3.2, taken from SpBase [3]). Related to this finding, a sequence within region 5, the region with the highest early activity, identified by Cluster-Buster [127] as an area where transcription factors might cluster was shown to have a ten closely spaced potential binding sites for mammalian Pax4, with some of these sites overlapping (Appendix D, Fig. D.1; Appendix E, Fig. E.1). Although the transcription factor binding sites identified by Cluster-Buster are not from *S. purpuratus*, human and mouse *Pax4* are both homologs to *Pax4* of *S. purpuratus* [51]. Therefore, the potential Pax4 binding sites identified by Cluster-Buster are putative binding sites for *Sp-Pax4*, which therefore may, by acting through region 5, medidate the expression of *Sp-CycD* in oral ectoderm. Region 5 and 17 have both been described as possibly contributing to the expression of *Sp-CycD* in the oral ectoderm, and they may divide their labor. Region 5 may function

early, as oral ectoderm is being specified, whereas region 17 may function later, as this territory becomes a discreet and mature part of the embryo.

Table 3.2 summarizes the major findings for each regulatory region discussed in both Chapter 2 and the current Chapter.

Table 3.2: Regulatory regions found in *Sp-CycD*, and their major points of interest
Note: This table encompasses three pages.

Region	**Location**	**Activity description and possible purpose**	**Subregions or CRMs found, and points of interest regarding them**	**Potential transcription factor binding sites of interest, and rationale for that interest**
2	Upstream	Begins by 10-12 hpf, peaks at ~21 hpf. May activate transcription at late blastula stage.	**Subregion 2-2**: May lack inhibitory **Gsc** binding sites. This may explain why this subregion appears to show more robust activity than region 2.	**Otx** binding sites are significantly over-represented, and may mediate activity in endoderm and mesoderm. Otx sites are also potential binding sites for inhibitory **Gsc**. **Gatac** binding sites, potentially activated via Delta-Notch signaling, may be responsible for activating these regions.
4	Upstream	Increases to relatively low but stable levels by 21-33 hpf, which is time of gastrulation. May contribute to maintaining activity during this time.	**4-1** and **4-2**	**Gatac** binding sites are significantly over-represented. **Runx** binding sites are not statistically over-represented, but a Runx site in subregion 4-1 was previously verified by ChIP to bind SpRunt1 and be functional.

Table 3.2 continued

5	Intronic	The most active region. Most active at 10-12 hpf, when *Sp-CycD* is becoming activated. Activity then declines somewhat but region 5 remains the most active of all regions. May divide labor with region 17. See below.	Contains inactive subregion **5-1**. 5-1 may be inactive due to not having significantly over-represented Otx binding sites and bearing no Bra sites.	Bears significantly over-represented binding sites for **Otx, Foxa, Gatac** and **TCF**. Region 5 is the only region to bear binding sites for endoderm-specifying **Foxa**. All these Foxa sites are within inactive subregion 5-1, so may be necessary but not sufficient for region 5's activity. Bra binding sites are not over-represented but may be required for activity, since inactive subregion 5-1 lacks a Bra binding site. Area in need of further investigation: The Otx binding sites are also potential binding sites for inhibitory **Gsc**. Gsc is expressed in oral ectoderm, where *Sp-CycD* is also known to be expressed. *Sp-CycD* may be able to be expressed in oral ectoderm because region 5's activity has declined by the time of specification of this domain. See further information regarding region 17 in this table.
6	Intronic	Has second strongest activity after region 5. Active early, when *Sp-CycD* is being activated, then remains stably active after ~33 hpf, perhaps contributing to maintaining activity after then.	Subregion **6-1** may bear all sequences needed for activity of region 6.	Bears almost the same contingent of transcription factor binding sites as region 5, but lacks **Foxa** sites. This could explain why this region is less active than region 5.

Table 3.2 continued

17	Intronic	Has the lowest activity of all regions but is of interest because its highest and maintained activity occurs after ~21 hpf through at least 45 hpf, when *Sp-CycD* expression is becoming restricted to gut and oral ectoderm.		Has sparsest number of binding sites for lineage-specifying transcription factors of all active regions. This may relate to this region playing a role in regulating *Sp-CycD* expression as it becomes spatially restricted. Regions, such as region 5, which many more transcription factor binding sites, may play role in activating *Sp-CycD* expression. In contrast to region 5, bears significantly fewer than predicted number of **Gsc** binding sites. Region 17 may therefore allow *Sp-CycD* to be expressed in oral ectoderm.
19	Intronic	Has reproducible activity pattern that peaks at ~21 hpf, shortly before gastrulation begins.	Bears inactive subregion 19-1.	3' end is rich in binding sites for various transcription factors. In particular, the **TCF** binding sites may be of interest, especially one that overlaps with a potential **Runx** binding site. Although Runx binding sites are under-represented, region 19 is the only region to show an overlap between a potential Runx and TCF binding site. This TCF site could function to regulate activity just before the onset of gastrulation, when this region reaches peak activity. This same Runx site also overlaps with potential **C/EBPalpha** site. Since Runx and C/EBPalpha transcription factors regulate development of myeloid cells, this Runx-C/EBPalpha site could regulate expression in blastocoelar cells, which ingress shortly after region 19's activity peak.

3.7 Some limitations to this study

It can be seen that of 22 potential regulatory regions identified by sequence conservation (Chapter 2, Fig. 2.3), only 6 were shown to be active during embryogenesis through gastrulation. There could be at least three reasons for this finding. First, it is possible for postulated regulatory regions that are identified computationally to be inactive in the analyses carried out here to still be functional [106]. In addition, some of the regions identified as inactive might function as repressors. This possibility was not tested during the *cis*-regulatory analysis of *Sp-CycD* because the method of Nam et al. used to test the activity of potential regulatory regions can only be used to identify positively acting regions, but not repressors [120]. Second, it is also possible that some of the regions shown to be inactive during embryogenesis could play a role in the expression of *Sp-CycD* in the adult. A third reason concerns the fact that all regions chosen to be tested for analysis were hypothesized, due to possession of various potential regulatory elements within their sequences, to be potentially regulatory. As described in Chapter 2, the activity values of all of these regions were used to determine a "background" level of region activity. Regions whose activities were at least 2.5 times greater than this background level were considered to have statistically significant activity. This statistical criterion was based on that used by Nam et al. in the 2010 high throughput identification of *cis*-regulatory modules [120]. However, in that study, the authors did not pre-select regions that were hypothesized to be active. Instead, regions to be tested for activity were selected at random. In this dissertation, then, only the most active regions in a population of regions already hypothesized to be active were being

tested. Therefore, it is possible that some regions with relatively low activity may have been scored as inactive.

3.8 Potential Future Work: Testing if *Sp-CycD* regulates the expression of developmental genes

Apart from acting as a regulator of the cell cycle, as introduced in Chapter 1, there is evidence that genes of the cyclin D family can regulate the transcription of other genes. The weight of the evidence indicates that cyclin D proteins accomplish this by undergoing protein-protein interactions with transcription factors and other DNA interacting proteins rather than directly binding to DNA. These interactions can then induce the transcription of genes whose regulatory regions are bound by these factors. For example, Bienvenu et al. [80] showed that cyclin D1 was associated with the promoters of genes that were being expressed in the tissues being examined. However, cyclin D1 was also shown to interact with transcription factors whose consensus binding sites were found within the promoters that were shown to be bound by cyclin D1. From this, it would be concluded that, rather than binding to these genes directly, cyclin D1 bound to these genes through recruitment by these transcription factors.

In a recent study by Paulkin and Vallier [156], the protein-protein interactions of cyclin D family genes were related to the two, at first thought, disparate roles of cyclin D genes in regulating both the cell cycle and development. Working with pluriopotent stem cells, the authors showed that these cells could be coaxed via growth factors to be more likely to differentiate into endoderm or into neuroectoderm, depending on the levels of cyclin D proteins within those cells. Moreover, these cyclin D proteins carried out their regulatory functions through their "classical" roles of activating cdks 4 and 6 within

the cytoplasm. When active, these SMAD proteins translocated to the nucleus and induced the transcription of genes whose protein products led to the development of endoderm. Phosphorylation of these SMAD proteins by the cdks led to their degradation and prevented them from translocating to the nucleus to contribute to the formation of endoderm. In this case, the cells would activate transcription factors that led instead primarily to the formation of neuroectoderm. However, if cyclin D protein levels were low, then endoderm-specifying transcription factors would be more able to translocate to the nucleus, and the stem cells would be more likely to differentiate into endoderm. Which developmental program – the formation of neuroectoderm or endoderm – was set in motion depended on levels of cyclin D proteins, which in turn, depended on the stage of the cell cycle. Therefore, cyclin D proteins, through protein-protein interactions, can function to link the stage of the cell cycle in which cells receive signals to the developmental program that those cells undergo.

From this summary, a larger theme emerges. Cyclin D family proteins interact with multiple proteins both within the nucleus and in the cytoplasm. Through these interactions, they can modulate the expression of genes, which in turn regulates developmental outcome. In this dissertation, the primary focus was on elucidating the inputs into *Sp-CycD* that regulate its expression. However, as is suggested from the above described studies, this gene, as a member of the cyclin D family of genes, likely has regulatory outputs into developmental regulatory genes. Within *S. purpuratus*, the cyclin D gene *Sp-CycD* also plays an important developmental role, as shown by Moore et al. [62].

It would also be important to identify and confirm the genes whose expression was regulated by cyclin D. This could be accomplished by using morpholino antisense oligonucleotides to knockdown *Sp-CycD*, similar to that done by Moore et al. [62], then using either quantitative RT-PCR or the more recently developed Nanostring technology [157] to measure the resultant levels of all developmental regulatory genes of the endomesoderm GRN. The data gained from these experiments could be related to those gained from the experiments just described where the protein binding partners of *Sp-CycD* were determined. In particular, it could be determined if the regulatory regions of genes whose expression was shown to be significantly affected by the knockdown of *Sp-CycD* have binding sites for any of the transcription factors shown to interact with *Sp-CycD*. These experiments would further complete our understanding of how *Sp-CycD* fits into the developmental GRNs of *S. purpuratus* by complementing the *cis*-regulatory analysis that was the primary focus of this dissertation.

3.9 Conclusions

This dissertation presented a *cis*-regulatory analysis of the *Sp-CycD* gene during embryogenesis in *S. purpuratus*. Regulatory regions that were proposed to regulate the expression of *Sp-CycD* during development were identified and characterized. In this chapter, further analysis was done to identify the developmentally regulated transcription factors that could mediate the expression of this regulatory gene. This work and analysis presented in this dissertation is pertinent because genes of the cyclin D family are developmental regulatory genes, acting as signal-controlled regulators of cell growth, the cell cycle, and development (Chapter 1).

This work is the first to provide a comprehensive *cis*-regulatory analysis across the entire locus of a cyclin D gene. The analysis identified several regions, both upstream and downstream of the locus, that were experimentally verified as regulatory regions. In this final Chapter, potential linkages between these regions and the developmental lineages where *Sp-CycD* is expressed were identified. This provides the foundation for experimentally testing each of these linkages in order to integrate this developmentally important gene into the GRNs that control embryogenesis in the important model organism, *S. purpuratus*.

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APPENDIX A: LIST OF GENES REFERENCED

Table A.1: Genes referenced in this dissertation

Notes: 1. The name used in the text is given, along with the species in which the gene being referenced was described, followed by the official name, provided by either NCBI Gene [2] for all genes except for those described in *Strongylocentrotus purpuratus*, or SpBase [3] for genes described in *S. purpuratus*. 2. If the name given in the text is a protein, or is written out in full, then that name is not italicized. Italicized names given under Official Names refer to genes rather than proteins. 3. If a gene family containing multiple members is mentioned, but the individual members are not individually described, then, generally, these are not provided in this table, although one example may sometimes be provided.

Name used in text	Species	Official name	GeneIdentier
AML1 (RUNX1)	*Mus musculus*	*Runx1*	12394
B-MYB	*Mus musculus*	*Mybl2*	1785
Bra	*S. purpuratus*	*Sp-Bra*	SPU_013015
Cdc25 phosphatase[1]	*Mus musculus*	*Cdc25c*	12532
C/EBPalpha	*Mus musculus*	*Cebpa*	12606
CLN3	*Saccharomyces cerevisiae*	*CLN3*	1201
cyclin A	Clam	Not found	
Cyclin A	*S. purpuratus*	*Sp-CycA*	SPU_003528
Cyclin A1	*Mus musculus*	*Ccna1*	12427
Cyclin A2	*Mus musculus*	*Ccna2*	12428
Cyclin B1	*Mus musculus*	*Ccnb1*	268697
Cyclin B2	*Mus musculus*	*Ccnb2*	12442
cyclin B	*Lytechinus pictus*	Not found	
Cyclin B	*S. purpuratus*	*Sp-Cycb*	SPU_015285
Cyclin D	*Arabidopsis*	Not found	
Cyclin D	*C. elegans*	*cyd-1*	174941
Cyclin D	*Drosophila*	*CycD*	32551
Cyclin D	*S. purpuratus*	*Sp-CycD*	SPU_007013

Table A.1 continued

Name used in text	Species	Official name	GeneIdentier
Cyclin D1	Chinese Hamster	*Ccnd1*	100689063
Cyclin D1	*Mus musculus*	*Ccnd1*	12443
Cyclin D1	*Xenopus laevis*	*ccnd1-a*	379937
Cyclin D2	*Mus musculus*	*Ccnd2*	12444
Cyclin D3	*Mus musculus*	*Ccnd3*	12445
Cyclin E	*Mus musculus*	*Ccne1*	12447
Cyclin E	*Drosophila*	*CycE*	34924
Cyclin E	*C. elegans*	*cye-1*	172399
cyclin dependent kinase 2 (CDK2)	*Schizosaccharo-myces pombe*	*cdc2*	2539869
Cyclin dependent kinase 2 (CDK2)	*C. elegans*	*cdk-2*	171911
Cyclin dependent kinase 2 (CDK2)	*Mus musculus*	*Cdk2*	12566
Cyclin dependent kinase 4 (CDK4)	*Mus musculus*	*Cdk4*	12567
Cyclin dependent kinase 4 (CDK4)	*C. elegans*	*cdk-4*	181472
Cyclin dependent kinase 4 (CDK4)	*Homo sapiens*	*CDK4*	1019
Cyclin dependent kinase 6 (CDK6)	*Mus musculus*	*Cdk6*	12471
Cyclin dependent kinase 6 (CDK6)	*Homo sapiens*	*CDK6*	1021
CyIIIa	*S. purpuratus*	*Sp-CyIIIa*	Not found
DP	*Drosophila*	*Dp*	36461
Delta	*S. purpuratus*	*Sp-Delta*	SPU_06128
E2F	*Drosophila*	Look up	
E2F[2]	*Mus musculus*	*E2f1*	13557

Table A.1 continued

Name used in text	Species	Official name	GeneIdentier
E2F1	*Mus musculus*	*E2F1*	13557
E2F4	*Mus musculus*	*E2f4*	104394
Endo16	*S. purpuratus*	*Sp-Endo16*	SPU_011038
ERB2	*Mus musculus*	*Esr2*	13983
Foxa	*S. purpuratus*	*Sp-FoxA*	SPU_006676
Gatac	*S. purpuratus*	*Sp-GataC*	SPU_027015
Gatae	*S. purpuratus*	*Sp-Gatae*	SPU_010635
GRIP1	*Mus musculus*	*Grip1*	74053
Gsc	*S. purpuratus*	*Sp-Gsc*	SPU_015982
HES6	*Mus musculus*	*Hes6*	55927
Histone deacetylase 1	*Mus musculus*	*Hdac1*	433759
Lef1	*Homo sapiens*	*LEF1*	51176
Lef1	*Mus musculus*	*Lef1*	16842
MEF2C	*Mus musculus*	*Mef2c*	17260
MTOR	*Mus musculus*	*Mtor*	56717
MTOR	Homo sapiens	*MTOR*	2475
MYT1	*Mus musculus*	*Myt1*	17932
Notch1	*Homo sapiens*	*NOTCH1*	4851
NOTCH1	*Mus musculus*	*Notch1*	18128
Notch	*S. purpuratus*	*Sp-Notchh_11*	SPU_015792 (1 of several homologs)
Nodal	*S. purpuratus*	*Sp-Nodal*	SPU_11064
Otx	*S. purpuratus*	*Sp-Otx*	SPU_010424
P16INK4a	*Mus musculus*	*Cdkn2a*	12578
P19ARF	This is derived from same locus as P16INK4A, but has alternative reading frame [158].		

Table A.1 continued

Name used in text	Species	Official name	GeneIdentier
P53	*Homo sapiens*	*TP53*	7157
P107	*Homo sapiens*	*RBL1*	5933
P107	*Mus musculus*	*Rbl1*	19650
P130	*Homo sapiens*	*RBL2*	5934
P130	*Mus musculus*	*Rbl2*	19651
Pax4	*S. purpuratus*	*Sp-Pax4*	Not listed
Retinoblastoma (Rb)	*Mus musculus*	*Rb1*	19645
Retinoblastoma (Rb)	*Homo sapiens*	*RB1*	5925
Runx1	*Mus musculus*	*Runx1*	12394
Runt1	*S. purpuratus*	*Sp-Runt1*	SPU_006917
SM50	*S. purpuratus*	*Sp-Sm50*	SPU_018811
Sp1	*Homo sapiens*	*SP1*	6667
Stat3	*Mus musculus*	*Stat3*	20848
STAT5	*Homo sapiens*	*STAT5*	50695
Su(H)	*S. purpuratus*	*Sp-SuH*	SPU_021566
TCF	*S. purpuratus*	*Sp-Tcf*	SPU_009520
Telomerase	*Homo sapiens*	*TERT*	7015
Telomerase	*Mus musculus*	*Tert*	21752
TGFA	*Homo sapiens*	*TGFA*	7039
WEE	*Mus musculus*	*Wee1*	22390
Wnt6	*S. purpuratus*	*Sp-Wnt6*	SPU_13570
Wnt8	*S. purpuratus*	*Sp-Wnt8*	SPU_020371

APPENDIX B: PRIMER SEQUENCES

Table B.1: Primer sequences

Notes: 1. In each case, the forward primer is shown before the reverse primer. **2.** The primers shown below were those used for linking potential regulatory regions of *Sp-CycD* to 13 tag reporters by fusion PCR. The nucleotides colored red in each reverse primer do not anneal with the *Sp-CycD* gene, but enable integration with a 13 tag reporter construct during fusion PCR. **3.** As noted in Materials and Methods, to generate PCR products for incorporation into EpGFPII rather than linkage to 13 tag reporters, the forward primer in each case is preceded on its 5' end with the modification 5'-CTATCGATAGGTACC. For the reverse primer, the 5'-TTGAAGTAGCTGGCAGTGACGT modification is replaced with 5'-ACAGTTTAACCCGGG.

A. For amplifying the indicated tested regions of *Sp-CycD*:

1: CAGATAAGATGTGAAGTGATGTTGG and
TTGAAGTAGCTGGCAGTGACGTAAGTAAATTTTGTTTTGGCCTGA
14: ACATGCAGTCAGGCCAAAAC and
TTGAAGTAGCTGGCAGTGACGTTTCCCCTGGCTACCAGTATG
2: GTAGCCAGGGGAATCGTGT and
TTGAAGTAGCTGGCAGTGACGTTCTGCAATCTTTGCTCACTTT
14: ACATGCAGTCAGGCCAAAAC and
TTGAAGTAGCTGGCAGTGACGTTTCCCCTGGCTACCAGTATG
15: GGTGTGGAACCATAGCCGTA and
TTGAAGTAGCTGGCAGTGACGTGAGAGAATGTGAAAGAGATAGAGAAGG
3: CGTTTCAAATGTACTTTTAATGAAGC and
TTGAAGTAGCTGGCAGTGACGTATTTGGCCTAGGCAACAGTG
16: ACAAAATGACGTGATCTATAGGC and
TTGAAGTAGCTGGCAGTGACGTTCAATATTGGGAGGACTGTGC
4: TTAATAAATGCGCACAGTCCTC and
TTGAAGTAGCTGGCAGTGACGTTGGAATGGGTTATTTATTTCTGTTC
17: AGTATTTTTCACTTTTCTCGGTTTCAA and
TTGAAGTAGCTGGCAGTGACGTCTGCAGAAAACAAACAAAAAGA
5: ACTCGTAAGTATTTCCATTTTTGG and
TTGAAGTAGCTGGCAGTGACGTCTAGGCTATTGAGGGCTTAGAG
18: AGAACAAAGAGACTGGTTTGTCG and
TTGAAGTAGCTGGCAGTGACGTAAGCTTTTGCACTTTGTATTTGG
6: CAGACGGAGTTGTCATAGTT and
TTGAAGTAGCTGGCAGTGACGTATTTCTGTGAATTGGGAAGAAAA
7: ACAGGTAAGCCAAACCCGTCCT and
TTGAAGTAGCTGGCAGTGACGTAGAGTAGAGGGGGAAAGAG
8: ATCTTCGGAATGGATTGTGG and
TTGAAGTAGCTGGCAGTGACGTAGAACCAGTGGAAGCACACC
19: AACCGTAAGTACATTTTATTTGTT and
TTGAAGTAGCTGGCAGTGACGTTTTACTTGGTACACTTCCAGCTT
9: TTTGATGATGCAATAAAGAAAGAAA and
TTGAAGTAGCTGGCAGTGACGTTAAATGTAACTTTGTACAGGCTGTTTG

20: CATCACGGATATCTCCAATTCC and
TTGAAGTAGCTGGCAGTGACGTCGAACCAGACTCAGAGACTATCAT

Table B.1 continued

10: TGAAGTCTCAACTTCCCAAGTAGT and
TTGAAGTAGCTGGCAGTGACGTTGTAAATGGCGAGAAGAAAAA
11: ATGTGCCATAATTCTAAAGAGACAA and
TTGAAGTAGCTGGCAGTGACGTTCGCTATCACCACCATCTTC
21: TGATTATGGGGATGATGCAC and
TTGAAGTAGCTGGCAGTGACGTTTCTGACATTCTGACAACGTG
12: TTAATGCACAAATCTTTGTTAAGTGC and
TTGAAGTAGCTGGCAGTGACGTCGAGAGGGAGAGAGAGGGAGAGAAAG
22: TCCCCTTTCTCTCCCTCTCT and
TTGAAGTAGCTGGCAGTGACGTCCCCTTAACTACGCCACGTC
13: GTTATCGACGTGGCGTAGTT and
TTGAAGTAGCTGGCAGTGACGTAACAAATAGAAAAGAAAGAAAGAACGA
2-2: GCCTTGCCCTAAATATTGAAATT and
TTGAAGTAGCTGGCAGTGACGTAGTTGACCCGACAAAGGAAG
4-1: TGAATACACAAATGAACAAAGG and
TTGAAGTAGCTGGCAGTGACGTCTACTGTACACATCGACCAC
4-2: GGAGCCTGGGTTGAAAGAA and
TTGAAGTAGCTGGCAGTGACGTGGGGAACAGCAGACGACCAG

B. For amplifying the versions of the 13 tag reporters used for this project

new_mNBP:
ACGTCACTGCCAGCTACTTCAACTTGGAAGGTAAGGTCTCAAGTATTTAAGATTGAGGGCTCACG
GGCACCTTCtcatcttacaagtgaatcacaa

end_core-polyA: CACAAACCACAACTAGAATGCA

APPENDIX C: LISTING OF REGULATORY REGIONS TESTED AND THE 13-TAG REPORTER TO WHICH EACH WAS LINKED

Table C.1: Listing of regulatory regions tested and the 13-tag reporter to which each was linked

Region or subregion	13-tag reporter to which region or subregion was linked
1	1308
2	1301
3	1314
4	1310
5	1308
6	1304
7	1305
8	1309
9	1307
10	1313
11	1305
12	1306
13	1314
14	1314
15	1308
16	1301
17	1309
18	1310
19	1306
20	1310
21	1307
22	1306
2-2	1306
6-1	1304
5-1	1308
19-1	1306

Notes:

1. Regions linked to the same 13-tag reporter were never analyzed in the same experiment.
2. 13-tag reporters 1303 and 1312 did not show expression when linked to active region 2 (data not shown), so were not utilized.

APPENDIX D: SEQUENCE DETAILS OF ACTIVE REGULATORY REGIONS

Figure D.1. Sequence details of active regulatory regions of *Sp-CycD*.
Each sequence is shown separately in FASTA format, respectively as region 2 (panel A), region 4 (panel B), region 5 (panel C), region 6 (panel D), region 17 (panel E) and region 19 (panel F). Sequences conserved with *Lv-CycD* are shown in red font; sequences that show at least 90% similarity to *Lv-CycD* are in red font; and sequences identified by Cluster-Buster [127] as having potential binding sites for clusters of transcription factors are highlighted in gray. Within each region, subregions described in the text are shown as composites of italic, bold and underlined font. (Note: The sequences for upstream regions 2 and 4 are from clones. Those of others are from GBrowse V3.1, at SpBase [3].) Other sites of interest include binding sites for transcription factors found in an endomesoderm-specifying subcircuit conserved between sea urchin and sea star [130, 131], and described in Chapter 3. These include the following transcription factors, whose potential binding sites are highlighted using the indicated colors: Otx (TAATCC, TAATCT, and the reverse complements GGATTA, AGATTA) (consensus binding sites provided in [159, 160]); Gatae (C/T)GATA(A/G), and the reverse complement (C/T)TATC(A/G) (cited in [161]); and Foxa (reverse complements of AAATGTTAATTT, GCCTATTGATTT, and ACCTATTTTTTC, as identified by Cluster-Buster [127] flagging of vertebrate Foxa2 sites but not identified by Transfac Public at the site [135]). The original (non-reverse complement) sequence binding sites identified by Cluster-Buster were not found in any sequence. There were no identified binding sites for Blimp1 (GTTCCCTTT, or its reverse complement AAAGGGAAC) (binding site given in 2008 paper by Robertson et al. [63]). Potential Su(H) binding sites were identified by searching for the consensus Su(H) sequences presented in a 2006 paper by Ransick and Davidson: CGTGAGAA, CGTGGGAA, GGTGGGAT, GGTGAGAA, and GATGGGAG [137], along with their reverse complements: TTCTCACG, TTCCCACG, ATCCCACC, TTCTCACC, and CTCCCATC. There were no identified potential binding sites for Hesc (CACGCGTG, and its reverse complement CACGCGTG) [cited in [123], whose transcription is activated by Su(H), as shown in the endomesoderm GRN [55]. There were also no potential binding sites ((ATGCGG(A/G)(T/C)) and reverse complement ((G/A)(C/T)CCGCAT)) for another direct transcriptional target of Su(H), Gcm [cited in [137]. Potential binding sites for another transcription factor whose expression is induced by Su(H), Six1/2, were searched for by querying for the consensus sequence TCAGGTTTC and its reverse complement GAAACCTGA, which is just one of several potential binding sites of this recently found to be promiscuous-binding transcription factor [cited in [162]. No such sites were found in any regulatory sequences. Potential binding sites for Bra were identified by searching for the consensus sequence (A/G)(A/T)(A/T)NTN(A/G)CAC(C/T)T and its reverse complement A(G/A)GTG(T/C)NAN(T/A)(T/A)(T/C)[134]. This consensus sequence was searched for using an online consensus sequence finder [163, 164]. The binding site TGGGTGGTC and its reverse complement GACCACCCA for the hedgehog signaling-induced transcription factor GliA were searched for based on the known binding site of the human homolog, Ci (binding sequence provided in [165]). No such sites were found in any active sequences of *Sp-CycD*. Transfac-identified [135] binding sites for Gata-1 ((T/G/A)(T/A)(G/C)AGACT(T/A)AGCT(T/G)), and its reverse complement), which is a

Figure caption of Fig. D.1 continued

homolog of Gatac (cited in [136]), are highlighted in dark green. Potential TCF sites (ACAAAG and its reverse complement CTTTGT) (cited in [63]) are highlighted in light green. The following consensus sequences, highlighted in yellow, were considered potential Runx binding sites: TGTGGT and its reverse complement ACCACA (based on consensus binding site provided in reference [63]); and (C/T)G(C/T)GGT(C/T) and its reverse complement (A/G)ACC(A/G)C(A/G), the consensus binding site for Runx an early paper characterizing these transcription factors [125]. Two other transcription factors discussed in the text include Gsc and Pax41. Gsc, a competitor with Otx, binds to the same binding sites as Otx [146]. Therefore, Otx binding site can also be considered as Gsc binding sites. Binding sites for **Pax4** are not shown individually in this figure. As shown in Appendix E, analysis of the sequences of the regulatory regions using Cluster-Buster [127] yielded potential binding sites for this transcription factor in region 5, at the following positions within this region: 509-538; 626-655; 1045-1074; 1047-1076; 1048-1077; 1210-1239; 1214-1243; 1490-1519; 1491-1520; and 1492-1521. These areas are distinguished in the figure by **increasing their font sizes to 16** rather than 11 used in the rest of the figure. These areas are also highlighted in the Cluster-Buster output from the analysis of region 5 in Appendix E. These regions appear within the bp identified by Cluster-Buster as potential areas where transcription factor binding sites might cluster, which, as noted above, are highlighted in gray.

Note: Sequences labelled as indicated in the above two page figure legend begin on the next page.

Fig. D.1 continued

A.

>Region 2 [derived from sequencing of reporter construct]

GTAGCCAGGGGAATCGTGTCAACATTTCTGTTTAATAGAAAAAACAGTCAAATATTCATATTTTA
ATCGCTCAAGATCTTGGATCCCGCCACCCCCCCCCCCCCCATATATTGCCTATACTTATAGAGAA
AGCAAATCAATATATTTGATATAGTCGTACACATATACATGCAATGACCTTTGAACAACCATCTA
GAGGCCTATAAAGCCATGACTGCGATGAAAGGAACCGGTAGGCCTTTCTGTGTTTAGAGCTACTT
TTGTCTTGTTGTGCTTTGTTGTTTATATGTTTTTGTTTATTGTAATCCTTGACGGCATAATATTG
AAGGTCTCTAATTATGAACCCTCGACCTATCCACCAAGGCACAATGTGTCGGAACGTGAGAAAGG
GCTGTAACTGATACGCTATTTCCTCTCATAAATGCTTTTTATGGGTAACATTAAATTAGAGAACC
CACGCAATGTGAAAAACCTTTTAGTGTATAATATTTTAATGCGCATTTCCGATTGTGGCATCGGC
AAATATACATGGACAAACAGGAAAGCCAGCGATATATACATACTTAATTCTATAGATATGGGATT
GCGTGATTTGTCTTGAATTCAGATGAGTGTAGAAGTTGTCAACTACGATGAAAAAGTGAAATTCC
GAGAAAACAAATGCTAAACTAAAGATCGCATACTCTGAAAGTATACATAGTTTGTCTGCATTATG
ATAATAATGCTCTGCAAAGCACATTACTATAATGAGCAATACGAGTTATTAGTTTTTTTCATATC
CTATAGTCACCGGTGCCCTTACAGTTGGAATAATTTGTTTCTTGCCTATTTTCATGAAATTATTT
GGAAAATAGGGTTTATACTTGATAAGTAAAGTTCATATCCCCTCAGAACTTTCCTGACCAATAAC
AATCGATAAAGTCCTGAGAAGAGGTAAACTTTATTTATCATGGGGAGCTAAATCATTATACTGCC
ACAATAAATTCATGAAAAGAGATTAAAAAAATATTATTCCAATTTTATGGGTTACAGTGTCGTTA
TAACTATAGTCATTTTATGCCCTATCACTCTTTATTATACATTATTGTGTACGAAATGTTCTTTC
ATTCATCAACATGGCTCAGTGGTAGAGCAGTGGTCCCGTAACCGGAAGGTCCCGGGTTCGAAATC
TATTCGATACGCTATATAGTGTCATTTTGTTAGGCATTGATCCTCATTGCCAAGTCCCTCCGAGA
AGAAGTTAAAGCCGTCGGCCCGCGTTGCTTATAATACATACACATTGTTTCTATGCAGTCGGAAA
AAAATTAACAAACCAATAATTATTTATAGATAATCAGGGCTTAAATTAATCCAAGGCCACCAAGG
CCATTGCCTTGGATGCCCCTTTGACTGGCCTCAATGCCCCTCTCATTGGCCTTGGAGATTTTTTG
TGCCCTCTCCAATTCTTCCCATTTTTGTGCTGTAATATAGGAATGTGCCCTACAGAAAAGTG***GCC***
TT*GCCCTAAATATTGAAATTT****AAGGCCTGGA****TAATAATTGAAAATCACCTTTCAATATTCCAATA*
GCTGGATGCACAGTGCCAATACCGGATGGAAGGGCTGTATGAGCACTTTGATAAAGGTAATGAGA
TAATAAAATCGCCACCAAAAGACGGGATATGTATAAATGTACAATTCCTGGAATCCATGACACGA
*CCCTGGACGTACTAATAACACTTTTCCGTTTGAAAGAAAGAAAGAAAAAAAAAAAT****GTCGGTCAA***
GATCCAACATGTTTGCATTGACCAGCATGGTATGATTTGATAATGGACGGGGCAAATCTGGATAT
AGAATGAGGGGCGTAGCATGGTCCAACCTATTGAAGGGGAGGGGCCACGATAGGGGGGGGGGGTA
ATAACTTACGTAGCCTGTGACGTCAGAGGGGCTGTTACCTCGATTAGTGCGGCGAGACATCGGTG
AAACAGGTGAATGGAATACCGGATGTAGGTTGTACCCTACTTCCGGTTCGCTCCTTGACCTTCCT
TTGTCGGGTCAACTCATTAATCTCGGGAAAATGAACTTTTCTGTTTTCATTGATCAAAAGACAAC
GATCGAATAACAGCAGTATAAATATAGAATGTGAGAAAAAAGTTTTATTGAACTGTTTTTCTAAC
ACACGCTGCATTTTCAACTCATTAATCTCGCATATTTCGTTTACCATAATATTCCTTTTCTTAGG
TAGGCCTAAGCATTTAACGAAGAACAGCGTAATTGCAGTAAATCCCCATCCCTCAACAACAACAA
CAACATAACATCTTTATAGCCGGTATATTTAGTTAACTCAAATTTTTGTATACAGAGTCTATTCT
TTTCTGACTCGCGGACTCAACACAACAGACGGACGATTCATGACCAGGATGTGTGGCGAAAAACC
TCACAGTCTCAAAAAAAAAAATCTATTTTGTTTGCAACTATAGATTGTAGGGCCTATTGATCGAG
ATTACGCCCCATGTCAGACCCAGCAACATCGTATACTGATAGGTAAGCCTACACATATACAATAC
AGAGGCCAATCTACTGAGCTTGGCTGTTCAATCATAATCCCTTTTTATGTCTGATTTGATCTATG
AACAATCATTATGAGTATTATTATTTAAGATTATTAATAAATGATTATTAGACGATATGGATAGT
GGACAAAAAGGCATTAGACAAACTGGGAATTAGACAGACTGATAAAATTAGACTAAATTTGCAAT
AGACCAAATGGGTAGTAGACTTATTGGAGATTTGACCGAATGGTCATTAGACCAAATGATACGTA
GACGAAATGATTATCAGCTTGATCAGACCATGGTTGTGGATAGTATAGACGGACATAATGTAGAC
CATATGGGAATAGACCAATTGGGTAGTAGACGAACTGATTGTAGACCAAACAGCAATACACTGAC
AGGATGAGCGTCAATCACAATGTTTGTATATAATAATAGTAGTGTATAATCATCAATACAATATA
CTTCTGCAATATATCTTTAAATCACACAATTGGGATAACGGGCATTGTCCAACTCTTGATCGAGT
AACATTGTAATCATTGGAATGGAAAGTCAACATCGAAATATCATCCCCAAATCCCGACGTCCGGA

Fig. D.1 continued

GAAGATGCCTCAAACTTCATTTATATTTAAAACGGTTCAGATTTAACGACTACACTACTTTTACC
CCCTTTTCCCCAGCTAGCTGAACACACACATTCGGCCGATGTATAAAACCACGATAAAACTTAAA
TTCCAACACGTTCACTCGTGCACTTTTCGTCTGCGGCGTAGTCTTGCGTTCATAGTCGCGTACAT
AATAGAGATGAAATCGAACCGCCCTTGCATTTAATTTCACTGATATAAACCCCTTGAAATATCAC
AGTAATTGAACAAACATAGAATATCACTAACATCAATCAGAAATTAACGCTGTGCTCACAAATCG
TTATATTGAAGTCACTTATTTACAACATTGCAGCATTTGGTGAGACTATGCTCGGCTCGTTACTA
AGGACGCTCAATACCGCGGCGCGCCATTTTGTATGTTGTGGTTTTGGGTGTGGAACCATAGCCGT
ATTCTCTAAAGTGAGCAAAGATTGCAGA

Fig. D.1 continued

B.

>Region 4 [derived from sequencing of reporter construct]

TTAATAAATGCGCACAGTCCTCCCAATATTGAATACACAAATGAACAAAGGTCCGAATTTGTTAT
TTATAATTCGATTGAGAAAGATAATGAAAAGGTTGAAAAAGATTATTCTCTGACCAAAATTTTTG
TTAGAGAAAAGGTAAAACGCATGAATCCATGATTATTAATTTTGTGTAAGGAAAATGAAACGTGT
AGAAAAATGGGCAATATCCTATCGATTAATATTGCTCATATATGATTATTTCATATTCGATCCTT
TTACAATGAATTCATTTATAGAAACGAATGTATCCGTGTGTTGTGAAATGAGCACTGTATCCGTG
TATTTTGCAATGAAAAGGCAGTAAAAAAAAAATCCCAATATTTGTATTCACCAGCGAGTTTTAAT
CATATACCGGGGAACTTTATCACCTTTATATATATCATTACTATATACGACAAAATCAATTACCA
ATTATTCAATTAATAACGAGCTCTCGACCTTCCATGGTATATTAACTTTGGCAGCGCTGAAAAGC
GAAGCCAAAGGGTCTTGCTTTTGTAGACTACAGATCTCGCTGTGGGCCGAGTTTTTTTTTCATTG
TACGCTACGCTACATGTTAGCACGATCAAGGAAGTTATGTCTCGCTTATGTACACCGTCTACGGG
AGAGAGCAATGTCTATAGAGTTAATGGCCATTCACTTTGTACACGTGTGTATGTTGTGTATGGGG
CTAGGCTGCCGTGGTCGATGTGTACAGTAGTGCAGTGAGATATGAATGCATTGGAGTGAGATACT
TCACTATAGCTGTACTGCACACAGTAAATTACATAGAGTAGTGCGTGGAGTCAAGTTGTATGCAG
CTAGCTAGTTTGCTGGAAAATATTTCAAAAATCATAAAATCGCTCATACATAACCAAAAGTGATA
ATCCAACCATTCATCATGTTCAGAAAAATATGATCTTTCCAATGAACTGATTTATTTTCAAAAAT
TTCACGATTATTTTTTTTTCGTGCATAGGCCTATACGCCTATTGTCATATTGAGTGTGTACTAAA
TATTTCTGGGCTAATACTAGAATAATTGATATAACTATTGAGAAGTGATACATTGAAAAGGAAGC
CGTTCATGATAATGCAAAAAGGTTAGAGATACATATTATAAGTTTTCAACCTTTTATCCTTCATG
ATCTGTCTTTCGTCATATGGACTGAACATGAAGCGTGTAATGATTTAGAATTATATTTTAATATA
TTAATTATAGCCAGTAATGATAAAGTGGTGCTGGAATGATTTGTTAGGGATTTTGGGGAAGTTCT
TGTTTCCGAAATTATTGGCAACCATAAGCGCTGGACACTTACATTTGACCATGGCCGCCCCAGCA
CTTCGGCCATTCCTAAACTAGTTGACCATTCAAAGCTAAACATTCCATCGAAAGATATAACCGGC
CCTAGCCAGTTTTCCACTACACACGTGAATACACCAGACCATATTACAAGGGACCGACAAGAGAC
TAGCTTGACCCAAATACTACCCCACCCCCTCATCTTCTCAAACTTCTCCCCCCCCCCCCCCCCG
ACTCCACCCTAGAAGGCGGAGCCTGGGTTGAAAGAAAAGACAGAGAGAGAGGGAGAGAGATAGAG
AGAATGAAGGAGAGAGCTTAGTGTGTGGTATATTACATGTAGCTCAGTGATATAGTACGGTACTA
AAATATATAGGCCAAGCTTTTGACCAATCAAATGGTAGCACGGTCTGATCTTATGCATATTCAAC
AACCACTAGTTGCCGGTCGAATGTACACGTTTTACACGTTGAAGCAATGTGTGCATCACAAGCAT
GCGTTGTGAAGGAAATATCAAAGCATTCGGCAAAGGGACAGCACCGAATACGTACAGGCCTAACA
GACAATCCCAGAACGAACGAGAAAAGTTTTGGAGTTTGGGTATTAGTGGTGATTTTACCCGTTTT
CGCCAATATTCTGATCTCCAATCTCCACTGGGTTTGTAGGTTCTGGTCGTCTGCTGTTCCCCTTG
TGTCAAGTCACCAAAACTATCCCATTTTCCCACCCCTTTTTCACATTGGAAGTTAAAAAAGAACA
GAAATAAATAACCCATTCCA

Fig. D.1 continued

C.

>Region 5 [derived from GBrowse V3.1]

ACTCGTAAGTATTTCCATTTTTGGGTGCTTTTTATGCTGATGATTTTTGAGCGATCAATTTTCAG
GCATTAAGTATTTTTAAATGGAAATATTAAACCGAATATGAGTTTGAGTTCTGAGGTAAAAACAC
GCGAGACGACATGAATCGTGAGGCCAGGCCTTCTTTTCTAATTTCAGTGAGCGGCAGAGTTGTTA
GAAAGTTACGGAAACGGGGTGTTTTCAGGAAGAAAGCTCCCGTGAATGAAAAAAAAAAAGCATTT
TTACACTTTGGTGTTTTGATGGTATCGGAAGTGTTTGAGAATGAATACAGTCGATATTTTCTGTC
AATGGAGTCGAAAGAAAAATCCGCTGTAAACATTCTCATGCATTTTTGATGAAGATGTGTTTTGA
AGTTGGATTATATTTCATGGATATTTTATTAATCATCAATCTATCAGGTAAGTTTTTGTTTATTT
ATTCGTTTAGGTTTTAATTTTCTTTATTGAGTGGACAATTTCTATGCCCTGTTGAAAAGGA
AAAAATAGGTTTTTAGCGCGCCGCGTTTCCGTCGCTTATCATGACTGTGTCCATT
GTTTATGTGTGTGTGTGTGTGTGTGTGTACACTTTGTCTGTGTGTATCATCGTTGACCAAT
TACACTCAATAATGACGGCGCGCATACTTTACGATGTGCGTGACTGGTTAGTCT
GTGCTTTGTGTAACGCGGTGATTGGCTGAAATTAACATTTTGCCCAGGGGCGCGCCAAATATAAA
ACTTTCGGCGCGCGCTGGAAGTACTCATTTCCACATTGTATTACATTTTATGCAGGGCGCAGTTC
ACCTCAAAAAAAGTACAGCTTTGTTTACATTATCTCGTCGGGGCTTTTGTACAAAATGTAGTTGC
TATTGTGTTGAATATTTTTCCAATCATTATTTTGCACTCTCCCGCACCTATATGCAGTGAAGTGA
TAAAAATTTGTACTGCTTAGACTTGTAATTTAACTAATAATTTGTAAAAGATGTTCAAGAATCTA
GATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAGGGGCGGGGGGTGAAGTTC
TACTTTTTCTTCTTTTTTTTTGTAGGTAGATTATTTCTCCCTTTTTTTTCTCTCTGTGTAAAA
TATTTAACTCTTCTGTTCATGTTTTGTAGATAAAGAATTAGTTTGGCTAATGGACTTCATTTACA
TGCAAACCTATTTTTATTTGAGGGGGGGGGGGGAGCTGTCTCGATTCAG
TTAGCACTTTGTCCATCCTTGGCAACTCGTAACAGTTCTGCGATTTCCTATCATCGATGATAGA
GGGACTAAATAAAACCTGGACAGAGATGGAGAGAAAGAAGAAAAAAAACCTCTGTAGGGACAACT
CCTTTTGAAATGGTATGGGTCATCAGGGCATCCAAATGAATGGCTTCCCTTTGTAAAGTTTGGGG
AAGAATGAGAAGCGATTGATTGAAGCATTGATTAGGTAGAGACAGAGAGCATGGGAAAATT
GTCCTGGGCCTTTTGTCATCCCCCCAAAGAGTCCTGTGCCTGTGTGTGGGAAGA
TGAGACCGGGGGAGATGGGATATAATGAAGAGAAAAATCAATAGGCTTCATGGGGAGAATGTTCA
AAGGATTAAAAGTACATTTTCTAAGCTCTGGGGTATTTTAATTTCCACAGCCACATTCCTTCGTC
GAAGGGGAACATGACCTGGTATGTGATACAAAAATACTCTGGATTAGTAAGAACCACAGTTTAAG
TTAACACTCGGCTTTAAACTGTTCTTAATTTGACATCCAAGTTAAACTATTCACCCCATTTATAT
TTTTTTTTATCAAAATTTATTTTTCAGTTTTTGTAGTCTAGTAGCATGGGATTTATCATAAGCC
CAGCCACCTTTTCACTCCACCACAGTCAGCTATACTGTTGAGAGCCAGAAGGGTTTTAACTCTTA
TACTTTTACCTCGCGTTAACGCCCTCCTGTTTCTCATCTTGTAGCGCCATATCCAGTTTCTCTGC
TCTGCTGAAGAGTTATGAGAACAAAGAGACTGGTTTGTCGTTTCTAAGAAAGAAGTGCTCTAGTG
ATCTTGTCTCATTTGCATAGTTTCAATAGCTTCAGGATCTTCCTACATCGAATTTAGGCTTAGTT
GTTGATCAAGCAACTTGGGGATTGAACTTTAAAATCATCATGACAAACTAGTTTCTATAAGGGGG
GGGGGGGCAGTATGTTGGCCAATTTTGATTAAGACTATTGTTCTGAACTTCTGTTGGGTTTTATC
TTTCCAAGGAGAGAAACTGAGTCCTACTCTGTTTTGTCTCTAATCCCTGAACAATGGATTTGAAA
AGAAGATTAAGGGCTCAACTCTGGGGTCTTTATTGGATATGTGTGAACTTGATGGCTCTAAGCCC
TCAATAGCCTAG

Fig. D.1 continued

D.

>Region 6 [derived from GBrowse V3.1]

CAGACGGAGTTGTCATAGTTAACTCTTATAAAACAAGGATTTTTTTTCTAAAATAGGAAAAAGCG
TATCAGTGACACCATCGTCCTTGCAAAAACAAGGTCAAGCAAAGTATACTGCAGATCAGTTTGTT
GTGGGTGTTATAAGTTCTTACTAAGTTATGATATCTTTTTGCCATAAATAATTTAATTTGCCACA
CTAGGAAATAGAAGCGACTTTTTAGATTTAATCAGCTTCAATAAAAATGACATAGAATTTATATT
TATAAATGTCACTTTGCATTTGCAGTATGCATGTCTTCAAAGAAAGGAAAATTTGTTTCATAGTT
ACTATTAGAATGGAATATATTTAATGAAAATCATCATTCATTTAAAGTGCATTTTTGAAGTTGGT
CTTTAATCTTTTCTCACATGTATTCAATTCAAGCGATTATCGTATCGATACCTTTAACGTCGAAA
CAGGATGTGGCATTTGATAATTAGCCTACGAGTGTGAAATATGAACTTCGGTCATTCCTTCTTTC
ATTTAACGAGACAGACGATTTATGAATGAGCCGGTTCATTTACTTGAGAAATAATTTCACTGGGA
TCTCAAGATAGATACTTTATTTGATTATTTTTAAGCAGTGACAAGTATGAAAATACAAACTGCAT
GGCCTCTGCTTTCATAGTTTTTACTCTTTAAACATATACCGGTAGAAAATAAACAGAACCAATTT
TTAGTTTAGCAATTTACTGGTTTCGTTTTATTCATAATTTAGTCTCAGCCGGGCCCAGAC***ACCAA***
ATACAAAGTGCAAAAGCTTTTCTTTCACTTAAACAAAAACAGGACTTGATTGAGAGTTGATCGAG
GAGGGATTCCGGATGTGATAGGATCCTTGTTATGTTTCAATTGATGTTAATTATTTCCCCTTTCT
TGTTCTTAATCCTCCTCTCAGTGTGCGTTAAAAAAAGTCACATGGATGAGAGGGGATTCTCGTTC
AGTGAGTATTTGTCAGAAATTGGAGATTGTGAAATGTTTGCTGGTGGTACTCTATGGACAGTTTA
GCCTGCAAGAGGGCAACGATTACATAAGCGTGTTTCCTCATTTAAAAACACTCAAAGTGAACATT
ACATAGATCATGTGATGAATTCACACCTATTTTTTTAAGCACATGTAGGGCCTGTTCGACCAAGA
AAATATGTGGGGAAAATGCATTTACTATAGCTATAACACAAAAATCTTATCCAATATGAAATGAA
CAAAAACACCACCCATTGTAGTGAAGGTTTCTGTTATTTATGTTCAATCGTGACTGCAATTTTAG
ATTTTCACAATTTTGTTGTAAATTTTAATAAATTCAATACAAAGTTTAATAATCAGAGTTCTTTT
GCCAAACTGCCAGAGATGATATGATCTGTGAAGAATCAGTAGGGTATTCATTCTGTGAGTAGTTC
ATCAGGCGTATCTGGCTCCGAACTGATTATTTCCCCCTCGTGTTATTTTAGGAGTGTCATTGACT
TGTGATAGAGATGAATGTGGTCACTCATGATCTACTTGGGTTTCATGCGGTCTGAGAAGACCGAT
GAAAATCCTGAAAAGGGCATTTGGTCTTCGCTAGCAAAAATGAAACAGGATCTAGGTTTTAATTT
TGACAAAAGTGACACTATTCACGTTAAGTGTTTCCTTTTCTTTAGTCTTGATGTGCAAGTAGAGA
TAAAGGATTTGATACCGAATGTGTGGTCAAATTATCTTGATAAAAAAAAAGCGGTCTTGTTCTTT
TGTTTCCCTCTTCGCACATTCGACCACAGAGTATGTAAATTGGACACTTCCATGCATGAGGATCA
TGTCTCTTCAGAGCAAAAATGGCGTTGGATCTGTGTTGAAAGTATATGATTCACCGGTAATGCCT
TTCTGGCATCAAAGAGTTAATTTATTTCATTTCCTTCATCAAAGTACAGTGTAGAGCCTAGATCA
AACTGTATAATGTGCTCTTATCACAAGATCTGAATATTATGCATTAATATTTTAAGGTGGGAGGC
TTTGTGTGTGTGTGTGTGTGTGGGGGGGGGGTGTTGTGGGTACAGTTAGGTACTAAGGTTATA
TTATACACCATGCCCTTTTTTTTTTATTAAATGGTATTATGCACATGATTGATTAACCCTTTCAAT
GATTTCGGAGCAGACAATGTTTTAATAAAAAAATTGATGGTAGTGAAACAGTGCTCAGGCGCTGC
ACATCATCGTATGATGGATATCGTCCAATAAGGAGCATACCAGTACTTGTAGGTTACCGTTTCTT
AGAAAAGACCCTTTTAGAATTATCAGGAATGTGTTTGGAAAGTAATTTTAGATTTTCATCATCAA
CAACTTTAAGATGTCATTAGTAATTGACTATAACTTGGCTAGCCAGATTGTAAAGGAGAAGTAAT
ATGTCATAATCTTACAATAAAAAAAACATGTCTGTTAGTTGCAACTTGCAAGTGTACTTTCTGTA
TTTGCATCATGTAAGATCTACCATAAAAATAGTTCAGCTCCCTAACATGGTCCAATTTTGTATAA
AGTTCAAAATGTGCGAAATACAATATTATTTGATGTACTCCATGTTTCTTCATTCTCTTAAGCAT
ACCCTAGATGGCGCTGTTTACAAAGTATTAACCTGAATGCTATTGTTTTTGTCTTCCTTTGTTTT
TCTTCCCAATTCACAGAAAT

Fig. D.1 continued

E.

>Region 17 [derived from GBrowse V3.1]

AGTATTTTTCACTTTTCTCGGTTTCAAAATCAAATAATTTGTATCTCAATCGCTAGAATACATCC
TTAGCTTTATTTTGACGTGTGATTTGTTCCTATTTTCTTCTTTTTTCTATTTTTGTCTGAATTTG
AATGAAATCGCAAATTTCCAGGAACAACCTGTTGACGTTTTGACCGCAACATGTCTAAGTTTGAC
TCAAGTTTTCTCTTAAATTTTGAAATGAAGAGGCTTGTGAAGCATATCATTAACTAGAATAGATC
TTTGGAATTATTTTGATATATATTTTGTCTTGTTTCCATGATTTTTCTTTTTTTGCTGATTAAAG
TAAACATTTTATCTTTATTTCATAAAGCGGGGCCGCGCGTGACGCGCCGTGGTTTTGCTCGTCAT
GAGACTCGGAATTATTCATAATTTCATTGATCTCGCCTTCGGCTTCTTGTGCTATTAATTGATGT
CATTCTAAAAATAATATTAACATTTGAATGTCGTGTTTCTATCATTTTATAATTGTTGACATGAT
TTGCTATGCAAAAAATATGTCGTCGTCATGTGCACTCTCTTTGTTGTAGATTTTAACATCAAACC
GATTTGTATGGTGCCGAAAGACATCGAATCTATCGCTTTTCAAAGAAAGTAATTGAAAATCTGTC
CATATAAAAAGTTAGAGAATATATTCTTCTTCCATTTGGTATTAAATTTGTCATATGTCGCGCCG
TCAATTAAATTTTTCGAATTTAACACGAATTTATGACAAGTCGACTTCTGAGAACGCAAGTCGCT
TCCTCTCCATTCAGGCATGTGTGATCGAGACGCAAAACTGCCAAGCCGTTTTTGCAACCTCCCTA
GAAAATATAAATTTAACAATTTTCCTATCCCTCTTTTGAAAATGTATTCATATGAAATCAAAGCA
TAGGCATTAGGCTTTCGAGCCATGCTATAAAAAATTACAACATGTGCCGGGTTTTATTTATTTCG
AATTAATTTACTCGTGTTTCAAGAAAAGTTCGTGTTGGTTTTGTCATGTAGTTTGCGCGAGCGAA
CATGCGTTGTGCAGCGTGTATGCACGGACAGACACATCGAGGCGAGCGCTAGCCCGTGTGTGCAG
TATGACATTACCGTATTGTACAGCAGACTTGACCGAGCCTGATGTATGAATTCTACATTGCATTG
GTCTTTTTCTGGCGCGCGCTTTTTACTTTTTCGGGGTAAATAGTTGGAACCTGGATTGCGCAGGA
TATCGGGCGGTAGATCGAGATCTATTTGCATTTCATGTTCATCAAATCTGAGAAATTATAATCAT
TATTAAGTCCGATCTTGTTTGGAATCGTCATCATTTATGTCAGAATCTCATTTTCTATATTTTGT
ATTTTTGTAAATCGACCATTCTTCATTTGATAGATGTTTCTCACGGATACAAATCCATCATCATT
CAATCATATTGACTCAACTGTTTCTTCAAAAACTTAGATCGGGGAAGTCCATTGTTTGGTTTGGA
TTTTGTAAACAGATCGTATGTCTTCTCTTTTGTAGAATGATATTAAAATAAAATTGTCAGTGCAT
CCTTGTTCGTAATTTTAATCATCTACAATTTTTACAACAGATATTTTAGTCAGTCGTGGGCTCAT
ATAAAAACATTCATAAACAGGACATGGCAAAGCTGGAGATATTATCGTCCTCCGCACTTTGAAAA
ATATAATAGACCAAAGCTAAAGCCGAAGGGGGAAATTGCATTTATTTAATAATTAGTTTTATAAA
TATGTAGGCCAAAGTTTAAGGCCAGATTTAGATATATTACAGACCTCTATGATCTTATCAGACCT
TCTTGTCTTCCTACCATGAAATTTGAAGATTTTTTTCGAAGGCAATTTAGCAATATTCTCATCAT
TTTTAAAAGACGTTTTGTTGCCCCTTCTGTTGGGGTTTGTTTCACATTTATATTGTCTTTCACTT
AATATGTCTTTTCTTCTTCTTCCTTCCTCTGTCATGTCCGCTGGGTATTATTGAAAATTATGATA
TTTATGATCATCTGCAGTTCATTAAAATATTTGTTTTTCTTTTTGTTTGTTTTCTGCAGgttt

Fig. D.1 continued

F.

>Region 19 [derived from GBrowse V3.1]

AACCGTAAGTACATTTTATTTGTTAAAAACATTTAAAAACATCAAAAGCAACTAGAATTTGTCTT
TTATGTAAATTTATGCAAGCATGACTGGATTTATGAGGTTGTCCTTGTACTGGGTTTTTCACCTT
GATTAAAGTTTTTTGCACCTTCGACCTTGTGCTTCAAAGGCATTGCAGTCTGTACCTTGTTTGCA
GACGTCGCTCAGTTCTTTCGATTAAGTGATCAATCTAAGTCCACGACAGGTCGTTCCAGTAACGC
CCCGCTAAATGGTGAAAATCTCCCCCATTAGAGCTAGGGGGAGAGACAAGATGGGACAATTACTA
TACATTGATGGATTACTGAATAAATATGAAAAGACCAAATTGGAGTGGCCTTGCGATGGTCTCAT
TATATTTAATTGAATCTTATGTGTGGTATTCATTTTGGGTCACAATATTTCAAGGGGTCAGAACT
CAGTCGAGGTTGATTTGGCTTGTAAGTTGTATCGTTTTTTTTTTTTAATGAAAGTAGCTTTTGGA
CTGTGTT***TGGTGTGCTTCCACTGGTTCTCTGGTTCCATACAGGACTCTGCATTCAACAGGATAGC***
ACAGACTTTCAAGAAATCAACACGAGGCAATTTTAATAGCCCTGTCTATCATTTGCAACATTACA
ATGAGATATAACATCATCCACATGATTTCTAATATGTACATGTTAGGCAATCGATATCGAAATTG
ACCATGAGAGGAATCTCCCAAGTCATTTCAATTGCGTAAATGGATTTGTAATCTTCACCCTCATA
TACTGAGTCTAGTCAGCCTACTGTCCAAAGCATTAGCAGAATCTAAGAATCTATCTGTATGGTAC
ACATAAAGCGATTAATTAGCCTGATACTTTCTGTGAGTTTTCAGATTGTTAGGGTTAAAAGCCTG
TCTCAAAAAAGGAAGTGTGTACTTAAGAAAAAAACTTGCTTCCACCCCCTTCAAACTCAAACATA
CCTTGTAGTCCAACAGAAGCTCCCCTAGCAACCTGACATGTCCTAGATAGTCGCAACAAAAGTAA
GACCAGGCTCCTTTTCTCTTTTTTCTTCTTGTTGAAGCCCTCCTGAGATAGCTCTTCAAGAATTC
CCAGTGGTTACTATGATTTATAAATTTTCTTTGAAGAGAACTGATTATTTTCTGTTAAGATTTCT
CTGGAGTCTTATGAAAAGAAAGAAAAATTTAATTTCCTGTTTGGTTTAGTGACTTTAGTCCTGAT
TGCCTCAACAACTTAGGCAGAGAGGATTTGGGACCTGAAAAGCAGATTTAATCAGGCTGTGTGTT
CGAGACTACGTTTTCGGCACAGCCTTCTTAAGCAAACCTTGTCATAAACGCTCAATGAATAGGCC
TTAAAACCATCCGCAAACATGGCCCCATTTTTGTGTCAGTTGTCTCTCAGTCTCCACCTCTTTTT
TTTTTAGTACCAACTCTCAAAGTACACACTTCCCCCTTTCTTTAGTTTGCAAATTTAGTCACATA
ATGGATCGGTTTTATTGTCTCCTACTTGTCTAGCTAGCATACCTCTCATTTGATCATTTTCCTTT
GATGCCAACCTGTATGTCTAATTGAACTACAAAAAAAAAGGAACAATTCTTTTTGGAAAGAATGG
GAGAGGGTTGGGACTTCGGGTCCGATGCACATGCCTTCGTTTTAGTCTTTGTTCAAAGACTTCCT
CGGTTTGTTTGTTTTCTAAACATGGGAAAAAAGAGGTGTTTGCACCCTTCGTTTAAAGCTCTGCT
CCAAAATTACACTGCCAAATTTAAGACGACCGTTTCTTGGATGTAAATGAGACAAGAGTACAGTT
CCACCATTGATTATTTCGCCCTCATTAGATCCCAAATACCATGAAAATCACAAATTTATATTACC
ATAAAGAACATAAAGCGTTGAATCCAATTTTGCTCTCCAAGCTTTTCATGAATTCATTTTAAAAA
AAAATGATAATTAGCTTATAACAATCATCTATTTTTGGAACATATTGCCAATTTGATGATGCAAT
AAAGAAAGAAATAAGTATAATGTGTTTTCTAGGTTAATTACACCAATGTAGGAAACAAGATTTAA
TTTTAATGGCATTTCATTTTTAAGTGTACTTGCAGATCTCGTCTTATGTCATAAAAAGAGATCAT
TACTGTCAAGTATTATGACATATGAAATCCAAATTAAAAGTAATGAATCATAGTTAATCAATTAC
TTTACCATACAACCATTTTAAATTCCCAGGGTTCTTTTCAATGACAGATATAAACACTTCATTTC
ACAAATTGAATTAAAAGAAGACAAAAGATATAAATAGATCATCTATTTATTTTCTTAATTTTTAT
AAAAAAAGAGCTTTTTAAGAATATGTGTCTGATTGATTGTCAACACTATTTCTTTTAAACGGGAA
TGGTTTTAACATATGGTCATTTACAGTGATACAGAGTTGAAGTTGAGGGCATGGAAACAGAAGGC
TGTTCACATATGGTGATTTTATGTTTCATTAAGAAGTACCTGGGGAATCATAAGGAGCAGTTTCA
GTGGTAATCATTGAGCACTGAATCTTTAGACGTTGAGTCGTCGTAGATCAAGAGTTTCTATCCTG
CAAAGCGGTAGAGTAATTGTTCTCAGAGAGTGAGCTATGAAAGACCGATCACCATCCAAATGTGA
ACCTCCCCTATTAGTAAGTTGATTAAGGGGAAACCCCATATATTTTAGAATCTTGTGATGGCTAT
CATGGCTGGCTTTGGAGTGTCATTTCCTGAAGTGAATCGGTTGTGATCCTCGGCCAAAAATGACT
TTCAATTCATCGTTTCGCCTTGCAACGTGATACGGAGAAAAGATAAAAAACAAGTGGTCACCAAG
AGTGTGCGATGATTGTCAGTGAGACTTTCGATTTCTGTCCGAGTTTTACTGGAAGTTTCACGTGA
TTGGAAGATCTGTGCTTTGAAAGGCCATAGGATTTAACATGGGAAATTGACCAGGCACATGCATG
GGCCCTTATGGCAAGGCATTAGATCAATTGTGATCAAACGCAATCGGGACGTAGAGGACTTTCAA
ATTATGGCACCAAATGCGTAATCAAAGCATGACGATGACGTCTTTCCTTCGTTTTTTAATGATTG

Fig. D.1 continued

GAAATGAAAGATGCATATATTTATAGCATTGAGATCTCTGTCAAATGCATCAAAATATGAATGAA
TCCATTTTTGTGAAGTATGAGGAATAAACACATGGAAATAGAAACAGTGACCTCTCTCTCTCTCT
CTCTCTTTCTCTCTCTCTCTTTCTCTCCACTTCTGACCAATCATTGTGAAGTTGTGTTAGGTGTC
CATTGTCAATGCCATTTACATCTTGAACTTGCCCCCAATGTGGTAAAAATTTCTGGAAACGCTGC
ATGCCACAGAACTAATAAACAAACTTGAGCTACATTGTACGAAGAAATAAAGTGCTATGATTATC
ATTTGTGGTCTAACACACATTGTATCCTCCTGCCTTTTCTTCCATCATCACACTCGCCCCTCTTC
GTCAGGAAAGGGGGGGGGGGGCGGTGAGGAACCCCTAGTCTGTGCCTGGGCATGTTATTAGCACT
TTAAAGGAGCGATTAGTTGAGATAAACCTACCTTTATTCTCCACTTGAGCTAAGCTAAGCTGAAT
AGTTCTAAGCTCTTCAGAGAATTTAATGATTCTTATAAAACATTGGGGTGAGACGATTAAATTGA
ATCTCATAGAAATGTTCCAGTTTTTCCAAAGCTAAGAATTTGACGGTCAACTTTGCCAAAGATCT
GCAACGATTCGGTCGGTATTCATGCGATGGGTATGATTGGTAGAAAACGTCTTGTAACCAGACTA
CATGTGAACACTTTGTGAACTCTTCTACGATGGCAAATGTCACAAGGGTTGGGTCATGTCTGGCC
CTCAGCAGAAAAAGAGACAAACTTTGTCCAAATCTGCGAAATTCCTGAGGCTCGCATACCATTCC
TGACCCCCAATATCCCCTTCGAGACCCAATTTCCCAAGATTTCTCAAGATTTTCTCACTCTATTG
ATGACAAAAAGAAAAAGGGTGAGTCATCCGCCCTGAAAAGTGGCTAGCAGAAAAGAAACAAATGG
TTGACCAAACAAGCTGTGAAGAGGATGCACCCTTTGAGGTTTTGAGTTCCCCTAATCTGTGTGAT
TAACCTTCCAAAGTCACACCACAAAGAATTGAGTATCAGGGACTAAAGAGGGTCCTCTTGCCAGA
AAGAACAAAAAAAATCCTTAGCAGTGCAATGAAAAGAGATACATAATTGGAACTTTCCCTCAACC
CTCCCCCAATGAAAAAAGATGAGTAATAAATACAAACAAGAACAAAACAGAACACAGATTGTAAA
AAACAACAACAAATAAAGTAACCAAATAAAATGGCTGAATTATGCTGAGTACATTTACAACCCTG
GATTTCTATTTCTATCCCGCTGCTGATTTTGTTTATAGAGCGAAAGCATCAAATCCTTGATTACT
CATGCGCTTTGTTATTATTTGTCTGCCTTCATGAGACTAATCAAGTCAACAGTCTTCAAATCAAC
AAAGGGTACTAGACTAGAAGGGCGTCAACTAAGAATTCTTTGTTTTTCCTGGAGTCAACAAATGT
AAACCAAGCTGGAAGTGTACCAAGTAAA

APPENDIX E: CLUSTER-BUSTER OUTPUT FOR REGIONS 5, 6 AND 19

Fig. E.1. Cluster Buster output for regions 5 (panel A), 6 (panel B) and 19 (panel C). Binding sites for Pax4, the significance of which is described in the text, are shown in larger font than for the other transcription factors.
Note: This figure continues for several pages.

A

>Cluster-buster output for region 5

Motif	Position	Strand	Score	Sequence
MA0045 HMG-IY HMG	506 to 521	+	9.95	gttgaaaaggaaaaaa
MA0013 Broad-complex_4 ZN-FINGER, C2H2	507 to 517	+	6.44	ttgaaaaggaa
MA0045 HMG-IY HMG	507 to 522	+	7.28	ttgaaaaggaaaaaat
MA0010 Broad-complex_1 ZN-FINGER, C2H2	508 to 521	+	6.23	tgaaaaggaaaaaa
MA0120 ID1 ZN-FINGER, C2H2	508 to 519	-	8.95	ttttccttttca
MA0010 Broad-complex_1 ZN-FINGER, C2H2	509 to 522	+	6.25	gaaaaggaaaaaat
MA0028 ELK1 ETS	509 to 518	+	6.76	gaaaaggaaa
MA0050 IRF1 TRP-CLUSTER	509 to 520	+	6.57	gaaaaggaaaaa
MA0068 Pax4 PAIRED-HOMEO	**509 to 538**	+	6.42	gaaaaggaaaaaataggtttttagcgcgcc
MA0120 ID1 ZN-FINGER, C2H2	509 to 520	-	6.12	tttttccttttc
Ets	510 to 520	+	7.33	aaaaggaaaaa
MA0039 Klf4 ZN-FINGER, C2H2	510 to 519	+	6.56	aaaaggaaaa
MA0021 Dof3 ZN-FINGER, DOF	511 to 516	+	6.03	aaagga
MA0039 Klf4 ZN-FINGER, C2H2	511 to 520	+	6.26	aaaggaaaaa
MA0081 SPIB ETS	511 to 517	+	7.1	aaaggaa
MA0013 Broad-complex_4 ZN-FINGER, C2H2	512 to 522	+	6.04	aaggaaaaaat
MA0026 E74A ETS	512 to 518	+	6.23	aaggaaa
MA0049 Hunchback ZN-FINGER, C2H2	512 to 521	+	6.95	aaggaaaaaa
MA0098 c-ETS ETS	513 to 518	-	6.77	tttcct
MA0039 Klf4 ZN-FINGER, C2H2	516 to 525	+	6.69	aaaaaatagg
MA0011 Broad-complex_2 ZN-FINGER, C2H2	519 to 526	-	6.1	acctattt
E2F	528 to 539	+	6.18	tttagcgcgccg
MA0024 E2F1 Unknown	528 to 535	+	6.5	tttagcgc
E2F	530 to 541	-	6.14	cgcggcgcgcta
MA0123 ABI4 AP2	530 to 539	-	7.49	cggcgcgcta
MA0079 SP1 ZN-FINGER, C2H2	531 to 540	-	8.21	gcggcgcgct
MA0123 ABI4 AP2	532 to 541	-	6.37	cgcggcgcgc
MA0028 ELK1 ETS	542 to 551	-	6.9	gcgacggaaa
GATA	548 to 560	-	6.52	tcatgataagcga
MA0089 TCF11-MafG bZIP	556 to 561	+	7.31	catgac
MA0077 SOX9 HMG	567 to 575	-	8.18	aaacaatgg
MA0040 Foxq1 FORKHEAD	568 to 578	+	8.5	cattgtttatg
MA0030 FOXF2 FORKHEAD	569 to 582	-	8.05	cacacataaacaat
MA0084 SRY HMG	569 to 577	-	6.08	ataaacaat
MA0087 Sox5 HMG	569 to 575	-	6.34	aaacaat
MA0021 Dof3 ZN-FINGER, DOF	601 to 606	-	6.02	aaagtg
CCAAT	623 to 638	+	6.32	gttgaccaattacact
MA0060 NF-Y CAAT-BOX	623 to 638	+	6.57	gttgaccaattacact
MA0068 Pax4 PAIRED-HOMEO	**626 to 655**	+	6.02	gaccaattacactcaataatgacggcgcgc
MA0075 Prrx2 HOMEO	630 to 634	+	7	aatta

Fig. E.1 continued

MA0122 Bapx1 HOMEO	633 to 641	-7.27	ttgagtgta
MA0110 ATHB5 HOMEO-ZIP	639 to 647	-8.13	tcattattg
MA0008 Athb-1 HOMEO-ZIP	640 to 647	-6.71	tcattatt
MA0089 TCF11-MafG bZIP	643 to 648	+6.88	aatgac
MA0123 ABI4 AP2	648 to 657	+6.08	cggcgcgcat
MA0006 Arnt-Ahr bHLH	669 to 674	+6.54	tgcgtg
MA0067 Pax2 PAIRED	670 to 677	-6.66	agtcacgc
MA0043 HLF bZIP	690 to 701	-6.81	cgttacacaaag
MA0025 NFIL3 bZIP	692 to 702	+6.22	ttgtgtaacgc
CCAAT	701 to 716	-10.6	ttcagccaatcaccgc
MA0060 NF-Y CAAT-BOX	701 to 716	-10.8	ttcagccaatcaccgc
MA0038 Gfi ZN-FINGER, C2H2	702 to 711	-7.86	ccaatcaccg
MA0041 Foxd3 FORKHEAD	715 to 726	-8.81	aaatgttaattt
MA0047 Foxa2 FORKHEAD	715 to 726	-6.42	aaatgttaattt
MA0040 Foxq1 FORKHEAD	716 to 726	-6.03	aaatgttaatt
MA0075 Prrx2 HOMEO	716 to 720	+6.46	aatta
MA0003 TFAP2A AP2	728 to 736	+7.03	gcccagggg
NF-1	729 to 746	-6.21	atttggcgcgcccctggg
MA0003 TFAP2A AP2	729 to 737	-6.91	gcccctggg
E2F	734 to 745	-7.83	tttggcgcgccc
MA0024 E2F1 Unknown	738 to 745	-10.1	tttggcgc
MA0082 SQUA MADS	741 to 754	+7.04	ccaaatataaaact
E2F	755 to 766	+6.48	ttcggcgcgcgc
MA0024 E2F1 Unknown	755 to 762	+6.29	ttcggcgc
MA0123 ABI4 AP2	757 to 766	+7.39	cggcgcgcgc
MA0017 NR2F1 NUCLEAR RECEPTOR	804 to 817	-6.11	tgaactgcgccctg
MA0114 HNF4 NUCLEAR	805 to 817	+ 6.5	agggcgcagttca
MA0049 Hunchback ZN-FINGER, C2H2	820 to 829	+6.62	tcaaaaaaag
MA0082 SQUA MADS	820 to 833	+ 7.5	tcaaaaaaagtaca
MA0013 Broad-complex_4 ZN-FINGER, C2H2	836 to 846	-6.17	atgtaaacaaa
MA0084 SRY HMG	836 to 844	-6.32	gtaaacaaa
MA0031 FOXD1 FORKHEAD	837 to 844	-8.11	gtaaacaa
MA0003 TFAP2A AP2	852 to 860	-6.47	gccccgacg
TATA	854 to 868	-6.78	gtacaaaagccccga
MA0108 TBP TATA-box	854 to 868	-6.81	gtacaaaagccccga
MA0020 Dof2 ZN-FINGER, DOF	858 to 863	-6.12	aaagcc
MA0078 Sox17 HMG	880 to 888	+6.58	gctattgtg
MA0008 Athb-1 HOMEO-ZIP	902 to 909	+7.36	caatcatt
MA0110 ATHB5 HOMEO-ZIP	902 to 910	-7.22	taatgattg
MA0114 HNF4 NUCLEAR	914 to 926	-6.33	gcgggagagtgca
MA0079 SP1 ZN-FINGER, C2H2	917 to 926	-6.18	gcgggagagt
MA0039 Klf4 ZN-FINGER, C2H2	918 to 927	-6.33	tgcgggagag
MA0062 GABPA ETS	918 to 927	- 6.1	tgcgggagag
MA0024 E2F1 Unknown	919 to 926	+6.89	tctcccgc
TATA	922 to 936	-8.76	gcatataggtgcggg
MA0108 TBP TATA-box	922 to 936	-8.79	gcatataggtgcggg
MA0103 deltaEF1 ZN-FINGER, C2H2	926 to 931	+7.36	caccta
MA0015 CF2-II ZN-FINGER, C2H2	929 to 938	+6.91	ctatatgcag
GATA	941 to 953	+6.04	aagtgataaaaat
MA0091 TAL1-TCF3 bHLH	992 to 1003	- 8	tgaacatctttt
MA0121 ARR10 TRP-CLUSTER	1003 to 1010	-6.11	agattctt
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1045 to 1054	+7.83	ttaggggcgg
MA0068 Pax4 PAIRED-HOMEO	**1045 to 1074**	-8.91	gaaaagtagaacttcacccccgcccctaa
Sp1	1046 to 1058	+15.3	taggggcgggggg
MA0039 Klf4 ZN-FINGER, C2H2	1046 to 1055	+8.63	taggggcggg
MA0079 SP1 ZN-FINGER, C2H2	1046 to 1055	+6.54	taggggcggg
MA0118 Macho-1 ZN-FINGER, C2H2	1046 to 1054	+7.12	taggggcgg
Sp1	1047 to 1059	+7.21	aggggcgggggt
E2F	1047 to 1058	+7.38	aggggcgggggg
MA0039 Klf4 ZN-FINGER, C2H2	1047 to 1056	+7.36	aggggcgggg

Fig. E.1 continued

MA0068 Pax4 PAIRED-HOMEO	**1047 to 1076** - 7.56 aagaaaagtagaacttcacccccgcccct
MA0079 SP1 ZN-FINGER, C2H2	1047 to 1056 +6.73 aggggcgggg
MA0111 Spz1 bHLH-ZIP	1047 to 1057 +6.45 agggcgggggg
MA0123 ABI4 AP2	1047 to 1056 - 8.94 ccccgcccct
Sp1	1048 to 1060 +6.27 ggggcgggggtg
MA0068 Pax4 PAIRED-HOMEO	**1048 to 1077** - 13 gaagaaaagtagaacttcacccccgcccc
MA0074 RXR-VDR NUCLEAR RECEPTOR	1048 to 1062 +6.69 ggggcgggggtgaa
MA0079 SP1 ZN-FINGER, C2H2	1048 to 1057 +11.5 ggggcggggg
MA0114 HNF4 NUCLEAR	1048 to 1060 +6.84 ggggcgggggtg
MA0118 Macho-1 ZN-FINGER, C2H2	1048 to 1056 +7.68 ggggcgggg
Myf	1049 to 1060 +6.33 gggcgggggtg
MA0007 Ar NUCLEAR RECEPTOR	1049 to 1070 - 6.67 agtagaacttcacccccgccc
MA0079 SP1 ZN-FINGER, C2H2	1049 to 1058 +7.55 gggcgggggg
MA0114 HNF4 NUCLEAR	1049 to 1061 +6.02 gggcgggggtga
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1050 to 1059 +8.59 ggcgggggt
MA0079 SP1 ZN-FINGER, C2H2	1050 to 1059 + 9.1 ggcgggggt
MA0123 ABI4 AP2	1051 to 1060 - 7.83 caccccccgc
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1052 to 1057 +7.56 cggggg
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1052 to 1061 +6.98 cgggggtga
MA0079 SP1 ZN-FINGER, C2H2	1052 to 1061 +8.68 cgggggtga
MA0113 NR3C1 NUCLEAR	1052 to 1069 + 6.7 cgggggtgaagttctac
MA0118 Macho-1 ZN-FINGER, C2H2	1052 to 1060 +10.3 cgggggtg
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1053 to 1058 +7.06 gggggg
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1053 to 1062 +6.57 gggggtgaa
MA0079 SP1 ZN-FINGER, C2H2	1053 to 1062 +7.82 gggggtgaa
MA0118 Macho-1 ZN-FINGER, C2H2	1053 to 1061 + 7.6 gggggtga
MA0018 CREB1 bZIP	1054 to 1065 +6.61 gggggtgaagtt
MA0016 CFI-USP NUCLEAR RECEPTOR	1055 to 1064 +7.56 ggggtgaagt
MA0114 HNF4 NUCLEAR	1055 to 1067 +6.45 ggggtgaagttct
MA0046 TCF1 HOMEO	1122 to 1135 - 6.16 agttaaatatttta
GATA	1151 to 1163 +6.27 tgtagataaagaa
MA0049 Hunchback ZN-FINGER, C2H2	1154 to 1163 +6.16 agataaagaa
MA0020 Dof2 ZN-FINGER, DOF	1158 to 1163 +6.44 aaagaa
MA0053 MNB1A ZN-FINGER, DOF	1158 to 1162 +6.16 aaaga
MA0075 Prrx2 HOMEO	1162 to 1166 +6.21 aatta
MA0019 Chop-cEBP bZIP	1189 to 1200 +6.08 acatgcaaacct
Mef-2	1197 to 1208 + 7.1 acctatttttat
MA0052 MEF2A MADS	1199 to 1208 +6.36 ctatttttat
MA0073 RREB1 ZN-FINGER, C2H2	1208 to 1227 - 6.7 ctccccccccccccctcaaa
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1210 to 1219 +7.55 tgagggggg
MA0068 Pax4 PAIRED-HOMEO	**1210 to 1239** - 7.97 gaatcgagacagctcccccccccccctca
Sp1	1211 to 1223 +6.59 gagggggggggg
Sp1	1212 to 1224 +7.34 agggggggggggg
Sp1	1213 to 1225 +7.49 ggggggggggggg
MA0079 SP1 ZN-FINGER, C2H2	1213 to 1222 +6.47 ggggggggg
Sp1	1214 to 1226 +6.11 gggggggggggga
MA0068 Pax4 PAIRED-HOMEO	**1214 to 1243** - 8.31 aactgaatcgagacagctcccccccccc
MA0079 SP1 ZN-FINGER, C2H2	1214 to 1223 +6.49 ggggggggg
MA0079 SP1 ZN-FINGER, C2H2	1215 to 1224 +6.49 ggggggggg
Sp1	1216 to 1228 +6.39 ggggggggggagc
MA0079 SP1 ZN-FINGER, C2H2	1216 to 1225 +6.49 ggggggggg
Sp1	1217 to 1229 + 6.7 ggggggggagct
MA0079 SP1 ZN-FINGER, C2H2	1217 to 1226 +8.19 ggggggggga
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1221 to 1226 +6.74 gggggga
MA0014 Pax5 PAIRED	1228 to 1247 - 6.04 tgctaactgaatcgagacag
MA0114 HNF4 NUCLEAR	1244 to 1256 - 6.61 tggacaaagtgct
MA0021 Dof3 ZN-FINGER, DOF	1246 to 1251 - 6.66 aaagtg
MA0078 Sox17 HMG	1246 to 1254 +6.03 cactttgtc
SRF	1253 to 1265 - 7.23 tgccaaggatgga
MA0095 YY1 ZN-FINGER, C2H2	1253 to 1258 +6.33 tccatc
MA0035 Gata1 ZN-FINGER, GATA	1254 to 1259 - 6.53 ggatgg

Fig. E.1 continued

MA0098 c-ETS ETS	1255 to 1260	+6.11	catcct
NF-1	1258 to 1275	+6.91	ccttggcaactcgtaaca
MA0045 HMG-IY HMG	1276 to 1291	-6.17	taggaaatcgcagaac
MA0038 Gfi ZN-FINGER, C2H2	1279 to 1288	-6.37	gaaatcgcag
MA0023 Dorsal_2 REL	1280 to 1289	+6.46	tgcgatttcc
MA0107 RELA REL	1280 to 1289	+ 6.5	tgcgatttcc
MA0098 c-ETS ETS	1285 to 1290	+6.09	tttcct
MA0037 GATA3 ZN-FINGER, GATA	1289 to 1294	- 6.2	tgatag
Mef-2	1310 to 1321	-7.34	ggttttatttag
MA0027 En1 HOMEO	1364 to 1374	-6.75	aaggagttgtc
MA0021 Dof3 ZN-FINGER, DOF	1370 to 1375	-6.04	aaagga
MA0071 RORA NUCLEAR RECEPTOR	1383 to 1392	+6.01	gtatgggtca
AP-1	1384 to 1394	-7.18	gatgacccata
MA0089 TCF11-MafG bZIP	1389 to 1394	-7.54	gatgac
MA0035 Gata1 ZN-FINGER, GATA	1398 to 1403	-6.49	ggatgc
MA0036 GATA2 ZN-FINGER, GATA	1399 to 1403	-6.09	ggatg
MA0050 IRF1 TRP-CLUSTER	1412 to 1423	-6.65	caaagggaagcc
MA0062 GABPA ETS	1412 to 1421	-6.59	aagggaagcc
MA0039 Klf4 ZN-FINGER, C2H2	1413 to 1422	-6.95	aaagggaagc
MA0028 ELK1 ETS	1414 to 1423	-6.22	caaagggaag
MA0039 Klf4 ZN-FINGER, C2H2	1414 to 1423	-7.16	caaagggaag
MA0080 SPI1 ETS	1414 to 1419	-8.36	gggaag
MA0098 c-ETS ETS	1414 to 1419	+ 6.2	cttccc
MA0081 SPIB ETS	1415 to 1421	- 6.2	aagggaa
MA0021 Dof3 ZN-FINGER, DOF	1417 to 1422	-6.89	aaaggg
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1431 to 1436	+6.26	tgggga
MA0070 Pbx HOMEO	1450 to 1461	-6.77	gcttcaatcaat
MA0068 Pax4 PAIRED-HOMEO	**1490 to 1519**	+6.22	gaaaattgtcctgggccttttgtcatcccc
MA0068 Pax4 PAIRED-HOMEO	**1491 to 1520**	+6.58	aaaattgtcctgggccttttgtcatccccc
MA0078 Sox17 HMG	1491 to 1499	+6.03	aaaattgtc
MA0068 Pax4 PAIRED-HOMEO	**1492 to 1521**	+7.03	aaattgtcctgggccttttgtcatcccccc
MA0078 Sox17 HMG	1505 to 1513	+6.23	ccttttgtc
AP-1	1506 to 1516	-6.57	gatgacaaaag
MA0010 Broad-complex_1 ZN-FINGER, C2H2	1506 to 1519	-8.62	ggggatgacaaaag
MA0045 HMG-IY HMG	1506 to 1521	-6.89	gggggatgacaaaag
NF-1	1507 to 1524	+6.31	ttttgtcatcccccccaaa
NF-1	1508 to 1525	-7.38	ctttgggggggatgacaaa
MA0018 CREB1 bZIP	1508 to 1519	-6.24	ggggatgacaaa
MA0084 SRY HMG	1508 to 1516	-6.07	gatgacaaa
MA0119 Hox11-CTF1 HOMEO/CAAT	1509 to 1522	-6.77	tgggggggatgacaa
MA0111 Spz1 bHLH-ZIP	1510 to 1520	-6.43	gggggatgaca
MA0119 Hox11-CTF1 HOMEO/CAAT	1510 to 1523	+6.95	tgtcatcccccccaa
Ets	1511 to 1521	-6.79	gggggggatgac
MA0089 TCF11-MafG bZIP	1511 to 1516	- 7.2	gatgac
MA0079 SP1 ZN-FINGER, C2H2	1512 to 1521	-7.68	gggggggatga
E2F	1513 to 1524	-8.32	tttgggggggatg
MA0039 Klf4 ZN-FINGER, C2H2	1513 to 1522	-6.28	tgggggggatg
MA0098 c-ETS ETS	1513 to 1518	+6.01	catccc
MA0118 Macho-1 ZN-FINGER, C2H2	1513 to 1521	-9.06	ggggggatg
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1514 to 1523	-6.69	ttgggggggat
MA0079 SP1 ZN-FINGER, C2H2	1514 to 1523	-7.96	ttgggggggat
MA0118 Macho-1 ZN-FINGER, C2H2	1514 to 1522	-8.32	tgggggggat
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1515 to 1520	-9.22	gggggga
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1515 to 1524	-6.99	tttgggggga
MA0118 Macho-1 ZN-FINGER, C2H2	1515 to 1523	-7.92	ttgggggga
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1516 to 1521	-7.85	gggggg
MA0116 Roaz ZN-FINGER, C2H2	1516 to 1530	+7.85	ccccccaaagagtcc
MA0116 Roaz ZN-FINGER, C2H2	1516 to 1530	-6.34	ggactctttgggggg
MA0024 E2F1 Unknown	1517 to 1524	-6.68	tttggggg
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1517 to 1522	- 7.7	tggggg
MA0047 Foxa2 FORKHEAD	1585 to 1596	-7.07	gcctattgatttt

Fig. E.1 continued

MA0077 SOX9 HMG	1586 to 1594	+6.82	aatcaatag
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1601 to 1606	+6.29	tgggga
Mef-2	1646 to 1657	+7.06	gggtattttaat
Tef	1667 to 1678	+9.99	cacattccttcg
MA0090 TEAD TEA	1667 to 1678	+10.1	cacattccttcg
MA0045 HMG-IY HMG	1677 to 1692	+6.72	cgtcgaaggggaacat
MA0066 PPARG NUCLEAR RECEPTOR	1680 to 1699	-6.07	ccaggtcatgttccccttcg
MA0120 ID1 ZN-FINGER, C2H2	1680 to 1691	-6.07	tgttccccttcg
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1681 to 1690	+ 6.4	gaaggggaac
MA0066 PPARG NUCLEAR RECEPTOR	1681 to 1700	+7.97	gaaggggaacatgacctggt
MA0112 ESR1 NUCLEAR	1681 to 1698	+6.92	gaaggggaacatgacctg
MA0113 NR3C1 NUCLEAR	1682 to 1699	+6.92	aaggggaacatgacctgg
ERE	1683 to 1696	+8.14	aggggaacatgacc
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1683 to 1688	+6.44	aggggа
MA0081 SPIB ETS	1683 to 1689	+6.84	aggggaa
MA0111 Spz1 bHLH-ZIP	1683 to 1693	+6.93	aggggaacatg
ERE	1684 to 1697	-6.66	aggtcatgttcccc
MA0080 SPI1 ETS	1685 to 1690	+6.34	gggaac
MA0106 TP53 P53	1685 to 1704	+ 11	gggaacatgacctggtatgt
MA0089 TCF11-MafG bZIP	1690 to 1695	+6.77	catgac
MA0071 RORA NUCLEAR RECEPTOR	1692 to 1701	-8.31	taccaggtca
MA0092 HAND1-TCF3 bHLH	1693 to 1702	+6.23	gacctggtat
Tef	1694 to 1705	-6.97	cacataccaggt
MA0090 TEAD TEA	1694 to 1705	-6.89	cacataccaggt

Fig. E.1 continued

B

>Cluster-buster output for region 6

Motif	Position	Strand	Score	Sequence
MA0073 RREB1 ZN-FINGER, C2H2	2039 to 2058	-	13.4	acccacaacacccccccccc
MA0079 SP1 ZN-FINGER, C2H2	2040 to 2049	+	7.67	gggggggggt
MA0079 SP1 ZN-FINGER, C2H2	2042 to 2051	+	6.26	ggggggtgt
ERE	2082 to 2095	+	6.59	tatacaccatgccc
MA0074 RXR-VDR NUCLEAR RECEPTOR	2082 to 2096	-	8.16	agggcatggtgtata
MA0017 NR2F1 NUCLEAR RECEPTOR	2084 to 2097	+	6.33	tacaccatgccctt
MA0114 HNF4 NUCLEAR	2084 to 2096	-	8.18	agggcatggtgta
Sp1	2086 to 2098	-	7.05	aaagggcatggtg
MA0078 Sox17 HMG	2086 to 2094	-	6.67	ggcatggtg
MA0111 Spz1 bHLH-ZIP	2086 to 2096	-	6.78	agggcatggtg
MA0079 SP1 ZN-FINGER, C2H2	2087 to 2096	-	10.3	agggcatggt
MA0095 YY1 ZN-FINGER, C2H2	2087 to 2092	+	6.64	accatg
MA0039 Klf4 ZN-FINGER, C2H2	2088 to 2097	-	6.93	aagggcatgg
MA0021 Dof3 ZN-FINGER, DOF	2093 to 2098	-	7.05	aaaggg

Fig. E.1 continued

C

>Cluster-buster output for region 19

Motif	Position	Strand	Score	Sequence
MA0022 Dorsal_1 REL	2692 to 2703	-	9.67	tggggtttcccc
MA0061 NF-kappaB REL	2692 to 2701	+	6.67	ggggaaaccc
MA0105 NFKB1 REL	2692 to 2702	+	9.39	ggggaaacccc
MA0105 NFKB1 REL	2692 to 2702	-	10.1	ggggtttcccc
MA0023 Dorsal_2 REL	2693 to 2702	-	9.95	ggggtttccc
MA0061 NF-kappaB REL	2693 to 2702	+	8.09	gggaaacccc
MA0061 NF-kappaB REL	2693 to 2702	-	10.1	ggggtttccc
MA0101 REL	2693 to 2702	-	6.41	ggggtttccc
MA0105 NFKB1 REL	2693 to 2703	+	7.52	gggaaacccca
MA0105 NFKB1 REL	2693 to 2703	-	7.98	tggggtttccc
MA0107 RELA REL	2693 to 2702	-	8.06	ggggtttccc
MA0023 Dorsal_2 REL	2694 to 2703	-	7.43	tggggtttcc
MA0101 REL	2694 to 2703	-	9.28	tggggtttcc
MA0107 RELA REL	2694 to 2703	-	9.75	tggggtttcc
MA0095 YY1 ZN-FINGER, C2H2	2722 to 2727	-	6.6	gccatc
MA0092 HAND1-TCF3 bHLH	2733 to 2742	+	6.17	tggctggctt
MA0020 Dof2 ZN-FINGER, DOF	2738 to 2743	-	6.39	aaagcc
MA0021 Dof3 ZN-FINGER, DOF	2738 to 2743	-	6.06	aaagcc
Ets	2751 to 2761	-	7.25	ttcaggaaatg
MA0026 E74A ETS	2753 to 2759	-	6.52	caggaaa
MA0081 SPIB ETS	2754 to 2760	-	6.44	tcaggaa
MA0044 HMG-1 HMG	2770 to 2778	+	6.56	gttgtgatc
NF-1	2772 to 2789	-	7.11	ttttggccgaggatcaca
SRF	2782 to 2794	+	6.35	ggccaaaaatgac
MA0051 IRF2 TRP-CLUSTER	2801 to 2818	-	6.58	gcaaggcgaaacgatgaa
MA0004 Arnt bHLH	2819 to 2824	+	6.42	aacgtg
MA0029 Evi1 ZN-FINGER, C2H2	2830 to 2843	+	10.8	gagaaaagataaaa
GATA	2833 to 2845	+	6.12	aaaagataaaaaa
MA0010 Broad-complex_1 ZN-FINGER, C2H2	2838 to 2851	+	9.46	ataaaaaacaagtg
MA0044 HMG-1 HMG	2841 to 2849	-	6.14	cttgtttttt
MA0012 Broad-complex_3 ZN-FINGER, C2H2	2842 to 2852	+	6.14	aaaacaagtgg
MA0016 CFI-USP NUCLEAR RECEPTOR	2849 to 2858	+	6.8	gtggtcacca
MA0110 ATHB5 HOMEO-ZIP	2867 to 2875	+	6.06	cgatgattg
MA0051 IRF2 TRP-CLUSTER	2879 to 2896	-	6.74	agaaatcgaaagtctcac
MA0050 IRF1 TRP-CLUSTER	2884 to 2895	-	9.2	gaaatcgaaagt
MA0082 SQUA MADS	2885 to 2898	-	7.14	acagaaatcgaaag
MA0076 ELK4 ETS	2908 to 2916	+	6.37	actggaagt
MA0058 MAX bHLH-ZIP	2918 to 2927	-	9.35	aatcacgtga
MA0093 USF1 bHLH-ZIP	2918 to 2924	-	8.49	cacgtga
MA0004 Arnt bHLH	2919 to 2924	+	8.17	cacgtg
MA0004 Arnt bHLH	2919 to 2924	-	8.17	cacgtg
MA0093 USF1 bHLH-ZIP	2919 to 2925	+	8.36	cacgtga
MA0104 Mycn bHLH-ZIP	2919 to 2924	+	8.25	cacgtg
MA0104 Mycn bHLH-ZIP	2919 to 2924	-	8.25	cacgtg

Fig. E.1 continued

D

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>Cluster-buster output for subregion 5-1
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Motif	Position	Strand	Score	Sequence
MA0045 HMG-IY HMG	7 to 22	+	11	gttgaaaaggaaaaaa
MA0013 Broad-complex_4 ZN-FINGER, C2H2	8 to 18	+	7.12	ttgaaaaggaa
MA0045 HMG-IY HMG	8 to 23	+	8.74	ttgaaaaggaaaaaat
MA0010 Broad-complex_1 ZN-FINGER, C2H2	9 to 22	+	7.41	tgaaaaggaaaaaa
MA0120 ID1 ZN-FINGER, C2H2	9 to 20	-	9.82	ttttccttttca
MA0010 Broad-complex_1 ZN-FINGER, C2H2	10 to 23	+	7.4	gaaaaggaaaaaat
MA0028 ELK1 ETS	10 to 19	+	7.31	gaaaaggaaa
MA0030 FOXF2 FORKHEAD	10 to 23	+	6.12	gaaaaggaaaaaat
MA0050 IRF1 TRP-CLUSTER	10 to 21	+	7.44	gaaaaggaaaaa
MA0068 Pax4 PAIRED-HOMEO	10 to 39	+	6.8	gaaaaggaaaaaataggttttagcgcgcc
MA0120 ID1 ZN-FINGER, C2H2	10 to 21	-	6.99	tttttccttttc
Ets	11 to 21	+	8.44	aaaaggaaaaa
MA0039 Klf4 ZN-FINGER, C2H2	11 to 20	+	7.52	aaaaggaaaa
MA0120 ID1 ZN-FINGER, C2H2	11 to 22	-	6.71	ttttttcctttt
MA0013 Broad-complex_4 ZN-FINGER, C2H2	12 to 22	+	6.23	aaaggaaaaaa
MA0020 Dof2 ZN-FINGER, DOF	12 to 17	+	6.07	aaagga
MA0021 Dof3 ZN-FINGER, DOF	12 to 17	+	6.28	aaagga
MA0039 Klf4 ZN-FINGER, C2H2	12 to 21	+	7.18	aaaggaaaaa
MA0081 SPIB ETS	12 to 18	+	7.52	aaaggaa
MA0013 Broad-complex_4 ZN-FINGER, C2H2	13 to 23	+	7.05	aaggaaaaaat
MA0026 E74A ETS	13 to 19	+	6.64	aaggaaa
MA0049 Hunchback ZN-FINGER, C2H2	13 to 22	+	7.83	aaggaaaaaa
MA0120 ID1 ZN-FINGER, C2H2	13 to 24	-	6.28	tatttttcctt
MA0049 Hunchback ZN-FINGER, C2H2	14 to 23	+	6.17	aggaaaaaat
MA0098 c-ETS ETS	14 to 19	-	6.99	tttcct
MA0049 Hunchback ZN-FINGER, C2H2	15 to 24	+	6.03	ggaaaaaata
MA0047 Foxa2 FORKHEAD	16 to 27	-	6.13	acctatttttc
MA0033 FOXL1 FORKHEAD	17 to 24	+	7.2	aaaaaata
MA0039 Klf4 ZN-FINGER, C2H2	17 to 26	+	7.59	aaaaaatagg
MA0011 Broad-complex_2 ZN-FINGER, C2H2	20 to 27	-	6.57	acctattt
MA0024 E2F1 Unknown	29 to 36	+	6.29	tttagcgc
MA0123 ABI4 AP2	31 to 40	-	6.34	cggcgcgcta
MA0079 SP1 ZN-FINGER, C2H2	32 to 41	-	6.72	gcggcgcgct
MA0028 ELK1 ETS	43 to 52	-	6.62	gcgacggaaa
GATA	49 to 61	-	6.53	tcatgataagcga
MA0089 TCF11-MafG bZIP	57 to 62	+	7.12	catgac

Fig. E.1 continued

MA0077 SOX9 HMG	68 to 76	-	8.25	aaacaatgg
MA0040 Foxq1 FORKHEAD	69 to 79	+	8.68	cattgtttatg
MA0030 FOXF2 FORKHEAD	70 to 83	-	8.31	cacacataaacaat
MA0084 SRY HMG	70 to 78	-	6.38	ataaacaat
MA0087 Sox5 HMG	70 to 76	-	6.58	aaacaat
MA0021 Dof3 ZN-FINGER, DOF	102 to 107	-	6.02	aaagtg
CCAAT	124 to 139	+	6.32	gttgaccaattacact
MA0060 NF-Y CAAT-BOX	124 to 139	+	6.57	gttgaccaattacact
MA0068 Pax4 PAIRED-HOMEO	127 to 156	+	6.02	gaccaattacactcaataatgacggcgcgc
MA0075 Prrx2 HOMEO	131 to 135	+	7	aatta
MA0122 Bapx1 HOMEO	134 to 142	-	7.27	ttgagtgta
MA0110 ATHB5 HOMEO-ZIP	140 to 148	-	8.13	tcattattg
MA0008 Athb-1 HOMEO-ZIP	141 to 148	-	6.71	tcattatt
MA0089 TCF11-MafG bZIP	144 to 149	+	6.88	aatgac
MA0123 ABI4 AP2	149 to 158	+	6.08	cggcgcgcat
MA0006 Arnt-Ahr bHLH	170 to 175	+	6.54	tgcgtg
MA0067 Pax2 PAIRED	171 to 178	-	6.66	agtcacgc
MA0043 HLF bZIP	191 to 202	-	6.81	cgttacacaaag
MA0025 NFIL3 bZIP	193 to 203	+	6.22	ttgtgtaacgc
CCAAT	202 to 217	-	10.6	ttcagccaatcaccgc
MA0060 NF-Y CAAT-BOX	202 to 217	-	10.8	ttcagccaatcaccgc
MA0038 Gfi ZN-FINGER, C2H2	203 to 212	-	7.86	ccaatcaccg
MA0041 Foxd3 FORKHEAD	216 to 227	-	8.81	aaatgttaattt
MA0047 Foxa2 FORKHEAD	216 to 227	-	6.42	aaatgttaattt
MA0040 Foxq1 FORKHEAD	217 to 227	-	6.03	aaatgttaatt
MA0075 Prrx2 HOMEO	217 to 221	+	6.46	aatta
MA0003 TFAP2A AP2	229 to 237	+	7.03	gcccagggg
NF-1	230 to 247	-	6.21	atttggcgcgcccctggg
MA0003 TFAP2A AP2	230 to 238	-	6.91	gcccctggg
E2F	235 to 246	-	7.83	tttggcgcgccc
MA0024 E2F1 Unknown	239 to 246	-	10.1	tttggcgc
MA0082 SQUA MADS	242 to 255	+	7.04	ccaaatataaaact
E2F	256 to 267	+	6.48	ttcggcgcgcgc
MA0024 E2F1 Unknown	256 to 263	+	6.29	ttcggcgc
MA0123 ABI4 AP2	258 to 267	+	7.39	cggcgcgcgc
MA0017 NR2F1 NUCLEAR RECEPTOR	305 to 318	-	6.11	tgaactgcgccctg
MA0114 HNF4 NUCLEAR	306 to 318	+	6.5	agggcgcagttca
MA0049 Hunchback ZN-FINGER, C2H2	321 to 330	+	6.62	tcaaaaaaag
MA0082 SQUA MADS	321 to 334	+	7.5	tcaaaaaaagtaca
MA0013 Broad-complex_4 ZN-FINGER, C2H2	337 to 347	-	6.17	atgtaaacaaa
MA0084 SRY HMG	337 to 345	-	6.32	gtaaacaaa
MA0031 FOXD1 FORKHEAD	338 to 345	-	8.11	gtaaacaa
MA0003 TFAP2A AP2	353 to 361	-	6.47	gccccgacg
TATA	355 to 369	-	6.78	gtacaaaagccccga
MA0108 TBP TATA-box	355 to 369	-	6.81	gtacaaaagccccga

Fig. E.1 continued

MA0020 Dof2 ZN-FINGER, DOF	359 to 364	-	6.12	aaagcc
MA0078 Sox17 HMG	381 to 389	+	6.58	gctattgtg
MA0008 Athb-1 HOMEO-ZIP	403 to 410	+	7.36	caatcatt
MA0110 ATHB5 HOMEO-ZIP	403 to 411	-	7.22	taatgattg
MA0114 HNF4 NUCLEAR	415 to 427	-	6.33	gcgggagagtgca
MA0079 SP1 ZN-FINGER, C2H2	418 to 427	-	6.18	gcgggagagt
MA0039 Klf4 ZN-FINGER, C2H2	419 to 428	-	6.33	tgcgggagag
MA0062 GABPA ETS	419 to 428	-	6.1	tgcgggagag
MA0024 E2F1 Unknown	420 to 427	+	6.89	tctcccgc
TATA	423 to 437	-	8.76	gcatataggtgcggg
MA0108 TBP TATA-box	423 to 437	-	8.79	gcatataggtgcggg
MA0103 deltaEF1 ZN-FINGER, C2H2	427 to 432	+	7.36	caccta
MA0015 CF2-II ZN-FINGER, C2H2	430 to 439	+	6.91	ctatatgcag
GATA	442 to 454	+	6.04	aagtgataaaaat
MA0091 TAL1-TCF3 bHLH	493 to 504	-	8	tgaacatctttt
MA0121 ARR10 TRP-CLUSTER	504 to 511	-	6.11	agattctt
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	546 to 555	+	7.83	ttaggggcgg
MA0068 Pax4 PAIRED-HOMEO	546 to 575	-	8.91	gaaaagtagaacttcacccccgcccctaa
Sp1	547 to 559	+	15.3	taggggcgggggg
MA0039 Klf4 ZN-FINGER, C2H2	547 to 556	+	8.63	taggggcggg
MA0079 SP1 ZN-FINGER, C2H2	547 to 556	+	6.54	taggggcggg
MA0118 Macho-1 ZN-FINGER, C2H2	547 to 555	+	7.12	taggggcgg
Sp1	548 to 560	+	7.21	aggggcgggggt
E2F	548 to 559	+	7.38	aggggcgggggg
MA0039 Klf4 ZN-FINGER, C2H2	548 to 557	+	7.36	aggggcgggg
MA0068 Pax4 PAIRED-HOMEO	548 to 577	-	7.56	aagaaaagtagaacttcacccccgcccct
MA0079 SP1 ZN-FINGER, C2H2	548 to 557	+	6.73	aggggcgggg
MA0111 Spz1 bHLH-ZIP	548 to 558	+	6.45	aggggcggggg
MA0123 ABI4 AP2	548 to 557	-	8.94	ccccgcccct
Sp1	549 to 561	+	6.27	ggggcgggggtg
MA0068 Pax4 PAIRED-HOMEO	549 to 578	-	13	gaagaaaagtagaacttcacccccgcccc
MA0074 RXR-VDR NUCLEAR RECEPTOR	549 to 563	+	6.69	ggggcgggggtgaa
MA0079 SP1 ZN-FINGER, C2H2	549 to 558	+	11.5	ggggcggggg
MA0114 HNF4 NUCLEAR	549 to 561	+	6.84	ggggcgggggtg
MA0118 Macho-1 ZN-FINGER, C2H2	549 to 557	+	7.68	ggggcgggg
Myf	550 to 561	+	6.33	gggcgggggtg
MA0007 Ar NUCLEAR RECEPTOR	550 to 571	-	6.67	agtagaacttcacccccgccc
MA0079 SP1 ZN-FINGER, C2H2	550 to 559	+	7.55	gggcgggggg
MA0114 HNF4 NUCLEAR	550 to 562	+	6.02	gggcgggggtga
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	551 to 560	+	8.59	ggcgggggt
MA0079 SP1 ZN-FINGER, C2H2	551 to 560	+	9.1	ggcgggggt
MA0123 ABI4 AP2	552 to 561	-	7.83	cacccccgc
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	553 to 558	+	7.56	cggggg
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	553 to 562	+	6.98	cgggggtga

Fig. E.1 continued

MA0079 SP1 ZN-FINGER, C2H2	553 to 562	+	8.68	cgggggggtga
MA0113 NR3C1 NUCLEAR	553 to 570	+	6.7	cgggggggtgaagttctac
MA0118 Macho-1 ZN-FINGER, C2H2	553 to 561	+	10.3	cgggggggtg
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	554 to 559	+	7.06	gggggg
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	554 to 563	+	6.57	gggggggtgaa
MA0079 SP1 ZN-FINGER, C2H2	554 to 563	+	7.82	gggggggtgaa
MA0118 Macho-1 ZN-FINGER, C2H2	554 to 562	+	7.6	ggggggggtga
MA0018 CREB1 bZIP	555 to 566	+	6.61	gggggtgaagtt
MA0016 CFI-USP NUCLEAR RECEPTOR	556 to 565	+	7.56	ggggtgaagt
MA0114 HNF4 NUCLEAR	556 to 568	+	6.45	ggggtgaagttct
MA0046 TCF1 HOMEO	623 to 636	-	6.16	agttaaatatttta
GATA	652 to 664	+	6.27	tgtagataaagaa
MA0049 Hunchback ZN-FINGER, C2H2	655 to 664	+	6.16	agataaagaa
MA0020 Dof2 ZN-FINGER, DOF	659 to 664	+	6.44	aaagaa
MA0053 MNB1A ZN-FINGER, DOF	659 to 663	+	6.16	aaaga
MA0075 Prrx2 HOMEO	663 to 667	+	6.21	aatta
MA0019 Chop-cEBP bZIP	690 to 701	+	6.08	acatgcaaacct
Mef-2	698 to 709	+	7.1	acctatttttat
MA0052 MEF2A MADS	700 to 709	+	6.36	ctatttttat
MA0073 RREB1 ZN-FINGER, C2H2	709 to 728	-	6.7	ctccccccccccccctcaaa
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	711 to 720	+	7.55	tgagggggggg
MA0068 Pax4 PAIRED-HOMEO	711 to 740	-	7.97	gaatcgagacagctcccccccccccccctca
Sp1	712 to 724	+	6.59	gagggggggggg
Sp1	713 to 725	+	7.34	agggggggggggg
Sp1	714 to 726	+	7.49	ggggggggggggg
MA0079 SP1 ZN-FINGER, C2H2	714 to 723	+	6.47	ggggggggg
Sp1	715 to 727	+	6.11	gggggggggggga
MA0068 Pax4 PAIRED-HOMEO	715 to 744	-	8.31	aactgaatcgagacagctcccccccccccc
MA0079 SP1 ZN-FINGER, C2H2	715 to 724	+	6.49	ggggggggg
MA0079 SP1 ZN-FINGER, C2H2	716 to 725	+	6.49	ggggggggg
Sp1	717 to 729	+	6.39	gggggggggagc
MA0079 SP1 ZN-FINGER, C2H2	717 to 726	+	6.49	ggggggggg
Sp1	718 to 730	+	6.7	gggggggggagct
MA0079 SP1 ZN-FINGER, C2H2	718 to 727	+	8.19	ggggggggga
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	722 to 727	+	6.74	ggggga
MA0014 Pax5 PAIRED	729 to 748	-	6.04	tgctaactgaatcgagacag
MA0114 HNF4 NUCLEAR	745 to 757	-	6.61	tggacaaagtgct
MA0021 Dof3 ZN-FINGER, DOF	747 to 752	-	6.66	aaagtg
MA0078 Sox17 HMG	747 to 755	+	6.03	cactttgtc
SRF	754 to 766	-	7.23	tgccaaggatgga
MA0095 YY1 ZN-FINGER, C2H2	754 to 759	+	6.33	tccatc
MA0035 Gata1 ZN-FINGER, GATA	755 to 760	-	6.53	ggatgg
MA0098 c-ETS ETS	756 to 761	+	6.11	catcct

Fig. E.1 continued

NF-1	759 to 776	+	6.91	ccttggcaactcgtaaca
MA0045 HMG-IY HMG	777 to 792	-	6.17	taggaaatcgcagaac
MA0038 Gfi ZN-FINGER, C2H2	780 to 789	-	6.37	gaaatcgcag
MA0023 Dorsal_2 REL	781 to 790	+	6.46	tgcgatttcc
MA0107 RELA REL	781 to 790	+	6.5	tgcgatttcc
MA0098 c-ETS ETS	786 to 791	+	6.09	tttcct
MA0037 GATA3 ZN-FINGER, GATA	790 to 795	-	6.2	tgatag
Mef-2	811 to 822	-	7.34	ggttttatttag
MA0027 En1 HOMEO	865 to 875	-	6.75	aaggagttgtc
MA0021 Dof3 ZN-FINGER, DOF	871 to 876	-	6.04	aaagga
MA0071 RORA NUCLEAR RECEPTOR	884 to 893	+	6.01	gtatgggtca
AP-1	885 to 895	-	7.18	gatgacccata
MA0089 TCF11-MafG bZIP	890 to 895	-	7.54	gatgac
MA0035 Gata1 ZN-FINGER, GATA	899 to 904	-	6.49	ggatgc
MA0036 GATA2 ZN-FINGER, GATA	900 to 904	-	6.09	ggatg
MA0050 IRF1 TRP-CLUSTER	913 to 924	-	6.65	caaagggaagcc
MA0062 GABPA ETS	913 to 922	-	6.59	aagggaagcc
MA0039 Klf4 ZN-FINGER, C2H2	914 to 923	-	6.95	aaagggaagc
MA0028 ELK1 ETS	915 to 924	-	6.22	caaagggaag
MA0039 Klf4 ZN-FINGER, C2H2	915 to 924	-	7.16	caaagggaag
MA0080 SPI1 ETS	915 to 920	-	8.36	gggaag
MA0098 c-ETS ETS	915 to 920	+	6.2	cttccc
MA0081 SPIB ETS	916 to 922	-	6.2	aagggaa
MA0021 Dof3 ZN-FINGER, DOF	918 to 923	-	6.89	aaaggg
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	932 to 937	+	6.26	tgggga
MA0070 Pbx HOMEO	951 to 962	-	6.77	gcttcaatcaat
MA0068 Pax4 PAIRED-HOMEO	991 to 1020	+	6.22	gaaaattgtcctgggccttttgtcatcccc
MA0068 Pax4 PAIRED-HOMEO	992 to 1021	+	6.58	aaaattgtcctgggccttttgtcatccccc
MA0078 Sox17 HMG	992 to 1000	+	6.03	aaaattgtc
MA0068 Pax4 PAIRED-HOMEO	993 to 1022	+	7.03	aaattgtcctgggccttttgtcatcccccc
MA0078 Sox17 HMG	1006 to 1014	+	6.23	ccttttgtc
AP-1	1007 to 1017	-	6.57	gatgacaaaag
MA0010 Broad-complex_1 ZN-FINGER, C2H2	1007 to 1020	-	8.62	ggggatgacaaaag
MA0045 HMG-IY HMG	1007 to 1022	-	6.89	gggggatgacaaaag
NF-1	1008 to 1025	+	6.31	ttttgtcatccccccaaa
NF-1	1009 to 1026	-	7.38	ctttgggggatgacaaa
MA0018 CREB1 bZIP	1009 to 1020	-	6.24	ggggatgacaaa
MA0084 SRY HMG	1009 to	-	6.07	gatgacaaa

Fig. E.1 continued

	1017			
MA0119 Hox11-CTF1 HOMEO/CAAT	1010 to 1023	-	6.77	tgggggggatgacaa
MA0111 Spz1 bHLH-ZIP	1011 to 1021	-	6.43	gggggatgaca
MA0119 Hox11-CTF1 HOMEO/CAAT	1011 to 1024	+	6.95	tgtcatccccccaa
Ets	1012 to 1022	-	6.79	gggggggatgac
MA0089 TCF11-MafG bZIP	1012 to 1017	-	7.2	gatgac
MA0079 SP1 ZN-FINGER, C2H2	1013 to 1022	-	7.68	gggggggatga
E2F	1014 to 1025	-	8.32	tttgggggggatg
MA0039 Klf4 ZN-FINGER, C2H2	1014 to 1023	-	6.28	tgggggggatg
MA0098 c-ETS ETS	1014 to 1019	+	6.01	catccc
MA0118 Macho-1 ZN-FINGER, C2H2	1014 to 1022	-	9.06	ggggggatg
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1015 to 1024	-	6.69	ttgggggggat
MA0079 SP1 ZN-FINGER, C2H2	1015 to 1024	-	7.96	ttgggggggat
MA0118 Macho-1 ZN-FINGER, C2H2	1015 to 1023	-	8.32	tgggggggat
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1016 to 1021	-	9.22	ggggga
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1016 to 1025	-	6.99	tttgggggga
MA0118 Macho-1 ZN-FINGER, C2H2	1016 to 1024	-	7.92	ttgggggga
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1017 to 1022	-	7.85	gggggg
MA0116 Roaz ZN-FINGER, C2H2	1017 to 1031	+	7.85	ccccccaaagagtcc
MA0116 Roaz ZN-FINGER, C2H2	1017 to 1031	-	6.34	ggactctttgggggg
MA0024 E2F1 Unknown	1018 to 1025	-	6.68	tttggggg
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1018 to 1023	-	7.7	tggggg
MA0047 Foxa2 FORKHEAD	1086 to 1097	-	7.07	gcctattgattt
MA0077 SOX9 HMG	1087 to	+	6.82	aatcaatag

Fig. E.1 continued

	1095			
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1102 to 1107	+	6.29	tgggga
Mef-2	1147 to 1158	+	7.06	gggtattttaat
Tef	1168 to 1179	+	9.99	cacattccttcg
MA0090 TEAD TEA	1168 to 1179	+	10.1	cacattccttcg
MA0045 HMG-IY HMG	1178 to 1193	+	6.72	cgtcgaaggggaacat
MA0066 PPARG NUCLEAR RECEPTOR	1181 to 1200	-	6.07	ccaggtcatgttccccttcg
MA0120 ID1 ZN-FINGER, C2H2	1181 to 1192	-	6.07	tgttccccttcg
MA0057 ZNF42_5-13 ZN-FINGER, C2H2	1182 to 1191	+	6.4	gaaggggaac
MA0066 PPARG NUCLEAR RECEPTOR	1182 to 1201	+	7.97	gaaggggaacatgacctggt
MA0112 ESR1 NUCLEAR	1182 to 1199	+	6.92	gaaggggaacatgacctg
MA0113 NR3C1 NUCLEAR	1183 to 1200	+	6.92	aaggggaacatgacctgg
ERE	1184 to 1197	+	8.14	aggggaacatgacc
MA0056 ZNF42_1-4 ZN-FINGER, C2H2	1184 to 1189	+	6.44	agggga
MA0081 SPIB ETS	1184 to 1190	+	6.84	aggggaa
MA0111 Spz1 bHLH-ZIP	1184 to 1194	+	6.93	aggggaacatg
ERE	1185 to 1198	-	6.66	aggtcatgttcccc
MA0080 SPI1 ETS	1186 to 1191	+	6.34	gggaac
MA0106 TP53 P53	1186 to 1205	+	11	gggaacatgacctggtatgt
MA0089 TCF11-MafG bZIP	1191 to 1196	+	6.77	catgac
MA0071 RORA NUCLEAR RECEPTOR	1193 to 1202	-	8.31	taccaggtca
MA0092 HAND1-TCF3 bHLH	1194 to 1203	+	6.23	gacctggtat
Tef	1195 to 1206	-	6.97	cacataccaggt
MA0090 TEAD TEA	1195 - 1206	-	6.89	cacataccagg

Appendix F: G-test to determine statistical significance of number of binding sites for each indicated transcription factor in each discussed regulatory region

Notes:

1. "Non_versions" of each transcription factor binding site are calculated because at least 1 degree of freedom is needed to determine if a G value is significant. Deg of freedom = N - 1, where N = number of each transcription factor binding site + its non_version.
2. If the value of either observed or predicted number of binding sites was zero, the number zero was not used in calculations, since the G value would then be undefined. Instead, a very small number, approaching zero, was used, e.g. 10^-100.
3. To achieve the most accurate values for G, the expected number of binding sites for each transription factor were not rounded up or down to the nearest whole number.
4. The p value cutoffs for statistically significant differences are shown only for p values of 0.10 and lower. G tests yielding p values of >0.10 were considered to yield non-significant results.
4. Sometimes, it could be determined without calculations that the difference between observed and expected values of transcription factors bindings sites was not significant, for example, if both values were the same number, or could be rounded to the same number.
   It was also sometimes able to be determined, based on comparison to preceeding calculations in this file, if a p value was <0.001.
6. Abbreviation: rc = reverse complement

Calculations for significance

Expected frequencies

How often both orientations are predicted to occur in each region and subregion (click on each cell to determime formula used to obain each value)

	2	**2_2**		**4**	**4_1**	**4_2**		**5**	**5_1**		**6**	**6_1**		**17**		**19**	**19_1**
Otx TAATCC TAATCT plus reverse complements	2.39689	0.31521	1.39546	0.4885	0.25942	1.60379	1.01358	1.83805	1.29655	1.4236	3.11002	0.99176					
Gatae (C/T)GATA(A/G) plus reverse complemement	10.1541	1.27622	5.90635	2.13804	1.05332	6.80313	4.25044	8.13051	5.64469	6.3208	13.2553	4.23741					
Bra (A/G)(A/T)(A/T)NTN(A/G)CAC(C/T)T plus rev comp	0.37038	0.04373	0.21532	0.07898	0.03638	0.24835	0.15391	0.30084	0.20826	0.23397	0.48516	0.15528					
Foxa (A/G)(A/C)(A/C)T(G/A)TT(A/G/T)(A/T)TT(T/C) + rc	0.31748	0.0238	0.18333	0.08423	0.02065	0.21496	0.12187	0.34168	0.21495	0.27149	0.4347	0.14157					
Gatac (T/G/A)(T/A)(G/C)AGACT(T/A)AGC(T/G) +rc	0.00516	0.00083	0.00301	0.00093	0.00067	0.00343	0.00227	0.00335	0.00251	0.00256	0.00653	0.00206					
Su(H) (C/G)(G/A)TG(A/G)GA(A/T/G) + rc	1.93846	0.35424	1.13389	0.33977	0.28258	1.28825	0.86687	1.22506	0.92214	0.93522	2.43858	0.76831					
Runx (C/T)G(C/T)GGTN +rc	3.87634	0.82113	2.27201	0.63811	0.64553	2.56864	1.77576	2.26166	1.74762	1.71667	4.81279	1.50863					
TCF ACAAAG + rc	2.39689	0.31521	1.39546	0.4885	0.25942	1.60379	1.01358	1.83805	1.29655	1.4236	3.11002	0.99176					
GC percentage	38.19	44.51	38.28	35.33	43.91	38.06	39.26	34.53	35.75	34.26	37.6	37.37					
G or C proportion	**0.19095**	**0.22255**	**0.1914**	**0.17665**	**0.21955**	**0.1903**	**0.1963**	**0.17265**	**0.17875**	**0.1713**	**0.188**	**0.18685**					
A or T proportion	**0.30905**	**0.27745**	**0.3086**	**0.32335**	**0.28045**	**0.3097**	**0.3037**	**0.32735**	**0.32125**	**0.3287**	**0.312**	**0.31315**					
Sequence length(bp)	3603	537	2100	716	435	2407	1546	2685	1905	2078	4643	1477					
Observed frequencies	**2**	**2_2**		**4**	**4_1**	**4_2**		**5**	**5_1**		**6**	**6_1**		**17**		**19**	**19_1**
Otx TAATCC TAATCT plus reverse complements	8	0	2	0	0	6	3	3	2	0	5	1					
Gatae (C/T)GATA(A/G) plus reverse complemement	12	3	3	2	2	6	5	7	4	5	4	2					
Bra (A/G)(A/T)(A/T)NTN(A/G)CAC(C/T)T plus rev comp	0	0	0	0	0	1	0	1	1	0	0	0					
Foxa (A/G)(A/C)(A/C)T(G/A)TT(A/G/T)(A/T)TT(T/C) + rc	0	0	0	0	0	3	3	0	0	0	0	0					
Gatac (T/G/A)(T/A)(G/C)AGACT(T/A)AGC(T/G) +rc	14	2	13	5	3	16	11	9	9	6	20	8					
Su(H) (C/G)(G/A)TG(A/G)GA(A/T/G) + rc	1	0	0	0	0	0	0	0	0	1	0	0					
Runx (C/T)G(C/T)GGTN +rc	2	0	2	1	1	2	2	4	4	2	4	0					
TCF ACAAAG + rc	2	1	2	2	0	6	6	5	5	1	7	1					

#bp/site	(for excel calcs)
6	otx
6	gatae
12	bra
12	foxa
13	gatac
8	su(H)
6	Runx
6	TCF

G test calculations to determine if observed numbers of each potential binding site in each region is significant. (For each region, G values for both each transcription factor binding site of interest, plus its non-version, were calculated, as shown in the columns designated "G."
These values were summed, and the resultant "Sum of G's" calculated in each case was compared to P values in the table below that starts at line 179. From this result, a level of statistical significance could be determined in each case).

Reg 2	**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**	Site	Expected
Otx	8	2.39689	19.2843	**Gatae**	12	10.1540705	4.00877	**Bra**	1E-133	0.37038	-6E-131	**Foxa**	0	0.31748		**Gatac**	14	0.00516	221.374	**Su(H)**	1	1.93846	-1.32378	**Runx**	2	3.87634	-2.64698	Otx	2.39689
Non Otx	592.5	598.103	-11.1536	Non Gatae	588.5	590.34593	-3.68608	Non Bra	300.25	299.88	0.74122					NonGatac	263.154	277.149	-27.2708	NonSu(H)	449.375	448.437	1.87888	NonRunx	598.5	596.624	3.75858	Gatae	10.1541
Sum of G's			8.13073	Sum of G's			0.32269	Sum of G's			0.74122					Sum of G's			194.103	Sum of G's			0.55509	Sum of G's			1.1116	Bra	0.37038
																												Foxa	0.15874
P<0.01				Not significant				Not significant				Not significant				P<0.001				Not significant in either tail.				Not significant.				Gatac	0.00516
																												Su(H)	1.93846
	Observed	**Expected**	**G**																									Runx	3.87634
TCF	2	2.39689	-0.7241																									TCF	2.39689
NonTCF	598.5	598.103	0.79405																										
Sum of G's			0.06994																										
Not significant																													

Reg 2_2	**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**	Site	Expected
Otx	1E-114	0.31521	-5E-112	**Gatae**	3	1.27622156	5.12825	**Bra**	0	0.04373		**FoxA**	0	0.0238		**Gatac**	2	0.00083	31.1406	**Su(H)**	0	0.35424		**Runx**	1E-111	0.82113	-5E-109	Otx	0.31521
Non Otx	89.5	89.1848	0.63153	Non Gatae	86.5	88.2237784	-3.41365									NonGatac	39.3077	41.3069	-3.89998	NonSu(H)				NonRunx	89.5	88.6789	1.64984	Gatae	1.27622
Sum of G's			0.63153	Sum of G's			1.7146									Sum of G's			27.2406					Sum of G's			1.64984	Bra	0.04373
Not significant either tail.				Not significant				Not significant				Not significant				P<0.001				Not significant				Not significant in either tail.				Foxa	0.0119
																												Gatac	0.00083
																												Su(H)	0.35424
																												Runx	0.82113
	Observed	**Expected**	**G**																									TCF	0.31521
TCF	1	0.31521	2.30904																										
NonTCF	88.5	89.1848	-1.36431																										
Sum of G's			0.94473																										
Not significant																													

Reg 4	**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**	Site	Expected
Otx	2	1.39546	1.4397	**Gatae**	3	5.90634754	-4.06449	**Bra**	0	0.21532		**FoxA**	0	0.18333		**Gatac**	13	0.09167	128.818	**Su(H)**	1E-97	1.13389	-4.5E-95	**Runx**	2	2.27201	-0.51008	Otx	1.39546
NonOtx	348	348.605	-1.20803	NonGatae	347	344.093652	5.83717	NonBra								NonGatac	148.538	161.447	-24.7559		262.5	261.366	2.27269	NonRunx	348	347.728	0.54424	Gatae	5.90635
Sum of G's			0.23166	Sum of G's			1.77268	Sum of G's								Sum of G's			104.062		Sum of G's		2.27269	Sum of G's			0.03416	Bra	0.21532
Not significant				Not significant in either tail				Not significant				Not significant				P<0.001					Not significant			Not significant in either tail				Foxa	0.09167
	Observed	**Expected**	**G**																									Gatac	0.09167
TCF	2	1.39546	1.4397																									Su(H)	1.13389
NonTCF	348	348.605	-1.20803																									Runx	2.27201
Sum of G's			0.23166																									TCF	1.39546
Not significant																													

Reg 4_1	**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**		**Observed**	**Expected**	**G**	Site	Expected
Otx	1E-101	0.4885	-5E-99	**Gatae**	2	2.13803723	-0.26696	**Bra**	0	0.07898		**FoxA**	0	0.08423		**Gatac**	5	0.00093	85.9273	**Su(H)**	0	0.33977		**Runx**	1	0.63811		Otx	0.4885

NonOtx	119.33333	118.845	0.979	NonGatae	117.333	117.195296	0.27624	NonBra								NonGatac	49.0769	55.076	-11.3196									Gatae	2.13804
Sum of G's			0.979	Sum of G's			0.00927									Sum of G's			74.6077									Bra	0.07898
Not significant				Not significant				Not significant				Not significant				p<0.001				Not significant				Not significant				Foxa	0.04211
																												Gatac	0.00093
	Observed	Expected	G																									Su(H)	0.33977
TCF	2	0.4885	5.63828																									Runx	0.63811
NonTCF	117.33333	118.845	-3.0037																									TCF	0.4885
			2.63458																										
Not significant																													
					Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G	Site	Expected
	Observed	Expected	G	Gatae	2	1.05331686	2.56481	Bra	0	0.03638		FoxA	0	0.02065		Gatac	3	0.00067	50.4045	Su(H)	0	0.28258		Runx	1	0.64553		Otx	0.25942
Reg 4_2	0	0.25942		NonGatae	70.5	71.4466831	-1.88077									NonGatac	30.4615	33.4609	-5.72139	NonSu(H)								Gatae	1.05332
Otx							0.68405												44.6831									Bra	0.03638
Not significant				Not significant				Not significant				Not significant				P<0.001				Not significant.				Not significant.				Foxa	0.01032
																												Gatac	0.00067
	Observed	Expected	G																									Su(H)	0.28258
TCF	0	0.25942																										Runx	0.64553
Not significant																												TCF	0.25942
	Observed	Expected	G	G tests checked, ok																									
Reg 5					Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G	Site	Expected
Otx	6	1.60379	15.8327	Gatae	6	6.80312576	-1.50747	Bra	1	0.24835	2.78585	FoxA	3	0.21496	15.8155	Gatac	16	0.00343		Su(H)	1E-82	1.28825	-3.8E-80	Runx	2	2.56864	-1.00092	Otx	1.60379
NonOtx	395.16667	399.563	-8.74386	NonGatae	395.167	394.363541	1.60789		199.583	200.335	-1.50048	NonFoxA	197.583	200.368	-5.53119					NonSu(H)	300.875	299.587	2.58202	NonRunx	399.167	398.598	1.1381	Gatae	6.80313
Sum of G's			7.08879				0.10041				1.28537				10.2843								2.58202				0.13717	Bra	0.24835
p<0.01				Not significant.				Not significant.				p<0.01				p<0.001				Not significant.				Not signficant.				Foxa	0.10748
																												Gatac	0.00343
	Observed	Expected	G																									Su(H)	1.28825
TCF	6	1.60379	15.8327																									Runx	2.56864
NonTCF	395.16667	399.563	-8.74386																									TCF	1.60379
	Sum of G's		7.08879																										
p<0.01																													
Reg5_1	Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G	Site	Expected
Otx	3	1.01358	6.51073	Gatae	5	4.25044273	1.62415	Bra	0	0.15391		FoxA	3	0.12187	19.2207	Gatac	11	0.00227	186.657	Su(H)	1E-160	0.86687	-7E-158	Runx	2	1.77576	0.47567	Otx	1.01358
NonOtx	254.66667	256.653	-3.95742	NonGatae	252.667	253.416224	-1.4969					NonFoxA	125.833	128.711	-5.69142	NonGatac	107.923	118.921	-20.9455		193.25	192.383	1.73764	NonRunx	255.667	255.891	-0.44828	Gatae	4.25044
Sum of G's			2.5533	Sum of G's			0.12725								13.5293				165.711				1.73764				0.02739	Bra	0.15391
Not significant				Not significant				Not significant				p<0.001				p<0.001				Not significant				Not significant				Foxa	0.06093
																												Gatac	0.00227
	Observed	Expected	G																									Su(H)	0.86687
TCF	6	1.01358	21.3392																									Runx	1.77576
NonTCF	251.66667	256.653	-9.87532																									TCF	1.01358
			11.4639																										
p<0.001																													
Reg6	Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G	Site	Expected
Otx	3	1.83805	2.93945	Gatae	7	8.13051233	-2.09599	Bra	1	0.30084	2.40234	FoxA	0	0.34168		Gatac	9	0.00335		Su(H)	1E-186	1.22506	-9E-184	Runx	4	2.26166	4.56158	Otx	1.83805
	444.5	445.662	-2.32087	NonGatae	440.5	439.369488	2.26393	NonBra	222.75	223.449	-1.39613									NonSu(H)	335.625	334.4	2.45461	NonRunx	443.5	445.238	-3.46989	Gatae	8.13051
Sum of G's			0.61858	Sum of G's			0.16794	Sum of G's			1.00621									Sum of G's			2.45461	Sum of G's			1.09169	Bra	0.30084
Not significant.				Not significant.				Not significant				Not significant				p<0.001				Not significant.				Not significant				Foxa	0.17084
																												Gatac	0.00335
	Observed	Expected	G																									Su(H)	1.22506
TCF	5	1.83805	10.0073																									Runx	2.26166
NonTCF	442.5	445.662	-6.30142																									TCF	1.83805
Sum of G's			3.70592																										
p<0.10																													
Reg 6_1	Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G	Site	Expected
Otx	2	1.29655	1.73376	Gatae	4	5.64469452	-2.75537	Bra	1	0.20826	3.13795	FoxA	0	0.21495		Gatac	9	0.00251	147.321	Su(H)	1E-191	0.92214	-9E-189	Runx	4	1.74762	6.62433	Otx	1.29655
Non Otx	315.5	316.203	-1.40533	Non Gata	313.5	311.855305	3.29805	NonBra	157.75	158.542	-1.57952					NonGatac	137.538	146.536	-17.4309	NonSu(H)	238.125	237.203	1.84785	NonRunx	313.5	315.752	-4.48866	Gatae	5.64469
Sum of G's			0.32843	Sum of G's			0.54267	Sum of G's			1.55843					Sum of G's			129.891	Sum of G's			1.84785	Sum of G's			2.13567	Bra	0.20826
Not significant.				Not significant.				Not significant				Not significant				p<0.001				Not significant				Not significant				Foxa	0.10748
	Observed	Expected	G																									Gatac	0.00251
TCF	5	1.29655	13.4973																									Su(H)	0.92214
NonTCF	312.5	316.203	-7.36335																									Runx	1.74762
Sum of G's			6.13395																									TCF	1.29655
p<0.025																													
Reg 17	Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G		Observed	Expected	G	Site	Expected
Otx	1E-258	1.4236	-1E-255	Gatae	5	6.32079962	-2.34408	Bra	0	0.23397		FoxA	0	0.27149		Gatac	6	0.00256	93.1009	Su(H)	1	0.93522		Runx	2	1.71667	0.61104	Otx	1.4236
NonOtx	346.33333	344.91	2.85308	NonGatae	341.333	340.012534	2.64672									NonGatac	153.846	159.844	-11.767					NonRunx	344.333	344.617	-0.56642	Gatae	6.3208
Sum of G's			2.85308	Sum of G's			0.30265									Sum of G's			81.3339					Sum of G's			0.04462	Bra	0.23397
p<0.10				Not significant.				Not significant.				Not significant.				p<0.001				Not significant.				Not significant.				Foxa	0.13574
																												Gatac	0.00256
TCF	Observed	Expected	G																									Su(H)	0.93522

	1	1.4236

Runx	1.71667
TCF	1.4236

Not significant.

Reg19	Observed	Expected	G
Otx	5	3.11002	4.7481
NonOtx	768.83333	770.723	-3.77533
Sum of G's			0.97277
Not significant			

	Observed	Expected	G
Gatae	4	13.2553231	-9.58484
NonGatae	769.833	760.57801	18.6228
Sum of G's			9.03798
p<0.01			

	Observed	Expected	G
Bra	0	0.48516	
Not significant			

	Observed	Expected	G
FoxA	0	0.4347	
Not significant			

	Observed	Expected	G
Gatac	20	0.00653	
p<0.001			

	Observed	Expected	G
Su(H)	1E-215	2.43858	-1E-212
NonSu(H)	580.375	577.936	4.88744
Sum of G's			4.88744
p<0.05			

	Observed	Expected	G
Runx	4	4.81279	-1.47986
NonRunx	769.833	769.021	1.62644
Sum of G's			0.14658
Not significant.			

Site	**Expected**
Otx	3.11002
Gatae	13.2553
Bra	0.48516
Foxa	0.21735
Gatac	0.00653
Su(H)	2.43858
Runx	4.81279
TCF	3.11002

	Observed	Expected	G
TCF	7	3.11002	11.3579
NonTCF	766.83333	770.723	-7.7603
Sum of G's			3.59765
p<0.10			

Reg19_1	Observed	Expected	G
Otx	1	0.99176	
Not significant			

	Observed	Expected	G
Gatae	2	4.23741354	-3.00322
NonGatae	244.167	241.929253	4.49546
Sum of G's			1.49223
Not significant			

	Observed	Expected	G
Bra	0	0.15528	
Not significant			

	Observed	Expected	G
FoxA	0	0.14157	
Not significant			

	Observed	Expected	G
Gatac	8	0.00206	
P<0.001			

	Observed	Expected	G
Su(H)	1E-216	0.76831	-1E-213
NonSu(H)	184.625	183.857	1.53982
Sum of G's			1.53982
Not significant			

	Observed	Expected	G
Runx	1E-185	1.50863	-9E-183
NonRunx	246.167	244.658	3.02654
Sum of G's			3.02654
p<0.10			

Site	**Expected**
Otx	0.99176
Gatae	4.23741
Bra	0.15528
Foxa	0.07079
Gatac	0.00206
Su(H)	0.76831
Runx	1.50863
TCF	0.99176

	Observed	Expected	G
TCF	1	0.99176	
	Not significant		

P value tables

Upper-tail critical values of chi-square distribution with *ν* degrees of freedom

Taken from http://www.itl.nist.gov/div898/handbook/eda/section3/eda3674.htm

	Probability less than the critical value				
ν	0.90	0.95	0.975	0.99	0.999
1	2.706	3.841	5.024	6.635	10.828
2	4.605	5.991	7.378	9.210	13.816
3	6.251	7.815	9.348	11.345	16.266
4	7.779	9.488	11.143	13.277	18.467
5	9.236	11.070	12.833	15.086	20.515
6	10.645	12.592	14.449	16.812	22.458
7	12.017	14.067	16.013	18.475	24.322
8	13.362	15.507	17.535	20.090	26.125
9	14.684	16.919	19.023	21.666	27.877
10	15.987	18.307	20.483	23.209	29.588
11	17.275	19.675	21.920	24.725	31.264
12	18.549	21.026	23.337	26.217	32.910
13	19.812	22.362	24.736	27.688	34.528
14	21.064	23.685	26.119	29.141	36.123
15	22.307	24.996	27.488	30.578	37.697
16	23.542	26.296	28.845	32.000	39.252
17	24.769	27.587	30.191	33.409	40.790
18	25.989	28.869	31.526	34.805	42.312
19	27.204	30.144	32.852	36.191	43.820
20	28.412	31.410	34.170	37.566	45.315
21	29.615	32.671	35.479	38.932	46.797
22	30.813	33.924	36.781	40.289	48.268
23	32.007	35.172	38.076	41.638	49.728
24	33.196	36.415	39.364	42.980	51.179
25	34.382	37.652	40.646	44.314	52.620
26	35.563	38.885	41.923	45.642	54.052
27	36.741	40.113	43.195	46.963	55.476
28	37.916	41.337	44.461	48.278	56.892
29	39.087	42.557	45.722	49.588	58.301
30	40.256	43.773	46.979	50.892	59.703
31	41.422	44.985	48.232	52.191	61.098
32	42.585	46.194	49.480	53.486	62.487
33	43.745	47.400	50.725	54.776	63.870
34	44.903	48.602	51.966	56.061	65.247
35	46.059	49.802	53.203	57.342	66.619

36	47.212	50.998	54.437	58.619	67.985
37	48.363	52.192	55.668	59.893	69.347
38	49.513	53.384	56.896	61.162	70.703
39	50.660	54.572	58.120	62.428	72.055
40	51.805	55.758	59.342	63.691	73.402
41	52.949	56.942	60.561	64.950	74.745
42	54.090	58.124	61.777	66.206	76.084
43	55.230	59.304	62.990	67.459	77.419
44	56.369	60.481	64.201	68.710	78.750
45	57.505	61.656	65.410	69.957	80.077
46	58.641	62.830	66.617	71.201	81.400
47	59.774	64.001	67.821	72.443	82.720
48	60.907	65.171	69.023	73.683	84.037
49	62.038	66.339	70.222	74.919	85.351
50	63.167	67.505	71.420	76.154	86.661
51	64.295	68.669	72.616	77.386	87.968
52	65.422	69.832	73.810	78.616	89.272
53	66.548	70.993	75.002	79.843	90.573
54	67.673	72.153	76.192	81.069	91.872
55	68.796	73.311	77.380	82.292	93.168
56	69.919	74.468	78.567	83.513	94.461
57	71.040	75.624	79.752	84.733	95.751
58	72.160	76.778	80.936	85.950	97.039
59	73.279	77.931	82.117	87.166	98.324
60	74.397	79.082	83.298	88.379	99.607
61	75.514	80.232	84.476	89.591	100.888
62	76.630	81.381	85.654	90.802	102.166
63	77.745	82.529	86.830	92.010	103.442
64	78.860	83.675	88.004	93.217	104.716
65	79.973	84.821	89.177	94.422	105.988
66	81.085	85.965	90.349	95.626	107.258
67	82.197	87.108	91.519	96.828	108.526
68	83.308	88.250	92.689	98.028	109.791
69	84.418	89.391	93.856	99.228	111.055
70	85.527	90.531	95.023	100.425	112.317
71	86.635	91.670	96.189	101.621	113.577
72	87.743	92.808	97.353	102.816	114.835
73	88.850	93.945	98.516	104.010	116.092
74	89.956	95.081	99.678	105.202	117.346
75	91.061	96.217	100.839	106.393	118.599
76	92.166	97.351	101.999	107.583	119.850
77	93.270	98.484	103.158	108.771	121.100
78	94.374	99.617	104.316	109.958	122.348
79	95.476	100.749	105.473	111.144	123.594
80	96.578	101.879	106.629	112.329	124.839
81	97.680	103.010	107.783	113.512	126.083
82	98.780	104.139	108.937	114.695	127.324
83	99.880	105.267	110.090	115.876	128.565
84	100.980	106.395	111.242	117.057	129.804
85	102.079	107.522	112.393	118.236	131.041
86	103.177	108.648	113.544	119.414	132.277
87	104.275	109.773	114.693	120.591	133.512
88	105.372	110.898	115.841	121.767	134.746
89	106.469	112.022	116.989	122.942	135.978
90	107.565	113.145	118.136	124.116	137.208
91	108.661	114.268	119.282	125.289	138.438
92	109.756	115.390	120.427	126.462	139.666
93	110.850	116.511	121.571	127.633	140.893
94	111.944	117.632	122.715	128.803	142.119
95	113.038	118.752	123.858	129.973	143.344
96	114.131	119.871	125.000	131.141	144.567
97	115.223	120.990	126.141	132.309	145.789
98	116.315	122.108	127.282	133.476	147.010
99	117.407	123.225	128.422	134.642	148.230
100	118.498	124.342	129.561	135.807	149.449
100	118.498	124.342	129.561	135.807	149.449

Lower-tail critical values of chi-square distribution with ν degrees of freedom

	Probability less than the critical value				
ν	0.10	0.05	0.025	0.01	0.001
1.	.016	.004	.001	.000	.000
2.	.211	.103	.051	.020	.002
3.	.584	.352	.216	.115	.024
4.	1.064	.711	.484	.297	.091
5.	1.610	1.145	.831	.554	.210
6.	2.204	1.635	1.237	.872	.381
7.	2.833	2.167	1.690	1.239	.598
8.	3.490	2.733	2.180	1.646	.857
9.	4.168	3.325	2.700	2.088	1.152
10.	4.865	3.940	3.247	2.558	1.479
11.	5.578	4.575	3.816	3.053	1.834
12.	6.304	5.226	4.404	3.571	2.214
13.	7.042	5.892	5.009	4.107	2.617
14.	7.790	6.571	5.629	4.660	3.041
15.	8.547	7.261	6.262	5.229	3.483
16.	9.312	7.962	6.908	5.812	3.942
17.	10.085	8.672	7.564	6.408	4.416
18.	10.865	9.390	8.231	7.015	4.905
19.	11.651	10.117	8.907	7.633	5.407
20.	12.443	10.851	9.591	8.260	5.921
21.	13.240	11.591	10.283	8.897	6.447
22.	14.041	12.338	10.982	9.542	6.983
23.	14.848	13.091	11.689	10.196	7.529
24.	15.659	13.848	12.401	10.856	8.085
25.	16.473	14.611	13.120	11.524	8.649
26.	17.292	15.379	13.844	12.198	9.222
27.	18.114	16.151	14.573	12.879	9.803
28.	18.939	16.928	15.308	13.565	10.391
29.	19.768	17.708	16.047	14.256	10.986
30.	20.599	18.493	16.791	14.953	11.588
31.	21.434	19.281	17.539	15.655	12.196
32.	22.271	20.072	18.291	16.362	12.811
33.	23.110	20.867	19.047	17.074	13.431
34.	23.952	21.664	19.806	17.789	14.057
35.	24.797	22.465	20.569	18.509	14.688
36.	25.643	23.269	21.336	19.233	15.324
37.	26.492	24.075	22.106	19.960	15.965
38.	27.343	24.884	22.878	20.691	16.611
39.	28.196	25.695	23.654	21.426	17.262
40.	29.051	26.509	24.433	22.164	17.916
41.	29.907	27.326	25.215	22.906	18.575
42.	30.765	28.144	25.999	23.650	19.239
43.	31.625	28.965	26.785	24.398	19.906
44.	32.487	29.787	27.575	25.148	20.576
45.	33.350	30.612	28.366	25.901	21.251
46.	34.215	31.439	29.160	26.657	21.929
47.	35.081	32.268	29.956	27.416	22.610
48.	35.949	33.098	30.755	28.177	23.295
49.	36.818	33.930	31.555	28.941	23.983
50.	37.689	34.764	32.357	29.707	24.674
51.	38.560	35.600	33.162	30.475	25.368
52.	39.433	36.437	33.968	31.246	26.065
53.	40.308	37.276	34.776	32.018	26.765
54.	41.183	38.116	35.586	32.793	27.468
55.	42.060	38.958	36.398	33.570	28.173
56.	42.937	39.801	37.212	34.350	28.881
57.	43.816	40.646	38.027	35.131	29.592
58.	44.696	41.492	38.844	35.913	30.305
59.	45.577	42.339	39.662	36.698	31.020
60.	46.459	43.188	40.482	37.485	31.738
61.	47.342	44.038	41.303	38.273	32.459
62.	48.226	44.889	42.126	39.063	33.181

63.	49.111	45.741	42.950	39.855	33.906
64.	49.996	46.595	43.776	40.649	34.633
65.	50.883	47.450	44.603	41.444	35.362
66.	51.770	48.305	45.431	42.240	36.093
67.	52.659	49.162	46.261	43.038	36.826
68.	53.548	50.020	47.092	43.838	37.561
69.	54.438	50.879	47.924	44.639	38.298
70.	55.329	51.739	48.758	45.442	39.036
71.	56.221	52.600	49.592	46.246	39.777
72.	57.113	53.462	50.428	47.051	40.519
73.	58.006	54.325	51.265	47.858	41.264
74.	58.900	55.189	52.103	48.666	42.010
75.	59.795	56.054	52.942	49.475	42.757
76.	60.690	56.920	53.782	50.286	43.507
77.	61.586	57.786	54.623	51.097	44.258
78.	62.483	58.654	55.466	51.910	45.010
79.	63.380	59.522	56.309	52.725	45.764
80.	64.278	60.391	57.153	53.540	46.520
81.	65.176	61.261	57.998	54.357	47.277
82.	66.076	62.132	58.845	55.174	48.036
83.	66.976	63.004	59.692	55.993	48.796
84.	67.876	63.876	60.540	56.813	49.557
85.	68.777	64.749	61.389	57.634	50.320
86.	69.679	65.623	62.239	58.456	51.085
87.	70.581	66.498	63.089	59.279	51.850
88.	71.484	67.373	63.941	60.103	52.617
89.	72.387	68.249	64.793	60.928	53.386
90.	73.291	69.126	65.647	61.754	54.155
91.	74.196	70.003	66.501	62.581	54.926
92.	75.100	70.882	67.356	63.409	55.698
93.	76.006	71.760	68.211	64.238	56.472
94.	76.912	72.640	69.068	65.068	57.246
95.	77.818	73.520	69.925	65.898	58.022
96.	78.725	74.401	70.783	66.730	58.799
97.	79.633	75.282	71.642	67.562	59.577
98.	80.541	76.164	72.501	68.396	60.356
99.	81.449	77.046	73.361	69.230	61.137
100.	82.358	77.929	74.222	70.065	61.918

BIOGRAPHY OF THE AUTHOR

Christopher M. McCarty was born in Bangor, Maine and graduated from Bangor High School in 1993. He obtained a Bachelor of Science in Biology at the University of Maine in 1997, and a Master of Science in Biochemistry in 2000, also from the University of Maine. His thesis focused on characterizing mutations in the G protein alpha subunit, Galpha2 in *Dictyostelium discoideum* in the lab of Dr. Robert Gundersen. After obtaining his M.S. degree, he spent time as an adjunct instructor of chemistry and biology lecture and lab courses at local colleges in Bangor. Following this, he worked as a research assistant in the lab of Dr. Qing Yin Zheng at the Jackson Laboratory, where he gained experience assisting in the characterization of mice with mutations in genes controlling hearing and balance. He then applied for and was accepted into the Ph.D. program in Biomedical Science in the University of Maine's Graduate School of Biomedical Sciences and Engineering (GSBSE). He and his advisor Dr. James Coffman published a paper that is described in Chapter 2 of this dissertation: C.M. McCarty, J.A. Coffman, Developmental cis-regulatory analysis of the cyclin D gene in the sea urchin Strongylocentrotus purpuratus, Biochem Biophys Res Commun 440 (2013) 413-418. He is a candidate for Doctor of Philosophy degree in Biomedical Sciences with a concentration in Cell and Molecular Biology from the University of Maine in August 2014.