REGULATION OF CELL PROLIFERATION AND DIFFERENTIATION
DURING DROSOPHILA NEUROGENESIS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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The Ohio State University
2003

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ABSTRACT

During *Drosophila* embryogenesis, tight coordination between cell proliferation and terminal differentiation is required to ensure the proper formation of the nervous system. However, little is known regarding the mechanism coordinating cell cycle proliferation and terminal differentiation. The main goal of my research was to analyze the transcriptional regulation of the cyclin-dependent kinase inhibitor (CKI) *dacapo (dap)* gene expression during *Drosophila melanogaster* neurogenesis. *dap* is the only identified G1 CKI in *Drosophila* and is a homolog of p27kip. I found *dap* expression to be regulated by a complex array of tissue-specific cis-regulatory elements. *prospero (pros)*, a pan-neural transcription factor, regulates *dap* expression in the embryonic nervous system. Furthermore, Pros and DmcytE, the *Drosophila* homolog of cyclin E, function cooperatively in regulating the expression of both *dap* and the neuronal differentiation marker-Even-skipped (Eve). A second goal of my research was to analyze the role of Pros in the regulation of mitotic activity and differentiation. Evidence is presented that cell cycle regulatory genes are downstream targets of Pros in regulating mitotic activity. In addition, Pros interacts with cell cycle regulatory genes to regulate the expression of neuronal differentiation markers in a lineage specific pattern.
Dedicated to my mother and husband
ACKNOWLEDGMENTS

I wish to thank my advisor, Dr. Harald Vaessin, for his guidance, support, and encouragement throughout this research. I would like to thank Drs. Amanda Simcox, Mark Seeger, and John Oberdick for serving on my dissertation committee and for their suggestions and comments. I thank Dr. Christine Beattie for critical reading of the manuscript. I wish to thank the past and present members of Vaessin lab, especially Ling Li and Shweta Chandra, for their friendship and for creating a supportive working environment, and Soo-Hyun Kim for continuing some of the studies. I also wish to thank members in Segger’s and Beattie’s labs, especially Vicki McGovern and Anilkumar Challa for their help and friendship.

I am indebted to Drs. B. Edgar, C. Doe, F. Mazusaki, C. Lehner, R. Duronio, N. Dyson, M. Seeger, H. Bellen, D. Kosman, and the Bloomington Stock Center for providing reagents and fly stocks critical for my work.

I am most thankful for the love and support of my family. I offer deepest thanks to my mother. Without her support I would not have made it this far. I also thank my husband, Bruce Schnepp, for his support and help. My family’s faith in me has helped me attain this goal.
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Te-Hui Liu, Ling Li, Harald Vaessin, “Transcription of the Drosophila CKI gene dacapo
is regulated by a modular array of cis-regulatory sequences”. Mech. of Dev. 2002 Mar;

Kenneth Wallace, Te-Hui Liu, and Harald Vaessin, “The pan-neural bHLH proteins
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Chiag-Shan Huang, Kun-Long Huang, May-Jan Huang, Yi-Ching Li, Te-Hui Liu, and
Tang K. Tang,“Neonatal jaundice and molecular mutation in glucose-6-phosphate
dehydrogenase deficient newborn infants”. American Journal of Hematology, 1996 Jan;
51(1): 19-25

Tang K. Tang, Te-Hui Liu, Vhieh-Ju Change Tang, Kb-Bik Tam
“Glucose-6-phosphate dehydrogenase (G6PD) mutations associated with F8C/G6PD
haplotypes in Chinese”. Blood, 1995 Jan; 85 (12): 3767-8

FIELDS OF STUDY

Major field: Molecular genetics
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CHAPTER 1
INTRODUCTION AND BACKGROUND

One of the fundamental questions in biology is how a multicellular organism develops from a single fertilized egg. To properly achieve this complex process, precise spatial and temporal coordination between cell proliferation and differentiation must occur. Cell proliferation is controlled by several cell cycle genes while differentiation is regulated by stage- or tissue-specific transcriptional factors. However, until recently little was known about the mechanism underlying the tight cooperation between cell cycle progression and the developmental program of multicellular organisms. To address this question, I chose *Drosophila* as a model organism to study the regulatory mechanisms that coordinate the cell cycle and differentiation.

*Drosophila melanogaster*, the so-called fruit fly, is one of several major organisms that has been used in the study of genetics and developmental biology. *Drosophila* is known for its short generation time, easy lab maintenance, and low cost. Even though the *Drosophila* genome is comparably simpler than vertebrates, the protein structure and gene function of key developmental regulators have been shown in many instances to be evolutionarily conserved between higher vertebrates. Furthermore, the completely sequenced genome and easy genetic manipulation make *Drosophila* an
excellent system to study genetics and development, and the knowledge gained from studying *Drosophila* can be readily applied to other systems.

To provide background for my work, I will briefly introduce the regulation of cell cycle progression and the development of embryonic nervous system in *Drosophila*.

**Cell cycle regulation during *Drosophila* embryogenesis**

Cell cycle progression is governed by an evolutionally conserved machinery. Most eukaryotic cell cycles can be divided into 4 phases: G1, S, G2, and M phase. The main regulators driving cell cycle progression are cyclin and its partners, the cyclin-dependent kinases (CDKs). The cell cycle-dependent accumulation of cyclins and the activation/inactivation of its partner, CDKs, drive the cell’s progression through different phases. The activity of cyclin/CDK complexes is controlled by transcriptional regulation, ubiquitin-dependent degradation, and phosphorylation/dephosphorylation of the kinase subunits (Hershko, 1997; Lees, 1995; Nurse, 1994; Peters, 1998). There are also negative regulators, called CDK inhibitors (CKIs), that can inhibit the activity of cyclin/CDK complexes (Harper and Elledge, 1996; Nakayama, 1998). In addition, members of the E2F family modulate transcription of cell cycle genes, and retinoblastoma protein (RB) physically regulates the cell cycle through targeting cyclin-CDK complexes (Dyson, 1998; Kaelin, 1999).

In *Drosophila*, the first 13 cycles of embryogenesis consist only of S phase and mitosis. These early cycles are driven by abundant maternally supplied gene products, and each cycle is less than 10 minutes long. However, during cycle 14, zygotic string
(stg), a homolog of cdc25 phosphatase, becomes the limiting factor due to exhausting maternal Stg supplies, while other cell cycle regulators are still in excess (Edgar et al., 1994; Edgar and Lehner, 1996; Edgar and O'Farrell, 1989). Stg activity is required for activation of Cdc2/CycB complex, thus, preventing the immediate entry into mitosis and creating a G2 phase (Figure 1.1)(Edgar and Datar, 1996; O'Farrell et al., 1989). stg mRNA has a very rapid turnover rate (T1/2< 15 minutes) and stg protein is very unstable after mitosis (Edgar, 1994; Edgar and Datar, 1996). Thus, Stg is the rate-limiting factor for mitotic cycles controlled at the G2/S transition. Loss-of-function (LOF) stg mutant embryos are arrested in G2 phase at embryonic cycle 14, while ectopic expression of Stg by heat shock induction induces G2 cells to rapidly enter mitosis (Edgar and O'Farrell, 1989).

stg is temporally and spatially expressed in a highly dynamic pattern (Edgar and O'Farrell, 1989). stg promoter studies show that the expression of stg transcript is controlled by a large (> 30 kb) array of many tissue-specific cis-regulatory elements which respond to developmental programs (Edgar et al., 1994; Lehman et al., 1999). In mutant embryos of pattern formation genes, such as bicoid, hunchback (hb), and neurogenic genes, such as Notch, Enhancer of split complex, there is altered transcription of stg, indicating developmental regulation of stg expression (Edgar et al., 1994). Stg has also been shown to be regulated by the developmental regulatory gene, pros (Li and Vaessin, 2000).
Cyclins and CDK

The main regulators driving *Drosophila* embryonic cell cycle progression, similar to the mechanism in mammals, are cyclins and their respective partners, CDKs. The level of CDK protein remains stable throughout the cell cycle while its activity is changed through binding to its partner cyclin, followed by phosphorylation of the cdk subunit of the cyclin/CDK complex, and associating with CKIs.

The G1 cyclins that have been identified in *Drosophila* are cyclin D (*cycD*) and cyclinE (*Dm cycE*) (Finley et al., 1996; Richardson et al., 1993). Although *cycD* mRNA is expressed in cells before entering S phase, recent studies showed that *Drosophila* CycD/Cdk4(6) functions primarily in regulation of cellular growth and is dispensable for cell cycle progression (Datar et al., 2000; Finley et al., 1996; Meyer et al., 2000). Dm cycE forms complexes with cdc2c and is essential for entering and progressing through S phase (Knoblich et al., 1994). LOF *Dm cycE* mutations result in cell cycle arrest in G1 phase after mitosis 16 and there is no BrdU incorporation in the epidermis during S phase of cycle 17 where it is normally observed in wild-type embryos (Knoblich et al., 1994). Down-regulation of Dm cycE is essential for the proper timing of exiting from mitosis. Ectopic expression of Dm cycE after the final mitotic cycle induces entry into S phase and results in progression through a complete additional cell cycle (Knoblich et al., 1994). Dm cycE function is also crucial for *Drosophila* eye development (Richardson et al., 1995). Ectopic expression of Dm cycE in eye imaginal discs also results in an extra round of cell division and a rough eye phenotype (Richardson et al., 1995). Dm cycE has been shown to interact physically with components of the Brahma
(Brm) complex, the SWI/SNF ATP-dependent chromatin remodeling complex, indicating that Dm cycE may function in remodeling the structure of chromatin and the alteration of expression of target genes (Brumby et al., 2002). Recently the cis-regulatory elements of Dm cycE have been mapped (Jones et al., 2000). Dm cycE transcription, like stg, is regulated by a large and complex cis-regulatory region containing tissue- and stage-specific elements. Li and Vaessin (2000) showed that Pros regulates transcription of Dm cycE during embryogenesis. Furthermore, ectopic Pros suppresses the expression of Dm cycE lacZ reporter genes containing the neuronal cis-regulatory elements (Li, 2000).

During Drosophila embryogenesis, CycA and CycB functions are required for the G2/M transition (Knoblich and Lehner, 1993; Stiffler et al., 1999). Both CycA and CycB bind to Cdk1. In the mammalian system, CycA function is required for the G1 and S phases, however in Drosophila, CycA has so far not been reported to function in G1 or S phase (Dong et al., 1997). LOF cycA, but not cycB mutations are embryonic lethal (Lehner and O'Farrell, 1990). Epidermal cells of LOF cycA embryos are arrested in G2 phase at cycle 16. Thus, this indicates that CycA function is required for cell cycle progression at the G2/M transition (Lehner and O'Farrell, 1990). Over expression of CycA has been shown to induce ectopic S phase in both embryos and eye imaginal discs (Dong et al., 1997; Sprenger et al., 1997). CycA function has also been indicated to prevent DNA re-replication in G2 due to the fact that in cycA mutant embryos, mitotic cycles of CNS and epidermis were transformed into endo-replication cycles (Sauer et al., 1995).
CycB functions in G2/M transition. However, LOF cycB mutations alone do not result in cell cycle arrest (Lehner and O'Farrell, 1990). Mild defects in mitotic spindle formation and delayed mitosis have been observed in LOF cycB mutant embryos (Sauer et al., 1995). Furthermore, double LOF cycA and cycB mutations result in cell cycle arrest in G2 of cycle 15, suggesting that CycA and CycB functions overlap in regulating the G2/M transition (Knoblich and Lehner, 1993; Lehner and O'Farrell, 1990). Stiffler et al. (1999) showed that during early embryonic cycles, CycA and CycB regulate different aspects of the cell cycle, which correlated with their localization in cells. CycB is mainly localized in the cytoplasm and is only detected in the nucleus during late prophase, while CycA is concentrated in the nucleus with only a low level occurring in the cytoplasm. Changes in the dosages of CycA or CycB reveal that CycA mainly functions to regulate the progression of the nuclear cycle while CycB regulates dynamic changes of the cytoskeleton (Stiffler et al., 1999).

**E2F family and dDP**

In mammals, in addition to G1 cyclins and its partners, a family of heterogenous transcription factors, known as the E2F family, also plays a critical role in regulation of the G1/S transition. The basic unit of E2F is composed of an E2F and a DP subunit which form a heterodimer. The activities of the E2F family members are regulated by the retinoblastoma (RB) family. As in mammalian cell cycles, the function of the E2F/DP heterodimers is critical for G1/S transition as well as progression through S phase in Drosophila cell cycles (Dynlacht et al., 1994; Hao et al., 1995; Ohtani and Nevins, 1994;
Sawado et al., 1998b). However, unlike mammalian cells that have six E2F genes and two DP genes, *Drosophila* has only two E2F genes, *de2f1* and *de2f2*, and one DP gene, *dDP* (Dynlacht et al., 1994; Hao et al., 1995; Ohtani and Nevins, 1994; Sawado et al., 1998b).

It has been shown that the DE2f1/dDP heterodimer binds to consensus E2F sites and activates the transcription of those E2F-regulated genes (Dynlacht et al., 1994). LOF mutations in *de2f1* result in G1 arrest after cycle 16 in most of the cells and almost a complete cessation of BrdU incorporation in the nervous system in stage 13 embryos (Duronio et al., 1995; Royzman et al., 1997). Furthermore, expression of downstream target genes of dE2F1, such as PCNA and DmRNR2, is absent in the LOF *de2f1* mutant embryos (Duronio and O'Farrell, 1994). Ectopic expression of dE2F1/dDP by heat shock induces an extra S phase in epidermal cells after the cells have finished their last mitotic division (Duronio et al., 1996). DE2F1/dDp function is also important for normal postembryonic development. Larvae that are homozygous for LOF *dE2f1* mutations show a significant delay in larval growth and die at the larval stage (Du, 2000; Royzman et al., 1997).

LOF *dDP* mutant embryos also result in absence of PCNA and DmRNR2 expression (Royzman et al., 1999, 1997). dDP function has been shown to play a role in oogenesis that it is required to shut off DNA synthesis in follicle cells (Myster et al., 2000; Royzman et al., 1999). In *dDP* mutants, defects in the patterns of DNA synthesis and cell proliferation are not as severe as *de2f1* mutants, indicating that the functions of
dE2F1 and dDP are overlapping but not equivalent (Frolov et al., 2001; Royzman et al., 1999).

dE2F2 has also been shown to cooperate with dDP to bind to consensus E2F-binding sites (Sawado et al., 1998). However, in contrast to dE2F1, dE2F2 represses the transcription of E2F-regulated genes and LOF de2f2 mutations result in increased expression (Frolov et al., 2001; Sawado et al., 1998a). Frolov et al. (2001) showed that normal dE2F2 functions as an antagonist to dE2F1 and that mutations in de2f2 can suppress most defects observed in de2f1 mutations such as defects in reduced expression of E2F-regulated genes, decreased DNA synthesis, and slow growth in de2f1 mutant larvae.

**RBF**

In mammals, there are three pRB family members, pRB, p107 and p130. pRB functions to repress transcription of E2F-regulated genes by directly binding to and blocking the activation domain of E2F protein (Mulligan and Jacks, 1998). The activity of pRB is regulated by the state of phosphorylation which is mediated by cyclin/CDK complexes and phosphatase (Mittnacht, 1998). In *Drosophila*, a single pRB related gene, rbf, has been identified (Du et al., 1996a). Rbf binds to and inhibits the activity of dE2F/dDP (Du et al., 1996b). In LOF rbf mutant embryos, ectopic expression of dE2F-related genes and an ectopic S phase is observed, indicating that RBF function is required for introduction of G1 (Du and Dyson, 1999). LOF rbf mutant embryos die at early larval stages (Du, 2000).
CKI

A major difference in cell cycle progression between mammals and *Drosophila* embryogenesis is that during *Drosophila* embryogenesis, cells only enter G1 phase after finishing their last mitotic division to go in G1 arrest and become terminally differentiated. In contrast, mammalian cells can progress through G1 phase and enter the next mitotic cycle. Thus, during most of the *Drosophila* embryonic cell cycle, S phase occurs immediately after mitosis, without a gap for G1 phase (Figure 1.1). Only after the final mitosis do cells arrest in G1 phase before entering terminal differentiation. However, as in the mammalian cell cycle, CKIs are one of the critical players regulating cell cycle withdrawal.

CKI can bind physically to and inhibit the activity of cyclin/CDKs. In mammals, two families of CKIs have been identified based on their structure and targets (Sherr and Roberts, 1999). The *p16^INK4a* families, including *p15, p16, p18*, and *p19*, are composed of multiple ankyrin repeats and specifically inhibit the catalytic subunits of the cyclin D-dependent kinases, *cdk4* and *cdk6*. Alternatively, the *p21 Cip/Kip* families, including *p21cip, p27kip1* and *p57kip2*, all contain a conserved amino-terminal domain that binds to and inhibits all kinases involved in G1/S transition.

The mammalian Cip/Kip family members are up-regulated during cell differentiation (Halevy et al., 1995; Parker et al., 1995), and are expressed in terminally differentiated tissues (Halevy et al., 1995; Lee et al., 1995), indicating that CKIs have a critical role in regulating cell cycle exit and initiating differentiation. This is further
supported by cell culture experiments. Overexpression of the Cip/Kip family members in
cultured cells arrest them in G1 phase (Harper et al., 1993; Lee et al., 1995; Toyoshima
and Hunter, 1994) and, in some cases, result in a differentiation phenotype (Kranenburg et
al., 1995; Liu et al., 1996). Mice deficient for one or more members of the Cip/Kip family
show tissue-specific defects, suggesting that the Cip/Kip family is required for proper
development of these tissues. For example, \( p57^{kip2} \) mutant mice have developmental
defects in the formation of the palate, intestine and endochondral bone (Yan et al., 1997).
\( p21^{cip} \) and \( p57^{kip2} \) double knockout mice show severe defects in skeletal muscle (Zhang
et al., 1999). Defects in lens fiber differentiation have been observed in \( p27^{kip1} \) and
\( p57^{kip2} \) double knockout mice (Zhang et al., 1998). The mechanisms underlying the tight
cooperation between cell cycle progression and the developmental programs of
multicellular organisms have only recently started to emerge. Recent studies suggest
developmental control of cell cycle progression could be achieved through the control of a
few core components of the cell cycle machinery by regulatory genes that are part of
tissue/lineage specific developmental programs. For example, transient expression of
mammalian proneural genes \( \textit{neuroD2}, \textit{mash1} \) and \( \textit{neurogenin-1} \) can convert mouse p19
embryonal carcinoma cells into differentiated neurons in association with activating the
expression of \( p27^{kip1} \) (Farah et al., 2000). HOXA10, a homeodomain transcription factor,
together with its partners, can activate \( p21^{cip} \) transcription resulting in differentiation of
myelomonocytic cells (Bromleigh and Freedman, 2000). In \textit{C. elegans}, \( \textit{lin-14} \), a
heterochronic gene, activates the transcription of \( \textit{cki-1} \), a member of the Cip/Kip family of
CKIs, to prevent the vulva precursor cells from dividing during the larval 2 stage (Hong et al., 1998). Thus, CKIs appear to be major effectors of differentiation pathways and appear to provide crucial links between developmental pathways and the cell cycle control machinery (see review by (Zhang, 1999)).

_**Drosophila** has one identified G1/S CKI gene, *dacapo* (*dap*), with structural similarity to the vertebrate Cip/Kip family. *dap* protein (Dap) has been shown to bind to and inhibit the activity of cdk2/cyclinE complexes and maintain G1 arrest (de Nooij et al., 1996; Lane et al., 1996). Loss-of-function mutations in *dap* cause an extra round of mitotic division in the embryonic epidermis (de Nooij et al., 1996; Lane et al., 1996). In addition, ectopic mitotic activity and disruption of the mitotic domain structure are observed in the optic lobes of third instar larvae (Wallace et al., 2000). Alternatively, premature expression of *dap* in embryos results in precocious G1 arrest (de Nooij et al., 1996; Lane et al., 1996). These observations suggest that *dap*, like vertebrate CKIs, is important for regulating cell cycle exit at the proper developmental time.

Consistent with its role as a CKI, *dap* expression is tightly correlated with cells exiting mitotic activity (de Nooij et al., 1996; Lane et al., 1996). In embryonic development, zygotic *dap* expression is first seen during cellularization and then in the amnioserosa during gastrulation. Following germband extension, *dap* RNA is observed in the developing central nervous system (CNS) and epidermis. In the developing CNS, *dap* RNA expression is generally initiated in ganglion mother cells (GMCs) before they enter the terminal division to give rise to differentiated neurons or glia. In the epidermis, *dap* transcripts increase abruptly just before mitosis 16, the last mitotic cycle for the majority
of the epidermal cells. After germband retraction, *dap* expression is restricted mainly to the developing CNS and peripheral nervous system (PNS). Expression of the protein closely matches *dap* RNA expression suggesting that regulation of *dap* transcription may be a major mechanism for the proper regulation of Dap activity.

**Development of the *Drosophila* embryonic nervous system**

*Drosophila* embryonic neurogenesis occurs in a highly organized pattern and has been intensely studied. The embryonic nervous system can be divided into the central nervous system (CNS), including the ventral nerve cord (VNC) and brain, and the peripheral nervous system (PNS). In the following, I will focus on the development of the VNC.

The first step in neurogenesis is a group of 3-5 cells in the ventral neuroectoderm become neurogenically competent by the expression of proneural genes. This group of cells is called the proneural cluster. Through lateral inhibition which is mediated by neurogenic genes, usually only one cell from each proneural cluster will become the neuronal precursor, called a neuroblast (NB) (Figure 1.2). Other cells in this proneural cluster will adopt an epidermal fate and become epidermoblasts (Campos-Ortega, 1993). The proneural genes include the *achaete-scute complex (AS-C)*, *daughterless (da)*, *ventral nervous system condensation defective (vnd)*, and *atonal (ato)* (Vaessin et al., 1990; Cronmiller and Cummings, 1993; Parras et al., 1996). These proneural genes all encode basic helix-loop-helix (bHLH) transcription factors (Alonso et al., 1988; Vaessin et al., 1990). LOF mutations in these genes result in neural hypoplasia, while ectopic
expression of these genes results in ectopic NB formation (Campos-Ortega, 1993; Giebel and Campos-Ortega, 1997; Parras et al., 1996). In contrast to the function of proneural genes, neurogenic genes function to limit the numbers of cells taking on neuronal fates. Thus, LOF mutations of neurogenic genes result in neural hyperplasia. The most studied neurogenic genes are members of the Notch signaling pathway (Vassin and Campos-Ortega, 1987; Artavanis-Tsakonas et al., 1999; Fortini and Artavanis-Tsakonas, 1993).

Immediately after one cell is selected as the NB at or after embryonic stage 9, this new NB will enlarge and delaminate toward the interior of the embryo. This process occurs in 5 waves with a stereotyped and spatiotemporal pattern (Campos-Ortega, 1993; Goodman and Doe, 1993). At the end of this process, there are up to 30 NB formed in each hemisegment. Each individual NB can be identified by its position, time of delamination and formation, and the profiles of gene expression (Doe, 1992; Goodman and Doe, 1993). In other words, each NB has acquired a unique fate by the time it has formed. There is evidence that several genes involved in pattern formation play important roles in determining the neuronal cell fate. These genes include gap genes such as kruppel (kr) and hunchback (hb), pair rule genes such as eve, fushi tarazu (ftz), and segment polarity genes such as wingless (wg) and engrailed (en) (Bhat, 1999; Doe et al., 1988a; Doe et al., 1988b; Dormand and Brand, 1998; Kambadur et al., 1998; McDonald and Doe, 1997; Romani et al., 1996).

NBs behave as stem cells and divide asymmetrically to produce another NB and a ganglion mother cell (GMC) (Figure 1.3). The daughter NB will repeat this asymmetrical division pattern up to 10 times but the GMC will only divide one more time to give rise
to two terminally differentiated neurons or glia (Figure 1.3) (Jan and Jan, 1998; Lu et al., 2000). At the end of neurogenesis, there are ~320 neurons and ~30 glia in each hemisegment (Bossing et al., 1996; Schmidt et al., 1997).

**Prospero (Pros)**

Each NB expresses a unique combination of neuronal identity genes to achieve their own lineage identity. *pros*, a pan-neural gene, is one of these neuronal identity genes (Hassan and Vaessin, 1996). Pros is expressed in all neuronal lineages in embryos. LOF of *pros* results in embryonic lethality and severe defects in the embryonic nervous system. In the CNS, loss of *pros* function results in loss of longitudinal axons and fusion of anterior and posterior axons, while in the PNS, loss of *pros* function causes axonal path-finding defects (Doe et al., 1991; Vaessin et al., 1991). Furthermore, Akiyama-Oda et al. (2000) showed that Pros function is also required for the glia-neuron cell fate switch of the NB 6-4 lineage. In *pros* LOF mutant embryos, transcriptional expression of *deadpan (dpn)*, *asense (ase)*, *even-skipped (eve)*, *fushi tarazu (ftz)* are affected (Doe et al., 1991; Vaessin et al., 1991). In addition to embryonic nervous system development, Pros function is also required for development of the adult eye and external sense organ precursor (SOP) lineages (Kauffmann et al., 1996; Manning and Doe, 1999; Reddy and Rodrigues, 1999a; Reddy and Rodrigues, 1999b).

*pros* mRNA and protein has been shown to localize asymmetrically in the NB in a cell cycle dependent manner (Broadus and Doe, 1997; Hirata et al., 1995; Knoblich et al., 1995; Li et al., 1997; Spana and Doe, 1995). In interphase cells, Pros protein forms a
crescent at the apical cortex of the NB and after division, Pros protein is segregated exclusively into the basal GMC daughter cell, where it is translocated into the GMC nucleus (Figure 1.4) (Hirata et al., Knoblich et al., 1995; Spana and Doe, 1995). Asymmetrical localization of Pros is essential for its function. In mutants the asymmetrical localization is disrupted and results in a defect similar to that of pros LOF mutants (Ikeshima-Kataoka et al., 1997). When pros mRNA localization is disrupted but Pros protein is not affected, there are no CNS defects observed suggesting that pros mRNA may function as a backup mechanism for Pros activity (Figure 1.4) (Li et al., 1997; Broadus et al., 1997).

The asymmetrical localization of pros mRNA and protein is regulated by a complex network. Genes involved in regulating Pros asymmetrical localization include miranda (mira), staufen (stau), inscuteable (insc), bazooka, and partner of inscuteable (pins) (Ikeshima-Kataoka et al., 1997; Kraut et al., 1996; Matsuzaki et al., 1998; Schober et al., 1999; Wodarz et al., 1999; Yu et al., 2000). Mira contains coil-coil domains and binds to Pros physically. Mira colocalizes with Pros in NB and also segregates into the GMC along with Pros, where Mira is rapidly degraded or delocalized from the GMC cell cortex while Pros is released and translocates into the nucleus (Ikeshima-Kataoka et al., 1997; Shen et al., 1997). Mira also interacts physically with Stau and is required for the asymmetrical localization of Stau, a double-strand RNA binding protein, which in turn is required for the asymmetrical localization of pros mRNA (Schuldt et al., 1998; Shen et al., 1998; St Johnston et al., 1991). Loss of mira function affects the localization of Stau, pros mRNA and protein. Thus, Mira functions as an adaptor for Stau and Pros.
localization (Matsuzaki et al., 1998; Broadus et al., 1998; Li et al., 1998; Schuldt et al., 1998; Shen et al., 1998).

The asymmetrical localization of Mira is regulated by Insc. Insc is apically localized and is required for proper orientation of the mitotic spindles, which in turn is essential for the asymmetrical localization of Mira, Pros and Stau (Knoblich et al., 1999; Kraut et al., 1996; Tio et al., 1999). The apical localization of Insc is regulated by bazooka, which encodes a multiple PDZ domain protein, and pins, which encodes a novel protein with multiple repeats of the TPR motif (Schober et al., 1999; Wodarz et al., 1999; Yu et al., 2000). Studies of both LOF and ectopic expression show that bazooka is necessary and sufficient for Insc localization (Schober et al., 1999; Wodarz et al., 1999). While Insc localization is initially bazooka-dependent and pins-independent, the maintenance of Insc apical localization requires Pins function (Yu et al., 2000). This complex network involved in the asymmetrical localization of Pros indicates that the localization and activity of Pros is critical for proper CNS development.

Pros is well known as a cell fate determinant; however, Li and Vaessin (2000) show that Pros also functions to regulate cell cycle progression during Drosophila embryonic neurogenesis. LOF pros mutations result in increased BrdU incorporation and in contrast, ectopic Pros suppresses mitotic activity. Furthermore, it was also observed that there was ectopic expression of cell cycle regulatory genes, including DmcycE, cycA, stg, e2f and rbf, in LOF pros mutants while ectopic Pros resulted in suppression of expression of these cell cycle regulatory genes. This indicates that Pros regulates mitotic
activity through regulating the expression of cell cycle regulatory genes (Li and Vaessin, 2000).

Pros is evolutionally conserved between vertebrates and invertebrates. Homologs of Pros have been identified in *C. elegans*, zebrafish, chicken, mice, and humans (Burglin, 1994; Glasgow and Tomarev, 1998; Oliver et al., 1993; Tomarev et al., 1996; Tomarev et al., 1998; Zinovieva et al., 1996). *Drosophila* Pros encodes an atypical homeodomain (HD) protein. Other than the HD, homologs of Pros also contain a conserved pros domain. Molecular dissection of the pros domain shows that Pros contains a nuclear export signal that is masked by the pros domain. This nuclear export signal mediates its nuclear export via the exportin pathway (Demidenko et al., 2001). Ryter et al. (2001) showed that the HD and pros domain constitute a single structure unit (a homeo-pros domain). They suggest that this homeo-pros domain coordinately regulates Pros nuclear location and DNA binding specificity.

Hassan et al. (1997) showed that Pros is a sequence-specific DNA binding protein and Pros can interact with other homeodomain proteins, including Deformed (Dfd) and Even-skipped (Eve), to differentially modulate their DNA binding properties *in vitro*. This suggested that Pros might function as a neuronal lineage-specific homeodomain protein cofactor during neuronal lineage development. Furthermore, this study also showed that Pros’ ability to interact with other homeodomain proteins is independent from its DNA-binding capacity (Hassan et al., 1997).

It is well known that *Drosophila* Pros function is required for the development of the embryonic nervous system and adult eye (Doe et al., 1991; Vaessin et al., 1991;
Interestingly, vertebrate homologs of Pros also have been shown through *in situ* hybridization and antibody staining to be commonly expressed in the nervous system and lens. This indicates that similar to *Drosophila* Pros, the vertebrate homologs of Pros may have similar functions in these tissues (Glasgow and Tomarev, 1998; Jeffery et al., 2000; Oliver et al., 1993; Tomarev et al., 1996; Tomarev et al., 1998; Zinovieva et al., 1996). The function of the mouse homolog of Pros, Prox1, is required for normal mouse development. Homozygous knock-out Prox1 mice die at E 14.5 and heterozygous Prox1 mice die 3 days after birth. (Wigle et al., 1999). In Prox1 knock-out mice, there are defects in lymphatic development and terminal fibre differentiation and elongation of the lens (Wigle et al., 1999; Wigle, 2000). Expression of two CKIs, P27kip1 and P57kip2, is down-regulated in the lens fibre of mice lacking Prox1, while ectopic S phase and apoptosis are observed (Wigle et al., 1999).

In the following chapters, I will present my research designed to understand the coordination between cell cycle proliferation and terminal differentiation. I approached this goal by studying the regulation of *dap* expression. I will present the evidence that (1) *dap* expression is regulated by a complex array of tissue-specific cis-regulatory elements, and that *dap* expression in the MP2 cells is regulated by Pros. (2) Pros and DmcytE cooperate to regulate *dap* expression in *Drosophila* embryonic neurogenesis. I also examined Pros’ function in cell proliferation and terminal differentiation. To achieve this goal, I analyzed the expression of cell cycle phase markers, cell cycle regulatory genes, and neuronal differentiation markers in double mutants for LOF *pros* and one of the cell cycle regulatory gene. Additionally, I also examined double ectopic expression of Pros.
along with one of cell cycle regulatory genes. Lastly, I show that Dpn and Pros regulate the size of larval brain lobes.
Figure 1.1 Schematic representation of *Drosophila* embryonic cell cycles. During *Drosophila* embryogenesis, the post-blastoderm mitotic cell cycles only consist of S/G2/M phases. Cell cycle progression is regulated at the G2/M transition by String. Entering S phase is regulated by cyclin E. Cells only enter G1 phase after they have completed their last cell division and become terminally differentiated. Dacapo activity is required for cells to enter G1 phase at the proper time.
Figure 1.2 Schematic representation of proneural cluster formation in the neuronal precursor selection. A group of 5-7 cells in the neuroectoderm (shown in yellow) acquire potential to become the neuronal precursors by expression of proneural genes. These cells are called proneural clusters. Through lateral inhibition mediated by neurogenic genes, only a single cell from each cluster (shown in red) will become the neuronal precursor, called the neuroblast.
Figure 1.3 Schematic representation of the asymmetric cell division in the CNS. After a neuroblast (NB) is formed (shown in red), it will enlarge and delaminate from the neuroectoderm. This NB will undergo asymmetrical cell division to generate another NB and a ganglion mother cell (GMC). Most NBs will continue several rounds of asymmetrical division while the GMC will only divide one more time to give rise to terminal differentiated neurons or glia.
During mitosis, pros protein is asymmetrically localized to the basal cortex of the NB by the adapter protein Miranda. Miranda also asymmetrically localizes Staufen which is bound to pros mRNA. After cytokinesis, Pros protein and mRNA, along with Miranda, are propagated to the GMC. Pros is released from the membrane and translocates to the nucleus while Miranda is degraded. Most NBs will repeat this division pattern several times.
CHAPTER 2
MATERIAL AND METHODS

Polymerase Chain Reaction (PCR)

PCR was performed following the guidelines of the manufacturer (Temp Tronic). PCR reactions were performed according to the suggested conditions of the manufacturer of the DNA polymerases. For PCR products > 2kb, the Expand TM long template PCR system (Boehringer Mannheim) was used; otherwise, Taq DNA polymerase (Invitrogen) was used.

Generation of dap-lacZ reporter gene constructs

Genomic DNA fragments used in dap-lacZ reporter genes are shown in Figure 3. 1. All DNA fragments were amplified with PCR, and first cloned into pCR II vectors (Invitrogen, CA). The fragments were subsequently transferred into the pCaSpeR-hs43-β-gal transformation vector. Normal 5’ to 3’ orientation was conserved in those constructs except dap-5’-2R, dap-4’2’, and dap-3’2’ which were reversed. Moreover, both forward, dap-5’-6F, and reversed, dap-5’-6R, orientation of reporter genes containing the 6.0 kb dap promoter region were generated. The primer pairs used for PCR were the
following: for dap-5’-6F and 6R (-6485 to –540), the primer sets were 5’-AAAGGTACCGT CCTATCGAATCTGATTCCCG-3’ and 5’-AAAGCGGCGCATATAGCGCTACCGATCGAAGC; for dap-4’2’ (-4482 to –2516), the primer sets were 5’-AAAGGTACCAAGATGGCAGTATCATTCTC GTGATCT-3’ and 5’-AAAAGCGGCCGCTCGGTAGAAACATTATCTCGTATCT-3’; for dap-3’2’ (-3437 to –2516), the primer sets were 5’-AAAAGGAATTCTAGCCAGTCCA AAATATTGCTACACATCGAAGC; for dap-3’-2R (-2539 to –540), 5’-AAAGGTACCCAGATACGAGATAATGTTCTACCGA-3’ and 5’-AAAAGCGGCCG CATATAGCGCTACCGATCGAAGC; for dap-5’-2R (-2539 to –540), 5’-AAAGGTACCGT CCTATCGAATCTGATTCCCG-3’ and 5’-AAAGCGGCGCATATAGCGCTACCGATCGAAGC; for dap-5’-2+UTR (-2539 to –6), the primer set were 5’-AAAAGCGGCGCAGATACGAGATAATGTTCTACACATCGAAGC; for dap-SB (+133 to +1995), 5’-AAAAGCGGCCGCTCCAAATCCGCCGAGGGTACA-3’ and 5’-AAAAGGATCCGACG TTGGCGCTCCTCTCATGA-3’; for dap-B (+588 to +1995), the primer set were 5’-AAAGCGGCGCTCCATAGTCCTCTCGC GCAAGAGA-3’ and 5’-AAAAGGATCCGACGGCGCTCCTCTCATGA-3’; for dap-BB (+588 to +1153), the primer set were 5’- AAAAGCGGCGCTCCATAGTCCTCTCGC GCAAGAGA-3’ and 5’-AAAAGGATCCGACG TTGGCGCTCCTCTCATGA-3’; for dap-HB (+1133 to +1995), 5’-AAAAGCGGCGCTCCATAGTCCTCTCGC GCAAGAGA-3’ and 5’-AAAAGGATCCGACG TTGGCGCTCCTCTCATGA-3’; for dap-PF (+1133 to +1995 minus +1383 to +1458), 5’-GCCACTAGCAGCATGTGTA-3’ and 5’-GAAAGCGGCAGATCCCCAGACCCCT-3’; for dap-DEL (+1459 to +1995), 5’-GCCACTAGCAGCATGTGTA-3’ and 5’-AATCTCCCACAAGCATGGCT-3’;
for dap-3’end (+2783 to +3494), the primer set were 5’-AAAAGCGGCCGAGGAATGCCTTTGAATTA-3’ and 5’-AAAAGATTTTCTACAAAGCGTCGTAATGGAA-3’.

Site-directed mutagenesis

The PCR-based site-directed mutagenesis was performed according to the instructions provided by the QuickChange™ Site-directed mutagenesis Kit (Stratagene). This kit was used to generate point mutations in Dap-Del lacZ reporter constructs to eliminate potential Pros binding sequences. A total of 18 cycles were performed, and the cycling reaction was done as following: 94 °C for 30 seconds, 55 °C for 1 minute, 68 °C for 9 minutes. The primers used for mutagenesis were listed as following:

MP1F, 5’-CATAATTTATGCACTTGGCTAGAGAACGACGGTCTTTGCTTTTCAACCG-3’,
MP1R, 5’-CGGTGAAAGCAAGACTGCTGGTCTAGCTAAAGTGCATAAAATTATG-3’,
MP2F, 5’-CTGCAACCCCTCTGTTACGACAAATTACTGCCGTCACGTGCC-3’,
MP2R, 5’-GGCACGTGGACGGCAGTATTTGCGTACCAGGGTTGCAG-3’;
MP3F, 5’-CCCCCATCGGGGTGTTAAGTAACGCTTTTGATCCACCGTGGG-3’,
MP3R, 5’-GCCGTGGGGGATTCAAAGGGTTAATATTGACCCGCATGCGGG3’;
MP4F, 5’-CCCCCAACCGCTCAAGCTCGGTGTTAAGTAAACGCTTTTGACCGGGGG-3’,
MP4R, 5’-GCAAAAAAGTGCACTTAAACTAAAACCGACTTTGAGCGTTGGG-3’.

After PCR, the product was digested with Dpn I to destroy the template before cloning
into PCR II vectors (Invitrogen, CA). The fragments were subsequently transferred into pCaSpeR-hs43-β-gal transformation vector.

**DNA sequencing**

All constructs generated were sequenced at the DNA sequencing facility at the Ohio State University Neurobiotechnology Center.

**Generation of transgenic flies**

To generate transgenic flies, P-element-mediated transformation to introduce the different reporter gene constructs into *Drosophila* was performed according to Spradling (1986) with the exception that the “wings clipped” helper plasmid was used. For each construct, at least four independent lines were examined for reporter gene expression.

**Fixation of *Drosophila* embryos for antibody staining / in situ RNA hybridization**

*Drosophila* embryos were collected and dechorionated in 50% bleach for 5 minutes. Embryos were fixed for 20 minutes in the following fixing solution. For antibody staining, the fixing solution contains 4% formaldehyde in 1x phosphate buffered saline (PBS) (140mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄), and equal volume of heptane. For in situ RNA hybridization, the fixing solution contained 10% formaldehyde in 1x PBS, and an equal volume of heptane. The aqueous fixative was replaced with methanol. Devitellinization of the embryos was done by shaking embryos vigorously for 1 minute. Unfixed embryos as well as heptane were removed. Fixed
embryos were rinsed with methanol and dehydrated with absolute ethanol before storing at 4°C for antibody staining, or −20 °C for future in situ hybridization.

Fixation of Drosophila third star larval brain lobes for antibody staining/ in situ RNA hybridization

Third instar larval brains were dissected in 1xPBS, then fixed in 4% freshly prepared paraformaldehyde in 1x PBS for antibody staining, or in 10% formaldehyde in PBS for in situ hybridization at room temperature for 20 minutes while rotating the tubes. The fixed larval brains were wash 4x in PBT for 1-2 minutes each then gradually dehydrated into absolute ethanol before storing at 4°C for antibody staining or −20 °C for future in situ hybridization.

Antibody staining of whole mount embryos and larval brains

Stored embryos or larval brains were re-hydrated back into 1x PBT. The embryos or larval brains were blocked in 1%BSA+PBT at room temperature for 1 hr. Supernatant was subsequentially removed and the embryos or larval brains were washed in 1x 1%BSA+PBT. The blocked embryos and brains were incubated with primary antibody (mouse anti-Eve 1:20, rat anti-Odd 1:10, mouse anti-DmcytE 1:5, mouse anti-En 1:2, guinea pig anti-Hb 1:400) at proper dilution in 1%BSA+PBT, at room temperature for 2 hrs or over night at 4°C. After incubating with the antibody, the embryos or larval brains were washed 4X 20 minutes in 1x PBT at room temperature before incubating with the secondary antibody at room temperature for 2 hrs. The secondary antibody was either
HRP-conjugated or fluorescent-conjugated. If fluorescent-conjugated antibodies were used, the tubes were kept in the dark after the antibodies were added. The embryos or brains were washed 4x 30 minutes with 1x PBT. For fluorescent-conjugated antibodies, the embryos or brains were directly mounted on slide using Vectashield mounting medium (H-1000, Vector Laboratories) to analyze with confocal microscopy. For HRP-conjugated antibodies, the substrate Diaminobenzoate (DAB) was added at a final concentration of 0.5 mg/ml and 0.03% hydrogen peroxide in 1x PBT. After the desired color developed, the reaction was stopped by several washes in PBT. The embryos or brains were dehydrated into ethanol, and then xylene, before mounting in Permount (Fisher Scientific).

**In situ hybridization and immunohistochemistry**

*In situ* hybridization to whole-mount embryos was performed as described previously (Vaessin *et al.*, 1991) using digoxigenin (Dig)-labeled anti-sense RNA probes and alkaline phosphatase (AP)-conjugated anti-Dig antibodies (Boehringer Mannheim). For *in situ* hybridization involving two probes we utilized Dig-labeled anti-sense RNA probes and fluorescein labeled anti-sense RNA probes. *In situ* hybridization was performed as with single probes. Antibody detection of the hybridized probes was performed with pre-absorbed AP-conjugated anti-Dig antibodies (Boehringer Mannheim) and pre-absorbed horseradish peroxidase (HRP)-conjugated anti-Fluorescein antibody (Boehringer Mannheim). Enzymatic detection of bound antibodies was performed sequentially. HRP detection was performed first, then the embryos were washed 4x 15
minutes in PBT, followed by AP detection. For \textit{in situ} hybridization analysis or immunohistochemistry in larval tissues, larval brains and eye discs were dissected from third instar larvae and fixed for 20 minutes in 10\% formaldehyde/PBT solution for RNA \textit{in situ} hybridization or 4\% paraformaldehyde/PBS solution for immunohistochemistry. Immunohistochemistry was performed as described in Vaessin et al. (1991) using rabbit anti-β-gal, 1:2000 dilution (Cappel), mouse anti-BP102 (Hybridoma Bank, University of Iowa), 1:20 dilution. The secondary antibodies used were fluorescein isothiocyanate (FITC) conjugated goat-anti-mouse 1:200 dilution (Cappel, Malverne, PA), Rhodamine conjugated goat-anti-rabbit 1:200 dilution (Cappel). AP conjugated anti-Dig antibodies in RNA in situ hybridization were visualized by light microscopy using Nomarski optics. Fluorescent conjugated secondary antibodies in immunohistochemistry were visualized using a Biorad MRC1024 system.

**Bromodeoxyuridine (BrdU) incorporation in larval brain**

The larval brains were dissected in S2 medium and incubated with 60 ug/ml BrdU/S2 medium for 1 hour. Subsequently the brains were fixed in 70\% ethanol for 30 minutes at room temperature with rocking. The fixed brains were rehydrated back to 1x PBT and hydrolyzed with 2 N HCl/PBS at room temperature for 1 hour with rocking. After hydrolysis, the brains were rinsed 2x in PBT before blocking in 1\% BSA + 1x PBT for 1 hr at room temperature. From this point, the steps were the same as antibody staining. The mouse anti-BrdU Antibody was used as a 1:10 dilution (Becton Dickinson)
**BrdU incorporation in embryos**

The embryos were collected and dechorinated with 50% bleach for 4 minutes. Bleach was washed out and embryos rinsed 4 x in S2 medium. The embryos were dried by tapping with kimwipe to remove excess liquid. S2 medium saturated-Octane was added to embryos for 5 minutes then removed. Embryos were dried with kimwipe until the remaining octane had been removed (the embryos will appear sparkly). The embryos were transferred into BrdU solution (1mg/ml in S2 media) and incubated for 30 to 40 minutes at room temperature. The BrdU-labeled embryos were fixed according to the antibody staining fixation procedure. After fixating, the embryos were rehydrated with PBT and incubated in PBT for 20-30 minutes. The embryos were hydrolyzed in 2 N HCl (in PBT) for 1 hour at room temperature with rocking. After hydrolysis, the embryos were rinsed 2x in PBT before blocking in 1x PBT + 1% BSA for 1 hr at room temperature. From this point, the steps were the same as antibody staining. The mouse anti-BrdU antibody was used as a 1:10 dilution (Becton Dickinson)

**Fly stocks and genetic crosses**

*pros* \textsuperscript{JO13}/TM3, Sb, Ubx-lacZ has been described in Vaessin et al. (1991) and Li and Vaessin (2000). Homozygous *pros* mutant embryos were identified based on the axonal defects associated with loss of *pros* function (Doe et al., 1991; Li and Vaessin, 2000) using BP102 monoclonal antibodies, or through absence of Ubx-lacZ reporter gene product. The pUAST/Gal4 system (Brand and Perrimon, 1993) was used for ectopic expression of full-length Pros by crossing UAS-pros flies (Manning and Doe, 1999) to
Krüppel (Kr) -Gal 4 driver lines (Figure 2.1). Ectopic expression of Pros was verified with anti-Pros antiserum (Spana and Doe, 1995; Vaessin et al., 1991).

The following *Drosophila melanogaster* mutations were used: cycEAr95 (Knoblich et al., 1994), dap^4^ (Lane et al., 1996), dap^{1377}, dap^2^, dap^3^ (fly stock center and de Nooij et al., 1996). The cycEAr95/Cyo, act-lacZ; prosjo13/TM3, Sb, act-lacZ double mutant flies were generated by standard genetic manipulation. The pUAST/Gal4 system (Brand and Perrimon, 1993) was used for ectopic expression of desired proteins by crossing UAS-Pros (Manning and Doe, 1999), UAS-DmcyE (gift from C. Lehner) or UAS-Dap flies (Lane et al., 1996) to Kr-Gal4 driver lines. A fly stock homozygous for both UAS-Pros and UAS-DmcyE was generated by standard genetic crosses. The expression of individual UAS constructs was verified with antibody staining (Vaessin et al., 1991; Richardson et al., 1995) or *in situ* hybridization. The double pros^jo13, stg 7B* mutant line was generated by standard recombination.

**Protein electrophoresis and transfer**

SDS-PAGE gels were performed according to Laemmli (1970). 7.5-12% of acrylamide: bis-acrylamide (29:1) gel was used for separating gels containing 0.375M Tris-HCl, pH 8.8, 0.1% SDS, 0.02% ammonium persulfate, and 0.04%TEMED. Stacking gels consisted of 3% acrylamide: bis-acrylamide (29:1), 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.1% ammonium persulfate, and 0.1% TEMED. Electrophoresis was performed following the manufacture’s guidelines for the Mini-V 8.10 Vertical Gel Eletrophoresis
Apparatus from BRL at 100V in a running buffer of 0.0025M Tris-HCl, 0.192M Glycine, 0.1%SDS.

SDS-PAGE gels were electronic transferred using the Mini-V 8.10 Blot Module from BRL. Gels were transferred to immobilon-P transfer membrane from Millipore in a buffer containing 0.025M Tris-HCl, 0.192M Glycine, 0.1% SDS, and 20% methanol.

**Preparation of embryonic extracts and immunoprecipitation assays**

Protein extracts from w embryos were prepared as following. *Drosophila* embryos were collected, bleached, washed and weighted. Embryos were subsequently homogenized in Lysis buffer (300 mM NaCl, 50 mM Tris pH8.0, 1% NP-40, 1mM CaCl$_2$, 1 mM MgCl$_2$) containing 1 mM PMSF and Protease Inhibitor Cocktail (P-2714, Sigma). Insoluble material was sendimented by centrifugation at 13,000 rpm for 15 minutes at 4$^\circ$C. Supernatants were pre-cleared with protein A-sepharose beads at 4$^\circ$C for 30 minutes. For immunoprecipitation, lysates were incubated with one of the following antibody at 4$^\circ$C for 1 hour: rabbit anti-HRP antibodies (1:3000) (Jackson Immuno Research lab) as control antibody, mouse monoclonal MR1 (1:4) (Spana and Doe, 1995), rabbit (1:1000) (Vaessin et al., 1991) or guinea pig (1:1000) polyclonal anti-Prox antibodies (Ling and Vaessin, unpublished), mouse monoclonal (8B10) (1:5) or rat polyclonal anti- DmCycE antiserums (1:500) (Richardson et al., 1995; Crack et al., 2002). Protein A-Sepharose beads (Sigma) were preblocked in Lysis buffer containing 5% BSA at room temperature for 1hour before added to lysates and incubated at 4 $^\circ$C for 1 hour. Lysates were washes 4 times 15 minutes each at 4 $^\circ$C in Lysis buffer. Proteins were
separated on 10% or 12% SDS-polyacrylamide gels and analyzed on Western blots using mouse anti-DmcycE (2B8) or mouse anti-cdc2c (Knoblish et al., 1994). Antibody labeling on Western blots was detected with a HRP-conjugated goat anti-mouse secondary antibody (Jackson Immunochemicals) and enhanced chemiluminescence (ECL) system (Amersham).

**Western blot analysis**

For Western blot analysis, the immoblin-P membrane was blocked by incubating in TBST (10mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween) plus 5% nonfat dry milk for 30 minutes. The membrane was then hybridized with the primary antibody, at the appropriate dilution (DmcycE 2B8 1:100; mouse cdc2c 1:100), directly in the blocking solution and incubated for 2 hours at room temperature or 4°C overnight. Following incubation, the blot was washed 3x briefly in TBST and 3x10 minutes in TBST. The blot was then incubated with the secondary HRP-or AP-conjugated antibody in TBST + 2.5% dry milk for 30 minutes at room temperature and washed as before. The NBT/BCIP color reaction (Promega) or ECL detection system (Amersham) was used to detect the proteins on the blot following the manufactures’ protocols.
Figure 2.1 Schematic representation of the UAS/GAL4 system. A transgenic fly carrying a GAL4 transcription activator is crossed to a transgenic fly carrying a gene of interest (the Pros ORF) cloned downstream of UAS binding sequences. Pros will be expressed by the GAL4 transcription activator in a tissue specific manner, which in this case is the Kruppel domain.
CHAPTER 3

TRANSCRIPTION OF THE DROSOPHILA CKI GENE DACAPO IS REGULATED BY A MODULAR ARRAY OF CIS-REGULATORY SEQUENCES

Introduction

During development, cell proliferation and differentiation must be precisely coupled to ensure the proper formation of an organism. Increasing or decreasing the numbers of a particular cell type, due to over proliferation or premature differentiation, respectively, results in the malformation of a tissue or even leads to the death of an organism. Cell proliferation is regulated by a family of cyclin-dependent kinases (CDKs) which, together with Cyclins, promote the transition of cell cycle phases. The activities of CDK-Cyclin complexes are regulated by Cyclin-dependent kinase inhibitors (CKIs). Two families of CKIs have been identified based on their structure and targets (Sherr and Roberts, 1999). The $p16^{INK4a}$ families, including $p15$, $p16$, $p18$, and $p19$, are composed of multiple ankyrin repeats and specifically inhibit the catalytic subunits of cyclin D-dependent kinases, $cdk4$ and $cdk6$. Whereas the $p21$ Cip/Kip families, including $p21^{cip}$, $p27^{kip1}$ and $p57^{kip2}$, all contain a conserved amino-terminal domain that binds to and inhibits all kinases involved in G1/S transition.
*Drosophila* has one identified G1/S CKI gene, *dacapo* (*dap*), with structural similarity to the vertebrate Cip/Kip family. Dap protein (Dap) has been shown to bind and inhibit the activity of cdk2/cyclinE complexes and maintain G1 arrest (de Nooij *et al*., 1996; Lane *et al*., 1996). Loss-of-function mutations in *dap* cause an extra round of mitotic division in the embryonic epidermis (de Nooij *et al*., 1996; Lane *et al*., 1996). In addition, ectopic mitotic activity and disruption of the mitotic domain structure are observed in the optic lobes of third instar larvae (Wallace *et al*., 2000). Alternatively, premature expression of *dap* in embryos results in precocious G1 arrest (de Nooij *et al*., 1996; Lane *et al*., 1996). These observations suggest that *dap*, like vertebrate CKIs, is important for regulating cell cycle exit at the proper developmental time.

Consistent with its role as a CKI, *dap* expression is tightly correlated with cells exiting mitotic activity (de Nooij *et al*., 1996; Lane *et al*., 1996). In embryonic development, zygotic *dap* expression is first seen during cellularization and then in the amnioserosa during gastrulation. Following germband extension, *dap* RNA is observed in the developing central nervous system (CNS) and epidermis. In the developing CNS, *dap* RNA expression is generally initiated in ganglion mother cells (GMCs) before they enter the terminal division to give rise to differentiated neurons or glia. In the epidermis, *dap* transcripts increase abruptly just before mitosis 16, the last mitotic cycle for the majority of the epidermal cells. After germband retraction, *dap* expression is restricted mainly to the developing CNS and peripheral nervous system (PNS). Expression of the protein closely matches *dap* RNA expression suggesting that regulation of *dap* transcription may be a major mechanism for the proper regulation of Dap activity.
A tight correlation of *dap* expression with cells exiting mitotic activity is also observed during larval development. In the optic lobes of third instar larvae, there are highly organized domains of proliferation and differentiation. Two of the major areas of mitotic activity in the optic lobes are the outer and inner proliferation centers (OPC and IPC, respectively). The IPC forms in a crescent shape at a more interior position of the brain with respect to the OPC. Ectopic expression and loss-of-function of two pan-neural transcription factors, Deadpan (Dpn) or Asense (Ase) changes mitotic activity in the optic lobes, illustrated by disruptions in the OPC. These disruptions appear, at least in part, to be mediated by associated changes in the expression pattern of *dap* (Wallace *et al*., 2000). These observations suggest that expression of *dap* needs to be precisely coupled with the developmental programs of specific cell lineages to ensure that cell cycle progression is coordinated with tissue and lineage specific growth and differentiation. Hence, developmental regulation of *dap* transcription may represent a critical mechanism for controlling Dap activity.

Recently the *cis*-regulatory elements of two *Drosophila* cell cycle regulatory genes, *string/cdc25* (*stg*), encoding a cdc25 type phosphatase, and *Drosophila cyclin E* (*DmcycE*), have been identified (Jones *et al*., 2000; Lehman *et al*., 1999; Edgar *et al*., 1994a). *stg* is the limiting factor of G2/M transition while DmcycE is essential for progression into S phase (Edgar and O'Farrell, 1989; Edgar *et al*., 1994b; Knoblich *et al*., 1994; Richardson *et al*., 1993). Both genes exhibit high complexity in transcriptional regulation that is controlled by tissue-specific *cis*-regulatory elements (Jones *et al*., 2000; Lehman *et al*., 1999; Edgar *et al*., 1994a). In mutant embryos of pattern formation genes,
such as *bicoid, hunchback*, and neurogenic genes, such as *Notch, Enhancer of split*, there is altered transcription of *stg*, indicating developmental regulation of *stg* expression (Edgar *et al.*, 1994a). *stg* has also been shown to be regulated by the developmental regulator gene, *prospero (pros)*, during *Drosophila* embryogenesis (Li and Vaessin, 2000).

*pros* is a pan-neural gene that encodes an atypical homeodomain protein (Pros) expressed in all neuronal lineages (Chu-Lagraff *et al.*, 1991; Doe *et al.*, 1991; Matsuzaki *et al.*, 1992; Vaessin *et al.*, 1991). Analyses of loss-of-function and ectopic expression of Pros in *Drosophila* embryos shows that *pros* function regulates mitotic activity and the RNA expression of several other cell cycle genes, including *stg, DmcycE*, and E2F (Li and Vaessin, 2000). In vertebrate, Prox1, the mouse Pros homologue, has been shown to play a role in cell cycle regulation. Expression of two CKIs, *p27kip1* and *p57kip2* is down regulated in lens fiber of mice lacking Prox1 (Wigle *et al.*, 1999).

It has been shown for various Cip/Kip CKIs in multicellular organisms that, in many instances, upregulation of Cip/Kip gene transcription immediately precedes the exit of mitosis in differentiating cells (de Nooij *et al.*, 1996; Halevy *et al.*, 1995; Hong *et al.*, 1998; Lane *et al.*, 1996; Lee *et al.*, 1995). However, the exact mechanism of the transcriptional regulation of Cip/Kip CKIs is poorly understood. I addressed this issue by studying the relationship between *Drosophila* embryonic development and the transcriptional regulation of *dap*. I first identified the *cis*-regulatory elements of the *Drosophila dap* transcription unit using a series of reporter gene constructs. My data indicated that *dap* transcription is controlled by a modular array of tissue-specific
elements. Furthermore, I showed that pan-neural Pros function is required for proper regulation of *dap* expression in the developing nervous system.

**Results**

*dap* transcription is controlled by a modular array of *cis*-regulatory elements

To identify the *cis*-regulatory regions critical for *dap* transcription, I first determined the genomic structure of the *dap* transcription unit. The sequence of *dap* cDNA (U68477) was aligned with the genomic DNA sequence, generated by the *Drosophila* Genomic project, in the 46B region of the *Drosophila* genome (AC005894, BDGP). These alignments showed that the *dap* transcription unit was interrupted at position 795 (U68477) by a small 59 base pair (bp) intron (position 104016 to 104074 of AC005894), and a 1344 bp intron (position 104476 to 105819 of AC005894) at position 1196 in the *dap* cDNA sequence (U68477). To map the elements controlling *dap* transcription, I tested sequences located upstream and downstream of the *dap* transcription unit as well as the intron sequences by generating a series of lacZ reporter constructs (Figure 3.1) and established transgenic lines. All map positions provided in the following are with regard to the first base of the ATG at the translation start site (position bp 103852 of AC005894, BDGP). LacZ mRNA and βgal protein expression was determined at different developmental stages for each reporter gene construct. At least four independent transgenic lines were generated and analyzed for each construct.

*cis*-regulatory sequences that account for all main spatial and temporal elements of *dap* expression were identified. *cis*-regulatory elements located within a 6 kb region
upstream of the *dap* transcription unit and in the *dap* introns can account for most, if not all, spatial and temporal pattern components of *dap* transcriptional expression (Figure 3.2). In contrast, sequences located immediately 3’ of the *dap* transcription unit appear not to contain any spatial and/or temporal specific transcriptional regulatory elements.

**Epidermal expression**

*dap* is expressed transiently in different tissues just before cells enter the last cell cycle (de Nooij *et al.*, 1996; Lane *et al.*, 1996). For instance, during early gastrulation, *dap* is expressed in the amnioserosa (Figure 3.3A), where cells are arrested in G2 phase at the beginning of embryonic cell cycle 14 and exit cell division earlier than most of the other cells. During germband extension, *dap* expression appears in the tracheal pits (Figure 3.3B) and epidermal cells, in which *dap* transcripts increase quickly right before the onset of mitosis 16 which is the last mitotic cycle (Figure 3.3C). *dap* transcripts are also observed in the mesodermal cells which become postmitotic during these stages (data not shown). The Dap-5’-2R construct, containing 2kb of sequence located immediately 5’ of the *dap* transcribed region, mediates expression in amnioserosa and tracheal pits in a pattern similar to that of endogenous *dap* (Figure 3.1, 3.2, 3.3A and B). LacZ expression of Dap-5’-2R was also detected in a subset of epidermal cells and muscle (Figure 3.2). The Dap-5’-2+UTR, containing the cis-regulatory fragment present in Dap-5’-2R plus an additional 536 bp toward the translational start site (5’ untranslated region), shows strong reporter gene expression in tracheal pits and developing epidermis (Figure 3.1, 3.2, 3.3D and F). This indicates that cis-regulatory sequences, located within the 536 bp of the
5’ untranslated region, are required for the epidermal expression of the Dap-5’-2+UTR. There is a second cis-regulatory fragment, located in Intron 2 (Dap-BB), that also drives reporter gene expression in the epidermis similar to the expression driving by Dap-5’-2+UTR (see below). At stage 13, the epidermal reporter gene expression of Dap-5’-2+UTR is strongly reduced, suggesting that the cis-regulatory sequences also contain elements required for termination of expression. However, in contrast to Dap-5’-2R this construct does not mediate lacZ expression in amnioserosa (data not shown). Thus, a negative element located within position –539 to –6 suppresses reporter gene expression in the amnioserosa that is seen in Dap-5’-2R lines.

**PNS expression**

When epidermal cells complete their final division, some cells in the PNS and CNS still undergo divisions. Consistent with this observation, after germband retraction, the majority of dap transcripts are detected in the developing PNS and CNS (Figure 3.3G, I and K). A 1kb cis-regulatory fragment extending from 2 to 3kb upstream of the of dap transcription unit, Dap-3’2’ (Figure 3.1 and 3.2), drives reporter gene expression in the entire developing PNS (Figure 3.3H) in a pattern similar to wild-type dap. In contrast, reporter construct Dap-4’2’, that contains a genomic fragment extending from 2 to 4 kb upstream of the dap transcription unit (Figure 3.1 and 3.2) and includes the 1kb fragment present in Dap-3’2’, drives reporter gene expression only in the developing chordotonal organs (Figure 3.3J). Thus, negative regulatory elements appear to be located within the distal 1kb of Dap-4’2’ construct, and can repress all non-chordotonal organ lineage
expression in the developing PNS driving by Dap-3’2’. There is no detectable expression in the CNS of Dap-3’2’ and Dap-4’2’, suggesting that dap expression in the CNS and PNS are regulated by separate cis-regulatory elements.

**Embryonic CNS expression**

In most neuroblast lineages, dap RNA expression begins just before GMCs enter the last division to give rise to two mitotically inactive cells that will enter terminal differentiation. In addition to epidermal expression, Dap-5’-2+UTR also drives lacZ expression in a subset of late dividing GMCs in the developing CNS (stage 13 and later) that mainly give rise to glia (data not shown).

I tested whether sequences in Intron 1, or sequences between Introns 1 and 2, can contribute to reporter gene expression. Since Intron 1 itself is rather small, I tested it together with Intron 2. Reporter gene expression driven by Intron 2 alone, Dap-B, and Intron 1 and Intron 2 together, Dap-SB, did not exhibit any obvious differences during embryogenesis. dap Intron 2 sequences, Dap-SB and DapB, mediated expression predominately in the developing CNS and in midgut visceral mesoderm (Figure 3.3L and data not shown), similar to the pattern observed for wild-type dap. When I dissected the 1344 bp Intron 2 into 529 and 840 bp fragments, Dap-BB and Dap-HB (Figure 3.1) respectively, I found Dap-HB directed reporter gene expression in most of the CNS and hindgut visceral mesoderm, while Dap-BB directs expression in migrating pole cells and in a subset of epidermal cells (data not shown). Since the expression of Dap-B and Dap-SB, both of which contain the entire Intron 2, was not seen in a subset of epidermal cells,
Dap-HB appeared to contain a negative regulatory element, which in the context of entire intron 2 sequence, suppressed reporter gene expression in the developing epidermis. Further deletion of the 5’ 295 bases of Dap-HB, limited reporter gene expression mainly to the midline glial lineages (Dap-DEL; Figures 3.1 and 3.2). This observation suggested separate sequences drive \textit{dap}-lacZ expression in cell type-specific lineages.

\textbf{Larval CNS expression}

During the third instar larval stage, \textit{dap} gene function is critical for proper formation of the larval optic lobes. Loss of \textit{dap} function results in a disruption of the mitotic domain structure as well as ectopic cell proliferation in the larval optic lobes (Wallace \textit{et al}., 2000). Consistent with a role in restricting cell proliferation, \textit{dap} expression in the larval optic lobes is mainly limited to cells entering G1 arrest and/or terminal differentiation (Wallace \textit{et al}., 2000). To identify the transcriptional regulatory elements required for \textit{dap} expression during larval CNS development, I analyzed reporter gene expression of the various constructs in the CNS of third instar larvae. \textit{dap} mRNA was expressed in neuronal lineage cells of the ventral nerve cord, and optic lobes of the third instar larval CNS (Wallace \textit{et al}., 2000). In optic lobes, \textit{dap} mRNA is expressed in the IPC, lamina furrow (LF) as well as a band of cells at the border between the OPC and the brain (Figure 3.4A). Analyzing the lacZ mRNA expression patterns, I found that the reporter gene constructs that drive expression in embryonic CNS were also active in the larval CNS (Figure 3.1, 3.2, and 3.4B).
Dap-SB appeared to contain all of the cis-regulatory elements required for proper larval CNS expression. However, more detailed analysis of this cis-regulatory region revealed an array of elements that were required for complete larval CNS expression. Dap-HB drove reporter gene expression in a pattern similar to wild-type dap in the IPC and LF of third instar larval optic lobes but, in addition to the posterior border of the OPC, Dap-HB drove reporter gene expression ectopically in the OPC where cells were in mitosis (Figure 3.4A and C). Compared to Dap-SB, reporter gene expression of Dap-HB in the OPC formed a broader band including one distinct area of high level expression (Figure 3.4C, indicated by arrows). This observation indicated some regulatory sequences essential to exclude dap expression from the OPC were absent in Dap-HB. Dap-PH, containing the genomic fragment of Dap-HB minus 75 bases, lost the ability to drive lacZ expression in LF while maintained the pattern in the IPC and OPC as observed in Dap-HB (data not shown). Dap-DEL, which deletes the 5’ 295 bases of Dap-HB, was only expressed posterior to the OPC, and abolished reporter gene expression in the IPC (Figure 3.4D). Furthermore, while both Dap-SB and Dap-B drove reporter gene expression in the optic lobes, lacZ mRNA expression of Dap-SB in the LF was more complete compared to Dap-B (data not shown). This indicated that the addition of sequences, including Intron 1, present in Dap-SB contributed to transcriptional regulation of dap in the larval CNS.
**Imaginal discs expression**

*dap* has been reported to be expressed in imaginal discs later in development. In eye discs, *dap* is expressed at the posterior edge of the morphogenetic furrow (MF), where cells have just withdrawn from mitotic activity and are entering G1 arrest (Figure 3.4E) (de Nooij *et al.*, 1996; Lane *et al.*, 1996). Expression of reporter constructs showed that the *cis*-regulatory elements driving expression in eye, wing and leg discs were located mainly in Intron 2. Further examination showed Dap-HB was able to mediate expression in eye discs similar to wild-type *dap* (Figure 3.4F). In Dap-DEL, the expression in eye discs was abolished, suggesting the 5’ 295 bases of intron 2 were essential for driving reporter gene expression in the eye discs. Contrasted with its ability to mediate expression in eye discs, Dap-HB showed no significant lacZ expression in either wing or leg discs. On the other hand, the construct containing 5’ 529 bases of intron 2, Dap-BB showed expression in wing and leg discs in a manner similar to endogenous *dap* expression (data not shown). There was consistency of expression of reporter constructs in similar tissues in both embryos and larva. The expression of Dap-BB in larval imaginal discs, which are derived from epithelial primordia, was consistent with its expression in epidermal cells during embryogenesis. Also, the Dap-4’2’ reporter gene exhibited expression in chordotonal organs during embryogenesis as well as in the chordotonal organ lineages of the leg discs in the larva (data not shown).
**dap transcription is regulated by developmental genes**

To understand the degree to which *dap* expression depended on cell cycle regulators, I tested *dap* transcriptional expression in homozygous *stg* mutant embryos. *stg*, a *Drosophila* cdc25 homologue, is required for the G2/M transition. Cell cycle progression in *stg* mutant embryos arrests at embryonic cell cycle 14 (Edgar *et al*., 1994a). While cell cycle arrest in *stg* mutant embryos results in severe morphological defects, the temporal and spatial pattern of *dap* RNA expression appears to be normal and at the proper development stage despite the absence of cell division after cell cycle 14 (data not shown). This observation is also consistent with the *dap* protein expression in *stg* mutant embryos (de Nooij *et al*., 2000). Thus, transcriptional regulation of *dap* expression does not depend on cell cycle progression or reaching certain number of cell cycles.

The *Drosophila* gene *pros* has previously been shown to regulate the transcription of cell cycle regulators during embryonic neurogenesis (Li and Vaessin, 2000). In most neuroblasts Pros protein is localized asymmetrically to the cytoplasmic membrane (Hirata *et al*., 1995; Knoblich *et al*., 1995; Spana and Doe, 1995). Upon neuroblast division Pros is distributed only to one daughter cell, the GMC, were it translocates into the nucleus of the GMC. The translocation of Pros into the GMC nucleus coincides with time when GMCs start to express *dap* and the developmental decision to initiate the exit from mitosis and to initiate terminal differentiation is made (de Nooij *et al*., 1996; Lane *et al*., 1996). To investigate whether *pros* also played a role in regulating *dap* expression, I analyzed *dap* expression in homozygous *pros* mutant embryos (Figure 3.5). There were at
least two phases of *dap* expression during embryonic neurogenesis that required Pros activity. During activation of *dap* expression in the developing CNS at stage 10, a distinct delay in the appearance of detectable amounts of *dap* transcripts was observed in homozygous *pros* mutant embryos as compared to wild type embryos (Figure 3.5A and B). This suggested that *pros* function was required for the timely activation of *dap* expression. However, *dap* expression, albeit delayed, ultimately was activated, indicating that redundant factors could activate *dap* expression in the absence of Pros. By stage 16, *dap* expression in the developing CNS became restricted to a small subset of cells (Figure 3.5C). However, in homozygous *pros* mutant embryos I observed continued high levels of *dap* expression (Figure 3.5D). Furthermore, ectopic Pros driven by Krüppel (Kr) Gal4 (borders indicated by arrows in Figure 3.5F), effectively suppressed *dap* expression within this domain (Figure 3.5E and F). These results indicated that during the late phase of embryonic neurogenesis Pros function was also required to terminate *dap* expression.

To determine if the requirement of *pros* function for *dap* expression could be reproduced by *dap* reporter gene constructs, I tested reporter gene expression in a *pros* loss-of-function background, as well as in embryos with ectopic Pros expression. In these experiments *cis*-regulatory regions were identified that recapitulated the two modes of Pros function. The expression of the Dap-SB lacZ construct was strongly reduced in the CNS of homozygous *pros* mutant embryos (Figure 3.6), supporting a role for Pros in the proper activation of this element. Ectopic mitotic activity and compensatory cell apoptosis have been shown in late-stage *pros* homozygous mutant embryos (Li and Vaessin, 2000). This raised the question whether the observed reduction of Dap-SB
reporter gene expression was a consequence of decreasing cell numbers. Double staining for DNA and lacZ showed no significant reduction in the cell numbers of the CNS in a pros mutant background, when dramatic reduction of Dap-SB reporter gene expression was observed (data not shown). This observation indicated that it is unlikely that the reduced expression of reporter gene in pros mutant embryos was the consequence of reduced cell numbers. However, I can not presently exclude the possibility that this reduction of reporter gene expression could have been due to changing cell fates in pros mutant embryos. However, consistent with the observation of reduced reporter gene expression in embryos lacking Pros activity, ectopic Pros expression resulted in ectopic activation of this reporter construct (Figure 3.7A and B). In contrast, the reporter gene construct Dap-5’-6F/R, exhibited the opposite effect in the presence of ectopically expressed Pros (Figure 3.7C and D). Here, Pros expression resulted in the suppression of lacZ expression, suggesting that Dap-5’-6F/R contained sequences that mediated a negative transcriptional response to the presence of ectopic Pros. Finally, Dap-DEL, a reporter construct expressed specifically in the nervous system, was ectopically expressed at stage 10 following misexpression of Pros (Figure 3.7E), whereas in stage 12 embryos, the same reporter gene construct was suppressed in the presence of ectopic Pros (Figure 3.7F). The two opposite responses of Dap-DEL toward ectopic Pros at different stages recapitulated the two modes of Pros function with regard to dap expression, observed in a loss-of-function pros mutant background. These observations supported the notion that Pros has a dual role in the regulation of dap transcription.
Pros binding sequences on dap-Del reporter gene

Pros is an atypical homedomain protein and has been shown to bind to the deformed (dfd) promoter (Hassan et al., 1997). The consensus Pros binding sequences (C A/t c/t N N C T/c) has been identified through target detection assay (Hassan et al., 1997). To investigate whether Pros could also bind to the dap promoter region, all potential Pros binding sites in the Dap-Del reporter gene were mutated using site-direct mutagenesis. This Dap-MP4 reporter construct carried the same cis-regulatory fragment as Dap-Del except all potential Pros binding sequences were mutated (Figure 3.8). The lacZ expression of the embryos carrying the Dap-MP4 reporter constructs could still be observed in MP2 cells of the developing nervous system. However, the lacZ expression of the Dap-MP4 appeared only in an irregular pattern and in a non-synchronized fashion with much lower intensity when compared to the lacZ expression of the Dap-Del reporter genes (Figure 3.8). This observation was consistent for 10 individual transgenic lines carrying Dap-MP4 reporter genes. Furthermore, the lacZ expression of the Dap-MP4 no longer responded to ectopic Pros (Figure 3.8 G and H). This indicated that the mutated sites are essential for Pros-mediated response.

Discussion

Complexity of transcriptional regulation of cell cycle regulatory genes

In an effort to understand the mechanism of controlling cell withdrawal from division at the proper time during developmental processes in Drosophila, I have mapped the cis-regulatory region of dap and showed a requirement for the pan-neural gene pros
for its regulation. The structural features of cis-regulatory elements of *dap*, as identified in this study, showed similarities to those of two other cell cycle regulatory genes, *stg* and *DmcycE* (Jones et al., 2000; Lehman et al., 1999). For instance, the cis-regulatory elements of all three genes are composed of modular tissue- and stage-specific cis-regulatory elements. In both, *dap* and *stg*, negative regulatory elements have been identified. During embryogenesis, progression of the developmental program moves very rapidly. Therefore, cell cycle regulators need to respond to a variety of developmental signals that direct the formation of different lineages and tissues at overlapping developmental times. It is, hence, not surprising that complex sets of regulatory elements are employed to regulate the expression of cell cycle regulatory genes, such as *dap*, *stg*, and *DmcycE*. A modular structure of cis-regulatory elements would allow tissue and/or lineage specific regulation in response to a wide range of tissue and/or lineage specific developmental signals.

**The dap cis-regulatory region**

The expression pattern of a series of Dap-lacZ constructs suggested that the transcriptional expression of *dap* was controlled through a complex array of cis-regulatory elements. My data indicated Intron 2 of *dap* contained critical cis-regulatory elements for *dap* expression in both the embryonic and larval CNS as well as imaginal discs. In addition, *dap* expression in a subset of CNS cells during late CNS development required sequences located upstream of the *dap* transcription unit. PNS expression appeared to be mediated mainly by a 1kb region located 5' to the epidermal element in
the promoter. Two epidermal regulatory elements were identified, one was within a 2.5 kb fragment located 5’ to the translation start site, and a second was located in the initial 529 bp of Intron 2. *cis*-regulatory elements mediating expression in tracheal pits, muscle, hindgut and amnioserosa were also identified in this study. Regulation of *dap* transcription also appears to involve tissue specific negative regulatory elements. Three segments containing negative regulatory elements have been identified in this study. One segment suppressed the expression of Dap-lacZ constructs in the PNS, except chordotonal organs. A second segment was found to suppress reporter gene expression in the amnioserosa. Finally, a third element that suppressed reporter gene expression in epidermal cells was identified. I suspect there may be more repressor elements hidden within the tested DNA sequences, since some of the tested DNA segments are still quite large and direct expression in multiple tissues. The observation of distinct, tissue and/or lineage specific negative regulatory elements in the cis-regulatory region of *dap* may appear surprising given that the internal *dap* transcription unit is transcribed in these tissues and/or lineages during development. Therefore, positive regulatory elements have to be present in the intact *dap* cis-regulatory region, and absent in the respective lacZ reporter gene constructs, that counteract individual negative regulatory elements in the correct lineages at the proper developmental stages.

**Developmental programs control cell cycle progression via *dap***

Evidence from knockout mice and cell culture systems indicates that the expression of mammalian CKIs of the Cip/Kip family is regulated by developmental
signals (review by Zhang, 1999). This hypothesis is further supported by studies of *Xenopus* and *C. elegans* CKIs, and *Drosophila* cell cycle regulatory genes *stg*, *DmcycE* and *dap* (de Nooij *et al*., 1996; Hardcastle and Papalopulu, 2000; Hong *et al*., 1998; Jones *et al*., 2000; Lane *et al*., 1996; Lehman *et al*., 1999). Although developmental regulation of the expression of a cell cycle regulator may be universal, there are presently only a few developmental genes implicated in regulating cell cycle progression (Ohnuma *et al*., 2001).

*pros*, a pan-neural gene, has previously been shown to regulate the expression of cell cycle regulatory genes including *stg* and *DmcycE* (Li and Vaessin, 2000). Here I showed that *pros* also played a role in regulating *dap* expression during embryonic neurogenesis. Studies in knock-out mice revealed that the mouse Pros homologue Prox1 was required for the expression of two CKIs, *p27kip1* and *p57kip2* in lens fiber development (Wigle *et al*., 1999). Thus, both *Drosophila* Pros and mouse Prox1 have been shown to regulate the expression of CKIs of the Cip/Kip family, pointing to a conservation in the regulation of Cip/Kip expression. However, differences are also evident. In Prox1 knockout mice, the expression of *p27kip1* and *p57kip2* is reduced in the lens fibers of mutant embryos, while in *Drosophila* homozygous *pros* mutant embryos, *dap* expression is initially delayed at an earlier developmental stage, but increases during later stages in the developing CNS. Thus in mice, Prox1 appears to function mainly to activate the expression of two CKIs of the Cip/Kip family, while in *Drosophila*, Pros functions first to initiate *dap* expression at the proper time and later to restrict *dap* expression. In *Drosophila*, *dap* is eventually expressed by early stage 11 in *pros* mutant
embryos, indicating the presence of redundant regulatory mechanisms in the activation of *dap* transcription. For the vertebrate Pros homologues no role in the suppression of Cip/Kip genes has been reported that would parallel the observation that Pros suppresses *dap* expression during late embryogenesis. However, it has been reported earlier that unlike vertebrate Kips which continue to be expressed after terminal differentiation, *dap* is only transiently expressed at high levels when cells exit from mitotic activity (Harper and Elledge, 1996; Lane et al., 1996). This may explain the differences in regulation of mouse Kips and *Drosophila dap* by Prox1 and Pros, respectively. However, the functional significance for a Pros mediated suppression of *dap* expression is presently not clear.

Pros protein and RNA have been shown to localize asymmetrically to the cytoplasmic membrane of neuroblasts (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995). When neuroblasts divide, Pros is distributed only to one daughter cell, the GMC. Pros then translocates from the cytoplasm into the nucleus of the GMC. The translocation of Pros into the GMC nucleus coincides with time when GMCs start to express *dap* and exit from mitosis to undergo terminal differentiation to generate neurons or glia (de Nooij et al., 1996; Lane et al., 1996). It was suggested that nuclear Pros and Dap function in concert to prevent further cell proliferation in the GMCs of the developing CNS (Li and Vaessin, 2000). While nuclear Pros suppresses the transcription of cell cycle regulatory genes, such as *stg* and *DmcyCE*, to prevent further cell proliferation, Dap promotes G1 arrest through inhibition of CDK2/cycE kinase activity. The observation that *dap* expression is delayed in *pros* homozygous mutant embryos at
early neurogenesis (stage 10) indicates that the activities of nuclear Pros and Dap do not function exclusively in a parallel way, but that Pros also regulates cell cycle exit via regulation of the CKI gene *dap* (Li and Vaessin, 2000). Furthermore, the ability to respond to ectopic Pros was abolished in the transgenic embryos carrying the Dap-MP4 reporter gene indicates that Pros does play a critical role in regulating *dap* expression, and that this role is likely at least in part mediated through direct regulation of *dap* expression via Pros response elements.

Two basic helix-loop-helix (bHLH) pan-neural genes, *dpn* and *ase*, have been shown to play critical roles in regulating *dap* expression during CNS development in third instar larvae (Wallace et al., 2000). Dpn negatively regulates *dap* expression, while Ase has a positive effect on *dap* expression. Changing Dpn and Ase dosage results in altered mitotic activity in brain lobes of third instar larvae that is paralleled by changes in *dap* expression (Wallace et al., 2000). In vertebrates, HES-1, a transcription factor with high similarity to the *Drosophila* Hairy and Dpn bHLH proteins, functions as a negative regulator of neuronal differentiation, and has been shown to suppress the transcription of the *p21cip* promoter in PC12 cells (Castella et al., 1999). This suggests the existence of a conserved mechanism of developmental control of CKIs by bHLH proteins. It is of interest in this context that Pros has been shown to be a negative regulator of *dpn* transcription in the larval CNS (Liu and Vaessin, not shown). It will thus be interesting to test the extent to which bHLH proteins and Pros interact in the transcriptional regulation of *dap* expression during development.
In summary, I have identified cis-regulatory elements located in the *dap* transcription unit. The identified cis-regulatory elements were organized in tissue specific modular arrays that contained both positive and negative components. Further, I identified Pros, a pan-neural transcription factor expressed in all neuronal lineages, as a regulator of *dap* transcription. My observation that Pros regulates the cell cycle via *dap* transcription provides a model for the linkage of developmental programs to cell cycle progression and differentiation through modulating cell cycle regulatory genes.
Figure 3.1 *dap* genomic region. Solid blocks indicate transcribed regions, and striped blocks represent introns. Scale on the top represents the distance in kb from the translation start site (0 kb). Genomic fragments used to generate lacZ reporter constructs are shown in relation to the *dap* map, with transgene names indicated to the right.
Figure 3.2 Identified cis-regulatory elements in the dap genomic region. The locations of tissue-specific cis-regulatory elements are shown in different color bars (main expression) or dots (minor expression) in relation to the genomic map of the dap transcription unit. 

- a: Epidermal elements are defined from the lacZ expression of dap-5'-2R (minor expression), dap-5'-2+UTR and dap-BB (major expression).
- b: PNS element is identified from the lacZ expression of dap 3'2'.
- c: Embryonic CNS elements are defined from the lacZ expression of dap-5'-2+UTR and dap-SB minus dap-BB.
- d: Larval CNS elements are defined from the lacZ expression of dap-5'-2+UTR and dap-SB minus dap-BB.
- e: Imaginal discs elements are defined by the lacZ expression of dap-BB and Dap-HB.
- f: Muscle elements are defined by the lacZ expression of dap-5'-6F/R minus dap-4'2'.
- g: Midgut visceral mesoderm element is identified by the lacZ expression of dap-B.
- h: Tracheal pit elements are defined from the expression of dap-5'-2+UTR and dap-5'6F/R minus dap-4'2'.
- i: Amnioserosa element is identified by the lacZ expression of dap-5'-2R.
- j: Hindgut element is defined by the lacZ expression of dap-5'-6F/R minus the expression of dap-4'2' and dap-5'-2R.
Figure 3.3 RNA expression of *dap-lacZ* reporter genes during embryogenesis. Wild-type *dap* mRNA expression is shown for comparison to the left (A,C,E,G,I,K) and lacZ mRNA expression in correspondingly staged embryos is to the right (B,D,F,H,J,L). The regulatory regions present in the five lacZ reporter genes, Dap-5’-2R (B), Dap-5’-2+UTR (D,F), Dap-3’2’ (H), Dap-4’2’ (J), and Dap-SB (L), used are shown in relation to the genomic map of *dap*. All embryos are oriented anterior to the left and dorsal up. At stage 7 embryos (A,B), Dap-5’-2R lacZ (B) is expressed in amnioserosa similar to the expression of *dap* mRNA (A). At early stage 11 embryos (C,D), *dap* (C) and Dap-5’-2+UTR lacZ (D) are expressed in tracheal pits. At late Stage 11 embryos (E,F), *dap* (E) and Dap-5’-2+UTR lacZ (F) are expressed in epidermal cells. During stage 13, *dap* is expressed in both the developing PNS (G,I) and CNS (K). Dap-3’2’ lacZ (H) is expressed in PNS, Dap-4’2’ lacZ (J) is expressed in chordotonal organs of PNS, and Dap-SB lacZ (L) is expressed in CNS.

CNS: central nervous system; PNS: peripheral nervous system; tb: tracheal pits
Figure 3.4 Dap-SB lacZ reporter gene expression in the third instar larval CNS and eye discs. Wild-type *dap* mRNA expression is shown for comparison in panels (A,E). Dap-SB lacZ mRNA expression is shown in panels B. Dap-HB (panel C,F) and Dap-DEL (panel D) lacZ mRNA expressions are shown. *dap* mRNA (A) and Dap-SB lacZ mRNA (B) expression in IPC, LF and outside of OPC of the third instar larval brain lobes. lacZ mRNA expression of Dap-HB (C) are observed in IPC, LF and OPC (arrows indicate high level lacZ expression in OPC), while Dap-DEL lacZ mRNA (D) is only observed in a band of cells outside of the OPC. In eye discs (E,F), *dap* mRNA (E) and Dap-HB lacZ mRNA (F) expression in posterior to MF indicated by arrowhead. IPC: inner proliferation center; OPC: outer proliferation center; LF: lamina furrow; MF: morphogenetic furrow.
Figure 3.5 Pros function is required for transcriptional expression of *dap*. In stage 10 embryos, *dap* expression (blue) is delayed in *pros* mutant embryos (B) compared with wild-type embryos (A). Examples of *dap* RNA expressing cells are indicated with arrows. Wild-type embryos are identified by the presence of lacZ mRNA (brown) from balancer chromosome TM3,Sb,Ubx-lacZ. In stage16 embryos, *dap* expression is increased in *pros* mutant embryos (D; ventral view) compared with wild-type embryos (C; ventral view). Ectopic Pros expression results in a suppression of *dap* expression (F) compared to *dap* expression in wild-type embryos (E). The effects of ectopic Pros expression are shown for Krüppel (Kr)-Gal4/UAS-Pros embryos. These embryos express ectopic Pros in the Kr domain (borders indicated by arrows).
Figure 3.6 Pros activity is required for activation of the Dap-SB lacZ reporter gene during embryonic neurogenesis. Expression of Dap-SB lacZ (red) is reduced in ventral cords of homozygous pros mutant (B) embryos compared to wild-type (A) embryos. Double labeling of embryos with axon-specific monoclonal antibody BP102 (green) was used to identify pros mutant embryos based on their axonal defects.
Figure 3.7 Overexpression of Pros has a dual effect on Dap-lacZ expression. A,C,E,G: Reporter gene expression in embryos without ectopic Pros expression. B,D,F,H: Kr-Gal4/UAS-Pros embryos. Kr-Gal4/UAS-Pros embryos express high level of Pros protein within the Kr domain (borders indicated by arrows). A,B: Dap-SB lacZ mRNA expression. Ectopic expression of Pros in Kr domain (B) increases lacZ mRNA expression of the Dap-SB reporter gene compared to reporter gene expression in embryos lacking ectopic Pros expression (A). C,D: Dap-5'-6F/R lacZ mRNA expression. In contrast to Dap-SB reporter gene, lacZ mRNA expression of the Dap-5'-6F/R reporter gene is reduced when Pros is ectopically expressed in the Kr domain (D). E,F,G,H: Dap-DEL lacZ mRNA expression. Ectopic expression of Pros in the Kr domain (F,H) increases lacZ mRNA expression of Dap-DEL in stage 10 embryos (F), while reduced lacZ mRNA expression of Dap-DEL is observed later in stage 12 embryos (H).
Figure 3.8 the lacZ expression of Dap-Del and Dap-MP4. (A, C, E) lacZ expression of Dap-Del reporter gene from 3 individual transgenic lines without ectopic Pros expression (B, D, F) lacZ expression of Dap-MP4 reporter gene from 3 individual transgenic lines without ectopic Pros expression (G) shows lacZ expression of Dap-Del with ectopic Pros expression in the Kr domain (H) shows lacZ expression of Dap-MP4 with ectopic Pros expression in the Kr domain. Arrows indicate the borders of Kr domain. The lower panel shows the clusters of potential Pros binding sequences. Red indicates the mutated sequence.
CHAPTER 4

COOPERATIVITY OF PROSPERO AND DM CYCE FUNCTION IN THE
REGULATION OF CKI, DACAPO, AND NEURONAL
DIFFERENTIATION/LINEAGE MARKER EVEN-SKIPPED EXPRESSION

Introduction

Precise coordination between cell proliferation and differentiation is essential for proper formation of multicellular organisms. Unrestrained proliferation or premature differentiation results in malformation or even death. Proliferation and differentiation are, in general, mutually exclusive. In almost all circumstances, cells undergoing terminal differentiation withdraw from mitotic activity prior to entering differentiation. Although the mechanism remains largely unknown, certain tissue-specific transcription factors have been shown to regulate mitotic activity through interacting with genes governing the cell cycle (Edgar and Lehner, 1996). During cell cycle progression, the decision to enter another proliferation cycle or undergo differentiation is made at the G1/S transition. Three classes of cyclins, cyclin D, A and E, together with their respective protein partners, cyclin-dependent kinases (CDKs), are essential for the G1 to S transition (see review by Ekholm and Reed, 2000). Cylin E and A form complexes with Cdk2 while cyclin D forms complexes with Cdk4 or Cdk6. It is also known that the activities of these cyclin-CDKs complexes are regulated by two families of cyclin kinase inhibitors (CKIs):
the p21\textsuperscript{cip/kip} and p16\textsuperscript{INK4a} families (Sherr and Roberts, 1999). The p21\textsuperscript{cip/kip} families, includes p21\textsuperscript{cip}, P27\textsuperscript{kip1}, and p57\textsuperscript{kip2}, inhibit all kinases involved in the G1 to S transition, while the p16 families, containing p15, p16, p18, and p19, specifically bind to and inhibit the activity of the cyclin D-dependent kinases, cdk4 and cdk6. Results from knock-out mice and cell culture studies suggest that CKIs play a critical role in regulating the timing of cell cycle withdrawal and, in some cases, affecting differentiation (Kranenburg et al., 1995; Liu et al., 1996; Yan et al., 1997; Zhang et al., 1998; Zhang et al., 1999b).

In *Drosophila*, the machinery regulating S phase entry is similar to the mammalian system. Homologs of numerous mammalian cell cycle genes have been identified in *Drosophila*, including cyclin A, cyclin D and E2f (reviewed by Edgar and Lehner, 1996). However, it has been shown that the *Drosophila* homologue of cyclin E, DmcycE, plays the most important role in G1/S transition. DmcycE expression is essential for progression into S phase both during embryogenesis as well as in larval imaginal discs (Knoblich et al., 1994; Richardson et al., 1995). DmcycE forms a complex with Cdk2. The activity of this complex is regulated by Dacapo (Dap), the only identified G1/S CKI in *Drosophila* (Knoblich et al., 1994; de Nooij et al., 1996; Lane et al., 1996). Dap shares structured similarity with the p21\textsuperscript{cip/kip} families and, as its mammalian homolog, has been shown to bind to and inhibit the activity of the Cdk2/CyclinE complex (de Nooij et al., 1996; Lane et al., 1996). *dap* is expressed immediately before epidermal cells enter G1 arrest. Ectopic expression of *dap* results in premature G1 arrest, while loss-of-function of *dap* cause an extra round of cell division in all epidermal cells (de Nooij et
al., 1996; Lane et al., 1996). This suggests that Dap plays an important role in regulating cell cycle withdrawal at the proper developmental time.

Recent studies on regulation of dap expression reveal a complex picture (de Nooij et al., 2000; Meyer et al., 2002; and this work). *dap* expression is regulated by a modular array of *cis*-regulatory elements that integrate spatial/temporal-specific signals (this work and Meyer et al., 2002). In addition to developmental cues, the cell cycle regulatory system, has also been shown to play a role in regulating *dap* expression (de Nooij et al., 2000). Thus, in *DmcycE* homozygous LOF mutant embryos, *dap* expression is dramatically reduced, while ectopic expression of DmcycE in wing and eye discs results in an increase of both, *dap* RNA and protein expression (de Nooij et al., 2000). The increase in *dap* protein expression in wing and eye discs, in response to ectopic DmcycE, is substantially stronger than the parallel increase in *dap* RNA expression (de Nooij et al., 2000). This suggests that, in addition to the increased transcription, post-transcriptional mechanisms are also involved in regulating *dap* activity. These data suggest that DmcycE is a positive regulator of *dap* expression. However, before cell cycle 16, *dap* expression is not detected even though the activity and level of DmcycE/Cdk2 complexes reach high levels in all preceding cell cycles. It has, therefore, been proposed that DmcycE/Cdk2 can induce *dap* expression, but only within a permissive window defined by developmental cues (de Nooij et al., 2000). Meyer et al. (2002), showed that during embryogenesis ectopic DmcycE does not increase or suppress *dap* expression, nor does it induce the early onset of *dap* expression, although prolonged *dap* expression in epidermal cells was noted when DmcycE is over-expressed (Meyer et al., 2002).
Pros, a pan-neural transcription factor (Hassan et al., 1997), has been previously described as a potential regulator of *dap* expression (Li and Vaessin, 2000; Meyer et al., 2002; Liu et al., 2002). *pros* encodes an evolutionarily conserved atypical homeodomain protein expressed in all neuronal lineages (Chu-Lagraff et al., 1991; Doe et al., 1991; Vaessin et al., 1991; Matsuzaki et al., 1992). Pros regulates mitotic activity and transcription of several cell cycle genes including *DmcycE*, *string*, and *E2f* (Li and Vaessin, 2000). It has been suggested that Pros is not likely to play a role in activation of *dap* expression, due to the observation that while co-expression of Pros and Dap was visible in MP2 precursors, Dap expression was also detectable in embryos lacking a functional *pros* gene (Meyer et al., 2002). Furthermore, pros protein expression was readily detectable in numerous early ganglion mother cells (GMCs) without a parallel detection of dap protein, and during later stages of neurogenesis there is no strict correlation between Dap expressing and Pros expressing cells (Meyer et al., 2002). It was shown (this work and Liu et al., 2002), in contrast, that *pros* gene function has a dual role in the regulation of *dap* transcription. During early neurogenesis, loss of *pros* function results in a delay in the transcriptional activation of *dap* in neuronal lineages. Thus, Pros function is required for the timely activation of *dap* transcription, but redundant factors are sufficient for the ultimate transcriptional expression of *dap*. Furthermore, during late neurogenesis, prolonged transcriptional expression of *dap* is readily observed in *pros* mutant embryos while ectopic expression of Pros results in a severe reduction of *dap* RNA starting during embryonic stage 12. This suggests that, in addition to timely activation of *dap* expression, Pros is also required for the proper termination of *dap*
transcription during later stages of embryonic neurogenesis (Liu et al., 2002 and this work).

Here I show that Pros and DmcycE cooperate in the activation of *dap* transcriptional expression in the developing nervous system. The two genes also act cooperatively in regulating the expression of the neuronal marker gene —*even-skipped* (*eve*). Although I observed that *eve* protein (Eve) expression was also affected by modulation of *dap* expression, data are presented that suggest that regulation of Eve expression by Pros and DmcycE is not likely to be exclusively mediated through regulating *dap*. Furthermore, evidence is presented that Pros can physically interact with the DmcycE/Cdk2 complex.

**Results**

**Cooperative action of Pros and DmcycE in activation of *dap* expression**

DmcycE is required for proper expression of *dap* in the developing nervous system (de Nooij et al., 2000). Furthermore, a critical role for the pan-neural transcription factor Pros in the regulation of *dap* expression has been shown (Liu et al., 2002 and this work). These observations raised the question whether DmcycE and Pros in fact functionally interacted in this process. To address this question I analyzed the expression of both the endogenous *dap* transcription unit, as well as various *dap* reporter constructs that mimicked different aspects of *dap* expression in developing CNS, such as Dap-5’-6R, Dap-DEL, and Dap-HB. These were examined in response to ectopic expression of
DmcyCE or Pros alone, or in response to the parallel ectopic expression of DmcyCE and Pros, as well as in response to loss-of-function of pros and/or DmcyCE.

To test the effect of ectopic DmcyCE and/or Pros on dap expression, full-length DmcyCE and/or Pros were expressed in embryonic tissue using the UAS/GAL4 system (Brand and Perrimon 1993). Region-specific driver lines, such as Kr-GAL4, were used to drive ectopic DmcyCE and/or Pros expression in a limited region of embryos. In this analysis, I mainly focused on the early stages of dap expression in the developing nervous system. It has been shown that dap RNA and protein expression are increased in the wing disc when DmcyCE is ectopically expressed (de Nooij et al., 2000). However, in my experiments, and in agreement with previous observations (Meyer et al., 2002), ectopic expression of DmcyCE did not result in significantly increased expression of dap RNA or protein during early embryogenesis (Figure 4.1 A, D, G). Nor did I detect precocious induction of dap expression (Figure 4.2 A and D). Similarly, ectopic DmcyCE expression did not result in a significant change of dap lacZ reporter gene expression (data not shown). I reported previously that ectopic Pros expression terminated dap transcript and protein expression starting the embryonic stage 12 (Liu et al., 2002 and this work). However, ectopic expression of Pros during the early stages of embryogenesis resulted in a slight increase of endogenous dap transcription (Figure 4.1 B and E). This result was in agreement with previous observations showing that ectopic Pros could activate the expression of the lacZ reporter constructs carrying dap cis-regulatory sequences that directed expression to neuronal lineages in the developing central nervous system (Liu et al., 2002). Increased expression of the endogenous dap gene by ectopic
Pros could also be seen at the protein level, where a more robust increase was evident (Figure 4.1 H). This suggested that *dap* expression in embryonic CNS lineages was regulated at the transcriptional level as well as at the post-transcriptional level. However, no precocious onset of expression of internal *dap* gene was detected in embryos expressing ectopic Pros (Figure 4.2 B and E). Although ectopic expression of DmcycE or Pros alone resulted in no or only slight activation of endogenous *dap* transcription respectively, parallel ectopic expression of Pros and DmcycE together resulted in a dramatic increase in *dap* transcripts in a subset of cells in the developing nervous system (Figure 4.1 C and F). Furthermore, precocious expression of endogenous *dap* was observed in these embryos (Figure 4.2 C and F). Thus, expression of the endogenous *dap* gene was readily detectable in segments with ectopic Pros and DmcycE expression, while no such expression was evident in flanking segments that lack ectopic Pros and DmcycE expression. Ectopic expression of both Pros and DmcycE in this experiment was achieved using Kr-Gal4 driver, a region-specific driver line, and was, therefore, not limited to the nervous system. However, no ectopic activation of *dap* expression was detectable outside the developing nervous system on the RNA or protein level (Figure 4.1 A-I). Furthermore, both precocious activation and ectopic *dap* expression was observed in only a subset of neuronal lineages. This indicated that Pros and DmcycE cooperated in regulating *dap* activity in a tissue- and/or lineage specific fashion, rather than representing a general phenomenon during embryonic development.

The two *dap* lacZ reporter gene constructs, Dap-HB and Dap-DEL, have previously been shown to mimic *dap* expression in a subset of cells in the developing
CNS during early stages and in response to ectopic Pros. These two dap reporter genes responded to ectopic expression of Pros and DmcycE in parallel the same as they responded to ectopic Pros alone. Dap-5’-6R, is a dap reporter gene construct which mimics dap expression in the epidermis and a subset of cells in the late developing CNS. Ectopic expression of Pros and DmcycE in parallel did partially rescue the suppression of the lacZ expression of Dap-5’-6R reporter constructs caused by ectopic Pros alone (data not shown and Liu et al., 2002). This indicated there are DmcycE response elements in the cis-regulatory region of dap contained in the Dap-5’-6R reporter gene construct. This was in agreement with previous observation of de Nooij et al.(2000) that the lacZ expression of the reporter constructs containing 2.7kb of the 5’ cis-regulatory region of dap could react to the change of ectopic DmcycE in wing and eye discs.

dap transcripts are strongly reduced, although not absent, in the developing nervous system of embryos that are homozygous mutants for loss-of-function DmcycE mutation (de Nooij et al., 2000). To determine whether Pros activity might have accounted for some of the remaining dap expression, I analyzed RNA expression of the internal dap gene in DmcycE and pros double mutants. In embryos that were homozygous mutant for the amorphic allele pros$^jo13$, dap expression was delayed at embryonic stage 10 and early stage11, while at embryonic stage 15 or later there was ectopic dap expression in the CNS (Figure 4.3 B; and Liu et al., 2002). In homozygous cycE$^{Ar95}$ mutant embryos, there was a dramatic reduction of dap transcripts in both CNS and PNS, however, dap transcripts could still be readily detected (Figure 4.3 C, de Nooij et al., 2000). In contrast, no dap transcript was observed in the developing CNS of double
homozygous cycE<sup>Ary5</sup> and pros<sup>j013</sup> mutant embryos (Figure 4.3 D). This suggested that both Pros and DmcycE activities are critical for dap expression in the embryonic CNS. Moreover, this result was consistent with the observed regulatory effects of ectopic of Pros and DmcycE expression on dap expression, and supported the notion that Pros and DmcycE act cooperatively to induce dap expression in the developing embryonic CNS.

**Pros and DmcycE function cooperatively in regulating expression of the neuronal differentiation marker-Eve**

Loss of pros function causes severe embryonic neuronal defects (Doe et al., 1991; Vaessin et al., 1991; Matsuzaki et al., 1992). Pros function is essential for proper expression of several neuronal lineage markers, including Mab 22c10, Eve, Engrailed (En) and Fushi tarazu (Ftz) (Doe et al., 1991; Vaessin et al., 1991). DmcycE loss-of-function results in a mild neuronal phenotype during embryonic neurogenesis, including thinner and/or occasionally interrupted longitudinal axons and loss of chordotonal organs, as revealed by anti-HRP and 22C10 staining (detail see chapter 5). The observation of functional cooperation of Pros and DmcycE in the activation of dap expression led me to wonder whether these two genes also function together in regulating the expression of neuronal differentiation markers. Ectopic expression of Pros lead to increased Eve expression, both in the number of Eve expressing cells and level of expression (Figure 4.4 B and F), although not in an earlier onset of Eve expression. Ectopic DmcycE did not cause detectable changes in Eve expression (Figure 4.4 C and G). In contrast, I observed significantly stronger and more expanded expression of Eve in segments with parallel
ectopic expression of Pros and DmcycE (Figure 4.4 B, D, F, H). Thus, DmcycE acted in concert with Pros in regulating Eve expression. To test whether the observed changes in Eve expression represented an artificial situation caused by forcing ectopic Pros and DmcycE protein expression, I next analyzed the effect of loss of pros and DmcycE gene function on Eve expression in pros\textsuperscript{jo13} and cycE\textsuperscript{Ar95} double homozygous mutant embryos.

Loss-of-function pros resulted in missing Eve expression in the RP2, aCC, pCC and most of CQ neurons, while Eve expression in the EL neuron clusters, located at lateral sides of the ventral cord, was not affected (Doe et al., 1991 and Figure 4.4 J). This suggested that pros plays a more prominent role in neuron differentiation and/or fate choice of RP2, aCC, pCC and CQ neurons than in the EL clusters. In contrast, DmcycE function was required for the proper expression of Eve in most of the CQ and EL neuronal clusters (Figure 4.4 K). In DmcycE loss-of-function mutants, Eve expression in most of CQ and EL neuron clusters was missing while eve expression in the inner RP2, aCC and pCC neurons appeared normal. Embryos that were double homozygous mutant for pros and DmcycE loss-of-function mutations exhibited an aberrant pattern of Eve expression (Figure 4.4 L), that neither resembled the homozygous loss-of-function phenotype of either pros or DmcycE mutation alone, nor did it appear to represent a straightforward additive phenotype. The specific identity of these Eve expressing cells in the CNS of the double pros and DmcycE mutant embryos is presently not clear. I also noted that the overall number of Eve positive cells was reduced in double DmcycE and pros mutant embryos compared to DmcycE or pros mutant embryos. Thus, the loss-of-function phenotype of DmcycE and pros double mutant embryos was not a
straightforward reversal of the overexpression phenotype, however, both experimental approaches supported the notion of close functional interactions between \textit{pros} and \textit{DmcycE} in Eve expression in selected neuronal lineages.

\textbf{Functional Cooperation between Pros and DmcycE is lineage-specific}

The functional cooperation of \textit{pros} and \textit{DmcycE} in regulation of neuronal differentiation, as indicated by neuronal differentiation marker expression appears to be limited to specific lineages. While I observed cooperative interactions in Eve expressing lineages, this appeared not to be a general theme in neuronal lineages regulated by Pros. For example, expression of Odd-skipped (Odd), another neuronal lineage specific marker, also required \textit{pros} gene function (Figure 4.5 B, and detail see chapter 5 Broadus et al., 1995 Li, 2000). There were four Odd-positive cells, two MP1 and two MP2 cells, per segment in WT embryos at stage 14 (Figure 4.5 A). In gain-of-function and loss-of-function \textit{pros} embryos, both resulted in ectopic expression of Odd (Figure 4.5 B and Figure 5.8). There was no detectable change of Odd expression in both gain-of-function and loss-of-function of \textit{DmcycE} mutant embryos (Figure 4.5 C and data not shown). Both the parallel ectopic expression and double loss-of-function of \textit{pros} and \textit{DmcycE} resulted in the same phenotypes that was observed by changing \textit{pros} dosage alone (Figure 4.5 D and data not shown). Thus, there was no detectable difference in expression level or number of cells expressing Odd in response to the modulation of either \textit{pros} alone or \textit{pros} and \textit{DmcycE} together suggesting that only \textit{pros} but not \textit{DmcycE} or \textit{pros} and \textit{DmcycE} together, function in regulating Odd expression. Hence, cooperative interaction of \textit{pros}
and *Dmcyce* in regulating the expression of neuronal differentiation markers was not a general phenomenon in all neuronal lineages.

**Ectopic Dap is not sufficient to mediate increased expression of neuronal differentiation markers**

The observation of precocious and increased *dap* expression and increased expression of *eve* in response to parallel ectopic expression of Pros and *Dmcyce* raised the question whether the observed changes in neuronal differentiation marker expression was a direct consequence of the induced *dap* expression. Change in *dap* expression may results in changed proliferation of neuronal lineage cells. This, in turn, could result in change in the number of Eve expressing cells. To test this possibility I analyzed Eve expression in embryos with regionally restricted ectopic Dap expression. Ectopic Dap expression did not induce increased Eve expression. In contrast, at stage 14 or later, there was decreased and/or missing Eve expression in RP2, and the lateral EL neurons clusters (Figure 4.6 B and E), compared to wild-type embryo (Figure 4.6 A and D). Thus, Dap expression alone was not sufficient to account for the observed increased Eve expression. It has been previously reported that in stage 14 *dap* mutants, there are excess Eve-positive neurons (Meyer et al., 2002). Meyer et al. did not, however, identify the *dap* allele used. In contrast, I observed that at stage 14 or later, the cell numbers in the Eve-positive EL neurons clusters were decreased, or in some cases, the entire neuron cluster was missing in *dap* and *dap*1377 homozygous mutant embryos (Figure 4.6 C and F). Both ectopic expression and loss-of-function of *dap* resulted in changes of Eve expression,
however, this change was much milder and also different from the phenotype observed in simultaneously over expression and double loss-of-function of Pros and DmcycE. Taken together, this suggested that, while *dap* gene function was required for proper Eve expression, the cooperative effect of Pros and DmcycE on neuronal differentiation marker Eve expression did not appear to be an exclusive consequence of induced Dap expression.

**Dap functions to regulate Odd expression**

Dap function was also required for the proper expression of another lineage specific marker-Odd. In loss-of function of *dap* mutant embryos, 57% of segments had only two to three Odd-expressing cells (Figure 4.7 B). In the remaining 43% of the segments which still had four Odd expressing cells, the position of these four cells were off (data not shown) compared to the “stereotypical” position of Odd-expressed cells in wild type embryos (Figure 4.7 A). Ectopic Dap did not significantly change the numbers of cells expressing Odd per segment, however, I did observe that the position of these four Odd –expressing cells were irregular (data not shown). Although both Pros and *dap* functioned to affect odd expression, there was an opposite phenotype between *pros* and *dap* mutants. This also suggested that Pros’ effect in neuronal markers expression, at least for Eve and Odd, was more likely not a direct consequence of inducing Dap expression.
Pros can physically associate with the DmcycE/cdc2c complex in vivo

The genetic interaction observed between pros and DmcycE lead me to speculate about the molecular mechanism of these two genes/protein. Recent studies reported that CCAAT/enhancer binding protein alpha (C/EBPα), a tissue-specific transcription factor, was able to interact with certain proteins that were involved in cell cycle progression, including cdk4 and retinoblastoma (Rb) (Chen et al., 1996; Wang et al., 2002). Thus, I decided to investigate whether there were physical interactions between Pros and DmcycE. For this purpose, I performed co-immunoprecipitation experiments using wild type embryonic extracts. Using different anti-Pros antisera, I found that DmcycE was co-precipitated with Pros from embryonic extracts (Figure 4.8 upper panel; and data not shown). In Drosophila, it has been shown that Cdc2c forms a complex with DmcycE (Knoblich et al., 1994). Hence I tested whether Pros could occur in a complex together with DmcycE and Cdc2c. Indeed, using anti-Pros antibodies, Cdc2c, the sole partner of DmcycE, was co-precipitated together with Pros from embryonic extract (Figure 4.8 lower panel). The expected co-precipitation of DmcycE with Cdc2c was also detected. Thus, Pros can physically associate with the DmcycE/Cdc2c protein complex in Drosophila embryos.

Discussion

Many studies have been devoted to understanding the mechanism of interaction between tissue-specific genes and cell cycle machinery to achieve the balance between cell proliferation and terminal differentiation to ensure the proper formation of an
organism. Recent studies of the cis-regulatory elements of several Drosophila cell cycle genes, suggest that the expression of cell cycles genes is controlled by a combination of inputs of developmental cues, such as the tissue-specific differentiation/determination factors, to ensure the coordination between cell proliferation and differentiation (Lehman et al., 1999; Jones et al., 2000; Meyer et al., 2002; Liu et al., 2002). Furthermore, results from studies in mice and other organisms, suggest that a role of differentiation/determination factors in regulation of both mitotic activity and expression of cell cycle genes represents a common developmental phenomenon (see review by Ohnuma et al., 2001; Zhang, 1999; Myster and Duronio, 2000). In addition, there is also evidence that cell cycle components regulate neuronal cell differentiation (see review by Ohnuma et al., 2001). These studies suggest that the mechanism coordinating cell cycle and differentiation is achieved by a complex network. The observed cooperative activity of a determination/differentiation factor, Pros, and a cell cycle regulator, DmcycE, in regulating the expression of the CKI gene dap in the developing nervous system points to additional regulatory mechanisms employed to ensure the precise coupling between cell cycle progression and developmental program. Furthermore, the observation that Pros and DmcycE also act cooperatively in regulating the neuronal differentiation marker Eve, suggests that this functional interaction extends and, either directly or indirectly, can be employed in regulating lineage specific differentiation/determination factors.

Pros function is required for proper termination of the DmcycE transcription (Li and Vaessin, 2000). In agreement with such a role, both the internal DmcycE gene, as well as expression of a DmcycE lacZ reporter construct carrying the DmcycE cis-
regulatory neuronal element, are suppressed by ectopic Pros expression (Li and Vaessin, 2000; and Liu and Vaessin, unpublished data). The co-immunoprecipitation data show that in addition to regulating DmcycE expression on the transcriptional level, Pros can also physically interact with DmcycE/Cdc2c protein complex. This observation raises the possibility that Pros can regulate mitotic activity and/or differentiation on different regulatory levels. Even though this is the first time it has been shown that Pros, or one of its homologs, such as mouse Prox1, physically interacts with any cyclin/cdk complexes, several studies present evidence that transcription factors may regulate cell proliferation through direct interactions with, and inhibition of cyclin-dependent kinases. For example, C/EBPα functions to enhance the proteasome–dependent degradation of cdk4, and MyoD functions to inhibit the cdk4-dependent phosphorylation of the retinoblastoma protein (pRb) (Wang et al., 2002; Wang et al., 2001; Zhang et al., 1999a). The biological significance of the protein-protein interaction observed between Pros and the DmcycE/Cdc2c complex is presently not clear and further research is required to examine these phenomena. It is possible that Pros, in addition to activating dap expression in order to drive neuronal lineage cells to enter G1 phase, may also function to inhibit the activity of other cell cycle genes to prevent the reentry of S phase. This would provide two levels of controlling the proper timing of exiting the cell cycle and entering differentiation.

Although LOF dap mutations are embryonic lethal, with only a few escapers, dap mutant embryos show no obvious morphological defects in the developing nervous system when stained with general neuronal marker, HRP (Lane et al., 1996; de Nooij et al., 1996; Wallace et al., 2000). In this study, by using lineage specific markers such as
Eve and Odd, I observed that *dap* mutant embryos do indeed have mild CNS defects. Meyer et al. (2002) also reported a change of Eve expression pattern in *dap* mutant embryos. However, the Eve phenotype in *dap* mutants reported by Meyer and coworkers is opposite from the Eve phenotype described here. Meyer et al. (2002) reported that in *dap* mutants there was increased numbers of Eve-positive cells, while I observed decreased Eve-positive cells in *dap*4 and *dap* 1377 null mutants at stage 14. Meyer et al. (2002) did not specify the *dap* mutant alleles used, therefore, it is not clear whether the difference in Eve-expression pattern was due to the different type of *dap* mutation used in the experiments.

I showed that Pros and DmcygE cooperatively regulate Eve expression. However this regulation is not likely exclusively mediated through their ability to regulate *dap* expression. Rather, Pros and DmcygE regulate of the expression and/or activity of other, presently not identified genes, which likely contributes to the observed altered expression of Eve. The apparent specificity observed in the modulated expression of neuronal lineage marker expression in the presence of both ectopic Pros and DmcygE supports this notion and suggests that the cooperative developmental functions of Pros and DmcygE also require the activities of other, lineage or spatial restricted regulators in the developing nervous system.

In summary, my data shows that Pros and DmcygE act cooperatively in the regulation of *dap*, a CKI, and *eve*, a neuronal differentiation marker gene, in subsets of neuronal cells. While *dap* activity is required for proper regulation of *eve* expression in a subset of neuronal linages, Pros- and DmcygE-mediated changes in *eve* expression appear
not to be an exclusive consequence of regulating *dap* expression. Furthermore, I show that Pros is capable of physically interacting with DmcycE/Cdc2c complex. This interaction may be of critical relevance for the observed cooperativity between Pros and DmcycE.
Figure 4.1 Pros and DmcycE function cooperatively in *dap* expression. (A, D, G) Krupple (Kr)-Gal4/UAS-DmcycE embryos. (B, E, H) Kr-Gal4/UAS-Pros embryos. (C, F, I) Kr-Gal4/UAS-Pros; UAS-DmcycE embryos. (A-C) ectopic *dap* RNA (B, C) (blue) and protein (E, F) (green) expression in embryos with ectopic Pros (B, E, H) in Kr domain or embryos with ectopic Pros and DmcycE together (F, I, L) in Kr domain. (G-I) show anti-Pro staining (red) of (D-F) embryos respectively. Ectopic Pros (red) is detected in (H, I) and corresponds to the region where ectopic Dap (green) is observed in (E, F).
Figure 4.2 Pros and DmcycE cooperate functionally to induce early onset of *dap* expression. (A, D) Krupple (Kr)-Gal4/UAS-DmcycE embryos. (B, E) Kr-Gal4/UAS-Pros embryos. (C, F) Kr-Gal4/UAS-Pros; UAS-DmcycE embryos. (D-F) are higher magnification of (A-C). Early induction of *dap* transcripts (blue) were detected in embryo with ectopic expression of Pros and DmcycE in parallel in Kr domain (borders indicated by arrows)(C, F), but not in embryos with ectopic Pros or DmcycE alone in Kr domain (A, B, D, E)
Figure 4.3 Pros and DmcycE are essential for dap RNA expression. *dap* RNA expression in stage 16 of wild-type (A), *pros* \(^{jo13}\) mutant (B), *DmcycE* \(^{Ar95}\) mutant (C) and *DmcycE* \(^{Ar95}; pros\) \(^{jo13}\) double mutant (D) embryos.
Figure 4.4. Pros and DmcycE cooperate to regulate Eve expression. Ectopic Eve expression is detected in Kr-Gal4/UAS-Pros (B, F) and Kr-Gal4/UAS-Pros; UAS-DmcycE (D, F) embryos but not in wild-type (A, E) or Kr-Gal4/UAS-DmcycE (C, G) embryos. Borders of Kr domains are indicated by arrows. (E, F, G, H) are higher magnifications of (A, B, C, D) respectively. Eve expression in stage 17 of wild-type (I), pros mutant (J), DmcycE mutant (K), and DmcycE; pros double mutant (L) embryo. Asterisks indicates aCC/pCC neurons. RP2s are indicated by arrows. Dots indicate CQ neurons. EL neuron clusters are indicated by arrowheads.
Figure 4.5 Pros, but not DmcyCE, regulates Odd expression. Odd expression in stage 16 of wild-type (A), pros jo13 mutant (B), DmcyCE Ar95 mutant (C) and DmcyCE Ar95; pros jo13 double mutant (D) embryos.
Figure 4.6 Dap regulates Eve expression. Dorsal (A-C) and ventral (D-E) views of stage 16 embryos for Eve protein expression in wild-type (A, D), Kr-Gal4/UAS-Dap (B, E), and dap mutant (C, F) embryos. Dosage of Dap affects Eve protein expression in RP2 (arrows) and EL neurons (arrowheads). Asterisks indicate missing expression.
Figure 4.7 Dap regulates Odd expression. Odd expression at stage 14 wild type (A), dap 4 (B) embryos. (B) dap 4 mutants show decreased numbers of Odd expression cells.
Figure 4.8 DmcycE and Cdc2c form a complex with Pros. Wild-type embryo extracts (lane 1) or immunoprecipitates obtained from wild-type embryo extracts with anti-DmcycE (8B10) (lane 2), with non-immune serum as control (lane 3), with guinea pig anti-Pros (lane 4), or with mouse anti-Pros antibodies were examined for the presence of DmcycE (upper panel) and Cdc2C (lower panel) by immunoblotting.
CHAPTER 5
THE FUNCTION OF PROS IN CELL PROLIFERATION AND DIFFERENTIATION

Introduction

Cell proliferation and differentiation have to be coupled precisely during embryogenesis of multiorganisms. In most circumstances, the cells withdraw from mitotic activity prior to entering differentiation. Thus, the two events are mutually exclusive. Many labs have devoted their effort to decipher the mechanism allowing the tight coordination between cell cycle progression and differentiation. Recent studies have shown that the expressions of cell cycle components are regulated by the developmental genes which are tissue-specific transcription factors. A former graduate student, Ling Li, observed that Pros, a pan-neural gene known as a neuronal fate determinator, also regulated mitotic activity (Li and Vaessin, 2000).

LOF pros mutations result in increased S phase-specific Histone 1A RNA expression and BrdU cooperation. Consistent with this observation in LOF pros mutations, ectopic expression of Pros, using UAS/Gal4 system, results in suppressed expression of both S phase markers (Li and Vaessin, 2000). Li and Vaessin (2000) also showed that Pros can regulate the expression of several cell cycle genes including stg, DmcyCE, and de2f1. stg, the Drosophila homolog of cdc25 phosphatase, is the limiting
factor of G2/M transition, while DmcytE is essential for progression into S phase (Edgar and O’Farrell, 1989; Edgar et al., 1994b; Knoblich et al., 1994; Richardson et al., 1993). dE2F1/dDP1 plays a role in G1/S transition as well as progression through S phase (Duronio et al., 1995). Loss of pros function results in ectopic expression of cell cycle genes, while ectopic expression of Pros results in suppression of cell cycle genes. However, the mechanism which Pros employs to regulate cell proliferation is not clear.

In addition to its role in mitotic activity, Pros is well known to function in neuronal cell fate specification of the developing CNS (Doe et al., 1991). During Drosophila embryonic neurogenesis, there are initially approximately 30 NBs in each hemisegment, and through several rounds of asymmetrical cell division, these 30 NBs will generate about 350 neurons and glia at end of embryonic neurogenesis (Bossing et al., 1996; Schmidts et al., 1997). It has been detailed that each NB acquires a distinct cell fate and gives rise to different GMCs with distinct combinations of differentiation marker expression. Pros function has been shown to be essential for proper differentiation of some neuronal lineages, such as Even-skipped (Eve), Engrailed (En) and Hunchback (Hb). In pros mutant embryos, the numbers of the Eve-, Odd-skipped (Odd)- and Hb-positive cells are decreased while the numbers of the En-positive cells are increased (Doe et al., 1991; Li, 2000). This suggests a possible change in the identity of GMCs and neurons (Doe et al., 1991). Recent studies have shown that cell cycle genes affect neuronal determination and differentiation (Akiyama-Oda et al., 2000; Cui and Doe, 1995; Ohnuma et al., 2001; Weigmann and Lehner, 1995). Thus, with the recent observation that Pros regulates the expression of cell cycle genes, it appears important to
reevaluate the role of Pros and its interaction with cell cycle genes upon neuronal differentiation.

To determine the functional interaction between Pros and cell cycle components upon cell proliferation and neuronal differentiation, a genetic approach was used. In collaboration with L. Li, I generated transgenic flies carrying two UAS constructs through standard genetic crosses. This facilitated ectopic expression of Pros together with one other cell cycle regulatory gene product, including Stg, DmcycE and dE2F1/dDP1, through utilization of the UAS-Gal4 system. This allowed the examination of whether ectopic expression of any one of the cell cycle genes could compensate for the effect of ectopic Pros upon cell proliferation and neuronal differentiation. To determine the effects of Pros and of cell cycle components, such as stg and DmcycE, upon neuronal differentiation, I also examined the expression of several neuronal differentiation markers in embryos that were homozygous mutant for pros and one of the cell cycle components, such as stg, DmcycE. No clear picture emerged regarding the effect of Pros and cell cycle regulatory genes upon neuronal differentiation.

Results

Parallel ectopic expression of cell cycle genes can rescue the suppression of BrdU incorporation, but not the H1 RNA expression, mediated by ectopic Pros

To examine the effect of ectopic cell cycle genes on DNA replication in the presence of ectopic Pros, two assays, BrdU incorporation and H1 RNA expression, were performed on UAS-Pros; UAS-Stg / Kr- GAL4 embryos, UAS-Pros; UAS-dE2F1/dDP1 /
Kr-GAL4 embryos, and UAS-Ps; UAS-DmcycE / Kr-GAL4 embryos. As a control, these two assays were also performed on UAS-Ps / Kr-GAL4 embryos, UAS-Stg / Kr-GAL4 embryos, UAS-dE2F1/dDP1 / Kr-Gal4 embryos and WT embryos. The results from the BrdU incorporation assay were consistent with previous reports that ectopic Stg, dE2F1/dDP1, and DmcycE alone resulted in ectopic BrdU incorporation (Edgar and O’Ferrell, 1989,1990; Knoblich et al., 1994; Duronio et al., 1995), while ectopic Pros resulted in suppression of BrdU incorporation (Figure 5.1 B), when compared to WT embryos (Figure 5.1A). When I ectopically expressed one of the cell cycle genes, such as stg, dE2F1/dDP1, and DmcycE, in parallel with ectopic Pros, the BrdU incorporation (shown in green) was restored in the Kr domain despite the existence of ectopic Pros (shown in red) (Figure 5.1 C, D, and data not shown). Therefore, ectopic expression of cell cycle gene products could bypass the suppression of BrdU incorporation caused by ectopic Pros. This indicated that these cell cycle genes act downstream of Pros during cell proliferation.

When I examined another S phase-specific maker, H1 RNA expression, I observed a different result from the BrdU incorporation experiment. As previously reported, ectopic Pros suppressed H1 RNA transcription in the Kr domain (Figure 5.2B, Li and Vaessin, 2000), compared to WT (Figure 5.2A). However, ectopic expression of Stg in parallel with Pros did not rescue the suppression of H1 RNA caused by ectopic Pros in the Kr domain (Figure 5.2D). Ectopic expression of DmcycE or dE2F1/dDP1 in parallel with ectopic Pros also did not restore H1 RNA expression (data not shown). Thus, parallel ectopic expression of one of these cell cycle genes could bypass Pros-
mediated suppression of BrdU incorporation, but not H1 RNA transcription. This indicated that ectopic expression of one of the cell cycle genes was only capable of partially inducing one round of S phase in the presence of ectopic Pros.

**Ectopic expression of cell cycle genes can not bypass the suppression of transcription mediated by ectopic Pros**

Ectopic DmcycE or Stg could restore the BrdU incorporation in the presence of ectopic Pros. I wondered whether ectopic DmcycE or Stg could also bypass the suppression of transcription of other cell cycle genes in the presence of ectopic Pros. To answer this question, in situ hybridization was performed on UAS-Pros; UAS-DmcycE/Kr-GAL4 and UAS-Pros; UAS-Stg/Kr-GAL4 embryos. *stg* and *cycA* transcription were still suppressed in the Kr domain where ectopic DmcycE was expressed simultaneously with ectopic Pros (Figure 5.3A and C). *DmcycE* and *cycA* transcription were also suppressed in the Kr domain where ectopic Stg was expressed in parallel with ectopic Pros (Figure 5.3B and D). These observations suggested that ectopic expression of these cell cycle gene products alone was not sufficient to alleviate the suppression of other cell cycle gene expression caused by ectopic Pros expression.

**Ectopic Stg can suppress the early onset of neuronal differentiation mediated by ectopic Pros**

It has been shown that ectopic Pros induces early onset of neuronal differentiation (Li, 2000). Ectopic pros could also increase expression of the neuronal differentiation
markers anti-HRP and anti-22C10, (Figure 5.4 A and D), while ectopic stg resulted in suppression of neuronal differentiation at earlier stages (Figure 5.4 B and E). To investigate the functional interaction between pros and stg on neuronal differentiation, I performed anti-HRP and anti-22C10 antibody staining on UAS-Pros; UAS-Stg/Kr-GAL4 embryos. UAS-Pros/Kr-GAL4 embryos and UAS-Stg/Kr-GAL4 embryos were used as control embryos (Figure 5.4 A, B, D, E). Suppression of HRP and 22C10 expression was observed at early stages in the Kr domain where ectopic Stg was expressed together with ectopic Pros (Figure 5.4 C and F). This suggested that ectopic Stg could suppress the early onset of neuronal differentiation induced by ectopic Pros.

Ectopic DmcycE had no obvious effect on neuronal differentiation (Figure 5.5 B). The phenotype of ectopic Pros and DmcycE embryos appeared to be identical to the phenotype of ectopic Pros alone when stained with anti–22C10 antibodies (Figure 5.5 C). Thus, unlike ectopic Stg, ectopic DmcycE could not block the increased or induction of early onset of neuronal differentiation expression by ectopic Pros (Figure 5.5 A and C).

**Additive phenotype of pros and DmcycE double mutants stained by anti-22C10 and anti-HRP**

Although ectopic DmcycE did not show obvious neuronal defects when stained with anti-HRP and anti-22C10, LOF DmcycE \(_{Ar95}\) mutant embryos showed mild CNS defects such as a thinner ventral cord and an occasional break of longitudinal axons, as revealed by anti-HRP (Figure 5.6 C). In \(pros^{jo13}\) mutant embryos, as previously reported, there was a fusion of anterical and posterical axons and lack of longitudinal axons, as was
revealed by anti-HRP staining (Figure 5.6B, Vaessin et al., 1991). In DmcyCE; pros double mutant embryos, the CNS defects had the same appearance as in pros mutants (Figure 5.6 D).

Anti-22C10 staining showed that in pros jo13 mutants, there were path finding defects for the PNS as previously reported (Figure 5.7B and Vaessin et al., 1991). In DmcyCE mutant embryos, there was a lack of chordotonal organ cells as revealed by anti-22C10 (Figure 5.7 C). I observed an additive phenotype in DmcyCE; pros double mutant embryos by anti-22C10 staining. Double mutants showed a path finding defect together with a lack of chordotonal organ cells (Figure 5.7D).

**The effect of Pros and the cell cycle regulatory genes, Stg, DmcyCE, on the expression of neuronal lineage markers**

Pros function has been shown to be essential for the expression of several neuronal lineage markers, such as Odd, Eve, En, and Hb (Doe, et al., Li, 2000). Recent studies also showed that mutations in cell cycle regulatory genes affect the expression of some neuronal lineage markers (Li et al., 1999; Weigmann and Lehner, 1995). In collaboration with L. Li, I observed that mutations in cell cycle regulatory genes, such as DmcyCE, resulted in neuronal defects. This led me to wonder whether there were functional interactions between Pros and cell cycle regulatory genes, such as Stg and DmcyCE, upon neuronal differentiation. Thus, I examined the expression of several neuronal markers, including Odd, Eve, En and Hb with UAS-Pros; UAS-DmcyE/Kr-GAL4 embryos and with UAS-Pros; UAS-Stg/Kr-GAL4 embryos and in double
DmcyCE; pros or pros, stg homozygous mutants. No clear picture emerged from these studies. Following, I have summarized the results from those analyses in order to provide a framework and experimental basis for future studies.

**Odd**

In wild type embryos, Odd is expressed in two MP1 and two dMP2 at end of stage 12. Therefore, there are only 4 Odd-positive cells in each segment from the end of stage 12 in wild type embryos (Figure 5.8 A, E, I, M) (Spana et al., 1995; Schuldt and Brand, 1999). In both ectopic Pros and LOF pros mutant embryos, I observed extra Odd-positive cells (Figure 5.8 B, F, J, N) (Li, 2000). Therefore, the increased numbers of Odd-positive cells observed in LOF pros mutants were not simply the result of additional cell proliferation due to the lack of functional Pros. This indicated that Pros plays an active role in terms of neuronal differentiation, in addition to its role in cell cycle termination.

Ectopic Stg also increased the numbers of Odd-positive cells (Figure 5.8 C and G). Thus, ectopic Stg, together with Pros, also resulted in increased numbers of Odd-positive cells in the Kr domain (Figure 5.8 D and H). In stg null embryos, stg7B, cells arrest in G2 phase of embryonic cycle 14 due to the exhaustion of maternal supplies of Stg after 13 mitotic cycles (Edgar and O’Ferrall, 1989, 1990). Homozygous stg 7B mutant embryos had Odd-positive cells even though the cells were arrested in G2 of cycle 14 (Figure 5.8 K and O). However, it appeared that the number of Odd-positive cells per segment were decreased in stg 7B mutant embryos. Since stg mutant cells arrested in G2 phase of cycle 14, the decreased numbers of Odd-positive cells were likely due to a block
of cell division. Odd-positive cells were also observed in stg 7B, pros$^{jo13}$ double mutant embryos (Figure 5.8 L and P), however, the severe neuronal phenotype made it difficult to determine the number of Odd-positive cells per segment.

**Eve**

In pros mutant embryos, Eve expression in the aCC, pCC, RP2 and most of CQ neurons were missing while the expression in the EL neurons were not affected (Figure 5.9 J)(Doe et al., 1991). Ectopic Pros resulted in ectopic expression of Eve (Figure 5.9 B and F), but not the early induction of Eve expression. There was no detectable change of Eve expression at early stages in the Kr domain of ectopic Stg embryos (Figure 5.9 C and G). Eve expression in the embryos with parallel ectopic expression of Pros and Stg appeared identical to embryos with ectopic Pros alone (Figure 5.9 D and H). Although ectopic Stg showed no detectable effects on Eve expression, in stg7B homozygous mutant embryos, there was no Eve expression in the CNS (Figure 5.9 K) (Weigmann and Lehner, 1995). Eve expression was also absent in the homozygous stg7B, pros$^{jo13}$ double mutant embryos (Figure 5.9 L). This observation was consistent with previous studies indicating that cell cycle progression is required for eve expression (Weigmann and Lehner, 1995). In stg 7B or stg 7B, pros$^{jo13}$ embryos, there was no cell division after cycle 13, and therefore no eve expression was expected in the CNS.
En has been used as a specific marker to trace the lineages of neuroblast 7-1 and 7-4 (Cui and Doe, 1995). It has been shown that in pros mutant embryos, there are additional En-positive neurons between the En-positive L and ML cluster in addition to the increased number of En-positive DM cells (Figure 5.10 J and N) (Doe et al., 1991). Ectopic Pros resulted in a suppression of En expression (Figure 5.10 B and F). Ectopic DmcycE in the Kr region showed no obvious effect on the pattern of En expression (Figure 5.10 C and G). It has been reported previously that ectopic expression of DmcycE by heat-shock resulted in increased cell density of En positive cells in the epidermal stripes within one hour of DmcycE induction. However, the cell density returned to normal values by 4 hours after DmcycE induction due to apoptosis (Li et al., 1999). Ectopic Pros and DmcycE suppressed En expression as observed in ectopic Pros alone (Figure 5.10 D and H).

Although ectopic DmcycE did not obviously change the pattern of En expression, loss of DmcycE gene function resulted a slightly decreased number of En-positive cells (Figure 5.10 K and O). I observed decreased numbers of En-positive DM cells, L and ML clusters. In DmcycE Ar95; pros jo13 double mutant embryos, there were additional En-positive cells in L and ML clusters but decreased numbers of DM cells in double DmcycE Ar95; pros jo13 mutant embryos compared to wild type embryos (Figure 5.8). Moreover, the numbers of additional En-positive L and ML neurons were less in DmcycE Ar95; pros jo13 double mutants compared to pros jo13 mutant embryos (Figure 5.10 J, N, C, P). The intermediate phenotype of En expression observed in DmcycE Ar95; pros jo13 double
mutant embryos suggested that Pros and Dmcyce both functioned in regulating En expression. In En-positive DM cells, Dmcyce function may play a more important role than Pros. It also appeared that in the En-positive L and ML clusters the differences in the lineages responding to changes of Pros and Dmcyce levels. Therefore, the effect of Pros on En expression was not exclusively through its ability to regulate Dmcyce expression. However, it was not clear whether there was functional interaction between Pros and Dmcyce in regulating En expression.

Ectopic Stg as does ectopic Dmcyce expression did not result in an obvious effect on En expression, even though ectopic Stg caused morphological defects in the developing CNS (Figure 5.11 C, G). En expression in embryos with parallel ectopic Pros and Stg appeared similar to embryos with ectopic Pros expression alone (Figure 5.11 D, H). In stg 7B mutant embryos, En-positive cells were observed (Figure 5.11 K and O). In the homozygous stg 7B, pros jo13 double mutant embryos, En expression appeared similar to stg 7B (Figure 5.11 L and P).

Hb

Hb encodes a zinc finger transcription factor and functions in embryonic pattern formation and then CNS development (Kambadur et al., 1998; Struhl et al., 1992). Hb is expressed most if not all neuroblasts and sequentially expressed in GMCs and in early-born neurons (Isshiki et al., 2001; Kambadur et al., 1998; Novotny et al., 2002). Ectopic Pros induced ectopic Hb expression and there were decreased numbers of Hb-positive cells in pros jo13 mutant embryos (Figure 5.12 B, F and J) (Li, 2000).
mutant embryos, I observed slightly decreased numbers of Hb-positive cells (Figure 5.12 G and K) compared to WT embryos, but no obvious change of Hb expression was observed upon ectopic expression of DmcycE (Figure 5.12 C). Hb expression in embryos with ectopic expression of both Pros and DmcycE appeared the same as in embryos with ectopic Pros alone (Figure 5.12 D). However, Hb expression in double $DmcycE^{Ar95}; pros^{jo13}$ mutant embryos showed an intermediate phenotype. Thus, in double $DmcycE^{Ar95}; pros^{jo13}$ mutant embryos, the number of Hb-positive cells was less compared to WT embryos, but higher than the number of cells observed in $pros^{jo13}$ mutant embryos (Figure 5.12 H and L).

Although ectopic DmcycE showed no obvious effect on Hb expression, ectopic Stg expression suppressed Hb expression (Figure 5.13 C and G). Ectopic expression of Pros and Stg in parallel also resulted in suppression of Hb expression (Figure 5.13 D and H). This suggests that Stg could override Pros’ effect on Hb expression. This was consistent with the earlier observation that ectopic Stg could block the early induction of neuronal differentiation caused by ectopic Pros. As shown in Figure 5.12 J and N, in $pros^{jo13}$ mutant embryos, there was decreased numbers of Hb-expressing cells in the CNS (Figure 5.13 J and N). In contrast, Hb expression in $stg\ 7B, pros^{jo13}$ double mutant appeared the same as the expression observed in $stg\ 7B$ mutants alone (Figure K, O, L, P).
Discussion

Cell cycle genes act downstream of Pros in the regulation of mitotic activity

Overexpression of a single cell cycle gene, such as DmcycE, stg, or de2f1, is able to bypass the suppression of BrdU incorporation caused by ectopic Pros expression. This suggests that cell cycle genes are downstream targets of Pros in the regulation of mitotic activity. However, transcription of H1 RNA remains suppressed in these embryos. Since H1 RNA transcription is normally initiated prior to DNA replication, as shown by BrdU incorporation, the lack of H1 RNA expression in embryos expressing both ectopic Stg and Pros indicates that ectopic expression of one of the cell cycle genes in parallel with ectopic Pros is not enough to restore an entire functional S phase. In agreement with this observation, expression of DmcycE, which is essential for entering S phase, is also still suppressed in the presence of ectopic Stg and ectopic Pros. Thus, ectopic expression of one of cell cycle genes can only push one more round of BrdU incorporation before ectopic Pros shuts it down.

Ectopic Stg, but not DmcycE, can block the early onset of neuronal differentiation caused by ectopic Pros

Ectopic Pros increases the expression of specific neuronal differentiation markers and induces early onset of neuronal differentiation, as revealed by anti-HRP and anti–22C10 staining. In contrast, ectopic Stg blocks neuronal differentiation. Neuronal differentiation is still blocked in embryos with parallel ectopic expression of Pros and Stg. These expression of cell cycle components needs to be shut down prior to entry into

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neuronal differentiation, and that Pros plays a critical role in preventing further cell proliferation. However, presently it is not clear why only ectopic Stg, but not ectopic DmcycE or E2F1, has the ability to suppress the terminal differentiation in the presence of ectopic Pros.

**Functions of Pros and cell cycle genes, Stg and DmcycE, on various neuronal differentiation markers appear to be lineage specific**

The observation of neuronal differentiation marker expression in embryos with ectopic Pros expression together with either ectopic Stg or DmcycE, and in double DmcycE^{Ar95}, pros^{jo13} or stg^{7B}, pros^{jo13} mutant embryos, indicates that the role of Pros and/or cell cycle genes in neuronal differentiation is lineage specific. The change of Odd expression in both ectopic and LOF stg embryos suggests that Stg plays a role in Odd expression. However, I did not detect any obvious changes of Odd expression corresponding to changes of DmcycE dosage. Thus, DmcycE may not play a significant role in Odd expression. In terms of Eve expression, Pros and DmcycE functionally cooperate in regulating Eve expression as mentioned in chapter 4. In Stg^{7B} mutant embryos, there is no Eve expression in the nervous system, suggesting Stg is required for Eve expression as previously reported (Weigmann and Lehner, 1995). However, the observation that Eve expression is increased in embryos with ectopic expression of Pros, which in turns suppresses the expression of stg, indicates that Pros can override the effects of Stg on Eve expression.
En expression in double $DmcyCE^{Ar^{95}}; pros^{jo13}$ mutant embryos shows an intermediate phenotype between $DmcyCE^{Ar^{95}}$ and $pros^{jo13}$ mutant embryos. It appears that in DM cells, DmcyCE function is more important than Pros. There are decreased numbers of En-positive DM cells in double $DmcyCE^{Ar^{95}}; pros^{jo13}$ mutant embryos, similar to $DmcyCE$ mutant embryos. However, in L and ML clusters, there are still additional En-positive cells between L and ML clusters, but the number of additional cells is decreased compared to $pros$ mutant embryos. This may indicate that in L and ML clusters, the effects of Pros and DmcyCE are lineage specific. In other words, DmcyCE function is more important than Pros in some cell lineages, while in other cell lineages Pros is more critical. Ectopic Stg has no obvious effect on En expression, and En expression in ectopic Stg together with ectopic Pros appears the same as ectopic Pros alone. Furthermore, in $stg^{7B}$ mutant embryos En expression was observed at approximately the right developmental stage. Taken together, this indicates that Stg may not play a significant role in regulating En expression.

There is a slight decrease in the Hb-expression number of cells in $DmcyCE^{Ar^{95}}$ mutant embryos, compared to wild-type embryos. Ectopic DmcyCE has no obvious effect on Hb expression. However, in $DmcyCE^{Ar^{95}}; pros^{jo13}$ double mutant embryos, Hb expression is different from that observed in embryos that are only mutant for $pros$. This could point to a functional interaction between DmcyCE and Pros in regulating Hb expression. Suppression of Hb expression was observed in both ectopic Stg alone and ectopic Stg in parallel with Pros, suggesting that Stg function is involved in the regulation of Hb expression. This observation is also in agreement with the observation made for
two other neuronal differentiation markers, anti-HRP and anti-22C10. Here, ectopic Stg blocked the effects of ectopic Pros on neuronal differentiation.

In summary, our data from the observation of the expression of several neuronal differentiation markers in embryos with double ectopic expression of Pros and either Stg or DmcycE, and double *DmcycE*^(Av95^, *pros*^jo13^ or *stg*^7B^, *pros*^jo13^) mutant embryos, show that the regulatory effect of Pros and/or cell cycle genes on the expression of the differentiation marker in question appears to be lineage specific. This observation further supports the notion that the regulatory mechanisms regulating neuronal differentiation and cell proliferation are complex. It appears likely that every lineage is regulated by an individual combination of different regulatory genes to gain its own identity. Thus, to determine the role of Pros and/or cell cycle genes in the regulation of neuronal differentiation, and in determining whether there is functional cooperativity between Pros and individual cell cycle genes, it will be necessary to identify individual lineages and trace the expression of neuronal differentiation makers in the particular lineages when the dosages of Pros and individual cell cycle genes are changed.
Figure 5.1 Ectopic expression of Stg or E2F/dDP in parallel with ectopic Pros can bypass the suppression of BrdU incorporation caused by ectopic Pros. (A) wild-type embryo, (B) Kr-GAL4/UAS-Pros embryo, (C) Kr-GAL4/UAS-Pros; UAS-Stg embryo and (D) Kr-GAL4/UAS-Pros; UAS-E2F1/dDP embryo have been double label for BrdU incorporation (Green) and Anti-Pros (Red). (B, C, D) these embryos have high level of Pros protein within the Kr domain (label in red in B, C, D)
Figure 5.2 Ectopic expression of Stg in parallel with ectopic Pros cannot bypass the suppression of H1 RNA transcription caused by ectopic Pros. H1 RNA expression of (A) wild type, (B) Kr-GAL4/UAS-Pros, (C) Kr-GLA4/UAS-Stg, and (D) Kr-GAL4/UAS-Pros; UAS-Stg embryos. Arrows indicate the borders of Kr domain.
Figure 5.3 Ectopic expression of one of cell cycle genes, DmcycE or Stg, in parallel with ectopic Pros can not bypass the suppression of RNA expression of other critical cell cycle genes. (A) Stg RNA expression in Kr-GAL4/UAS-Pros; UAS-DmcycE embryo. (B) DmcycE RNA expression in Kr-GAL4/ UAS-Pros; UAS-Stg embryo. (C, D) Cyc A RNA expression in (C) Kr-GAL4/ UAS-Pros; UAS-DmcycE and (D) Kr-GAL4/UAS-Pros; UAS-Stg embryos. Arrows indicate the borders of Kr-domain.
Figure 5.4 Ectopic Stg can block the increasing and induction of neuronal differentiation caused by ectopic Pros. (A, E) wild type embryo. (B, F) Kr-GAL4/UAS-Pros embryo (C, G) Kr-GAL4/UAS-Stg embryo (D, H) Kr-GAL4/UAS-Pros; UAS-Stg embryo. (A, B, C, D) embryos stain with anti-HRP. (E, F, G, H) embryos stain for anti-22C10. Arrows indicate borders of Kr domain.
Figure 5.5 Ectopic DmcycE cannot block the increasing expression of neuronal differentiation marker, 22C10, caused by ectopic Pros. (A) Kr-GAL4/UAS-Pros, (B) Kr-GAL4/UAS-DmcycE, and (C) Kr-GAL4/UAS-Pros; UAS-DmcycE embryos. Arrows indicate the borders of Kr domain.
Figure 5.6 Loss-of-function *pros* or/and *DmcycE* results in CND defects. (A) wild-type, (B) *pros* jo13, (C) *DmcycE* Ar95, and (D) *DmcycE* Ar95; *pros* jo13 double mutant embryos stain for anti-HRP
Figure 5.7 Loss-of-function pros or/and DmcycE results in PNS defects. (A) wild-type, (B) pros jo13, (C) DmcycE Ar95, and (D) DmcycE Ar95; pros jo13 double mutant embryos stain for anti-22C10. Arrows indicate path finding defects. Arrowheads shows position of chordotonal organ cells and stars indicate missing of chordotonal organ cells.
Figure 5.8 Pros and Stg regulate Odd expression. (A, E, I, M) wild type embryo (B, F) Kr-GAL4/UAAS-Pros embryo (C, G) Kr-GAL4/UAAS-Stg embryo (D, H) Kr-GAL4/UAAS-Pros; UAS-Stg embryo (E, F, G, H, M) embryos are higher magnification of (A, B, C, D, I) embryos respectively. (J, N) pros\textsuperscript{jo13} mutant embryo (K, O) stg 7B mutant embryo (L, P) pros\textsuperscript{jo13}, stg 7B double mutant embryos (N, O, P) embryos are higher magnification of (J, K, L) embryos respectively.
Figure 5.9 Eve expression in change dosage of Pros or/and Stg. (A, E, I) wild type embryo (B, F) Kr-GAL4/UAAS-Pros embryo (C, G) Kr-GAL4/UAS-Stg embryo (D, H) Kr-GAL4/UAAS-Pros; UAS-Stg embryo (E, F, G, H) embryos are higher magnification of (A, B, C, D) embryos respectively. Arrows indicate the borders of Kr domain. (J) pros jo13 mutant embryo (K) stg 7B mutant embryo (L) pros jo13, stg 7B double mutant embryos.
Figure 5.10 Pros and DmcyE regulate En expression. (A, E, I, M) wild type embryo (B, F) Kr-GAL4-UAAS-Pros embryo (C, G) Kr-GAL4/UAS-DmcyE embryo (D, H) Kr-GAL4/UAS-Pros; UAS-DmcyE embryo (E, F, G, H, M) embryos are higher magnification of (A, B, C, D, I) embryos respectively. (J, N) pros\_jo13 mutant embryo (K, O) DmcyE\_Ar95 mutant embryo (L, P) DmcyE\_Ar95; pros\_jo13 double mutant embryos (N, O, P) embryos are higher magnification of (J, K, L) embryos respectively. Small arrows indicate L neurons. Big arrows indicate ML neurons and arrowheads indicate DM cells.
Figure 5.11 En expression in change dosage of Pros or/and Stg. (A, E, I, M) wild type embryo (B, F) Kr-GAL4/UAS-Pros embryo (C, G) Kr-GAL4/UAS-Stg embryo (D, H) Kr-GAL4/UAS-Pros; UAS-Stg embryo (E, F, G, H, M) embryos are higher magnification of (A, B, C, D, I) embryos respectively. (J, N) pros jo13 mutant embryo (K, O) stg 7B mutant embryo (L, P) pros jo13, stg 7B double mutant embryos (N, O, P) embryos are higher magnification of (J, K, L) embryos respectively.
Figure 5.12 Pros and DmcycE interact in regulating Hb expression. (A, E, I) wild type embryo (B) Kr-GAL4/UAS-Pros embryo (C) Kr-GAL4/UAS-DmcycE embryo (D) Kr-GAL4/UAS-Pros; UAS-DmcycE embryo Arrows indicate the borders of Kr domain. (F, J) pros\(^{jo13}\) mutant embryo (G, K) DmcycE\(^{Ar95}\) mutant embryo (H, L) DmcycE\(^{Ar95}\); pros\(^{jo13}\) double mutant embryos (I, J, K, L) embryos are higher magnification of (E, F, G, H) embryos respectively
Figure 5.13 Pros and Stg regulate Hb expression. (A, E, I, M) wild type embryo (B, F) Kr-GAL4/UAAS-Pros embryo (C, G) Kr-GAL4/UAS-Stg embryo (D, H) Kr-GAL4/UAS-Pros; UAS-Stg embryo (E, F, G, H, M) embryos are higher magnification of (A, B, C, D, I) embryos respectively. Arrows indicate the orders of Kr domain. (J, N) pros jo13 mutant embryo (K, O) stg 7B mutant embryo (L, P) pros jo13, stg 7B double mutant embryos (N, O, P) embryos are higher magnification of (J, K, L) embryos respectively.
CHAPTER 6
MITOTIC ACTIVITY AND GENE REGULATION IN THE LARVAL OPTIC LOBES

Introduction

The optic lobes of *Drosophila* provide a good model for studying regulatory relationship between cell proliferation and differentiation due to highly organized domains of proliferation and differentiation. In the optic lobes of third instar larvae, there are two major areas of mitotic activity, the outer and inner proliferation centers (OPC and IPC, respectively). The OPC is one of the major areas of mitotic activity and is located at the outer most part of the brain lobe. Next to the OPC is the larminal furrow in which cells are arrested in G1 phase. The lamina lays next to the larminal furrow and cells in the lamina are going through their last mitotic division before entering differentiation. The IPC forms a crescent shape at a more interior position of the brain with respect to the lamina.

*dpn* is a pan-neural genes which is expressed in all neuronal lineages during embryonic neurogenesis (Bier et al., 1992; Gonzales et al., 1989; Brand et al., 1993). *dpn* encodes a HES type bHLH protein (Dpn) with similarities to the *Drosophila* Hairy and the Enhance of split complex bHLH proteins. LOF *dpn* mutations are embryonic lethal. A former graduate student, Ken Wallace, observed that Dpn negatively regulates *dap*
expression in the optic lobes of the third instar larvae (Wallace et al., 2000). This led me to wonder whether Dpn also regulates mitotic activity in the optic lobes of the third instar larvae.

Pros is a pan-neuronal transcription factor and encodes an atypical homeodomain protein (Vaessin et al., 1991; Doe et al., 1991). Pros regulates mitotic activity through regulating the expression of cell cycle genes, including stg and DmcycE, during embryonic neurogenesis (Li and Vaessin, 2000). A former graduate student, Ling Li, has shown that Pros can regulate the expression of reporter constructs containing stg cis-regulatory neuronal elements during embryonic development (Li, 2000). However, it is not clear whether at the post embryonic stage if Pros also has the same effect on the expression of the same stg cis-regulatory neuronal elements. Thus, I examined the expression of the reporter constructs of stg cis-regulatory neuronal elements in the brains of the third instar larvae in an ectopic Pros background.

**Results**

**Ectopic expression of Dpn increases mitotic activity in the optic lobes**

A former graduate student, Ken Wallace, observed that ectopic expression of Dpn in the larval brains dramatically increased the brain size compared to the wild-type (Figure 6.1 A and B) (Wallace et al., 2000). In this experiment, Dpn was ectopically expressed using the UAS/GAL4 system. 71B-GAL4 line drove UAS-Dpn expression in the larval brains. BrdU incorporation assays showed that ectopic expression of Dpn in the larval optic lobes increased the number of cells that were mitotically active (Figure 6.1
B). Furthermore, compared to wild-type, optic lobes which displayed a highly organized array of mitotic regions, such as the OPIC, IPC, and lamina, the optic lobes of UAS-Dpn/71B-GAL4 showed a breakdown of the mitotic domain structure (Figure 6.1 A and B). This suggested that ectopic Dpn increased brain size through increasing the number of cells that were mitotically active.

**Ectopic Pros suppresses the expression of reporter genes containing Stg cis-neuronal elements in the optic lobes**

*stg*, a Cdc25-type phosphatase, is the limiting factor for G2/M progression in most stages of *Drosophila* development (Edgar and O’Farrell, 1989, 1990; Neufeld et al., 1998). Ectopic expression of Stg drives G2 cells rapidly into mitosis while loss-of-function *stg* mutation results in G2 arrest in both embryonic and imaginal disc cells (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990; Johnston and Edgar, 1998; Milan et al., 1996a; Milan et al., 1996b; Neufeld et al., 1998). The expression of Stg has been shown to be regulated by developmental cues including Kr and Notch (Edgar and O’Farrell, 1990). The *cis*-regulatory elements of *stg* have been identified (Lehman et al., 1999). More than 30 kb of *cis*-regulatory elements control *stg* expression spatially and temporally (Lehman et al., 1999). StgE- 6.4, 5.3, 4.9 reporter genes all can drive lacZ expression in the brains of third instar larvae, although only StgE-6.4 and 5.3, but not 4.9, can drive lacZ expression in the embryonic CNS (Lehman et al., 1999).

Pros has been shown to regulate *stg* expression during embryonic neurogenesis (Li and Vaessin, 2000). To further investigate whether Pros could also regulate *stg*
expression in larval CNS, I analyzed lacZ expression of Stg E-6.4, 5.3, and 4.9 reporter genes in the presence of ectopic Pros in the brains of UAS-Pros/71B-GAL4 larvae.

Stg E-4.9 directed lacZ expression in the central brain (CB), OPC, and ventral nerve cord (VNC) (Figure 6.2 A and C) (Lehman et al., 1999). I observed that ectopic Pros suppressed lacZ expression of Stg E-4.9 (Figure 6.2B and D). Stg E-5.3 directed lacZ expression in the VNC, CB and lamina (Figure 6.3A) (Lehman et al., 1999). Ectopic Pros suppressed lacZ expression of Stg E-5.3 in VNC and CB but not in lamina (Figure 6.3B). Stg E-6.4 directed lacZ expression in the VNC, CB, and interior optic lobe cells (Figure 6.4A) (Lehman et al., 1999). Ectopic expression of Pros also suppressed lacZ expression of Stg E-6.4 in CB but had less of an effect on lacZ expression of interior optic lobe cells of the third instar larval CNS (Figure 6.6B). Thus, similar to embryonic stages, ectopic Pros suppressed the expression of Stg reporter gene constructs. However, this effect was not uniform. It was unclear whether this was due to the incomplete nature of the individual reporter gene constructs.

Discussion

BrdU incorporation assays shows that ectopic Dpn, driven by 71B-GAL4, increases the mitotic activity in the optic lobes and results in increasing the size of optic lobes in the third instar larvae. Ectopic Dpn has been shown to suppress dap expression in the larval brain. Thus, this indicates that Dpn regulates mitotic activity at least in parts through regulating dap expression.
Although Pros has been shown to regulate the expression of *stg* during embryonic neurogenesis, it is not clear whether Pros plays a similar role in regulating the expression of *stg* in the larval CNS. The observation that ectopic Pros is able to suppress the expression of reporter genes containing *stg* cis-neuronal elements suggests that Pros may also regulate the expression of cell cycle genes in the developing larval CNS, as it does during embryonic neuronogenesis. Loss-of-function *pros* is embryonic lethal. Therefore mosaic analysis or RNAi assays will be necessary in the future to investigate Pros function in the developing larval CNS.
Figure 6.1 Ectopic expression of Dpn results in increased mitotic activity in the larval brain. Optic lobes of (A, C) wild-type and (B, D) 71B-GAL4/UAS-Dpn (A, B) shown BrdU incorporation as S phase marker (Green). (C, D) shown M phase marker, anti-phospho Histone H3, labeled cells (Red)
Figure 6.2 Ectopic Pros suppresses the lacZ expression of Stg E-4.9 reporter gene. LacZ expression (Red) of Stg E-4.9 reporter gene in the optic lobes of (A and C) wild-type, and (B and D) 71B-GAL4/UAS-Pros
Figure 6.3 Ectopic Pros suppresses the lacZ expression of Stg E-5.3 reporter gene. LacZ expression (Red) of Stg E-5.3 reporter gene in the optic lobes of (A) wild-type, and (B) 71B-GAL4/UAS-Pros
Figure 6.4 Ectopic Pros suppresses the lacZ expression of Stg E-6.4 reporter gene. LacZ expression (Red) of Stg E-6.4 reporter gene in the optic lobes of (A) wild-type, and (B) 71B-GAL4/UAS-Pros
CHAPTER 7
CONCLUSIONS

The main goal of my research was to investigate the regulation of the expression of *dap*, a *Drosophila* CKI gene. A second goal of this study was to analyze the function of Pros in regulating mitotic activity and differentiation.

In this context, I showed that transcriptional expression of *dap* is regulated by a complex array of tissue-specific cis-regulatory elements. I found that the *dap* neuronal element was mainly localized in the second intron. Pros, a pan-neural transcription factor, was found to be both a positive and negative modulator of *dap* transcription. Furthermore, I identified Pros response elements on *dap* regulatory region. Several clusters of potential Pros binding sequences on Dap-DEL, a Dap lacZ reporter gene containing part of *dap* intron, were found to be essential for Pros-mediated response.

Using double ectopic expression and double mutations, I observed that Pros and DmcycE functionally cooperated in the activation/induction of *dap* expression. DmcycE and Pros also acted cooperatively to regulate the neuronal differentiation marker Eve. Although Dap is required for the regulation of Eve expression, the ability of Pros and DmcycE to regulate Eve expression appeared not to be mediated exclusively through their ability to regulate *dap* expression. Furthermore, I showed that Pros physically interacted with DmcycE/Cdc2c complexes.
Pros has been shown to regulate mitotic activity and the expression of cell cycle regulatory genes. I found that cell cycle regulatory genes, including \textit{stg}, \textit{DmcycE}, \textit{dE2F}, act downstream of Pros in regulating mitotic activity. Furthermore, the effect of Pros and the cell cycle regulatory genes, \textit{stg} and \textit{DmcycE}, on the expression of several neuronal markers, such as Eve, Odd, En, Hb, were lineage specific.

In summary, results from this work point to a bi-directional mechanism coordinating cell proliferation and differentiation. In agreement with such a view, I showed that the cell cycle regulatory genes \textit{dap} and \textit{DmcycE} regulate the expression of neuronal determination/ differentiation factors (Eve) and likewise, that the neuronal determination factor Pros regulates the expression of cell cycle regulatory genes (e.g. \textit{dap}). Moreover, the observation that Pros and DmcycE cooperated functionally to regulate \textit{dap} expression suggested that a complex network is used to ensure the coordination between cell proliferation and differentiation.

Further experiments are necessary to determine whether Pros binds to \textit{dap} cis-regulatory elements directly and also what cofactors, if any, assist Pros binding. More experiments are also required to determine the biological significance of the protein-protein interaction between Pros and DmcycE/Cdc2c complex, and more specifically which of the domains of Pros is responsible for the interaction with DmcycE/Cdc2c complex. In the future, it will be necessary to trace the expression of a neuronal marker in a specific lineage to further identify the effects of Pros and cell cycle genes on this specific lineage.
LIST OF REFERENCES


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