Cyclin dependent kinase activity is necessary for thyroid hormone induced tail regression in the *Rana catesbeiana* tadpole

by

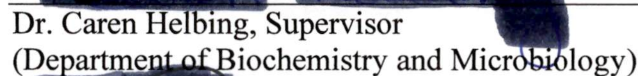
Rachel Carrie Skirrow
B.Sc., University of Victoria, 1999

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

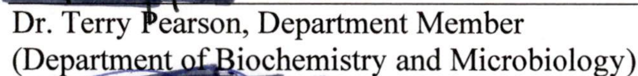
MASTER OF SCIENCE

In the Department of Biochemistry and Microbiology

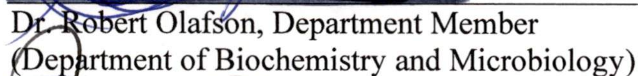
We accept this thesis as conforming to the required standard



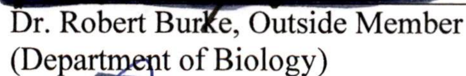
Dr. Caren Helbing, Supervisor
(Department of Biochemistry and Microbiology)



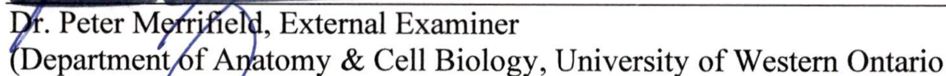
Dr. Terry Pearson, Department Member
(Department of Biochemistry and Microbiology)



Dr. Robert Olafson, Department Member
(Department of Biochemistry and Microbiology)



Dr. Robert Burke, Outside Member
(Department of Biology)



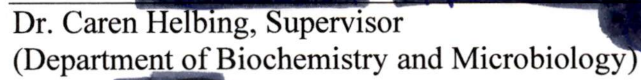
Dr. Peter Merrifield, External Examiner
(Department of Anatomy & Cell Biology, University of Western Ontario

© Rachel Carrie Skirrow, 2003
University of Victoria

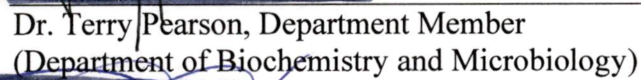
All rights reserved. This thesis may not be reproduced in whole or in part, by photocopying or by other means, without the permission of the author

QL669.2
S55

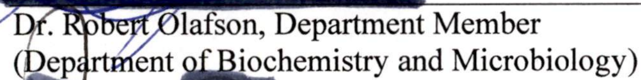
Examiners:



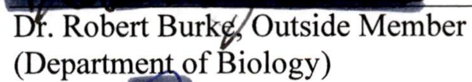
Dr. Caren Helbing, Supervisor
(Department of Biochemistry and Microbiology)



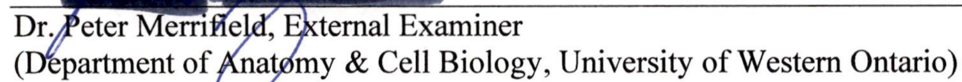
Dr. Terry Pearson, Department Member
(Department of Biochemistry and Microbiology)



Dr. Robert Olafson, Department Member
(Department of Biochemistry and Microbiology)



Dr. Robert Burke, Outside Member
(Department of Biology)



Dr. Peter Merrifield, External Examiner
(Department of Anatomy & Cell Biology, University of Western Ontario)

Supervisor: Dr. Caren C. Helbing

Abstract

Maintaining a balance between proliferation and programmed cell death (apoptosis) is necessary for the development and homeostasis of eukaryotic organisms. These processes represent contrasting cell fates; however, experimental evidence has suggested that the execution of each program may rely on common molecular machinery. Cell proliferation is controlled by cyclin-dependent kinases (Cdks) whose activities are regulated by cyclin subunits. Recent evidence has supported a role for these kinases in the process of apoptosis, although the mechanism of this regulation is poorly understood. The postembryonic model of amphibian metamorphosis was therefore used to study the function of these proteins in the apoptotic process of metamorphic tail regression.

Using thyroid hormone (TH)-dependent metamorphosis of the *Rana catesbeiana* tadpole, it was shown that the Cdks are essential for the apoptotic process of tail regression. Their activity was inhibited *in vitro* by the Cdk inhibitor, roscovitine, which also completely prevented TH-induced regression of cultured premetamorphic tail tips. The mitogen activated protein kinase (MAPK) and the phosphatidylinositol-3 kinase (PI-3K) signal transduction pathways both regulate entry into the cell cycle and yet neither of those pathways showed any involvement in TH-induced tail regression. Roscovitine can inhibit transcription by acting on Cdk7 and Cdk9. The thyroid hormone receptor β (TRβ) is transcribed as an early response to T_3 treatment and roscovitine inhibits the TH-induced upregulation of TRβ in cultured tail tips. These data present the first evidence that TH-dependent apoptosis is dependent upon Cdk activity.

Table of Contents

ABSTRACT II

TABLE OF CONTENTS IV

LIST OF TABLES VIII

LIST OF FIGURES IX

CHAPTER 1. INTRODUCTION 1

I. CELL CYCLE OVERVIEW 1

i. Cyclin-dependent kinases (Cdks) 4

ii. Cyclins 7

iii. Cell cycle regulation, p53 and cancer 10

iv. Substrate specificity 13

II. MOLECULAR MECHANISM OF APOPTOSIS 16

i. Caspases 17

ii. Caspase activation 18

iii. Caspase-independent apoptosis 19

iv. Cell cycle regulators and apoptosis 20

III. ALTERNATIVE FORMS OF CYCLINS AND CDKS 22

IV. CYCLINS/CDKS REGULATE TRANSCRIPTION 24

i. Cdk7 25

ii. Cdk8 25

iii. Cdk9 26

iv. Additional CTD kinases 26

V. THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) PATHWAY 27

i. MAPK regulation of cell cycle progression 31

ii. Additional regulation of G_1 progression 33

VI. THE AMPHIBIAN MODEL 33

i. The tadpole tail as a model for studying apoptosis 35

VII. THYROID HORMONES 39

i. Thyroid hormone receptors 40

ii. Mechanism of TR transcriptional regulation 42

iii. Nongenomic effects 44

VII. RESEARCH HYPOTHESIS AND THESIS OUTLINE 45

CHAPTER 2. CYCLIN DEPENDENT KINASE ACTIVITY IS REQUIRED FOR THYROID HORMONE-DEPENDENT TAIL REGRESSION IN THE FROG TADPOLE 47

1. INTRODUCTION 47

2. MATERIALS AND METHODS 49

2.1 Animal care. 49

2.2 Tail organ culture. 50

2.3 Tail measurement and stastical analysis. 50

2.4 *In vivo* T_3 tadpole treatment. 51

2.5 Tissue homogenization. 51

2.6 Extraction of cytoplasmic and nuclear proteins. 52

2.7 Immunoprecipitation (IP) and kinase assay 52

2.8 *In vitro* roscovitine inhibition assay 54

2.9 Immunoblotting 54

2.10 Calf Intestinal Alkaline Phosphatase (CIP) Reactions 55

3. RESULTS 56

3.1 Roscovitine inhibits T_3-induced tadpole tail regression 56

3.2 Roscovitine inhibits tail regression in a stage-specific manner 56

3.3 The requirement for Cdk activity coincides with the commitment point for the T_3-induced response 59

3.4 Maximal cyclin A-associated kinase activity precedes metamorphic climax during natural metamorphosis and is inhibited by roscovitine *in vitro* 62

3.5 Generation of a 35 kDa cyclin A protein correlates with apoptosis in natural and T_3-induced metamorphosis 67

3.6 Cdk1 and Cdk2 steady state levels are reduced during natural metamorphosis 69

3.7 Roscovitine reduces the steady state levels of the 35 kDa cyclin A, Cdk1 and Cdk2 proteins 69

3.8 Roscovitine inhibits ERK activity *in vitro* but has no effect on steady state protein levels in cultured tail tips 72

3.9 Neither PD098059 nor Wortmannin inhibit T_3-induced tail tip regression...... 75

4. DISCUSSION 78

CHAPTER 3. ROSCOVITINE INHIBITS TH-INDUCED GENE EXPRESSION IN THE TAIL TIP OF *R. CATESBEIANA* TADPOLES........ 82

1. INTRODUCTION 82

2. MATERIALS AND METHODS 84

2.1 Animal care. 84

2.2 Tail organ culture. 85

2.3 *In vivo* T_3 tadpole treatment. 85

2.4 Tissue homogenization. 86

2.5 Immunoprecipitation (IP) and kinase assay. 86

2.6 *In vitro* roscovitine inhibition assay 87

2.7 Immunoblotting 87

2.8 Tissue Biopsy 89

2.9 Preparation of total RNA 89

2.10 Preparation of total cDNA 89

2.11 Primer design 90

2.12 PCR amplification and isolation of gene fragments 91

2.13 Cloning and sequencing 92

2.14 Quantitative PCR Analysis 93

2.15 Statistical analysis 94

3. RESULTS 94

3.1 Gene expression in the tail is altered during natural metamorphosis. 94

3.2 RNA polymerase II is phosphorylated during natural metamorphosis. 97

3.3 Roscovitine affects protein steady state levels 100

3.4 Roscovitine differentially alters the steady state levels of gene transcripts... 102

3.5 Roscovitine affects the steady state levels of "normalizer" gene transcripts suggesting a lower RNA yield 104

4. DISCUSSION 106

SUMMARY AND CONCLUSION 111

REFERENCES.......... 115

APPENDIX I. ABBREVIATIONS.......... 161

List of Tables

TABLE 2.1. CHARACTERIZATION OF ANTIBODIES USED FOR IMMUNOPRECIPITATION AND IMMUNOBLOTTING........ 53

TABLE 3.1. CHARACTERIZATION OF ANTIBODIES USED FOR IMMUNOBLOTTING........ 88

TABLE 3.2. PRIMER SEQUENCES USED IN THE ISOLATION OF *R.CATESBEIANA* GENE FRAGMENTS........ 90

TABLE 3.3. PRIMER SEQUENCES DESIGNED AGAINST *R.CATESBEIANA* GENE SEQUENCE FOR AMPLIFICATION OF GENE FRAGMENTS BY QUANTITATIVE PCR........ 91

List of Figures

FIGURE 1.1. SIMPLIFIED CELL CYCLE SCHEMATIC.. 2
FIGURE 1.2. CDK TERTIARY STRUCTURE AS REPRESENTED BY CDK 2. 5
FIGURE 1.3. CYCLIN/CDK COMPLEX STRUCTURE AS EXEMPLIFIED BY CYCLIN A AND CDK2. 6
FIGURE 1.4. OSCILLATIONS IN MAJOR CYCLIN LEVELS DURING THE CELL CYCLE................ 8
FIGURE 1.5. CYCLIN TERTIARY STRUCTURE AS EXEMPLIFIED BY CYCLIN A...................... 11
FIGURE 1.6. SUBSTRATE TARGETING SITES ON CYCLIN/CDK COMPLEXES......................... 14
FIGURE 1.7. SCHEMATIC REPRESENTATION OF THE MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) PATHWAY, FOCUSING ON THE MEMBERS OF THE ERK1,2 PATHWAY. 29
FIGURE 1.8. CROSS-TALK BETWEEN THE RAS/RAF/MEK/ERK SIGNALING NETWORK AND THE PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) PATHWAY........................... 34
FIGURE 1.9. TADPOLE TAIL CROSS-SECTION. 37
FIGURE 1.10. EPIDERMAL CELL FATE.. 38
FIGURE 1.11. GENERAL MODEL FOR THYROID HORMONE ACTION IN THE NUCLEUS........... 41
FIGURE 1.12. MODULAR STRUCTURE OF THYROID HORMONE RECEPTOR AND THE DNA-BINDING DOMAIN.. 43
FIGURE 2.1. ROSCOVITINE INHIBITS T_3-INDUCED TAIL TIP REGRESSION IN CULTURE........ 57
FIGURE 2.2. CULTURED TAIL TIPS SPONTANEOUSLY REGRESS AT A RATE DEPENDENT UPON DEVELOPMENTAL STAGE. 58
FIGURE 2.3. ROSCOVITINE INHIBITION OF TAIL REGRESSION IS STAGE SPECIFIC............... 60
FIGURE 2.4. ROSCOVITINE INHIBITS T_3-INDUCED TAIL REGRESSION IN CULTURED TK STAGE XV AND XX TAIL TIPS. 61
FIGURE 2.5. ROSCOVITINE INHIBITION OF T_3-INDUCED TAIL REGRESSION CORRELATES WITH THE COMMITMENT POINT.. 63
FIGURE 2.6. CYCLIN A-CONTAINING COMPLEXES EXHIBIT DIFFERENTIAL KINASE ACTIVITY IN THE TAIL TIP OF TADPOLES UNDERGOING NATURAL METAMORPHOSIS..... 65
FIGURE 2.7. CDK2-CONTAINING COMPLEXES EXHIBIT DIFFERENTIAL KINASE ACTIVITY IN THE TAIL TIPS OF TADPOLES UNDERGOING NATURAL METAMORPHOSIS........ 66

FIGURE 2.8. ROSCOVITINE INHIBITS THE KINASE ACTIVITY OF CYCLIN A- AND CDK2-CONTAINING COMPLEXES. 68

FIGURE 2.9. STEADY STATE LEVELS OF A 46 KDA CYCLIN A PROTEIN DO NOT VARY IN THE TAIL TIP DURING EITHER NATURAL OR T_3-INDUCED METAMORPHOSIS, WHILE A 35 KDA PROTEIN INCREASES UPON INDUCTION OF TAIL REGRESSION. 70

FIGURE 2.10. THE STEADY STATE LEVELS OF CDK1, CDK2 AND PCNA ALL DIMINISH FOLLOWING METAMORPHIC CLIMAX 71

FIGURE 2.11. TREATMENT OF CULTURED TAIL TIPS WITH ROSCOVITINE AFFECTS THE STEADY STATE LEVELS OF CDK1, CDK2, PCNA AND THE 35 KDA CYCLIN A PROTEIN 73

FIGURE 2.12. ROSCOVITINE PARTIALLY INHIBITS ERK KINASE ACTIVITY *IN VITRO* BUT DOES NOT AFFECT PROTEIN STEADY STATE LEVELS. 74

FIGURE 2.13. THE MAPK INHIBITOR, PD098059, AND THE PI3K INHIBITOR, WORTMANNIN, HAVE NO EFFECT ON T_3-INDUCED TAIL REGRESSION 76

FIGURE 2.14. THE MAPK INHIBITOR, PD098059, AND THE PI3K INHIBITOR, WORTMANNIN, PARTIALLY INHIBIT ERK ACTIVITY 77

FIGURE 2.15. PROPOSED MODEL FOR THE ROLE OF CDKS DURING T_3-DEPENDENT TAIL REGRESSION. 80

FIGURE 3.1. GENE EXPRESSION IN THE TAIL CHANGES DURING NATURAL METAMORPHOSIS. 96

FIGURE 3.2. RNA POLYMERASE II IS PHOSPHORYLATED DURING NATURAL METAMORPHOSIS 98

FIGURE 3.3. CTD OF RNAP II IS PHOSPHORYLATED BY ERK1 99

FIGURE 3.4. ROSCOVITINE AFFECTS PROTEIN STEADY STATE LEVELS 101

FIGURE 3.5. ROSCOVITINE AFFECTS THE STEADY STATE LEVELS OF TRβ AND ERK2 103

FIGURE 3.6. ROSCOVITINE MAY AFFECT TOTAL RNA YIELD OBTAINED FROM BIOSPIES OBTAINED FROM CULTURED TAIL TIPS 105

Chapter 1. Introduction

I. Cell Cycle Overview

Multicellular organisms are composed of interacting communities of cells whose proliferation is tightly controlled to ensure that new cells are made only when they are needed. Extracellular signals that control entry into a new cell cycle and intracellular signals that dictate progression through cell cycle checkpoints regulate cell division in the tissues of these organisms.

The duplication of chromosomes and their separation into two daughter cells constitute the two central events of the cell cycle. These two processes occur in discrete, non-overlapping phases of the cell cycle known as the S and M phases, respectively **(Figure 1.1)**. DNA is replicated in the S phase and separated into two daughter cells in the M phase. The M phase typically includes two distinct events: chromosome separation and cytokinesis. Duplicated chromosomes are first pulled to opposite poles of the cell by the mitotic spindle during mitosis. The cell is then split into two daughter cells during cytokinesis, with each new cell receiving a nucleus and roughly equal cytoplasmic content. Proteins, RNA and macromolecules are continuously synthesized throughout the progression of the cell cycle and are evenly distributed between the two daughter cells during cytokinesis. By contrast, membrane-bounded organelles are reproduced by continuous growth and division of pre-existing organelles and are not synthesized *de novo* from individual components during the cell cycle. During mitosis, the smaller organelles, such as mitochondria and lysosomes, are evenly distributed between daughter cells, while the larger organelles, such as the Golgi apparatus, are fragmented into smaller vesicles and then evenly distributed with other cytoplasmic components [100].

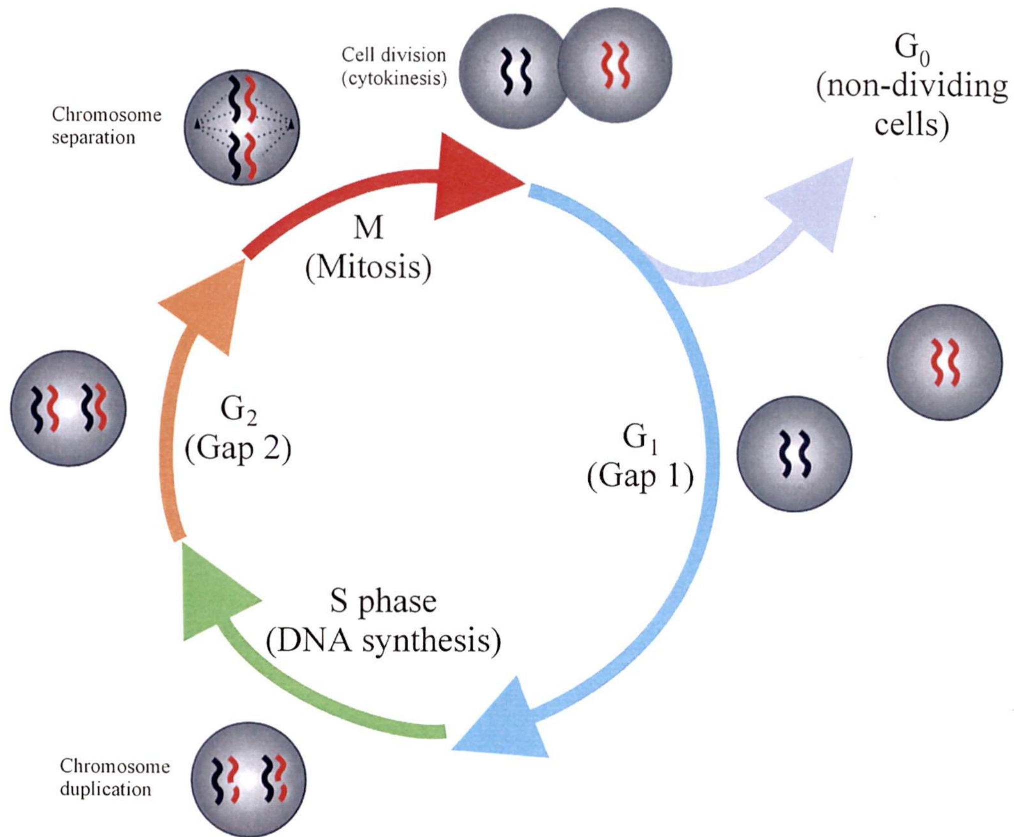


Figure 1.1. Simplified cell cycle schematic.

Depiction of the four phases of the standard eukaryotic cell cycle. The central events of cell reproduction include chromosome duplication (S phase), chromosome separation, and cell division (M phase). Gap phases separate the S and M phases. Adapted from Morgan [269].

The period between the end of one M phase and the beginning of the next is known as interphase and it comprises the S phase and two gap phases known as G_1 and G_2. Gap phases provide additional time for cell growth and allow for regulation of cell cycle progression by intracellular and extracellular signals. Cells that are not actively dividing may be either terminally differentiated or in a state of temporary arrest known as G_0 [190].

Progression through the cell cycle is controlled by a series of proteins that execute cell cycle events through phosphorylation. This control system turns the cell cycle machinery on and off at the appropriate times and also responds to a variety of intra- and extracellular cues to ensure that cell cycle events are coordinated under a variety of conditions [142].

The primary function of the cell cycle control system is to trigger cell cycle events at the appropriate time, in the correct order, and only once per cycle. This process is extensively monitored at each phase of the cell cycle, known as checkpoints, to ensure completion of each phase prior to progression to the next phase. There are 3 major checkpoints, including G_1/S, G_2/M, and M/G_1 [54, 75]. At the G_1/S boundary, entry into the cell cycle is blocked when cell growth or environmental conditions are inappropriate for continued division. Cell cycle arrest occurs at the G_2/M boundary if DNA replication is incomplete or if DNA is damaged, while arrest at the M/G_1 checkpoint occurs if chromosomes are not attached to the mitotic spindle. Periods of cell cycle arrest allow for repair of defects; however, if repair proves impossible, the cell will undergo apoptosis. Malignant transformation can also occur if multiple genetic abnormalities are

incurred leading to the activation of oncogenes and/or the inactivation of suppressor genes [85].

i. Cyclin-dependent kinases (Cdks)

The central components of the cell-cycle control system are a family of small protein kinases (~34-40 kDa) known as cyclin-dependent kinases (Cdks). Nine Cdks have been identified in mammalian cells. Of these, only 5 are directly involved in cell cycle control (Cdk 1, 2, 3, 4, 6). These proteins contain a catalytic core sequence that is shared by all protein kinases. Furthermore, like all protein kinases, Cdks have a tertiary structure comprising a small N-terminal lobe and a larger C-terminal lobe [68, 268]. ATP fits in the cleft between the two lobes with the phosphates oriented outward, toward the mouth of the cleft. Protein substrates bind at the entrance of the cleft and interact with the surface of the C-terminal lobe. Nearby residues catalyze the transfer of the γ-phosphate of ATP to a hydroxyl oxygen in the protein substrate (typically serine, threonine and tyrosine) resulting in changes in the substrate's enzymatic activity or its interactions with other proteins **(Figure 1.2)**.

By definition, Cdks require the binding of a cyclin regulatory subunit for enzymatic activation **(Figure 1.3)**. In most cases, full activation also requires phosphorylation of a threonine near the kinase active site [38, 269]. These requirements are the result of structural constraints within the Cdk. The structure of an inactive Cdk includes a large, flexible loop known as the "T-loop" (the "T" referring to the activating threonine located within the loop) that rises from the C-terminal lobe to block protein substrate binding at the entrance of the active site cleft.

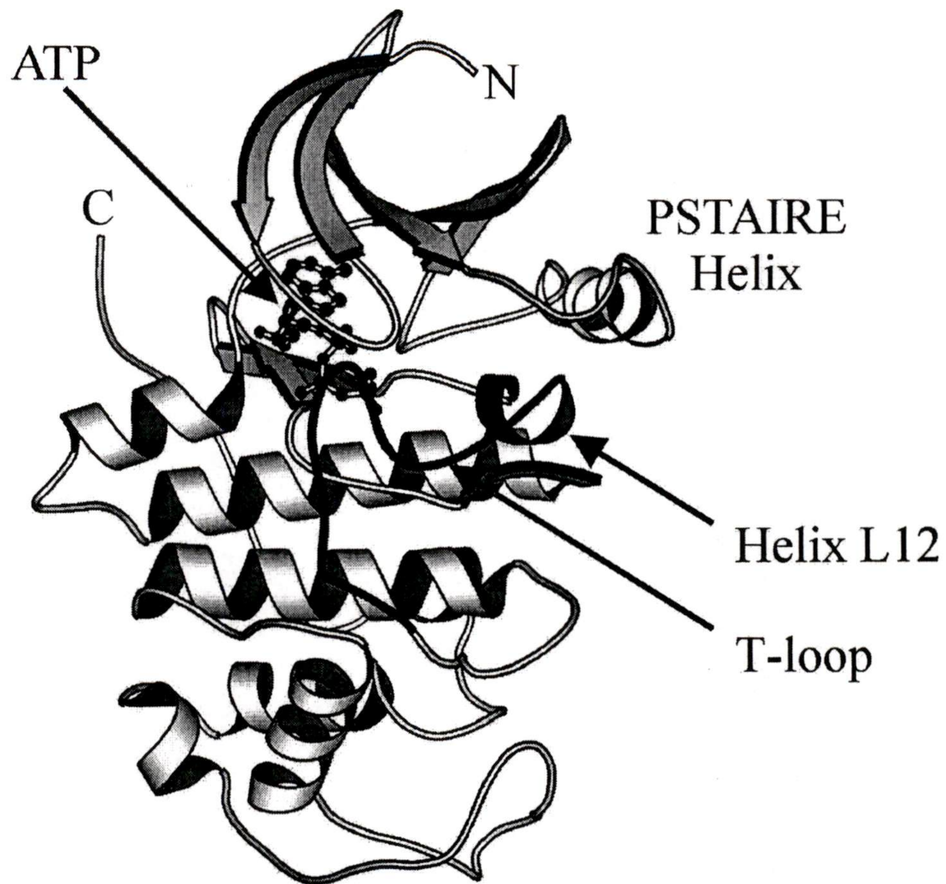


Figure 1.2. Cdk tertiary structure as represented by Cdk 2.

Cdk2 contains two lobes: a smaller N-terminal lobe (top) that is composed primarily of β-sheet and a single large helix (PSTAIRE) and a large C-terminal lobe (bottom) that is primarily composed of α-helices. The ATP substrate is shown within the active site cleft between the two lobes. The phosphates are oriented outward, toward the mouth of the cleft, which is blocked in this structure by the T-loop (highlighted in black). The L12 and PSTAIRE helices undergo major conformational changes during Cdk activation. Modified form De bondt et al [68] and Morgan [269].

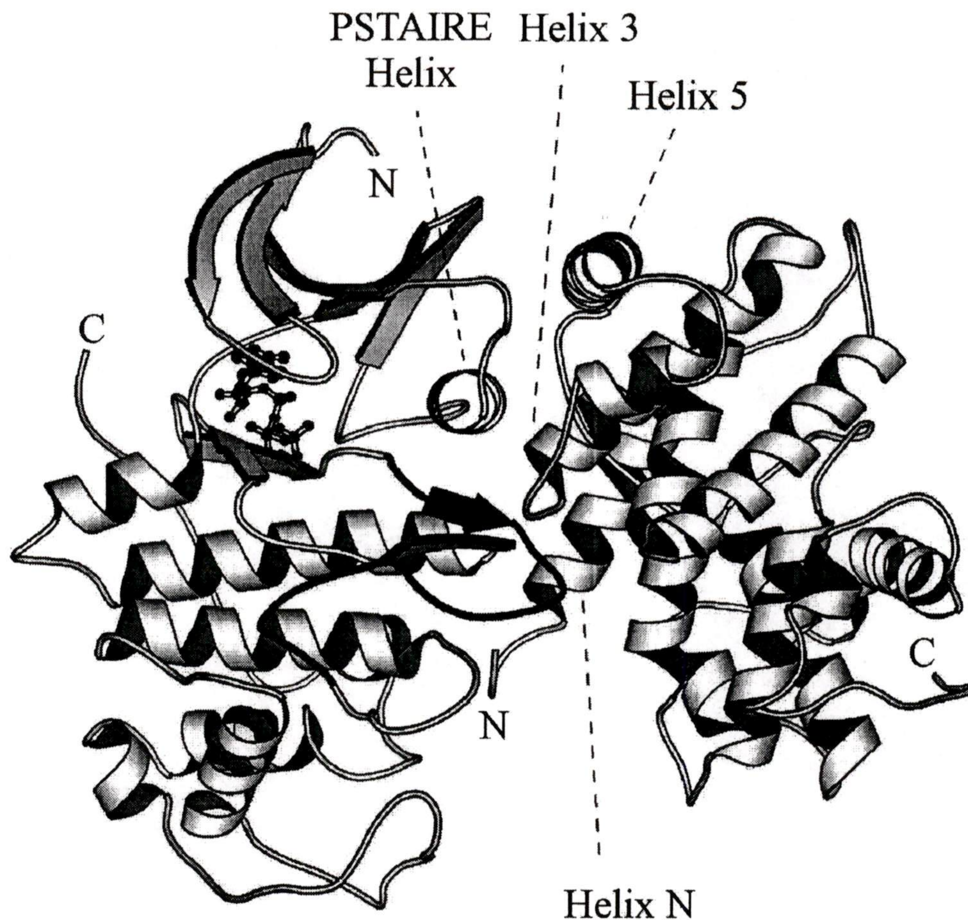


Figure 1.3. Cyclin/Cdk complex structure as exemplified by cyclin A and Cdk2.

Cdk2 is shown on the left with the T-loop highlighted in black. The binding of cyclin A to Cdk2 involves helices 3 and 5 in the cyclin box domain of cyclin A and the PSTAIRE helix of Cdk2. Another major interaction involves the C-terminal lobe of Cdk2 and the nonconserved N-terminal helix of cyclin A. Cyclin A structure is unaffected by Cdk2 binding [37], however, it induces dramatic conformational changes in Cdk2. The L12 helix in the T-loop is replaced with a beta strand, allowing the PSTAIRE helix to move inward, leading to the correct positioning of the side chains involved in ATP phosphate orientation. Modified from Jeffrey [178] and Morgan [269].

The inactive Cdk also demonstrates incorrect positioning of key residues within the active site which prevent the appropriate orientation of the ATP phosphates for the kinase reaction [68]. Consequently, Cdk activation requires extensive structural changes to allow ATP and substrate access to the Cdk active site.

These structural changes are mediated by phosphorylation within the T-loop and by binding with the cyclin partner [298]. In addition to regulation by phosphorylation and cyclin binding, Cdk activity is also modulated through interaction with Cdk inhibitory subunits (CKIs) [269, 298]. These include the Cdk2- and Cdk4/6-cyclin complex Cip/Kip inhibitor family (p21, p27, and p57) and the Cdk4/6-cyclin D complex Ink4 inhibitor family (p15, p16, p18, and p19) [135].

ii. Cyclins

The activities of cell-cycle-regulatory Cdks rise and fall as the cell progresses through the cell cycle. These oscillations lead directly to cyclical changes in the phosphorylation of key components of the cell cycle machinery, resulting in the initiation of cell cycle events. The abundance and subcellular localization of the cyclins govern the temporal activation of the Cdks, while cyclin abundance is regulated at both the transcriptional and post-translational level [336]. The generation of cyclin mRNA is tightly coupled to protein synthesis, while the cyclin protein levels are regulated by post-translational modifications involved in mediating both the stabilization and degradation of the cyclin [336].

There are four classes of cyclins **(Figure 1.4)**: the G_1, the G_1/S, the S, and the M phase cyclins. The G_1 cyclins include D1, D2, and D3 which help coordinate cell division with cell growth [132].

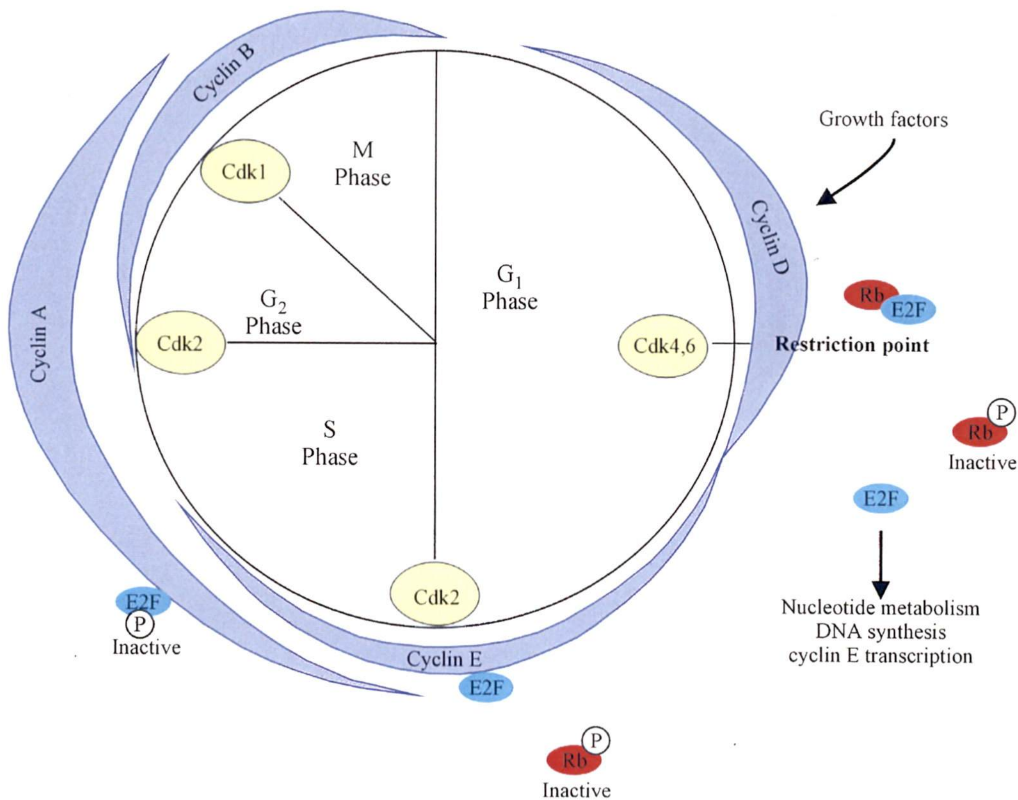


Figure 1.4. Oscillations in major cyclin levels during the cell cycle.

Simplified view of changes in cyclin concentration during the cell cycle. The cyclin-dependent kinases (Cdks) and their regulatory cyclin partners are indicated at each of the 4 phases. G_1 progression is regulated by the E2F transcription factor. The inactive E2F protein is bound to hypophosphorylated retinoblastoma protein (pRb). Cyclin D/Cdk4, 6 complexes phosphorylate the Rb-E2F complex at the "restriction point", liberating E2F for subsequent regulation of nucleotide metabolism, DNA synthesis and cyclin E transcription. Cyclin E/Cdk2 and cyclin A/Cdk2 complexes maintain pRb in a hyperphosphorylated state, allowing E2F to remain active into the S phase. Active cyclin A/Cdk2 complexes phosphorylate E2F following S phase initiation, abolishing its DNA-binding activity. The thickness of the cyclin crescents indicates the relative abundance of the protein. Adapted from Sherr [348].

Their levels do not oscillate dramatically during the cell cycle, but appear to be controlled by cell size and external growth-regulatory signals [302]. They control cell cycle entry by regulating the generation of the G_1/S cyclins when bound to Cdk4 and Cdk6. This is achieved by phosphorylation of the retinoblastoma protein (pRb) and liberation of the bound E2F transcription factor at the "restriction point" [211, 237]. The E2F family of transcription factors are required for the transcription of several genes needed for DNA synthesis, including cyclin E, cyclin A and E2F itself [15]. The G_1/S cyclins include E1 and E2 [300] and are characterized by regulation of the G_1/S transition [347]. When bound to their kinase partner, Cdk2, they stimulate the assembly of the replication complex through cooperation with Cdc6 [58, 107]. They also initiate other early cell-cycle events, such as the duplication of the centrosome [283]. It is further speculated that in addition to Cdk2, the G_1 kinase Cdk3, may also regulate the G_1/S transition when associated with cyclin E [420, 430]. The active cyclin E complex facilitates progression to the S-phase by further phosphorylating pRb, maintaining the active state of E2F. E2F then mediates the transcription of the S-phase cyclin A [336], which later binds and phosphorylates it, abolishing its DNA-binding activity. When bound to Cdk2, cyclin A stimulates DNA replication [235]; however, it also forms complexes with Cdk1 to regulate the induction of mitosis at a later step in the cell cycle [287]. Cyclin A remains high throughout the S phase, G_2 and early mitosis when its activity is important in preventing DNA re-replication [305]. Mitosis is primarily controlled by the B-type cyclins. These cyclins interact with Cdk1 to initiate the cellular changes that lead to the attachment of sister chromatids to the mitotic spindle. Their destruction brings on mitotic exit and cytokinesis [294, 315].

Distant members of the cyclin family show dramatic differences in their primary sequence; however, all classes of cyclins share a limited homology in a 100-residue region termed the cyclin box. This region has been shown to be required for Cdk and substrate binding [167, 215, 236]. Despite variations in their primary structure, all cyclins are thought to possess a similar tertiary structure, comprising a core of two compact domains each containing five alpha helices **(Figure 1.5).**

The first five-helix bundle corresponds to the conserved cyclin box. This core cyclin fold is also found in other proteins, including members of the Rb family, which regulate G_1/S gene expression, and the RNA polymerase II transcription factor, TFIIB [116, 205, 277]. The second five-helix bundle displays the same arrangement of helices as the first, despite limited sequence homology between the two subdomains [37]. Outside of the core cyclin fold, cyclin sequences are highly divergent. The length of the amino-terminal region is particularly variable, containing regulatory and targeting domains that are specific for each cyclin class. For example, the amino-terminal regions of the S- and M-phase cyclins (cyclins A and B) contain short destruction box motifs that target these proteins for proteolysis by the anaphase-promoting complex/cyclosome to allow exit from mitosis [123, 173, 183, 209, 217, 428]. By contrast, a C-terminal PEST sequence, a region rich in proline, glutamate, serine, and threonine [77, 106], regulates the ubiquitin-mediated proteolysis of the remaining cyclins.

iii. Cell cycle regulation, p53 and cancer

Cell division and the maintenance of genomic integrity are intimately associated processes whose uncoupling may lead to cancer. Families of positive and negative regulators control cell cycle progression and may be targeted by cancer-causing

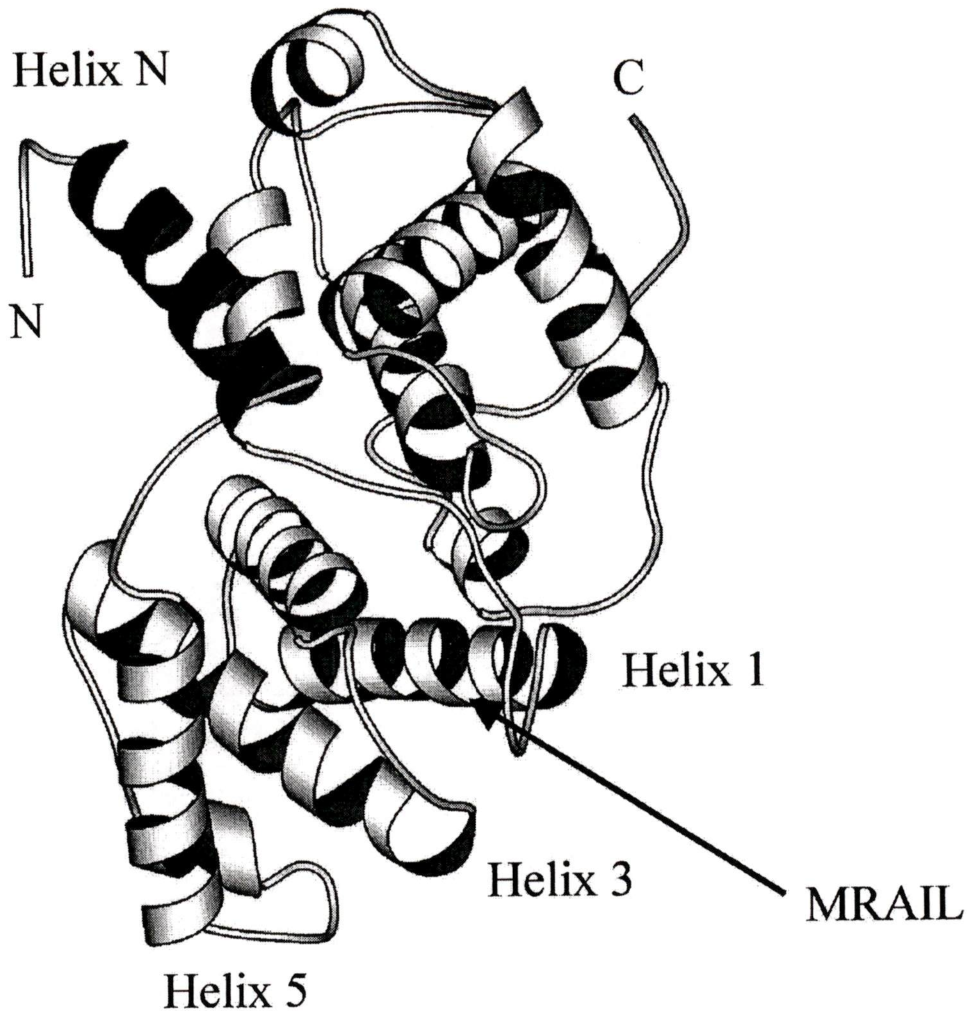


Figure 1.5. Cyclin tertiary structure as exemplified by cyclin A.

Structure of truncated human cyclin A (lacking the N-terminal 172 residues). The structure comprises two bundles of 5 α-helices flanked by single α-helices on either side. The N-terminal bundle of α-helices is the conserved cyclin box containing the Cdk2-interacting helices 3 and 5. The second 5-helical bundle differs from the first in its primary structure; however, it is structurally similar. The large N-terminal helix (helix N, indicated in black*)* and the small C-terminal helix (hidden behind the second core domain) are also shown. Modified from Jeffrey [178] and Morgan [269].

mutations [278]. The most common mutations affect the tumour suppressor protein, p53. The p53 protein functions primarily as a transcription factor that translates stress stimuli into cellular outcome. Activation of p53 induces the expression of more than 50 proteins, including p21^{Waf1}, GADD45, MDM2, and BAX which all mediate antiproliferative events *via* modulation of cell cycle progression, apoptosis, DNA repair, cell differentiation and senescence [83]. The diverse activities of p53 allow the supervision of every checkpoint in the cell cycle.

Progression through G_1/S is inhibited by p53-dependent induction of the CKI, p21^{Waf1} [82, 84]. This inhibitor blocks the kinase activity of cyclin E/Cdk2 and cyclin D/Cdk4 and 6 complexes, preventing the phosphorylation of pRb and the liberation of E2F [274]. In S-phase, p53 exerts several effects that may inhibit DNA replication and cell cycle progression. In intact cells, p53 binds to the basal transcription-repair complex TFIIH and modulates the activity of the associated helicases, ERCC3/XPB and ERCC2/XPD [334, 404]. S-phase progression may be further hindered by downregulation of cyclin A expression by p53 interaction with its promoter [71]. The overexpression of p53 can also inhibit entry into mitosis, leading to an accumulation of cells in the G_2 phase of the cell cycle [3, 274, 361]. The mechanism of action is unclear; however, the effectors appear to include p21^{Waf1}, 14-3-3σ, cdc2, CAK and GADD45. Given the extent of p53 involvement in cell cycle regulation, the loss of p53 function by mutation is likely to have consequences at multiple levels of cell-cycle control [278].

Aberrant expression of the cyclins, particularly those involved in the G1 phase of the cell cycle, has been associated with a variety of human cancers, including breast and colorectal cancer, B-lymphoma, prostate and non-small cell lung cancer [41]. While rare,

changes in cyclin A expression have also been implicated in transformation. The development of hepatoma has been shown to arise from the insertion of the hepatitis B virus (HBV) into the cyclin A gene to generate a chimeric protein lacking the destruction box [402]. Inhibition of Cdk kinase activity in these cancers has been shown to result in the induction of apoptosis [11, 41, 214, 266, 396]. Therefore, the study of Cdk inhibitors as a means of treating cancers has been proposed as productive strategy for the discovery and design novel anticancer agents specifically targeting the cell cycle.

iv. Substrate specificity

Cdks are proline-directed serine/threonine kinases as their substrate consensus phosphorylation sequence typically includes a serine/threonine residue followed by a proline. A basic amino acid is also typically found within the consensus sequence. The consensus phosphorylation site for Cdk1 and Cdk2 is defined as S/T-P-X-K/R, where 'S/T' indicates the phosphorylated serine or threonine, 'X' represents any amino acid, and 'K/R' represents the basic amino acids lysine or arginine [269]. While a general consensus sequence is proposed for recognition by Cdks, the substrate specificity of a Cdk extends beyond a primary sequence. The substrate specificity of a given Cdk is further altered through interaction with the cyclin subunit, which targets the Cdk partner to different substrates by directly binding the target [341]. These substrate preferences are due in part to the presence of a short hydrophobic patch of amino acids within the cyclin box (MRAIL) that binds with moderate affinity to substrate proteins that contain a complementary hydrophobic sequence known as the RXL (or LFG) motif [341] **(Figure 1.6)**. This interaction concentrates the substrate in the vicinity of the active kinase, leading to increased reaction rate. The MRAIL pocket also functions to regulate Cdk

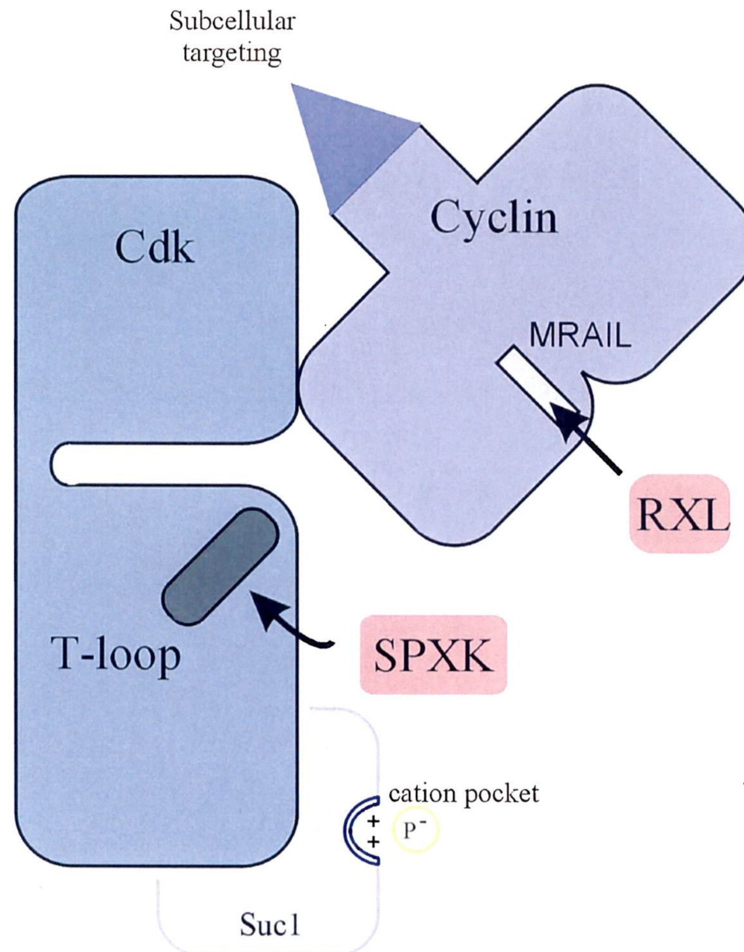


Figure 1.6. Substrate targeting sites on cyclin/Cdk complexes.

The central recognition site on Cdks lies in the active site T-loop, which interacts with the SPXK consensus sequence that is adjacent to the phosphorylation site. The presence of an RXL motif on some Cdk substrates allows interaction with the MRAIL motif on the cyclin, increasing the local substrate concentration and thereby enhancing the rate of phosphorylation. Sequences in the amino-terminal region of some cyclins control the subcellular location of the protein, thereby affecting access to certain substrates. Finally, the presence of a phosphate-binding pocket on Suc1 further contributes to substrate targeting by enhancing interactions with targets that contain multiple phosphorylation sites. Based on Morgan [269].

activity by binding to Cdk inhibitory proteins, including members of the Cip/Kip family. The inhibitors compete with substrates for binding, thereby reducing the interaction between kinase and substrate [107].

Homology within the cyclin box sequence cannot fully account for substrate specificity. Cyclins A and E retain the highest identity of all cyclin members throughout their cyclin box domains [167, 279] and both cyclins have been shown to bind Cdk1 and Cdk2 [218, 306, 328, 330]; however, they show significant differences in substrate preference. For example, Cdk2 can phosphorylate histone H1 and the retinoblastoma susceptibility gene product (pRb) *in vitro* when bound by either cyclin E or cyclin A. However, Cdk2 can only phosphorylate lamin B when bound by cyclin A [161]. Despite similar binding partners, overlapping expression, and the ability to promote S phase entry there are important differences in the abilities of cyclins A and E to activate kinase and recognize substrate. Substrate recognition, Cdk binding and Cdk activation have been shown to depend on both the cyclin box and the C-terminal domains of the cyclin [161].

Cyclins further modulate Cdk specificity by regulating subcellular localization of the active complex. Some cyclins contain sequence information that targets them to specific subcellular locations. Cyclin B1, for example, possesses both nuclear import and export signals within the amino-terminal half of the protein, outside the core Cdk-binding domain [128, 307]. During prophase, several residues within this region are phosphorylated, thereby inactivating the nuclear export signal and activating the import signal. This event promotes the rapid net movement of the cyclin B1 complex into the nucleus where it phosphorylates nuclear lamins to trigger nuclear envelope breakdown

[387]. The F-box protein, cyclin F, has also been shown to interact with cyclin B and promote its translocation into the nucleus [220].

Substrate targeting is further facilitated by the use of adaptor proteins. Suc 1, a small (9-13 kDa) protein binds the C-terminal lobe of the Cdk and interacts with phosphorylated residues on certain Cdk targets [295, 296]. This interaction is particularly important in the phosphorylation of mitotic Cdk substrates that contain clusters of multiple Cdk phosphorylation sites. After the cyclin-Cdk-Suc1 complex phosphorylates one site in a cluster, the ability of Suc1 to bind phosphate increases kinase-substrate affinity, leading to enhanced phosphorylation of the remaining sites **(Figure 1.6).**

It is also possible that the accessibility of some Cdk substrates changes during the cell cycle, such that certain substrates become available for phosphorylation only during a specific cell-cycle stage. The quantity of active cyclin-Cdk complexes may also be important in determining substrate specificity.

The list of candidate cyclin/Cdk substrates is extensive, including a number of chromatin-associated proteins, cytoskeletal proteins, transcription factors, protein kinases, tumour suppressors, phosphatases and the cyclins and Cdks themselves [275]. There are many more suspected substrates that have yet to be identified. Given that protein phosphorylation affects cellular outcome, the identification and characterization of the substrates involved in both proliferation and apoptosis may provide critical insight into the molecular mechanisms governing both processes.

II. Molecular mechanism of apoptosis

Apoptosis, or programmed cell death, is an evolutionarily conserved process found in all eukaryotic organisms, including yeast [97], and essential to the normal

development and homeostasis of those organisms. Cell death is marked by a series of prominent hallmarks including nuclear events such as chromatin condensation, DNA fragmentation, and breakdown of the nuclear envelope [393, 416]. These events are followed by the reorganization of the plasma membrane associated with blebbing, shrinkage, and loss of membrane phosphatidylserine asymmetry [92, 201]. Mitochondrial integrity is also compromised during cell death and cytochrome c is released concurrent with a loss of membrane potential [368, 421].

i. Caspases

Most morphological changes associated with apoptosis are caused by a set of aspartate-specific cysteine proteases known as caspases [149]. A caspase's distinct substrate specificity is determined by four residues amino-terminal to the cleavage site [383]. Currently, over a dozen caspases have been identified in humans, of which two-thirds are believed to function in apoptosis [81, 382]. These caspases have been classified into subfamilies according to their substrate preference, extent of sequence identity and structural similarity [149]. Caspases are synthesized as inactive precursors and undergo proteolytic maturation upon induction of apoptosis [318]. Once activated, caspases function to selectively cleave a restricted set of structural and regulatory proteins in a manner that can result in either the activation or inactivation of a particular protein. The CKIs, $p21^{Cip1/Waf1}$ [179] and $p27^{Kip1}$ [91, 240] are both inactivated by caspase cleavage, resulting in increased Cdk activity associated with apoptosis, while the p21-activated kinase 2 (PAK2) [25] and the CAD/ICAD dimer [89, 333] are activated by caspase cleavage. DNA laddering results from the caspase-3-mediated cleavage of ICAD (inhibitor of caspase-activated DNase), liberating the ICAD-bound CAD (caspase-activated DNase) [272], while cleavage of PAK2 seems to mediate the active blebbing

observed in apoptotic cells [331]. Caspase cleavage of numerous structural proteins induces the characteristic morphological changes of the apoptotic cell. Cleavage of the nuclear lamins is required for nuclear shrinking and budding [39, 317] while loss of the overall cell shape results from the cleavage of cytoskeletal proteins, fodrin and gelsolin [221].

ii. Caspase activation

There are two main pathways leading to the activation of caspases. One involves the interaction of a death receptor, such as the tumour necrosis factor receptor-1 (TNFR-1) [47] or the Fas receptor [400] with its ligand, and the second pathway depends on the participation of mitochondria [318]. Pro- and anti-apoptotic members of the Bcl-2 family regulate the mitochondrial pathway [2]. Cellular stress induces pro-apoptotic Bcl-2 family members to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome c, while the anti-apoptotic Bcl-2 proteins work to prevent cytochrome c release from mitochondria, and thereby preserve cell survival [2, 9]. Once in the cytoplasm, cytochrome c catalyzes the ATP-dependent oligomerization of apoptotic protease activating factor-1 (Apaf-1), which recruits and facilitates the processing of the initiator caspase, procaspase-9, to form an active complex [324] known as the apoptosome [245]. The apoptosome then activates the effector caspases, procaspase-3 and –7, which are primarily responsible for dismantling the cell during the execution phase of apoptosis [31]. However, this activation of the effector kinases can only occur if the caspase-binding cytosolic inhibitor of apoptosis proteins (IAPs) are first inactivated by interaction with either Smac/Diablo (second mitochondria-derived activator of caspases and direct IAP binding protein with low pI) or Omi/htra2 [94, 372]. Alternatively, ligation of death receptors causes the activation of the initiator caspase,

procaspase-8 [222]. The mature caspase may now either directly activate procaspase-3 or cleave the pro-apoptotic Bcl-2 homology 3-only protein Bid [243], which then subsequently induces the release of mitochondrial pro-apoptotic factors, including cytochrome c and Smac/Diablo [222]. Nevertheless, the end result of either pathway is caspase activation and the cleavage of specific cellular substrates, resulting in the morphological and biochemical changes associated with the apoptotic phenotype.

iii. Caspase-independent apoptosis

While caspases are clearly important in mediating the apoptotic process, several caspase-independent mechanisms of apoptosis have also been described. In 1996, Zamzami *et al.* isolated a factor that induced apoptosis independently of caspases [369, 429]. This protein was recovered from mitochondria treated with the mitochondrial adenine nucleotide translocator ligand atractyloside and named apoptosis inducing factor (AIF). It was found that induction of apoptosis triggers the translocation of AIF from the mitochondria to the cytosol and the nucleus where it induces peripheral chromatin condensation and high molecular weight (50 kb) DNA fragmentation. Another mitochondrial factor was recently implicated in mediating caspase-independent apoptosis [244]. Endonuclease G (Endo G) is a mitochondrial nuclease encoded by a nuclear gene that is released from the mitochondria following apoptotic stimulation. Endo G translocates to the nucleus where it generates oligonucleosomal DNA fragmentation even in the presence of caspase inhibitors [244]. In addition to mitochondria-regulated factors, a number of proteases have been implicated in mediating both caspase-dependent and independent apoptosis. The translocation of cathepsin proteases from lysosomes to the cytosol and/or nucleus correlates with the appearance of gross morphological changes indicative of apoptosis [186, 187]. The serine protease granzyme A induces death by

generating DNA single-strand breaks by activating a DNase through proteolytic cleavage [20], while granzyme B triggers a slower necrosis-like form of cell death in the presence of caspase inhibitors [374]. Granzyme B has also been shown to induce apoptosis by activating cyclin A/Cdk1 and cyclin A/Cdk2 complexes [349], suggesting a role for the cell cycle regulators in mediating apoptosis.

iv. Cell cycle regulators and apoptosis

Caspases cleave a series of cell cycle regulators including: the inhibitory wee 1 kinase; the APC component, CDC27; the CKIs, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$; and the E2F regulator, pRb [1, 91, 113, 120, 174, 179, 240, 435], among others. Cleavage of these factors has been shown to promote the activation of downstream Cdks. For example, the induction of apoptosis in human hepatoma SK-HEP-1 cells following treatment with ginsenoside-Rh2 resulted in caspase 3-mediated cleavage of $p21^{Cip1/Waf1}$ and selective activation of cyclin A/Cdk2 [179]. The upregulating effects were blocked by treatment with olomoucine, a specific Cdk inhibitor. Conversely, cyclin A/Cdk2 complexes have been implicated in the activation of downstream caspases. Hiromura *et al* [152] found that UV-induced apoptosis of mouse mesangial cells was associated with translocation of the cyclin A and Cdk2 proteins from the nucleus to the cytoplasm, where they formed an active complex. Caspase 3 activity was also significantly increased in UV irradiated cells. Treatment with the Cdk inhibitor, roscovitine, reduced both Cdk2 and caspase 3 activity. The exact mechanism of caspase 3 activation remains to be determined. Hiromura *et al.* examined Bcl-2, BAD and BAX, but were unable to show that these proteins colocalized with Cdk2 or that they were phosphorylated. The relationship between Cdk and caspase activity remains controversial. Several investigators have shown that Cdk activity functions upstream of the caspases [131, 152, 271], while others

have shown that it functions downstream of caspase activation [1, 96, 113, 137, 240, 422, 435]. Therefore, the role of Cdk activity in apoptosis may depend on the cell type and and/or the nature of the apoptotic stimuli.

The involvement of cell cycle regulators in mediating apoptosis is well established in the literature; however, the exact nature and function of these proteins in regulating apoptosis remains to be determined. The Cdk inhibitors, roscovitine, olomoucine and flavopiridol have been used to implicate the activation of Cdks in mediating apoptosis in a variety of cell systems, including neurons [115, 255, 289, 291], *Drosophila* cells [271] and mammalian mesangial cells and fibroblasts [152]. Numerous studies have further identified a role for active Cdk1 and Cdk2 in apoptotic cells [1, 27, 53, 66, 108, 120, 131, 137, 152, 163, 240, 263, 264, 349, 350, 435]. The regulatory cyclin partner has not been identified in all cases of Cdk activation; however, cyclin A has been most commonly linked to the onset of apoptosis [1, 27, 96, 152, 164, 240, 263, 264, 349, 384]; while cyclin B has shown only occasional involvement [164, 358]. Cyclin D complexes have also shown some involvement in the induction of apoptosis in neurons [101, 175, 286, 290, 293] and *Xenopus* embryos [96].

To date, the exact function of Cdk complexes in mediating apoptosis remains uncertain. As serine/threonine kinases, it is likely that the Cdks act in concert with the caspases in modifying structural and regulatory proteins within the cell, resulting in an apoptotic outcome. However, the caspases have also shown a direct effect on the activation of cyclin/Cdk complexes through cleavage of the cyclin subunit in an activational capacity.

III. Alternative forms of Cyclins and Cdks

In recent years, alternative forms of various cyclins and Cdks have been described. Either alternative splicing of the mRNA or proteolytic cleavage of the protein generates these forms. The functional significance of these proteins remains to be fully elucidated; however, they have been implicated in such diverse roles as stimulating the onset of disease, including cancer [23, 138, 202, 309, 310] and Alzheimer's [139]; slowing cell cycle progression [88, 242, 254]; stimulating apoptosis [96, 260]; and contributing to the onset of differentiation [411].

Of particular interest are the apoptosis-promoting truncations. The truncation of cyclins D, E and cyclin A has been implicated in the promotion of apoptosis [96, 260]. Cyclin E truncations are commonly associated with cancer progression [23, 138, 202, 309, 310]; however, an 18 kDa form of cyclin E has been implicated in apoptosis of hematopoietic tumor cells [260]. This cleavage product was generated in a caspase-dependent manner, eliminating interaction with Cdk2 and resulting in a loss of the associated kinase activity. By contrast, many of the previously described truncated cyclin E proteins have demonstrated increased Cdk2-dependent kinase activity [23, 138, 202, 309, 310].

An N-terminal truncated form of cyclin A (cyclin A^t) was found *in vitro*, in cultured mammalian cells, and *in vivo*, in mouse tissues. This truncated form was 5 kDa smaller than the full-length 60 kDa protein, was induced by cell density and localized to the cytoplasm where it interacted with Cdk2 [196]. Cyclin A overexpression in tumor cells has been shown to correlate with a low survival rate [134, 169, 431]. For this reason, it is interesting to note that cyclin A^t was undetectable in transformed cells such as A549,

SW2, and PC3 while it was found at high levels in the untransformed human primary Hs68 cells. This could indicate a relationship between abundance of the N-terminally cleaved cyclin A and transformation.

Another truncated cyclin A protein was recently identified in apoptotic, irradiated *Xenopus* embryos [96]. This truncated protein resulted from the cleavage of cyclin A2 by caspases and was found to form a kinase-active complex with Cdk2 (ΔN-cyclin A2-Cdk2) that was resistant to inhibition by $p27^{Xic1}$ and insensitive to degradation by the ubiquitin-mediated proteasome pathway. This complex demonstrated an expanded substrate specificity that included histone H2B. Phosphorylation of histone H2B at S-32 was proposed to facilitate DNA cleavage. Consistent with a role for cyclin A2 in apoptosis, it was found that the addition of ΔN-cyclin A2-Cdk2, but not full-length cyclin A2-Cdk2, to *Xenopus* egg extracts triggered apoptotic DNA fragmentation even when caspases were not activated. Cyclin D1 was similarly targeted by caspases, generating a product with higher affinity for $p27^{Xic1}$. This interaction lead to reduced phosphorylation of the retinoblastoma protein (pRb) during apoptosis [96].

The effector caspases are thought to induce the morphological and biochemical changes associated with the apoptotic phenotype, while the cyclin/Cdk contribution remains more elusive. Attempts at determining the exact function of the cyclin complexes in mediating apoptosis have proven inconclusive and have focused only on the cell cycle regulatory kinases. While it seems evident that the cell cycle kinases are involved in the apoptotic process, it remains important to consider the actions of the transcriptional Cdks. Studies utilizing the Cdk inhibitors, roscovitine, olomoucine and flavopiridol, have focused on their inhibition of Cdk1 and Cdk2. It has only recently

been determined that these Cdk inhibitors are equally effective against the transcriptional Cdks [401]. The involvement of the transcriptional Cdks in apoptotic processes has not been extensively examined.

IV. Cyclins/Cdks regulate transcription

Cdks were originally identified as cell cycle regulating enzymes; however, members of the Cdk family share more diverse functions. Among the nine previously characterized enzymes, Cdk 7, 8, and 9 are known to regulate transcription through phosphorylation of RNA polymerase II (RNAP II) [33].

In eukaryotes, RNAP II is responsible for transcribing protein-encoding genes. The RNAP II preinitiation complex includes both the RNAPII catalytic core (composed of 12 subunits) and a set of associated general transcription factors (GTFs) that include the TATA box binding protein (TBP), TBP associated factors ($TAF_{II}s$), TFIIB, TFIIE, TFIIF and TFIIH [133, 414]. The mammalian RNA polymerase II large subunit (RNAP II-LS) consists of 1970 amino acids and contains an essential carboxy-terminal domain (CTD) composed of 52 tandem repeats of a heptapeptide with the consensus sequence, YSPTSPS. The CTD repeats may or may not be perfect iterations of the consensus heptapeptide [33]. Five out of seven amino acids in the CTD have the potential to be modified by phosphorylation.

The recruitment and assembly of active transcription/RNA processing complexes within the nucleus is regulated by phosphorylation of the RNAP II-LS CTD. Phosphorylation of this subunit is further thought to coordinate transcription with cell cycle progression. RNAP II-LS binds to the basal promoters of eukaryotic genes as a hypophosphorylated protein, known as RNAP-IIa. During the pre-initiation and initiation

stages of transcription, transcription factors are recruited to the hypophosphorylated CTD, resulting in its phosphorylation to the hyperphosphorylated form, RNAP-IIo [56, 194]. Phosphorylation of the CTD allows promoter clearance, transcript elongation and recruitment of additional proteins involved in the processing of pre-mRNA [154-156, 194, 204].

i. Cdk7

The CTD is phosphorylated on a portion of RNAP II molecules *in vivo* and is phosphorylated by the general transcription factor TFIIH *in vitro.* TFIIH is composed of at least 9 subunits, which include Cdk7, cyclin H and MAT1 [335]. Cdk7, cyclin H and Mat1 also form a ternary complex known as Cdk-activating kinase (CAK), which functions to activate Cdks through phosphorylation of a threonine residue within the T-loop [188, 344, 356, 359]. TFIIH phosphorylates IIa shortly after the pre-initiation complex assembles at the promoter, allowing promoter clearance and the onset of transcript elongation [102]. Furthermore, alteration in the phosphorylation status of the CTD changes the affinity of its binding proteins. The general transcription factors involved in initiation (TFIID, E, and F) bind the IIa form [56, 194], while proteins involved in pre-mRNA capping, polyadenylation, and splicing bind preferentially to the more highly phosphorylated IIo form [154-156, 194, 204].

ii. Cdk8

The Cdk8/cyclin C complex has been found to associate with the RNAP II holoenzyme in mammalian cells; however, neither the level of this cyclin/Cdk complex or its associated CTD kinase activity have shown any change during the cell cycle [321]. Both Cdk7/cyclin H and Cdk8/cyclin C are associated with RNAP II early in the transcription cycle (at or near pre-initiation complex formation) and appear to

phosphorylate consensus YSPTSPS heptapeptides at the S-5 position [148, 320] and the S-2 position [79, 80, 366]. Despite similarities in substrate specificity and temporal activation, the two holoenzymes remain biochemically distinct [320]. Cdk7/cyclin H has a greater affinity for non-consensus heptapeptides than Cdk8/cyclin C. Cdk8/cyclin C has further shown an inhibitory function in regulating the transcription of a subset of genes involved in control of cell type specificity, meiosis and sugar metabolism [224, 367, 399]. Cdk8/cyclin C inhibits transcription by phosphorylating the CTD before the RNAP II holoenzyme has bound to the promoter to form the initiation complex [148, 366]. Cdk8/cyclin C can also regulate transcription through repressive phosphorylation of cyclin H [4].

iii. Cdk9

Cdk9/cyclin T forms a complex known as P-TEFb [303]. P-TEFb promotes CTD phosphorylation after promoter clearance to regulate elongation. To date, two cyclin T genes, T1 and T2, and cyclin K have been found in association with Cdk9 [104, 303]. In most eukaryotic cells examined, the level of cdk9 and cyclin T proteins as well as the associated kinase activity does not vary appreciably during the cell cycle [110]; however, the induction of cdk9/cyclinT1 CTD kinase activity was found to be sensitive to mitogenic stimulation [110].

iv. Additional CTD kinases

PITSLRE, casein kinase II (CKII), Cdk1, the mitogen-activated protein kinases (MAPK), extracellular-regulated protein kinases, ERK-1 and -2 and the tyrosine kinase, c-Abl have also been shown to phosphorylate CTD [281]. PITSLRE is another member of the Cdk superfamily and has been shown to bind to the hyperphosphorylated form of CTD as well as to CKII [259]. CKII phosphorylates three non-consensus heptapeptide

repeats toward the carboxyl-terminus of human CTD [388] and is essential for cell cycle progression in the G_1 phase of the cell cycle [304]. Cdk1 activates and stabilizes CAK through phosphorylation [229], while possibly inactivating TFIIH during mitosis [253]. The MAPK proteins, ERK-1 and -2, are mitogenically activated to phosphorylate CTD [26, 78]. *In vitro* studies have shown a preference for phosphorylation of the S-5 residues within the consensus sequence [258, 389], resulting in the generation of a novel form of RNAP II-LS denoted IIm [26]. The physiological role of the IIm forms remains to be determined; however, it does not appear to be engaged in transcription and is exclusively phosphorylated at position S-5 [26, 389], whereas the pool of transcriptionally active IIo in unstimulated mammalian cells is phosphorylated on both S-5 and S-2 [32, 44, 297]. While the amino acids targeted for phosphorylation within the CTD are primarily serine residues, c-Abl has been shown to phosphorylate tyrosine residues in response to DNA damage [403].

Given the extensive number of kinases involved in phosphorylating and regulating the CTD of RNAP II, it is not surprising that the CTD requirement for RNAP II transcription *in vitro* has been shown to vary between promoters, suggesting that the relative phosphorylation RNAP dictates the specificity of gene transcription [5, 40, 193].

V. The Mitogen-Activated Protein Kinase (MAPK) pathway

The involvement of the MAPK effectors in regulating transcription *via* direct phosphorylation of RNAPII is of particular interest given the extensive overlap between activation of the MAPK pathway and cell cycle control.

Growth factors and the extracellular matrix provide the environmental cues that control the proliferation of most cell types. These factors bind to surface receptors and

activate a series of cytoplasmic signal transduction cascades. Activation of the ERK subfamily is a well-characterized response to both receptor tyrosine kinase (RTK) and integrin activation [55, 181, 241, 338] and this activation has been implicated in diverse cellular activities including cell proliferation, differentiation, migration, and apoptosis [219].

MAPK activity is regulated through three-tiered cascades composed of three kinases. In this cascade, a MAPK kinase kinase (MAPKKK) phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates a MAPK **(Figure 1.7).** To date, six MAPK modules have been identified which share structurally related components, but seem to mediate specific biological responses. These include the ERKs, c-Jun N-terminal kinase, p38, ERK5, nemo-like kinase and ERK3α, β pathways [301].

The ERK pathway initiates cell cycle progression by both direct and indirect regulation of transcription [26]. In this pathway, the GTPase, Ras, transduces extracellular stimuli through activation of the MAPKKK, Raf. Raf then activates the MAPKK, MEK, which in turn activates ERK through dual phosphorylation of the conserved TEY sequence in the activation loop [299]. ERK1 and ERK2 are proteins of 44 and 42 kDa, respectively, that are nearly 85% identical overall, with much greater identity in the core regions involved in binding substrates [30]. They are ubiquitously expressed, although their relative abundance in tissues is variable. They are activated by numerous stimuli including, serum, growth factors, cytokines, certain stresses, ligands for GPCRs and transforming agents.

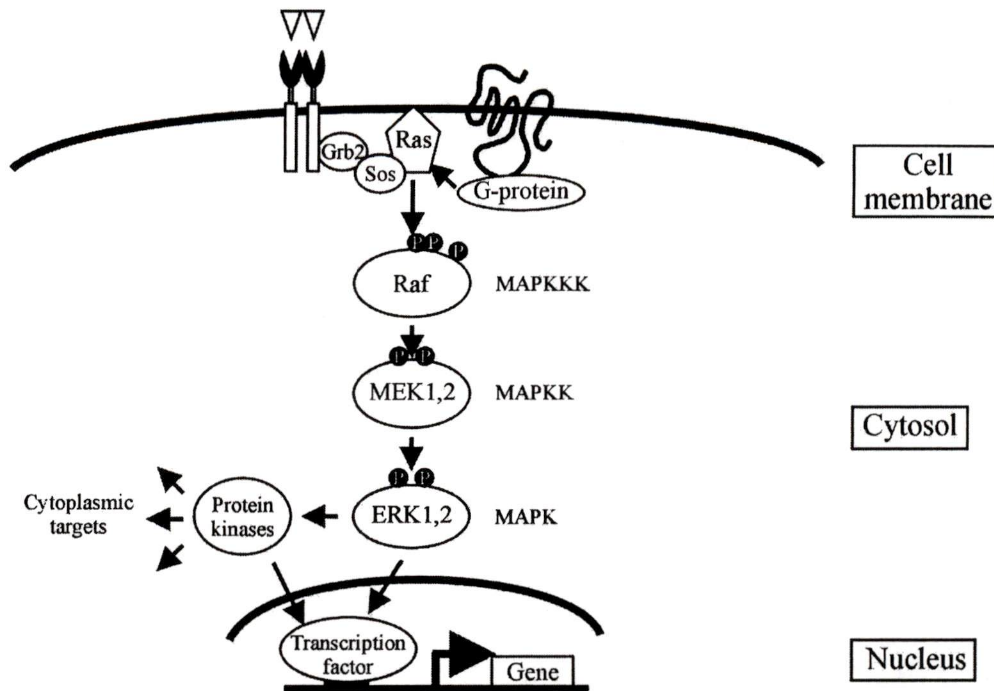


Figure 1.7. Schematic representation of the mitogen-activated protein kinase (MAPK) pathway, focusing on the members of the ERK1,2 pathway.

G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTK) transduce extracellular stimuli into the cytoplasm *via* the Ras proteins situated at the inner surface of the plasma membrane. Ras activity is controlled by a regulated GDP/GTP cycle. Signaling through RTKs activates the MAPK pathway through the adaptor protein, Grb2. Grb2 interacts and activates the guanine nucleotide exchange factor, Son of Sevenless (SOS), which promotes the formation of the active GTP-bound, Ras. Ras recruits the MAPK kinase kinase (MAPKKK), Raf, to the plasma membrane where it is activated. Upon activation, Raf phosphorylates and activates two MAPK kinases (MAPKK), MEK-1 and -2. Activated MEKs function as dual specificity kinases, and phosphorylate tandem threonine and tyrosine residues (TEY motif) in the two MAPKs, ERK-1 and -2. Once activated, the MAPKs phosphorylate various cytoplasmic substrates and then translocate into the nucleus where they activate transcription factors to induce changes in gene expression. Activated cytoplasmic protein kinases act in a similar manner. Adapted from Frodin *et al* [103].

Once active, the ERKs phosphorylate a variety of cytosolic substrates including a number of protein kinases, such as the p90 kDa ribosomal S6 kinases, Rsk1, Rsk2, Rsk3, MAPKAP kinase-2, the MAPK-integrating kinases-1 and -2 (Mnk-1, Mnk-2) [105, 112, 162, 362, 364, 407, 434], and the mitogen- and stress-activated protein kinases-1 and –2 (MSK-2 and MSK-2) [69]. Once activated, the cytoplasmic protein kinases can also phosphorylate cytoplasmic targets, including glycogen synthase kinase-3 (GSK-3) [370], the Ras GTP/GDP-exchange factor, Son of Sevenless (Sos) [76], the transcription factors NFκB and IκBα [114], and the estrogen receptor alpha, ERα [180]. Both the activated protein kinases and the ERKs then translocate into the nucleus where they phosphorylate various transcription factors and chromatin structural components to modulate gene expression.

In the nucleus, the ERKs phosphorylate the activating protein-1 (AP-1) family of transcription factors (c-Jun, c-Fos, ATF-2) [48, 49, 70, 125, 151], the ternary complex factors (TCFs) (Elk-1, SAP-1 and SAP-2) [122, 176, 177, 312, 363], the thyroid hormone receptor beta 1, TRβ1 [65], as well as many other transcription factors such as the signal transducers and activators of transcription (STAT)[342]. ERK2 has also recently been shown to modulate hormone-dependent gene transcription by phosphorylating the steroid receptor coactivator-1 (SRC-1). This protein possesses an intrinsic histone acetyltransferase activity while also functioning as a steroid nuclear receptor coactivator [329].

The Rsks also phosphorylate a number of transcription factors in the nucleus, including cAMP response element-binding protein (CREB) [417] and c-Fos. Further, the

Rsk proteins associate with the transcriptional coactivator proteins CREB-binding protein (CBP) and p300 [273] to modulate gene expression.

The cascade of phosphorylation events resulting from ERK activation serves to initiate a variety of gene expression profiles dependent upon cell type and stimulus. The predominant outcome of ERK activation is the induction of cellular proliferation through regulation of the cell cycle.

i. MAPK regulation of cell cycle progression

ERK regulation of cell cycle progression is extensive; however most studies have focused on the G_1 phase of the cell cycle. At this time point, the ERKs have been shown to regulate progression by controlling the expression of cyclin D1 [6, 12, 51, 231, 327, 345, 408, 412] and/or the Cdk-inhibitory proteins, $p21^{Cip1}$ and $p27^{kip1}$ [29, 197]. The intensity and duration of an ERK signal seems particularly important in determining whether cells will induce cyclin D1 or the Cdk2 inhibitor, $p21^{Cip1}$ [326].

Numerous studies have shown that a prolonged ERK signal is necessary for cyclin D1 induction [12, 29, 327, 408]. Balmanno and Cook [12] showed that the duration of the ERK signal affected gene expression. In their study, they showed that individual members of the *fos* gene family are differentially expressed in response to transient versus sustained ERK signals. A transient ERK signal (<1hr) induced c-fos, Fra-2, and c-jun in CCL39 fibroblasts, whereas a sustained ERK signal (5-20 hrs) was required to induce Fra-1 and JunB. This result is particularly relevant when considering cyclin D1 regulation. Fos and jun family members heterodimerize and bind to AP-1 sites in promoters [129] and the cyclin D1 promoter contains an AP-1 site [6]. Furthermore, some jun and fos family members also heterodimerize with certain ATF family members to activate the cAMP-response element (CRE)[129]. A CRE is present in the cyclin D1

promoter, and this site has been implicated in the regulation of cyclin D1 gene expression [150].

In addition to inducing cyclin D1 expression, ERK stimulates p21^{Cip1} induction in an intensity dependent manner [28, 29, 246, 250, 256, 345]. Low intensity signals were shown to lead to cyclin D1 induction while high intensity signals induced p21 expression [345] While possibly counterintuitive, this induction of the p21-family of CKIs is not inhibitory of cell cycle progression. These proteins bind and inhibit the activity of cyclin E/Cdk2 complexes, while promoting the assembly of cyclin D/Cdk4/6 complexes without inhibiting their kinase activity [50, 226]. This interaction seems to result in the redistribution of the total Cip/Kip pool, by titrating the Cip/Kips away from cyclin E/Cdk2 and facilitating the activation of those complexes [50, 346]. The Cip/Kip family of inhibitors promotes cell cycle progression as long as their levels are downregulated in mid-late G_1 phase. Long-term induction of p21^{Cip1} and/or failure to downregulate p21^{Cip1} has been repeatedly linked to the inactivation of cyclin E/Cdk2 and G_1 phase arrest [29, 93, 252, 313, 345, 412, 436].

The ERKs further promote cell cycle progression through the G_1/S transition by phosphorylating p27^{Kip1}, thereby preventing its interaction with Cdk2 [197]. ERK regulation of Cdk2 activation extends to control of Cdk2 subcellular localization. Blanchard *et al.* [24] found that Cdk2 associated with ERK *in vivo* in Kit 225 T lymphocytes and that this interaction was necessary for the nuclear translocation of Cdk2.

While the ERK pathway has been fully elucidated, the regulation of that pathway and, consequently, the regulation of cell cycle progression, extends beyond the canonical members of the MAPK module to additional regulatory pathways.

ii. Additional regulation of G_1 progression

The phosphatidylinositol 3-kinase (PI3K) pathway has also been reported to contribute to cyclin D1 mRNA induction [121, 373] as well as regulating the translation and stability of the cyclin D1 protein *via* glycogen synthase kinase 3 (GSK-3) [72, 270]. This pathway is also intimately linked to the activation of the MAPK signal transduction pathway **(Figure 1.8).** Ras has been shown to activate Raf indirectly through the PI3K pathway. Activation of this pathway results in the generation of phospholipid products that can activate Rac, a small G-protein that binds and activates the p21cdc42/rac1-activated serine/threonine kinase (PAK) [365]. PAK-3 has recently been shown to phosphorylate Raf-1 on serine-338, one of the sites whose phosphorylation is required for activation [208]. In addition, PI3K may also supply an inhibitory signal *via* Akt, which has been reported to suppress Raf-1 activity by phosphorylation of serine-259 [437].

VI. The Amphibian Model

The interactions among the numerous signaling networks regulating proliferation and apoptosis are complex. Numerous studies have utilized cell lines to investigate the functions of those pathways in various contexts including, most often, chemical and UV exposure. Alternatively, models of embryonic development have been applied using *Xenopus laevis* oocytes [46, 52]. However, the postembryonic amphibian presents an excellent system for studying proliferation, differentiation and apoptosis in a developmental context as the entire organism is remodeled during metamorphosis in response to a single hormonal signal [74].

Amphibian metamorphosis is an intricate postembryonic developmental process during which a larval tadpole is transformed into a frog [378]. During this process many

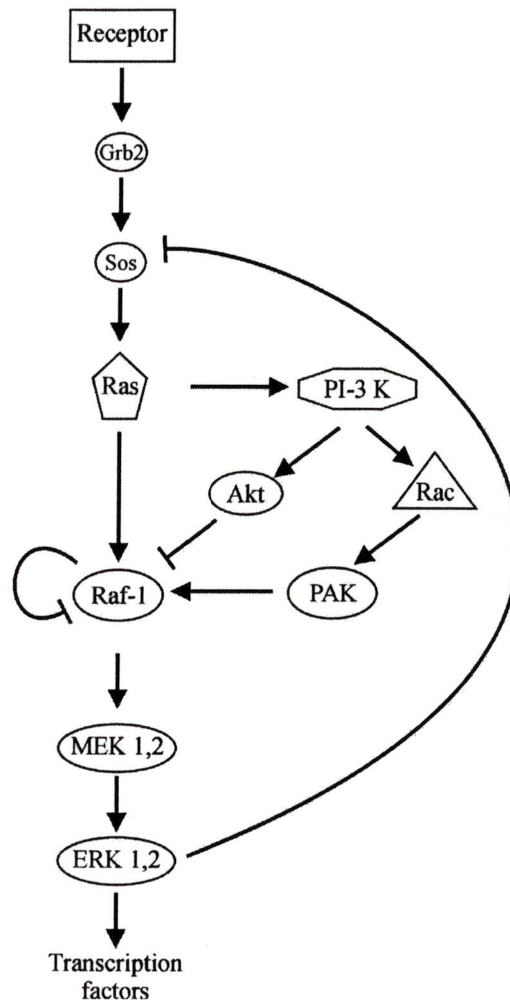


Figure 1.8. Cross-talk between the Ras/Raf/Mek/ERK signaling network and the phosphatidylinositol 3-kinase (PI3K) pathway.

Ras can regulate both the PI3K and the MAPK pathways. Extracellular stimuli activate the membrane-associated G-protein, Ras, via the GDP/GTP exchange factor, Son of Sevenless (SOS) and the adaptor protein, Grb2. The MAPK pathway is activated as described in figure 1.7; however, it can be further regulated by the PI-3K pathway. Ras activation of PI-3K results in the generation of phospholipids that recruit the small G-protein Rac and the Ser/Thr kinase, Akt. Akt suppresses Raf-1 activity, while Rack stimulates Raf-1. Both ERK-1 and –2 and Raf regulate the activation of the MAPK pathway by negative feedback. Adapted from Kolch [219].

morphological and biochemical changes occur within the tadpole to ensure that every organ system is adapted to a terrestrial lifestyle from an aquatic one. The metamorphic process induces dramatic changes in the structural organization of the animal, requiring not only synthesis, but also carefully controlled degradation and removal of structural elements. During this process, tadpole specific organs, such as the tail and gills, are completely resorbed, while frog specific organs, such as the limb, develop *de novo* [170]. However, the vast majority of organs are present in both tadpoles and frogs and undergo extensive remodeling in order to function in a post-metamorphic frog [117, 353, 427].

Amphibian metamorphosis is induced by thyroid hormone (TH) and is one of the best-studied hormone-regulated developmental processes [74, 117, 118, 203]. Early studies demonstrated that blocking the synthesis of endogenous TH inhibited natural metamorphosis while adding exogenous TH to the rearing water of premetamorphic tadpoles induced precocious metamorphosis [74, 375]. Furthermore, this regulation by TH was shown to target each organ independently, suggesting that TH directly affects gene expression in each organ [189].

i. The tadpole tail as a model for studying apoptosis

Thyroid hormones play important roles in vertebrate homeostasis, differentiation and development and these effects are mediated genomically *via* the nuclear thyroid hormone receptors (TRs) and non-genomically *via* phosphorylation of the nuclear receptor or through a putative membrane receptor. TH-mediated effects are extensive and the postembryonic amphibian presents an ideal model for studying those effects.

The regressing tail of the metamorphosing tadpole was one of the first systems in which apoptosis was described and represents an excellent model for the study of that process [200, 355, 376, 377]. This is due to the easy manipulation of the free-living

animal and the ease of inducing precocious metamorphosis in both the live animal or in cultured organs [145, 207].

The tadpole tail is a complex tissue composed primarily of epidermis, muscle, connective tissue, nerve tissue and notochord **(Figure 1.9)**. The tail epidermis is of particular interest as it is structurally similar to the body epidermis and yet demonstrates a dramatically different fate during metamorphosis. The tail epidermis undergoes apoptosis in the normal course of development [276, 427], while the body epidermis becomes transformed into an adult skin type that undergoes proliferation and differentiation **(Figure 1.10)** [172, 323]. The differences in tissue fate are thought to be due largely to the cell types present in each structure. The tail skin is composed of two tissues of developmentally different origin: epidermis and dermis (mesenchymal tissue) [427]. These two tissues are separated by basement membrane. The tail epidermis is made up of two cell types: the apical and skein cells. The apical cells make up the outermost layer of the epidermis and differ from the underlying skein cells by the lack of figures of Eberth [427]. The figures of Eberth are threadlike structures in the epidermal cells of frog tadpoles composed of intermediate filaments (11 nm in diameter) [323] that facilitate the attachment of the skein cells to the basement membrane. The body epidermis also contains apical and skein cells, but has an additional layer of basal cells located beneath the skein cells [265]. Mitotic activity in the body epidermis is limited to these basal cells which subsequently differentiate into granular and cornified cells of the froglet [172]. These differences facilitate the study of proliferation and apoptosis in similar developmental systems.

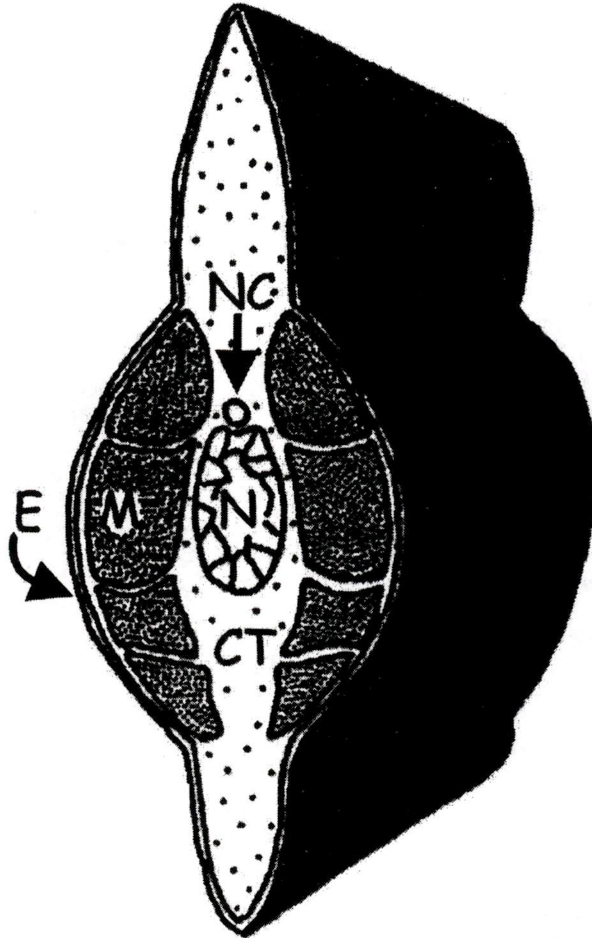


Figure 1.9. Tadpole tail cross-section.

The epidermis (E), muscle (M), connective tissue (CT), nerve chord (NC) and notochord (N). Modified from Yoshizato 1989 [427].

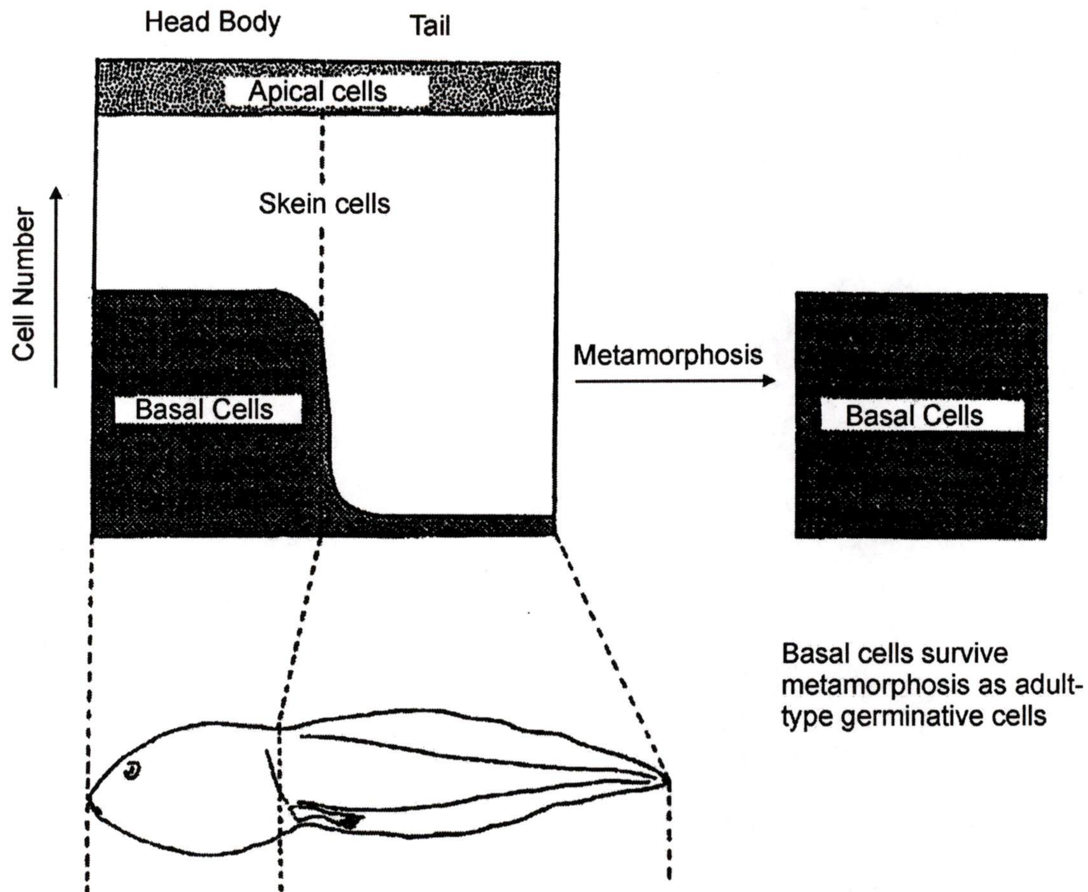


Figure 1.10. Epidermal cell fate.

The body epidermis is composed of apical, skein and basal cells, while the tail epidermis consists of apical and skein cells alone. During metamorphosis, the body basal cells are mitotically active and develop into adult-type germinative cells, while the apical and skein cells undergo apoptosis. Adapted from Yoshizata 1989 [427].

TH-dependent amphibian metamorphosis is largely induced by changes in gene expression following TH binding to nuclear TRs, which act as transcription factors. The metamorphic dependence on these receptors was shown by Schreiber *et al.* [340] who developed a transgenic tadpole expressing a dominant negative form of TRα. These animals showed resistance to a wide range of metamorphic events induced by TH. Up-regulation of the TRβ gene by TH has also been shown to contribute to the establishment of tissue-specific genetic programs necessary for metamorphosis [192, 198, 284, 316, 332]. Changes in gene expression leading to tail resorption have been studied extensively in *Xenopus laevis* [35, 36, 86, 394, 406] and *Rana catesbeiana* [111, 282, 371, 395, 419]. Using a novel 420-gene frog cDNA array (MAGEX) and real-time quantitative polymerase chain reaction (QPCR), Veldhoen *et al* [394] recently identified 79 genes whose steady-state mRNA expression levels were altered in the tail of *Xenopus laevis* tadpoles during natural metamorphosis. The significance of these changes in mRNA transcripts have yet to be correlated to significant changes in protein steady state levels, although those studies are currently underway [147].

VII. Thyroid hormones

Two naturally occurring thyroid hormones, T_4 (thyroxine) and T_3 (3,5,3'-triiodothyronine) are synthesized by the thyroid gland. Although T_4 is the main form of thyroid hormone secreted from the thyroid gland into circulation, T_3 is more active and is the predominant form that interacts with receptors in target organs [189]. T_4 is converted to T_3 in the thyroid gland and target organs through the actions of a 5'-deiodinase [354]. Both forms of TH can also be inactivated through the action of 5-deiodinases, producing 3,3'-diiodothyronine (T_2) and reverse T_3 (rT_3) from T_3 and T_4, respectively. Both 5'- and

5-deiodinase show distinct developmental patterns of regulation in various organs of the metamorphosing tadpole, suggesting that T_3 and T_4 levels can vary in different organs and tissues, likely contributing to their tissue-specific biological function [61].

i. Thyroid hormone receptors

The biological effects of TH are primarily mediated by the regulation of gene expression through interaction with the nuclear thyroid hormone receptors (TRs). These receptors are ligand-dependent, DNA-binding transcription factors that induce a cascade of gene expression following ligand-binding **(Figure 1.11)**, leading to the metamorphosis of individual tissues [233, 257, 353, 391, 426]. TRs can form monomers, homodimers and heterodimers with retinoid X receptors (RXRs) on the thyroid hormone response element (TRE) [212]. Unliganded TR generally represses basal transcription, while ligand binding triggers a conformational change in the TR, resulting in activated transcription of the target gene [257].

The TRs are encoded by two separate genes, designated TRα and TRβ, located on different chromosomes [232]. Alternative splicing from each gene generates multiple TR isoforms, including TRα1, TRα2, TRα3 from the TRα gene and TRβ1 and TRβ2 from the TRβ gene [432]. The TR structure is modular with five regions and 4 functional domains (A-E) (Figure 1.10). The amino terminal A/B domain is of variable length and sequence divergent among TR isoforms and among different species [216]. The exact function of the amino-terminal domain is controversial; however, some studies have suggested that it may be important for transcriptional activation and interaction with the general transcription factor TFIIB [13, 126]. It may also modulate ligand-independent

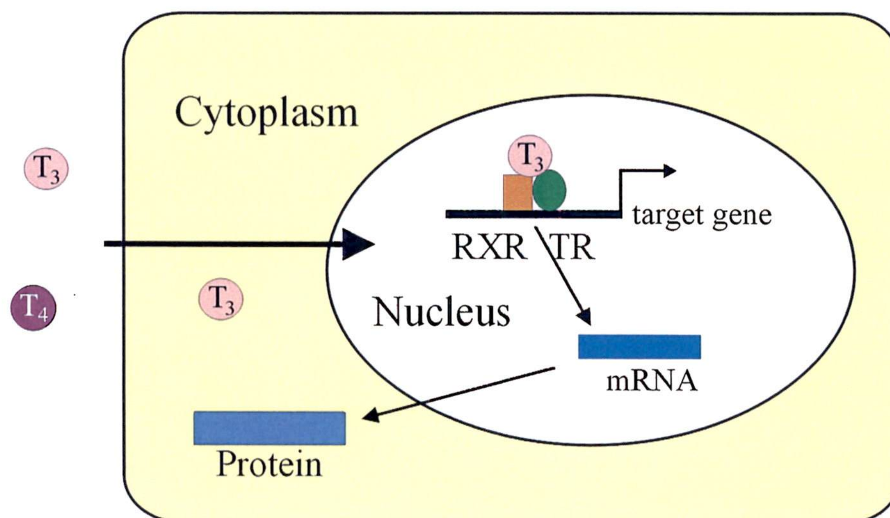


Figure 1.11. General model for thyroid hormone action in the nucleus.

The bioactive hormone, T_3, passes into the nucleus where it binds to the thyroid hormone receptor-retinoid X receptor (TR-RXR) heterodimer, already bound to a thyroid hormone response element (TRE). Interaction of the T_3 ligand with the TR-RXR heterodimer induces gene transcription, resulting in the production of a newly synthesized protein. Adapted from Yen [425].

repression by interacting with corepressors [158] and influence the conformation of the DNA-binding domain (DBD) to affect the TRE-binding specificity [127, 185]. The DBD is located in the central portion of the TR in the "C" module. This structure has two zinc fingers, each composed of four cysteines coordinated with a zinc ion and functions to mediate interactions with the TRE and dimerization **(Figure 1.12).** The hinge region follows the DBD and is believed to contain a nuclear localization signal (NLS) as well as corepressor binding sites [90, 159]. The T_3 ligand binds the receptor at the carboxy-terminal ligand-binding domain (LBD) in a hydrophobic pocket formed by discontinuous stretches of amino acids [398]. In addition to ligand-binding, the LBD also plays critical roles in dimerization, transactivation and basal repression by unliganded TR [98, 99].

ii. Mechanism of TR transcriptional regulation

As a transcription factor, the key function of the TR is to regulate gene expression in response to ligand binding. The TR constitutively binds to DNA response elements in both the absence and presence of the ligand. Unliganded TR represses basal transcription while ligand binding enhances transcriptional activation through the association of protein cofactors with the receptor [433]. Corepressors and coactivators interact with the receptor and alter chromatin structure to either facilitate or inhibit transcription. This is achieved through the histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities of the cofactors [234]. Acetylation of histones in the nucleosome octamer loosens the interaction between DNA and the nucleosome by reducing the net positive charge. This induces a more open structure that allows assembly of basal transcriptional machinery and increased transcription. By contrast, deacetylation of the histone octamer creates a more condensed chromatin structure that blocks access of transcription factors to the DNA [432].

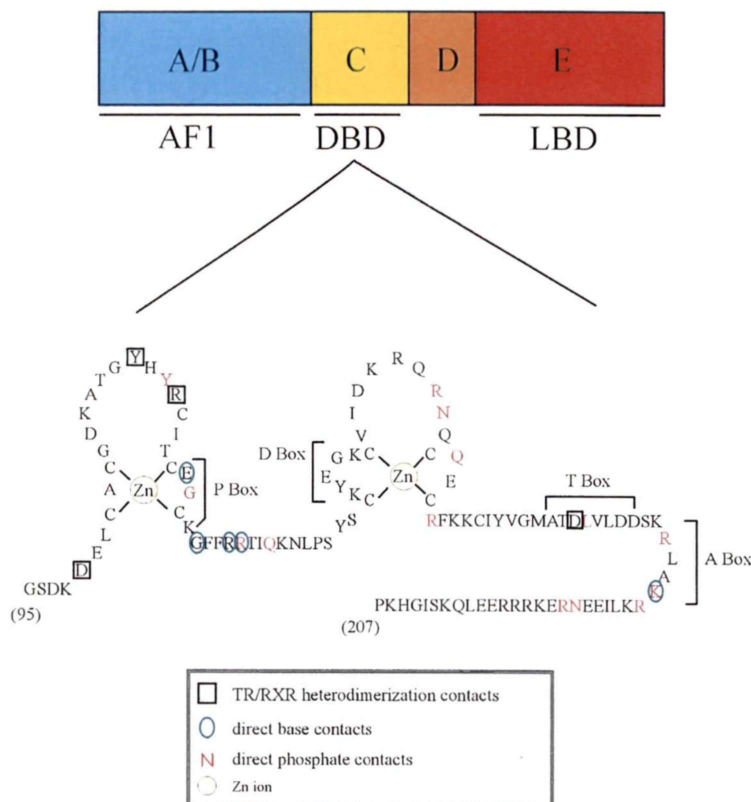


Figure 1.12. Modular structure of thyroid hormone receptor and the DNA-binding domain.

The amino-terminal A/B domain contains coactivator and corepressor interaction sites and modulates interactions with basal transcriptional factors. The DNA-binding domain (DBD) dictates the specificity of gene activation. It contains two zinc fingers of human and various subdomains. The "P box" is involved in the sequence-specific recognition of hormone response elements. The "D box" and "T box" are important for dimerization. The "A box" denotes residues that interact with the minor groove of the thyroid response element (TRE). The D domain, located between the DBD and the ligand-binding domain (LBD), is known as the hinge region. The hinge region is believed to contain a nuclear localization signal as well as functioning as a binding site for corepressor proteins. The E domain is the LBD. This domain is necessary for TH binding and also plays critical roles for dimerization, transactivation and basal repression of unliganded TR. Modified from Yen PM [425].

iii. Nongenomic effects

Posttranslational modification of proteins by phosphorylation has been known to play an important regulatory role in the functions of the protein [168, 225, 285]. Nuclear hormone receptors are substrates for a variety of protein kinases, and phosphorylation can have profound effects on protein stability, subcellular localization, DNA binding, and the transcriptional activity of these receptors [184, 195, 385, 386, 410]. Serine phosphorylation of the TRs has been described [238, 385, 386, 392] and has been most commonly attributed to protein kinase A (PKA) and CKII; however, a recent study by Davis *et al.* [65] identified the ERK as the kinase responsible for the phosphorylation of TRβ1 in 293T cells. This phosphorylation resulted in the dissociation of TR and the corepressor, silencing mediator of retinoid and thyroid hormone receptors (SMRT). Activation of ERK was mediated nongenomically by T_4 within 10-20 minutes following treatment and resulted in an interaction between ERK and TRβ1. T_4 was further shown to induce nuclear complexing of p53 and ERK, as well as TRβ1 and p53 [357]. Both of these interactions were dependent on ERK activation, as treatment with the ERK inhibitor, PD098059 prevented the interactions. Interestingly, nuclear complexing of p53 and ERK occurred in HeLa cells, which lack functional TR. This interaction resulted in the phosphorylation of p53, modulating the transcriptional activity of the tumour suppressor protein and leading to the accumulation of the immediate-early gene product, c-Jun. This accumulation was inhibited by T_4, while the co-application of PD098059 and T_4 abrogated the T_4-inhibitory effect, suggesting that the transcriptional activity of p53 was altered by T_4-directed ERK-p53 interaction.

Numerous studies have suggested that the THs may modulate cellular response in receptor-independent processes. Lin *et al.* [248, 249] found that T_4 could induce the tyrosine phosphorylation and nuclear translocation of STAT1α and STAT3 in the $TR^{-/-}$ HeLa and CV-1 cells in as little as 10-20 minutes. This effect was reproduced by treatment with T_4-agarose and blocked by numerous inhibitors, including the protein kinase C (PKC) inhibitor, CGP 41251; the protein tyrosine kinase (PTK) inhibitor, genistein; the ERK inhibitor, PD 098059; and, the Raf-1 inhibitor, geldanamycin. These studies suggest that T_4 can activate the STAT1α and STAT3 transcription factors by a receptor-mediated process requiring the activities of PKC, PTK and ERK. Lin *et al.* [247] subsequently implicated a GPCR in the activation of the ERK pathway. They found that activation of ERK by T_4 or agarose-T_4 in HeLa or CV-1 cells was pertussis toxin-sensitive and guanosine 5'-*O*-(3'thiotriphosphate)-sensitive, suggesting GPCR involvement.

These nongenomic actions have been found to be most strongly induced by T_4; however, rT_3 and T_2 have also shown some nongenomic effects [34]. The use of agarose-bound T_4, which cannot penetrate the cell membrane, indicates that the effector of the T_4 signal must reside in the membrane, while the preference for T_4 over T_3 suggests that the T_4 receptor is distinct from the nuclear receptor. No nongenomic effects have been currently ascribed to T_3.

VII. Research hypothesis and thesis outline

Cell cycle regulators have been implicated in the contrasting processes of cell proliferation and apoptosis. The function of the cyclins and cdks in regulating cell cycle

progression has been extensively studied over the past 30 years; however, the involvement of those proteins in apoptosis is a recent discovery and is not well understood. Therefore, the purpose of this thesis was to determine the function of the cyclins and the cdks in TH-dependent apoptosis using the tail of the metamorphosing tadpole as a model. It was hypothesized that changes in cdk activity would be evident during metamorphosis and that these changes would correlate with the induction of tail regression.

In the introductory chapter, the molecular mechanisms governing cell cycle progression and apoptosis are described. This discussion includes the involvement of cell cycle regulators and signal transduction pathways in regulating both processes. Hormonally-regulated apoptosis was studied in the tail of metamorphosing tadpoles; therefore, the amphibian model is described, with emphasis on the changes in gene expression induced by TH leading to the execution of the metamorphic program.

Chapter 2 examines the activity of cyclin A-containing complexes in the tail during natural metamorphosis. Cyclin A can interact with Cdks 1,2, and 3; therefore, the involvement of those kinases in mediating tail regression was examined. The Cdk inhibitor, roscovitine, was found to inhibit tail regression in a stage-dependent manner; however, it also inhibited T_3-induced tail regression at all developmental stages examined.

Roscovitine inhibits the activities of the transcriptional regulators, Cdk7 and Cdk9; therefore, the effect of roscovitine on phosphorylation of the transcriptional machinery and on the induction of the TH-dependent genetic program in the tail is examined in chapter 3.

Chapter 2. Cyclin dependent kinase activity is required for thyroid hormone-dependent tail regression in the frog tadpole

1. Introduction

Protein phosphorylation is a critical signaling mechanism that integrates a variety of stimuli to elicit an appropriate cellular response. Several distinct phosphorylation-dependent signaling cascades contributing to cell proliferation control have been described, including the extracellular signal-regulated kinase (ERK), cyclin dependent kinase (Cdk) and phosphatidylinositol-3-kinase (PI3K) pathways. Cell proliferation requires the activation of Cdks by periodically synthesized activators (cyclins) during each phase of the cell cycle [346]. Cdk4 and 6 are activated by D-type cyclins during early G_1, Cdk2 is activated by cyclin E in G_1/S and by cyclin A during S and G_2/M, and cyclin B activates Cdk1 during mitosis. Phosphorylation of Ser/Thr residues in target protein substrates results in an alteration of substrate function and cell cycle progression [346]. Although many substrates are still unidentified, Cdks are highly selective in their target substrates. Regulation of Cdk activity is complex and includes the use of cyclins, inhibitor proteins, phosphorylation, and subcellular localization. Several Cdks whose function is associated with critical non-cell cycle-related functions have recently been identified. These functions include regulation of RNA transcription, neural differentiation and insulin secretion [337]. Cdk hyperactivation by overexpression of particular cyclins is common in tumor cells and inhibition of Cdk activity promotes apoptosis in these cells [337].

Recent studies have also shown that Cdks can promote apoptosis in cultured cells and in transgenic mice [1, 27, 73, 119, 146, 157, 240, 263, 264, 292, 350, 422]. Cyclin

A/Cdk2 complexes promote apoptosis in the absence of DNA synthesis [263] suggesting that the regulation of proliferation and apoptosis can be uncoupled. Despite the pivotal role that Cdks appear to play in these disparate processes, it is still unclear how this control is accomplished.

In order to identify the molecular mechanisms whereby a cell fate is decided, the hypothesis that Cdk activity is required for apoptosis in a normal developmental context was examined. To address this issue, postembryonic development of the frog was employed as a model for studying developmental apoptosis. During this process, a marked elevation of endogenous thyroid hormone (TH) levels triggers the dramatic metamorphosis of the aquatic tadpole to a (semi-)terrestrial juvenile frog over a relatively short period of time (reviewed in [352]). As in mammals, thyroxine is the main secretory product of the thyroid gland [42] and it is converted by the deiodinase activity in peripheral tissues to the bioactive form, 3,5,3'-triiodothyronine, T_3 [18, 166, 199]. Virtually all tissues are targets for T_3 action and, depending upon the context, cells will proliferate, differentiate, redifferentiate, or undergo apoptosis. Postembryonic development is divided into three phases: premetamorphosis, prometamorphosis, and metamorphic climax. Premetamorphosis (TK [381] stages VI-XII) is the interval of development that precedes thyroid gland function and is mainly a period of growth. At this stage, the tadpole is competent to respond to TH, but is functionally athyroid. Prometamorphosis begins with maturation of the thyroid gland and the low-level secretion of TH that initiates the first overt metamorphic changes such as limb growth (TK stages XIII-XIX). TH levels rise and peak dramatically at metamorphic climax, which is characterized by rapid remodeling of the tadpole (TK stages XX-XXV). The

major metamorphic change of tadpole tail regression is accomplished through apoptotic pathways [352]. Many metamorphic changes can be precociously induced by exogenous TH administration *in vivo* or in organ and cell culture, and can be enhanced or inhibited by various agents including environmental contaminants [60, 143-145, 171, 191, 314, 379, 395, 397].

The major mechanism of TH action at the cellular level is mediated by specific nuclear receptors (TRs) that regulate gene expression [136, 352, 425, 432]. Previous work has shown that ERKs and protein kinases A and C influence TH signaling. Phosphorylation of TR by ERK leads to TR stabilization and enhanced activity in mammalian cells ([65] and references therein). Furthermore, TH-induced cell proliferation is mediated by changes in Cdk activity in GC cells [14]. This evidence suggests that phosphorylation may be an important part of the TH signaling pathway. The role that Cdks may have in the initiation of TH-dependent apoptosis in the tadpole tail was examined. Results indicate that Cdk activity is required for tail regression and that changes in the activity of cyclin A-containing complexes precedes metamorphic climax.

2. Materials and Methods

2.1 Animal care.

Taylor and Kollros (TK) [381] stage VI-XXI *Rana catesbeiana* tadpoles were either locally caught or purchased (Wm A Lemberger Co, Oshkosh, WI, USA). Animals were housed in the University of Victoria aquatics facility and maintained in dechlorinated, aerated tap water at 15°C in a constant flow-through system with exposure to natural daylight. Tadpoles were fed Nutrafin® flakes (Rolf C. Hagen Inc, Montreal, QC) daily.

2.2 Tail organ culture.

The procedure used for tail culture was adapted from previously described procedures [144]. Tadpoles were sacrificed in 0.1% tricaine methanesulfonate (MS-222) (Syndel Laboratories, Vancouver, B.C.). The animals were then immersed in sterile distilled water for 10 seconds, followed by a 5 second immersion in 70% ethanol and two subsequent 10 second rinses in sterile distilled water. Tail tips (2 cm) were removed and placed into Tadpole Minimal Essential Medium (TMEM: 55% strength solution of MEM (Invitrogen) adjusted to pH 7.1 and supplemented with 25 mM HEPES, 3 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 1.2 mM NaH_2PO_4, 20 mM NaCl, 2mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml neomycin) at a density of 4 tips per 10 ml media. Tips were incubated for 24 hours at 25°C prior to addition of treatment reagents. After 24 hours, cultured tail tips were treated with equal volumes of dimethyl sulfoxide (DMSO) (vehicle), 3,5,3'-triidothyronine (T_3) (Sigma), roscovitine (Sigma), PD098059 (Sigma) or Wortmannin (Calbiochem). Tails were preincubated for 3 hours with the inhibitors prior to addition of T_3 where appropriate. Final concentrations of the chemical modifiers were: 100 nM T_3, 50 μM PD098059, 60 μM roscovitine, 50 μM Wortmannin. Media and chemicals were replenished daily for the duration of the time course.

2.3 Tail measurement and stastical analysis.

Photographs were taken of cultured tails every 24 hours using a digital camera (DVC Company, Austin, TX) and tail area was measured using Northern Eclipse v5.0 (Empix Imaging Inc., Mississauga, ON). Statistical analysis was conducted using In-Stat v3.01 (GraphPad Software Inc., San Diego, CA). The One-way analysis of variance (ANOVA)

was conducted to determine the p-value and the Tukey post-test was conducted if the p-value was greater than 0.05.

2.4 *In vivo* T_3 tadpole treatment.

Prior to treatment with T_3, TK stage VI tadpoles (6 per 8L water) were acclimated at 25°C for 2 days without feeding. After two days, the animals were immobilized on ice and injected intraperitoneally through the tail muscle with either vehicle solution (400 μM NaOH) or T_3-containing solution at a dose of 3×10^{-10} moles/g body weight [145]. Animals were maintained at 25°C for the duration of the experiment and water was changed daily. Following the exposure time course, animals were euthanized in 0.1% MS-222 and tail tips were either aseptically removed for tail culture or homogenized for protein collection. Cultured tail tips were treated with equal volumes of DMSO, ethanol (EtOH), cycloheximide in EtOH (CHX; Sigma) or roscovitine in DMSO, for 2 days. The final concentrations of each of the chemical modifiers were: 20 μg/ml CHX and 60 μM roscovitine. Media and chemical reagents were replenished daily.

2.5 Tissue homogenization.

Tail tips were homogenized on ice in a buffer containing 25 mM HEPES, 10 mM EDTA, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM DTT, 100 μM PMSF, 4 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain and 300 μg/ml benzamidine using a volume of 3 ml buffer per gram tissue [267]. Homogenates were centrifuged at 12,000 x g for 20 min at 4°C and the collected supernatant was stored at –70°C. The homogenate concentration was determined using the Bio-Rad Protein Assay (Bio-Rad).

2.6 Extraction of cytoplasmic and nuclear proteins.

Tail tips were homogenized in low salt lysis buffer (20 mM HEPES, 5 mM KCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 0.1% sodium deoxycholate, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM NaF, 100 μM PMSF, 4 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, 300 μg/ml benzamidine) at 2 ml buffer per gram tissue [146]. The cytoplasmic fraction was obtained by centrifuging the homogenate at 500xg for 2 minutes at 4°C and collecting the supernatant. The pellet was then washed with low salt buffer (400 μl per ml of original volume) and centrifuged at 500xg for 2 minutes at 4°C. The nuclear fraction was obtained by then resuspending the pellet in high salt lysis buffer (250 mM NaCl, 20 mM HEPES, 5 mM KCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 0.1% sodium deoxycholate, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM NaF, 100 μM PMSF, 4 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, 300 μg/ml benzamidine) at 500 μl per gram of original tissue and incubating on ice for 15 minutes. The high salt homogenate solution was then centrifuged at 1000xg for 2 minutes at 4°C and the supernatant collected as the nuclear fraction.

2.7 Immunoprecipitation (IP) and kinase assay.

Tissue homogenates (500 μg) were diluted to a 1 ml volume in IP buffer containing 50 mM HEPES (pH 8.0), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, and 0.1% Tween-20. Homogenates were precleared by rotation with 20 μl/ml of protein G-Sepharose beads (Amersham) for 20 minutes at 4°C. Following preclearing, the mixture was centrifuged at 3,000xg for 10 min at 4°C and the supernatant transferred to microfuge tubes containing 20 μl of fresh beads and 5 μg of polyclonal antibody. Immunoprecipitations were carried out using rabbit polyclonal antibodies against cyclin

A (H-432), Cdk1 (H-297), Cdk2 (M2), ERK1 (K-23), and ERK2 (C-14) (Santa Cruz Biotechnology).

Table 2. 1. Characterization of antibodies used for immunoprecipitation and immunoblotting.

Antibody	Species	Epitope	Specifity
Cdk1 (H-297)	Rabbit polyclonal	Full length Cdk1 of human origin	Broad interspecies reactivity, including mouse, rat, human
Cdk2 (M2)	Rabbit polyclonal	Carboxy terminus of Cdk2 of human origin (identical to rat sequence)	Mouse, rat, human and hamster reactive.
ERK1 (K-23)	Rabbit polyclonal	Subdomain XI of ERK1 of rat origin	Broad interspecies reactivity, including murine, human, chicken and frog.
ERK2 (C-14)	Rabbit polyclonal	Carboxy terminus of ERK2 of rat origin	Broad interspecies reactivity, including murine, human, chicken and frog.
PCNA (PC10)	Mouse monoclonal	Full length protein	Mouse, rat, human, insect and yeast reactive.
Cyclin A (H-432)	Rabbit polyclonal	Full length cyclin A of human origin	Mouse, rat and human reactive.
Cyclin A (ICRF)	Rabbit polyclonal	Full length cyclin A2 protein of *Xenopus laevis* origin.	Frog.

The antibody-bead-homogenate solution was mixed by rotation at 4°C for 2 hours and the beads were washed 3 times with 1 ml IP buffer. The complexes were further washed in 1 ml kinase reaction buffer containing 50 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM dithiothreitol

and 20 μM reduced glutathione. The immunoprecipitates were then incubated in 25 μl kinase reaction buffer containing 1.2 μM cold ATP (Invitrogen), 10 μCi γ^{32}P-ATP (Amersham) and either 1 μg histone H1 (Roche), or 1 μg Myelin Basic Protein (MBP) (Sigma) for 40 minutes at 30°C. The reactions were terminated by boiling for 3 minutes in sodium dodecyl sulfate (SDS) sample buffer [227]. The protein substrates were then resolved by SDS-PAGE. Proteins in MBP reactions were separated by 15% SDS-PAGE, while the proteins in the histone H1 reactions were separated by 12% SDS-PAGE. The resulting gels were dried and exposed to phosphor imaging screens (Amersham) for 16 hours on average. Radiographic data was obtained using a Storm 820 optical scanner phosphorimaging system at 50 μM resolution (Amersham).

2.8 *In vitro* roscovitine inhibition assay.

Immune complexes were immunoprecipitated from tadpole tail homogenate and incubated in kinase reaction buffer containing equal volumes of either DMSO or 50 μM roscovitine for 40 minutes at 30°C. Kinase reactions were resolved by SDS-PAGE and visualized by exposure to phosphor imaging screens.

2.9 Immunoblotting.

Equal quantities of tail tissue homogenate (30 μg/lane) in SDS sample buffer were electrophoresed through 12% SDS-polyacrylamide gels and transferred to 0.2 μm nitrocellulose (Bio-Rad) [146]. Protein loading was verified by membrane staining with 0.1% Ponceau S (Sigma) in 5% acetic acid. Membranes were blocked with 5% nonfat milk in 0.2M NaCl, 4.2 mM KCl, 12.7 mM Na_2HPO_4, 2.3 mM KH_2PO_4, and 0.15% Tween 20 (PBST) overnight at 4°C and then probed with antibody solutions diluted in 1% nonfat milk/PBST for 1h at room temperature, shaking. Cyclin A was detected using

a rabbit polyclonal antibody obtained from Tim Hunt (Imperial Cancer Research Fund, UK). Cdk1 (H-297), Cdk2 (M2), ERK1 (K-23), ERK2 (C-14), and PCNA (PC10) antibodies were all obtained from Santa Cruz Biotechnology. Antibody dilutions were as follows: rabbit anti-cyclin A, 1/1000; rabbit anti-Cdk1, 1/200; rabbit anti-Cdk2, 1/200; rabbit anti-ERK1, 1/2500; rabbit anti-ERK2, 1/2500; and mouse anti-PCNA, 1/5000. Following the primary antibody incubation, blots were washed for 1h at room temperature in PBST, shaking. Washed blots were then incubated with secondary antibodies diluted in 1% nonfat milk/PBST for 30 minutes at room temperature, shaking. Goat anti-rabbit and goat anti-mouse HRP-conjugated antibodies were diluted 1/3000 in 1% nonfat milk/PBST. Blots were washed for an additional hour in PBST following incubation with secondary antibodies and then processed using the enhanced chemiluminescence (ECL) method, as described by the manufacturer (Amersham) and exposed to Kodak Biomax film.

2.10 Calf Intestinal Alkaline Phosphatase (CIP) Reactions

Two hundred micrograms of stage XV tail tip homogenate were diluted to 100 μl in a digestion buffer of 50 mM HEPES, pH 8.0 and 1mM $MgCl_2$ and incubated at 30°C for 10 minutes. Twenty-five units of CIP (New England BioLabs) were added to the homogenate solution and incubated for a further 30 minutes at 30°C. The reactions were terminated by boiling for 3 minutes in sodium dodecyl sulfate (SDS) sample buffer [227] and resolved by 12% SDS-PAGE.

3. Results

3.1 Roscovitine inhibits T_3-induced tadpole tail regression

To determine the involvement of Cdks in hormone-dependent, developmentally-regulated apoptosis, tail tips from premetamorphic (TK stages VI-XII) *R.catesbeiana* tadpoles were cultured over 72 hours and treated with either T_3 alone or T_3 with the Cdk inhibitor, roscovitine. Tails treated with T_3 showed a 51±5% reduction in tail area over 72 hours in culture (**Figure 2.1**). This regression was completely inhibited by the addition of roscovitine to the culture media, while tails cultured with vehicle alone, showed only a small degree of regression (13±5%). There was no significant difference between vehicle-treated tails and tails treated with a combination of T_3 and roscovitine.

3.2 Roscovitine inhibits tail regression in a stage-specific manner

Tail tips cultured from tadpoles at different developmental stages will spontaneously regress in serum-free media in a manner relative to the endogenous TH levels [409]. To determine the extent of natural regression, stage VI (premetamorphic), XV (prometamorphic) and XX (metamorphic climax) tail tips were cultured over 72 hours and the area was measured at 24 hour intervals (**Figure 2.2**). Stage VI and XV tail tips demonstrated similar patterns of area reduction over 72 hours with stage VI tips regressing by 13±5% and stage XV tail tips regressing by 24±10%, which was not statistically significant. In contrast, stage XX tail tips demonstrated significant reduction in tail area over 72 hours in culture (49±13%; $p<0.05$ compared to stage VI).

Endogenous TH levels remain low throughout pre- and early prometamorphosis, increasing only at the late stages of prometamorphosis (TK stages XVIII-XIX) and reaching maximal levels at the onset of metamorphic climax (TK stages XX-XXI).

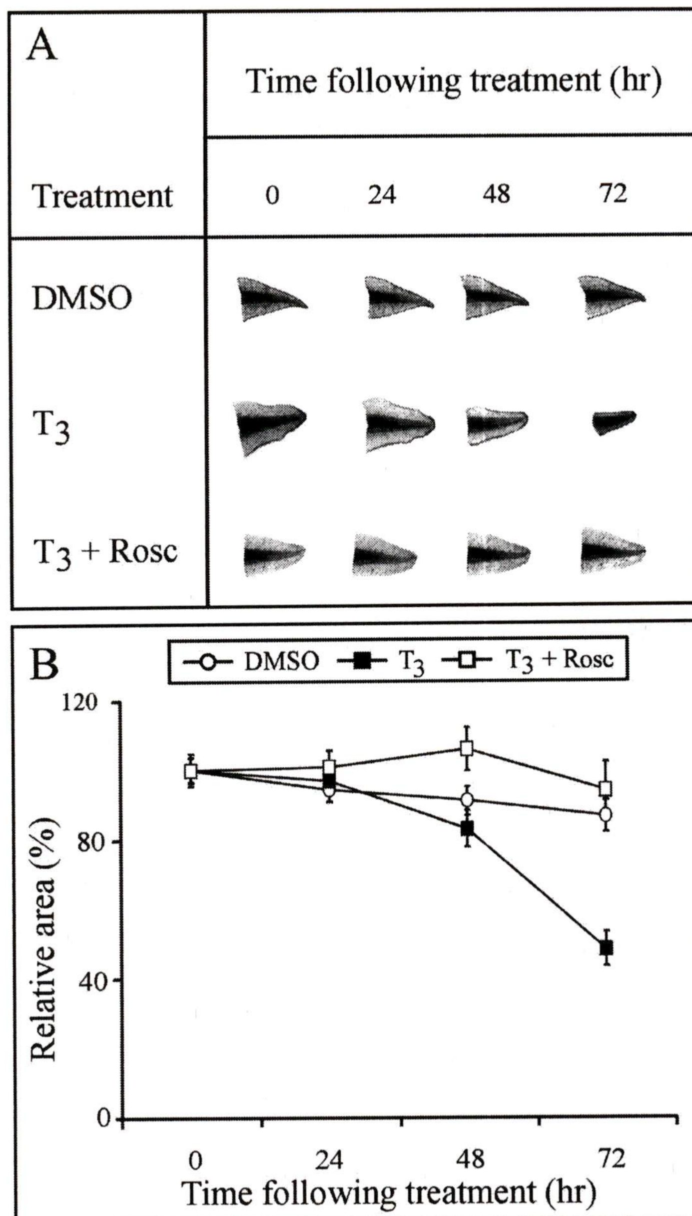


Figure 2.1. Roscovitine inhibits T_3-induced tail tip regression in culture.

Two centimetre tail tips were removed from premetamorphic TK stage VI tadpoles and cultured in TMEM over 3 days. Tail tips were treated with vehicle (DMSO), T_3, or T_3 with roscovitine (T_3+Rosc). (A) Representative pictures of tail tips are shown. (B) The tail tip area was measured at each time point and plotted relative to the 0 hour (hr) time point (DMSO and T_3: n=49 for day 0 and 1, n=35 for day 2 and n=25 for day 3; T_3 with roscovitine: n=26 for day 0 and 1, n=20 for day 2 and n=14 for day 3). The error bars represent SEM ($p<0.0001$ at 72 h).

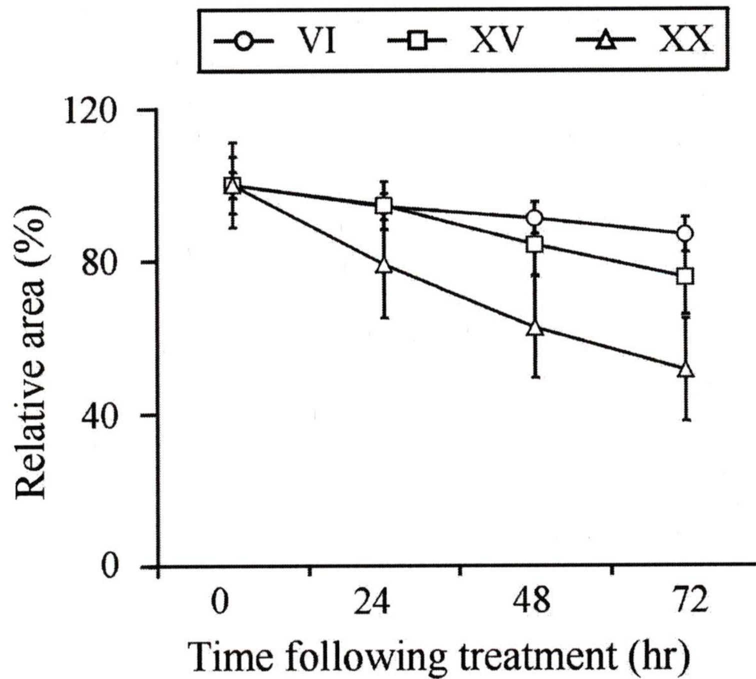


Figure 2.2. Cultured tail tips spontaneously regress at a rate dependent upon developmental stage.

The area of cultured TK stage VI (premetamorphic), XV (prometamorphic) and XX (metamorphic climax) tail tips was measured every 24 hours for 72 hours. Tail tip area was then plotted relative to the 0 hour time point. (Stage VI: n=49 for day 0 and 1, n=35 for day 2 and n=25 for day 3; Stage XV and XX: n=6 at each time point).

Given that the T_3-induced regression of cultured tails was inhibited by roscovitine, it was hypothesized that roscovitine would inhibit natural tail regression in successively older tadpoles, as the levels of ciruclating T_3 increased. To determine the effect of roscovitine on natural regression, stage VI, XV, and XX tail tips were cultured over 72 hours in the presence of roscovitine (**Figure 2.3**). The addition of roscovitine to the culture media significantly inhibited the regression of stage VI tail tips ($p<0.05$ at 48 hours; $p<0.1$) (**Figure 2.3A**). The effect of roscovitine on stage XV tail tip regression was inhibitory; however, this effect was not statistically significant (**Figure 2.3B**). By contrast, roscovitine had no effect on the natural regression of stage XX tail tips (**Figure 2.3C**).

To determine the effect of roscovitine on stage-specific T_3-induced tail regression, stage XV and XX tail tips were cultured in the presence of vehicle (DMSO), T_3 alone or T_3 with roscovitine (**Figure 2.4**). When stage XV and XX tail tips were treated with T_3 alone, tail regression was significantly accelerated compared to the DMSO control (At 72 hours: XV, $p<0.01$; XX, $p<0.05$). Stage XV tail tips regressed by 74±4% (**Figure 2.4A**), while stage XX tail tips regressed by 83±3% over 72 hours in culture (**Figure 2.4B**). This T_3-induced acceleration of tail regression was completely inhibited by the addition of roscovitine to the culture medium at both stages examined (comparing T_3 with T_3 + rosc at 72 hours: XV, $p<0.001$; XX, $p<0.01$).

3.3 The requirement for Cdk activity coincides with the commitment point for the T_3-induced response

Previous studies in *Xenopus laevis* have shown that T_3 induces a genetic program that regulates tail regression [406]. This program requires the continuous presence of T_3 during the early stages of induction; however, between 24 and 48 hours following T_3 treatment, the T_3-induced program has become established in the tadpole tail and the

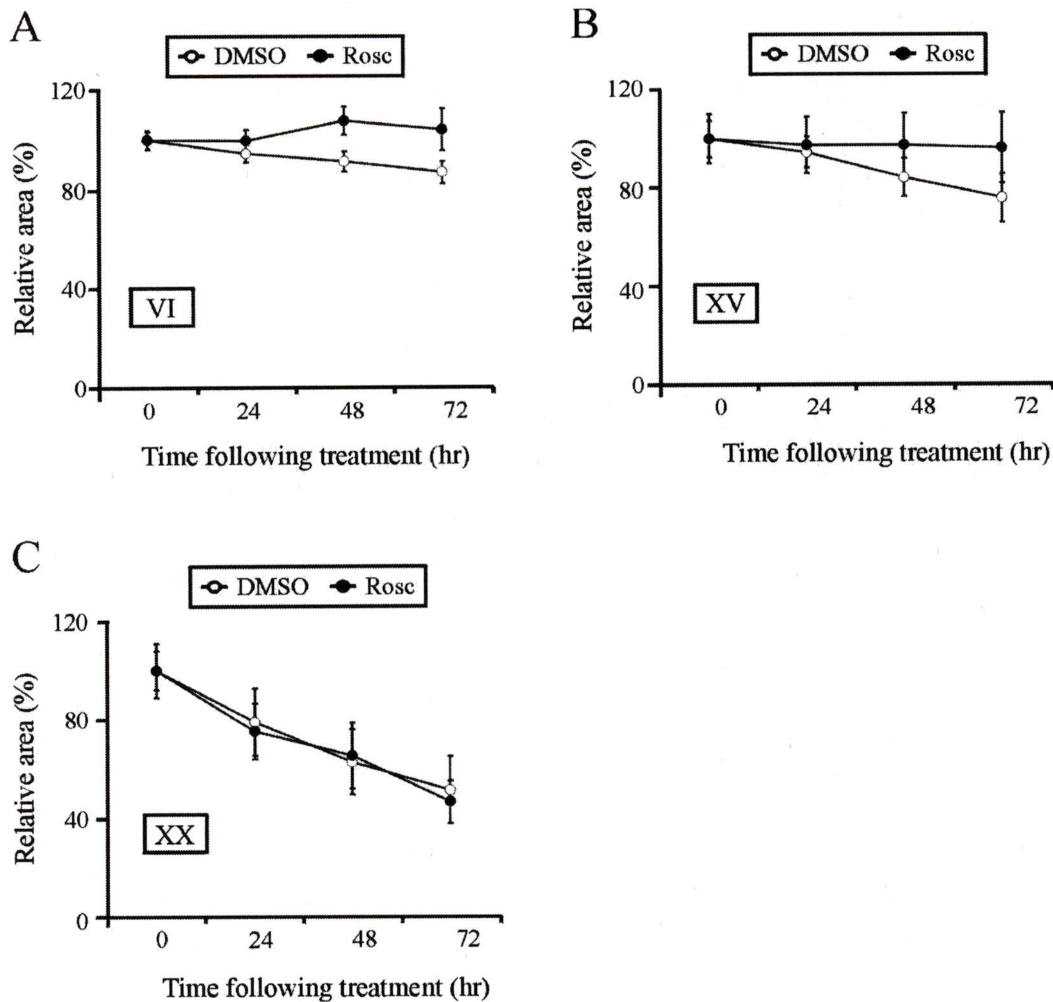


Figure 2.3. Roscovitine inhibition of tail regression is stage specific.

TK stage (A) VI, (B) XV and (C) XX tail tips were cultured over 72 hours in the presence of either DMSO or roscovitine (Rosc). The tail tip area was measured every 24 hours and plotted relative to the 0 hour time point. (Stage VI DMSO: n=49 for day 0 and 1, n=35 for day 2 and n=25 for day 3; Stage VI roscovitine: n=20 at day 0 and 1, n=14 at day 2 and n=8 at day 3; Stage XV and XX: n=6 at each time point).

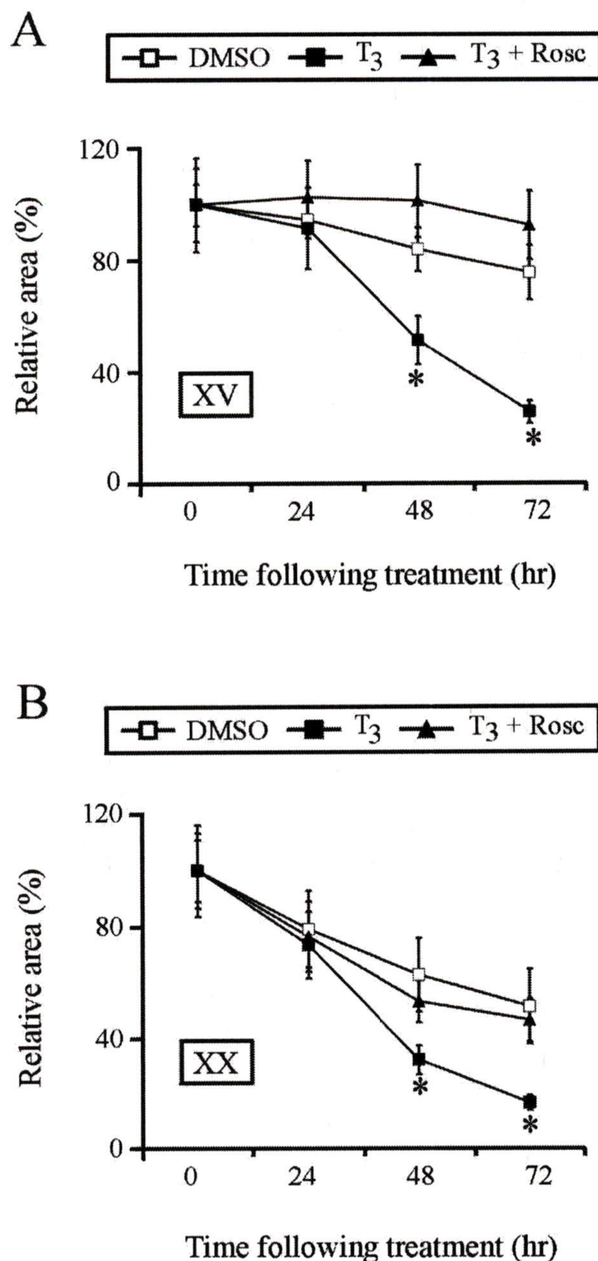


Figure 2.4. Roscovitine inhibits T_3-induced tail regression in cultured TK stage XV and XX tail tips.

TK stage (A) XV and (B) XX tail tips were cultured over 72 hours in the presence of either DMSO, T_3 or T_3 with roscovitine (T_3 + Rosc). The tail tip area (n=6 at each time point) was measured at 24 hour time points and plotted relative to the 0 hour time point. The error bars represent SEM. Measurements showing statistically significant differences are indicated by a "*". The p-values are indicated in the text.

requirement for T_3 after this commitment point is released. At this point, application of the protein synthesis inhibitor, cycloheximide (CHX) cannot fully inhibit tail regression [406]. To determine if Cdk activity affects this commitment point, stage VI tadpoles were injected with vehicle or T_3. These animals were then maintained at 25°C for either 24 hours or 48 hours before collection and culture of their tail tips in the presence of vehicle (EtOH or DMSO) or inhibitors (CHX or roscovitine). Tail tips collected from vehicle-injected tadpoles showed no regression, irrespective of treatment in subsequent organ culture (**Figure 2.5A**). Tail tips collected from T_3-injected tadpoles at the 24 hour time point showed two major trends with regard to tail regression (**Figure 2.5B**). The tail tips regressed substantially when exposed to vehicle solutions in culture, whereas application of either cycloheximide or roscovitine resulted in complete inhibition of regression. By contrast, both cycloheximide and roscovitine were unable to inhibit tail regression to any great extent in tails obtained from 48 hour T_3-injected tadpoles (**Figure 2.5C**). Furthermore, the effectiveness of roscovitine was significantly reduced compared to cycloheximide ($p<0.01$).

3.4 Maximal cyclin A-associated kinase activity precedes metamorphic climax during natural metamorphosis and is inhibited by roscovitine *in vitro*

Given that roscovitine inhibited tail regression in a stage-specific manner and given that cyclin A and E complexes have been implicated in apoptosis [157, 263], the kinase activity of those complexes was examined during natural metamorphosis. The histone H1 kinase activity of cyclin A- and cyclin E-containing complexes was examined following immunoprecipitation from the tail tips of stage XII tadpoles (**Figure 2.6A**). The kinase activity of cyclin E-containing complexes was not greater than background in stage XII tail tips. The kinase activity of those complexes was examined at other developmental

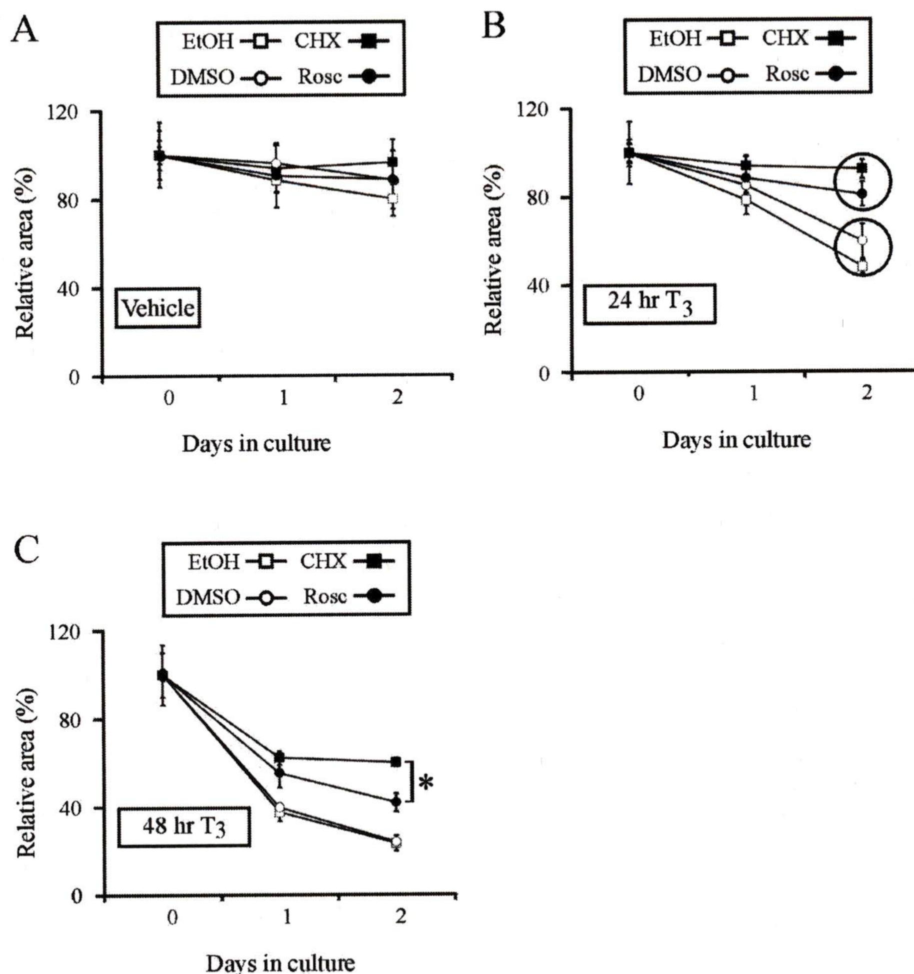


Figure 2.5. Roscovitine inhibition of T_3-induced tail regression correlates with the commitment point.

(A) Tail tips were collected from premetamorphic stage VI tadpoles injected with NaOH *in vivo* for 24 hours prior to tail tip removal (Vehicle), (B) T_3 *in vivo* for 24 hours (24 hr T_3), or (C) T_3 *in vivo* for 48 hours (48 hr T_3). Tail tips were cultured for 2 days in serum-free medium containing EtOH, cycloheximide (CHX), DMSO or roscovitine (Rosc). Tail tip area (n=3 per treatment) was normalized to the 0 hour measurement for each treatment. The vehicle and inhibitor treatments are highlighted by circles at the 2 day time point to accentuate similar responses seen in the 24 hr T_3-graph. The area of tail tips treated with vehicle are significantly different from those treated with inhibitor ($p<0.001$). The asterisk indicates significant difference between Rosc and CHX treatments in the 48 hr T_3-graph ($p<0.05$). The error bars represent SEM.

stages; however, little histone H1 kinase activity was detected at any stage examined (data not shown). Cyclin A-containing complexes were then immunoprecipitated from tail tip homogenates collected from tadpoles at various stages and their activity against histone H1 was determined (**Figure 2.6B**). Cyclin A-containing complexes displayed a differential pattern of activity that correlated with tail growth and preceded detection of circulating levels of serum T_3 (**Figure 2.6C**). This kinase activity achieved maximal levels at stage XV and remained high at stage XVIII. Substantial cyclin A-associated kinase activity was also evident at stages XIX and XX (58% and 65% of the activity seen at stage XV, respectively).

Only Cdk1 and Cdk2 have been reported to form an active complex with cyclin A *in vivo* [346]. Therefore, the kinase activity of those complexes was examined in tail tip homogenates derived from tadpoles at various stages of metamorphosis. Cdk1 complexes showed little kinase activity at all developmental stages examined, with only a slight increase in activity evident at stage XV (**Figure 2.7A**). Cdk2 complexes, by contrast, showed increasing kinase activity between stages VI and XV. This kinase activity was substantially reduced by stage XVIII and was not detectable by stage XXI. Cdk3 is known to associate with cyclin A *in vitro*; therefore, the activity of Cdk3 complexes was also assessed (**Figure 2.7B**). Cdk3 complexes exhibited little kinase activity against histone H1 and was maximal at stage XV. The activity in stage VI and stage XVIII tail tips was comparable, while the activity at XXI was minimal. This suggests that there is a kinase associating with cyclin A at stages XVIII through XX that is neither Cdk1, Cdk2 nor Cdk3.

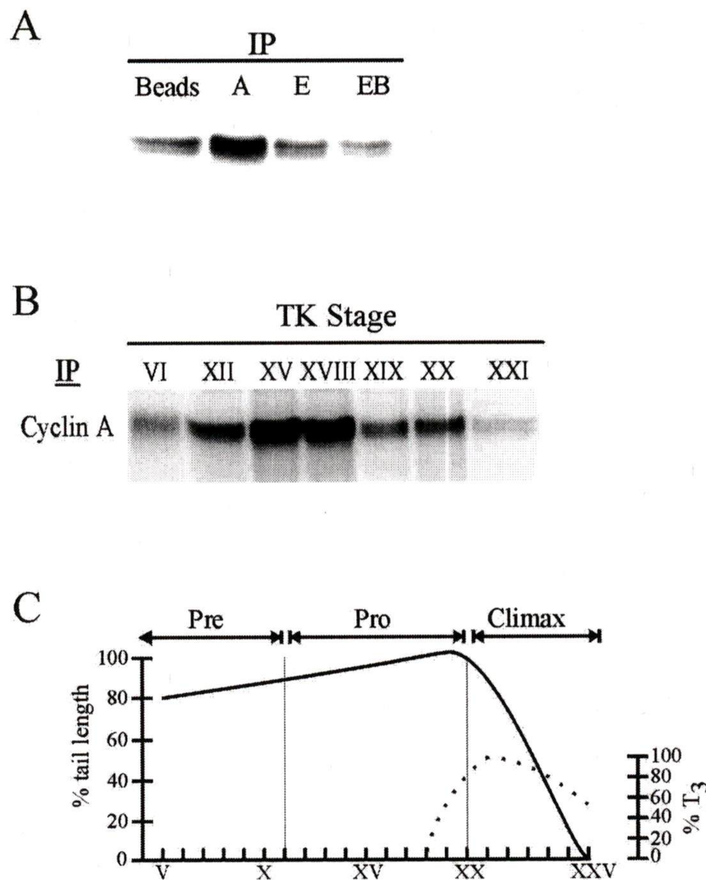


Figure 2.6. Cyclin A-containing complexes exhibit differential kinase activity in the tail tip of tadpoles undergoing natural metamorphosis.

Cyclin E-containing complexes display no detectable kinase activity. (A) Cyclin A- and cyclin E-containing complexes were immunoprecipitated from TK stage XII tail tips and assayed for activity against a histone H1 substrate. (B) Cyclin A-containing complexes display stage-dependent kinase activity corresponding with previously published serum TH levels (adapted from [319]). Tail tip homogenates were collected from tadpoles (n=2-9) at different developmental stages from TK stage VI (premetamorphic) through to TK stage XXI (metamorphic climax). Cyclin A-containing complexes were immunoprecipitated and assayed for activity against a histone H1 substrate. (C) Depiction of tadpole tail length (solid line) and relative T_3 serum levels (dotted line) during metamorphosis of the *R.catesbeiana* tadpole. Adapted from Krug [223].

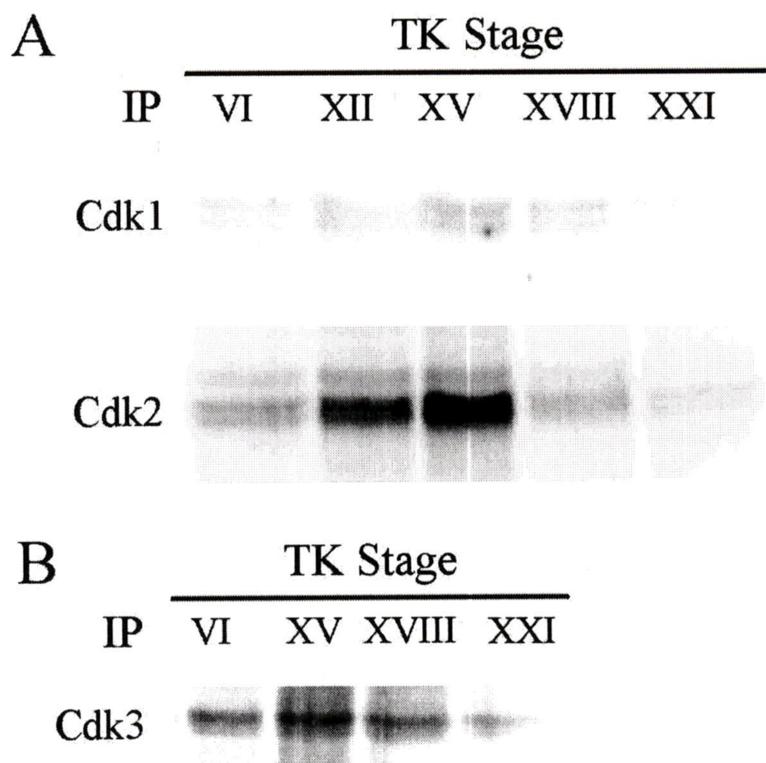


Figure 2.7. Cdk2-containing complexes exhibit differential kinase activity in the tail tips of tadpoles undergoing natural metamorphosis.

(A) Cdk1 and Cdk2 complexes display stage-dependent kinase activity. Cdk1 and Cdk2 proteins were immunoprecipitated from tail tip homogenates and assayed for activity against a histone H1 substrate. (B) Cdk3 complexes display modest stage-dependent kinase activity. Cdk3 protein was immunoprecipitated from tail tip homogenates and subjected to kinase activity analysis as described above.

To determine if this kinase was susceptible to Cdk-specific inhibition, cyclin A-containing complexes were immunoprecipitated from stage VI, XV and XVIII homogenates and treated with roscovitine *in vitro* (**Figure 2.8A**). Roscovitine was found to substantially inhibit the kinase activity of cyclin A-containing complexes at all three stages examined, suggesting that the kinase associated with cyclin A at stage XVIII is mechanistically similar to a Cdk. To determine if roscovitine could also inhibit the kinase activity of Cdk2-containing complexes, Cdk2 immunoprecipitates were isolated from stage XV tail tip homogenate and treated with roscovitine *in vitro* (**Figure 2.8B**). Roscovitine was found to inhibit Cdk2 activity by 86%.

3.5 Generation of a 35 kDa cyclin A protein correlates with apoptosis in natural and T_3-induced metamorphosis

Finkielstein *et al.* [96] recently identified a cyclin A2 proteolytic cleavage product generated in UV-treated *Xenopus laevis* embryos that correlates with apoptosis. This alternative form of cyclin A2 was cleaved by caspases to generate a truncated 35 kDa protein that formed an active kinase complex with Cdk2 [96]. Immunoblot analysis on nuclear and cytoplasmic protein fractions isolated from the tail tips of naturally metamorphosing tadpoles identified a similar 35 kDa protein (**Figure 2.9A**). This protein was found to increase in steady state levels during metamorphosis while a slightly smaller protein remained unchanged during that time. These proteins were more prominent in the nucleus. This contrasts with the detection of a 46 kDa protein that did not change between stages. This protein was more abundant in the cytoplasmic fraction compared to the nuclear fraction. The relative subcellular distribution of this protein corresponds with the kinase activity of immunoprecipitated cyclin A-containing complexes (**Figure 2.9B**). A similar relationship between the 46 kDa protein and the 35

To determine if this kinase was susceptible to Cdk-specific inhibition, cyclin A-containing complexes were immunoprecipitated from stage VI, XV and XVIII homogenates and treated with roscovitine *in vitro* (**Figure 2.8A**). Roscovitine was found to substantially inhibit the kinase activity of cyclin A-containing complexes at all three stages examined, suggesting that the kinase associated with cyclin A at stage XVIII is mechanistically similar to a Cdk. To determine if roscovitine could also inhibit the kinase activity of Cdk2-containing complexes, Cdk2 immunoprecipitates were isolated from stage XV tail tip homogenate and treated with roscovitine *in vitro* (**Figure 2.8B**). Roscovitine was found to inhibit Cdk2 activity by 86%.

3.5 Generation of a 35 kDa cyclin A protein correlates with apoptosis in natural and T_3-induced metamorphosis

Finkielstein *et al.* [96] recently identified a cyclin A2 proteolytic cleavage product generated in UV-treated *Xenopus laevis* embryos that correlates with apoptosis. This alternative form of cyclin A2 was cleaved by caspases to generate a truncated 35 kDa protein that formed an active kinase complex with Cdk2 [96]. Immunoblot analysis on nuclear and cytoplasmic protein fractions isolated from the tail tips of naturally metamorphosing tadpoles identified a similar 35 kDa protein (**Figure 2.9A**). This protein was found to increase in steady state levels during metamorphosis while a slightly smaller protein remained unchanged during that time. These proteins were more prominent in the nucleus. This contrasts with the detection of a 46 kDa protein that did not change between stages. This protein was more abundant in the cytoplasmic fraction compared to the nuclear fraction. The relative subcellular distribution of this protein corresponds with the kinase activity of immunoprecipitated cyclin A-containing complexes (**Figure 2.9B**). A similar relationship between the 46 kDa protein and the 35

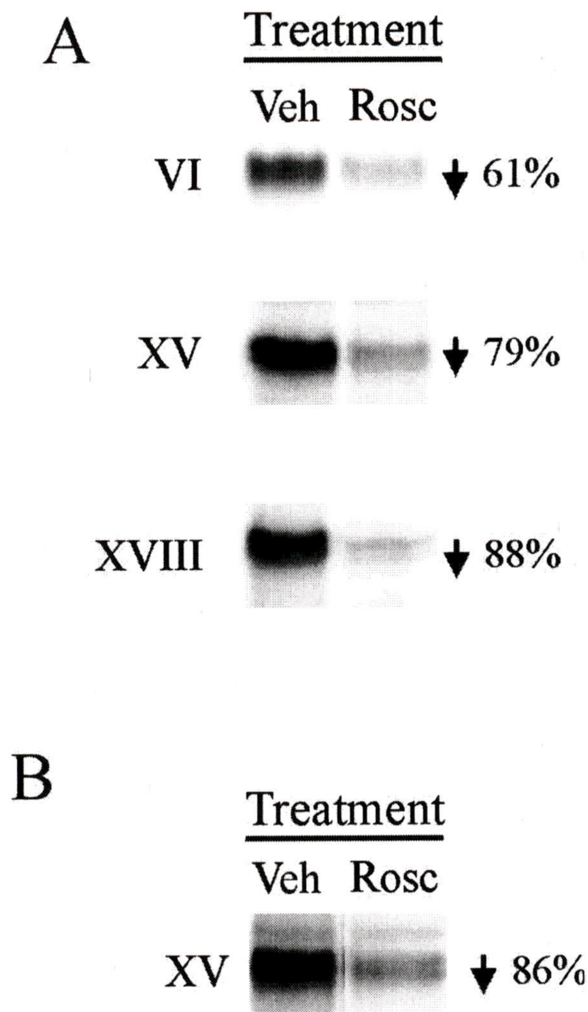


Figure 2.8. Roscovitine inhibits the kinase activity of cyclin A- and Cdk2-containing complexes.

(A) Roscovitine inhibits the kinase activity of cyclin A-containing complexes immunoprecipitated from TK stage VI, XV and XVIII tail tip homogenates. Immunoprecipitates were incubated in the presence of DMSO vehicle (Veh) or 50 μM roscovitine (Rosc) during the kinase reaction. The extent of inhibition is indicated for each stage. (B) Roscovitine inhibits the kinase activity of Cdk2-containing complexes. The same reaction conditions were performed on Cdk2-containing complexes immunoprecipitated from stage XV tail tips as described in "A".

kDa form was observed during precocious metamorphosis. The 35 kDa protein was induced during precocious metamorphosis while the other two proteins remained constant, suggesting that this 35 kDa protein is TH-responsive and may contribute to apoptosis (**Figure 2.9C**). Proliferating cell nuclear antigen (PCNA) was visualized as a marker of active proliferation and the steady state levels were reduced by 96 hours following T_3 treatment.

3.6 Cdk1 and Cdk2 steady state levels are reduced during natural metamorphosis

Cyclin A protein levels do not correlate with the pattern of kinase activity observed for cyclin A-containing complexes during natural metamorphosis. The protein levels of the potential kinase partners, Cdk1 and Cdk2, were therefore examined (**Figure 2.10**). Cdk1 and Cdk2 protein levels demonstrate a similar pattern of expression. Cdk1 protein levels increase slightly at stage XII and XV before dropping at stage XVIII through to stage XXI. Cdk2 protein levels are relatively high at stage VI and remain at that level until stage XVIII where they are dramatically reduced. Proliferating cell nuclear antigen (PCNA) was visualized as a marker of active proliferation and shows a pattern of reduction in steady state levels by stage XVIII and XXI, similar to that observed for Cdk1 and Cdk2.

3.7 Roscovitine reduces the steady state levels of the 35 kDa cyclin A, Cdk1 and Cdk2 proteins

Roscovitine was found to inhibit tail regression while also inhibiting the activity of cyclin A- and Cdk2-containing complexes. To determine if roscovitine affects the protein levels of cyclin A and the known *in vivo* partners, premetamorphic tail tips cultured over 24 hours were collected and the protein levels assessed by immunoblot analysis. The 46 kDa cyclin A protein was unaffected by roscovitine treatment (**Figure 2.11A**).

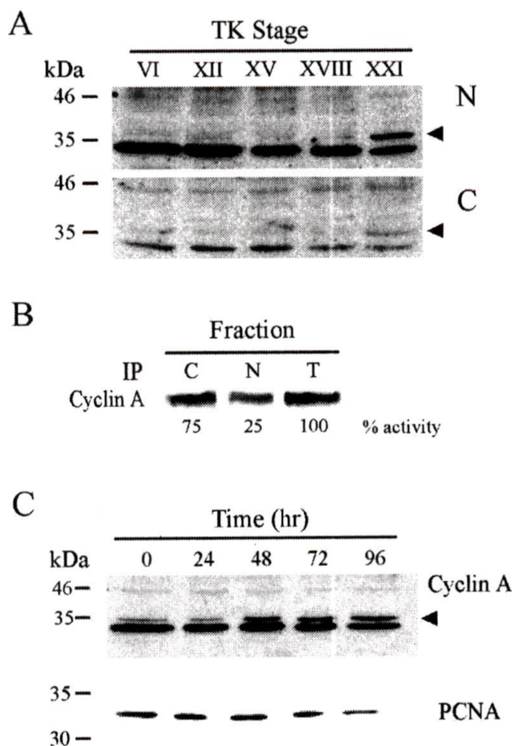


Figure 2.9. Steady state levels of a 46 kDa cyclin A protein do not vary in the tail tip during either natural or T_3-induced metamorphosis, while a 35 kDa protein increases upon induction of tail regression.

(A) The steady state levels of the 35 kDa form of cyclin A increase following metamorphic climax. Nuclear ("N") and cytoplasmic ("C") fractions were separated by 12% SDS-PAGE and immunoblotted using an antibody generated against *Xenopus laevis* cyclin A2. The 35 kDa protein that appears at later stages during metamorphosis is indicated by the arrow. Relative molecular weights are indicated to the left. (B) The kinase activity of cyclin A-containing complexes is 3-fold greater in the cytoplasm compared to the nucleus. Cyclin A-containing complexes were immunoprecipitated from the nucleus ("N"), the cytoplasm ("C") and total cell contents ("T") of tail tips collected from TK stage XV animals and assayed for activity against a histone H1 substrate. The percent kinase activity is indicated. (C) The steady state levels of the 35 kDa form of cyclin A increase upon T_3 induction. Tadpoles were injected with T_3 and housed at 25°C for up to 96 hours. Total tail tip homogenates from the indicated time points were immunoblotted for cyclin A. The arrow indicates the 35 kDa protein.

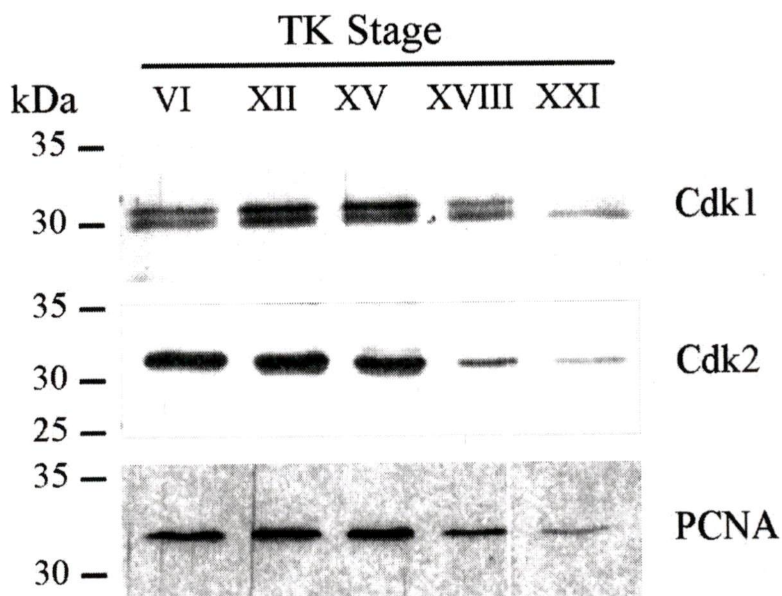


Figure 2.10. The steady state levels of Cdk1, Cdk2 and PCNA all diminish following metamorphic climax.

Immunoblotting was used to examine the protein levels of Cdk1, Cdk2 and PCNA in total tail tip homogenates during natural metamorphosis.

In contrast, the 35 kDa protein disappeared upon roscovitine treatment. The upper band representing Cdk1 was also decreased. This band may correspond with the phosphorylated form of Cdk1 [390]. With respect to the 35 kDa cyclin A protein, it is unlikely that this protein is merely a phosphorylated form of the lower band since calf intestinal alkaline phosphatase (CIP) treatment did not alter its relative migration pattern (**Figure 2.11B**). Cdk2 protein expression was decreased by roscovitine treatment only in the presence of T_3. PCNA was again included as an indicator of cell proliferation. Both roscovitine alone and the combination of T_3 with roscovitine resulted in a reduction in PCNA protein levels.

3.8 Roscovitine inhibits ERK activity *in vitro* but has no effect on steady state protein levels in cultured tail tips

In the experiments described above, roscovitine was applied to cultured tails at a dose sufficient to partially inhibit both ERK1 and ERK2 activities [67, 261]. It is therefore possible that the effect of roscovitine on tail regression is dependent upon inhibition of ERK activity as well as Cdk-associated inhibition. To determine if roscovitine was able to inhibit the kinase activity of amphibian ERKs, both ERK1 and ERK2 activity was examined with and without the addition of exogenous roscovitine in an *in vitro* kinase reaction (**Figure 2.12A**). ERK1 and ERK2 were immunoprecipitated from stage VI tail tip homogenate and allowed to phosphorylate an MBP substrate *in vitro*. Roscovitine inhibited ERK1 activity by 24% and ERK2 activity by 31%. To determine if this inhibition had any effect on protein levels, tail tips cultured over 24 hours were collected and the proteins examined by immunoblot analysis (**Figure 2.12B**). No change in protein levels was observed between treatments for either ERK1 or ERK2.

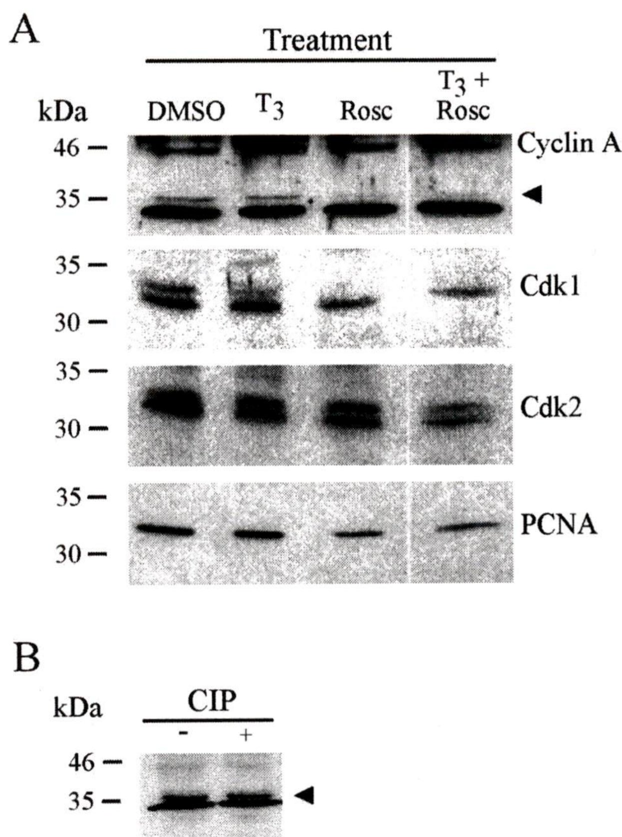


Figure 2.11. Treatment of cultured tail tips with roscovitine affects the steady state levels of Cdk1, Cdk2, PCNA and the 35 kDa cyclin A protein.

(A) Tail tips cultured for 24 hours in the presence of DMSO, 100 nM T_3, 60 μM roscovitine (Rosc) or 100 nM T_3 with 60 μM roscovitine (T_3+Rosc) were homogenized and the proteins were immunoblotted for the indicated proteins. The arrow indicates the 35 kDa protein. (B) The 35 kDa form of cyclin A is not a phosphoprotein. Homogenates from the tail tips of TK stage XV animals were incubated with Calf Intestinal Alkaline Phosphatase (CIP) and separated by 12% SDS-PAGE.

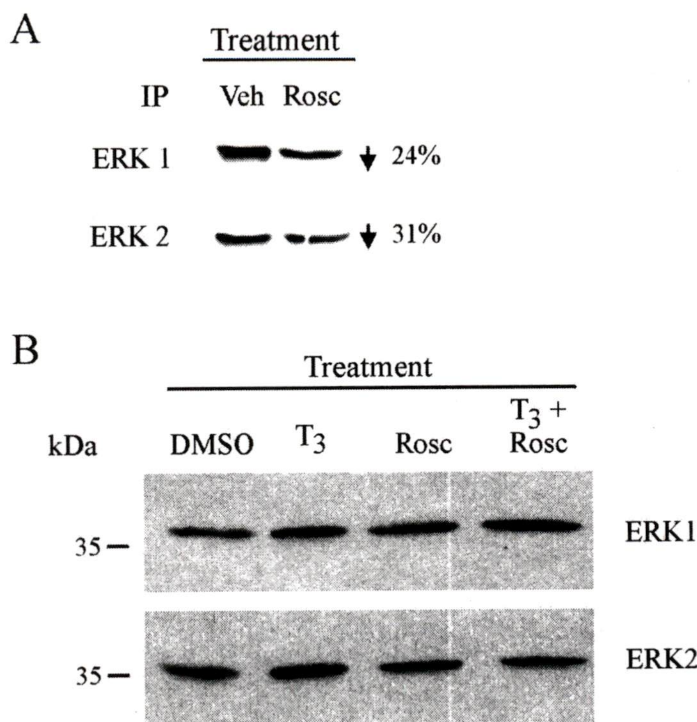


Figure 2.12. Roscovitine partially inhibits ERK kinase activity *in vitro* but does not affect protein steady state levels.

(A) ERK1 and ERK2 protein complexes were immunoprecipitated from tail tip homogenates and incubated in the presence of DMSO vehicle (Veh) or 50 μM roscovitine (Rosc) during the kinase reaction. The MBP substrate was resolved by 15% SDS-PAGE and its phosphorylation visualized by phosphor imaging. The extent of inhibition is indicated for each kinase. (B) ERK1 and ERK2 steady state levels in the tail tip are unaffected by 24 hour treatment with DMSO, 100 nM T_3, 60 μM roscovitine or T_3 with roscovitine (T_3 + Rosc). Tail tip homogenates were immunoblotted for ERK1 and ERK2.

3.9 Neither PD098059 nor Wortmannin inhibit T_3-induced tail tip regression

Given that roscovitine can partially inhibit ERK activities at the dose used in these studies, the extent of ERK involvement in tail regression was examined by the application of a specific inhibitor of ERK activation, PD098059, to cultured tail tips. In addition, the PI3K inhibitor, Wortmannin, was also tested to determine if cyclin D expression is involved in tail regression. Cyclin D is a known target of both the PI3K and ERK pathways [288]. Stage VI tail tips were cultured over 72 hours with the inhibitors alone (**Figure 2.13A**) or T_3 plus inhibitors (**Figure 2.13B**). Neither PD098059 nor Wortmannin had any effect on the regression of untreated tail tips (**Figure 2.13A**). Addition of T_3 to the cultured tail tips induced regression that was not significantly inhibited by either PD098059 or Wortmannin (**Figure 2.13B**). Both PD098059 and Wortmannin affect the activities of the ERK proteins. PD098059 is a noncompetitive inhibitor of the ERK activating kinase, MEK [7], while Wortmannin is a fungal metabolite that acts as a cell-permeable irreversible inhibitor of PI3K [59]. The PI3K pathway can regulate ERK activation *via* activational phosphorylation of Raf-1 by PAK [208] and inhibitory Raf-1 phosphorylation by Akt [437]; therefore, inhibition of the PI3K pathway could affect ERK activation. To determine the effect of those inhibitors on ERK kinase activity, tail tips were cultured in the presence of each compound for 48 hours and homogenized for protein isolation. ERK kinase reactions using MBP as a substrate were conducted on immunoprecipitated proteins (**Figure 2.14**). PD098059 inhibited ERK1 activity in cultured tail tips by 30% while ERK2 activity was inhibited by 65%. Treatment of the tail tips with Wortmannin in conjunction with T_3 also resulted in a substantial reduction in ERK1 activity (58%), although ERK2 was unaffected (when corrected for background signal).

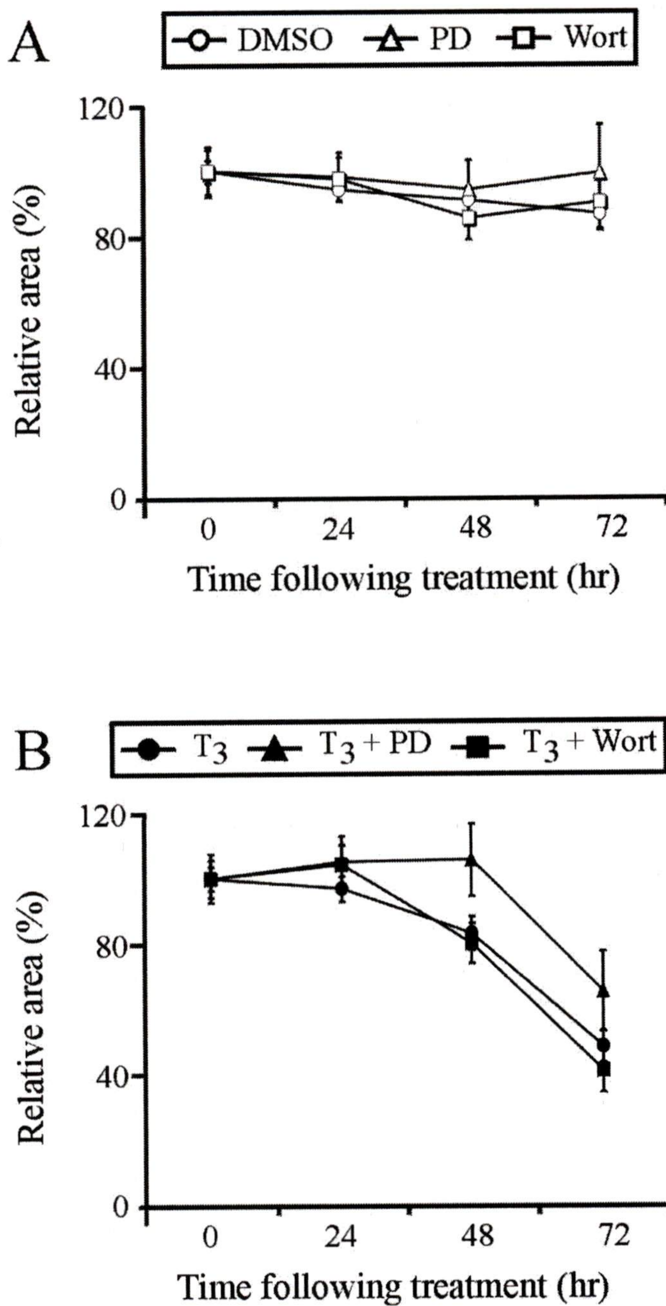


Figure 2.13. The MAPK inhibitor, PD098059, and the PI3K inhibitor, Wortmannin, have no effect on T_3-induced tail regression.

Tail tips from premetamorphic stage VI tadpoles (n=6 per treatment) were cultured in the presence of DMSO, PD098059 (PD), or Wortmannin (Wort) over 72 hours in the (A) absence or (B) presence of 100 nM T_3. Tail area was normalized relative to the 0 hour measurement for each treatment. The error bars represent SEM.

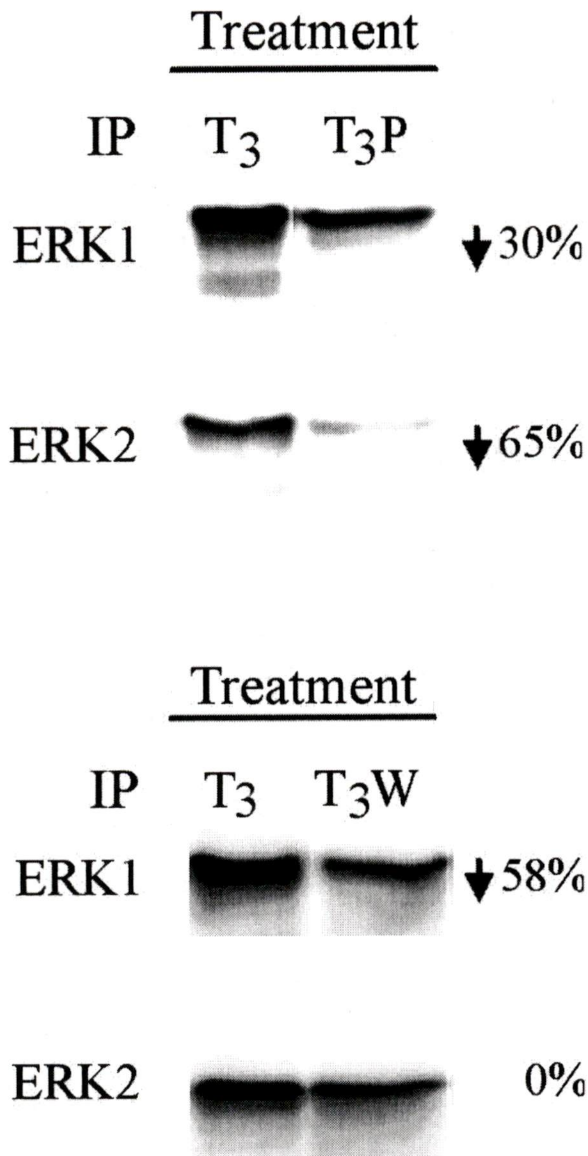


Figure 2.14. The MAPK inhibitor, PD098059, and the PI3K inhibitor, Wortmannin, partially inhibit ERK activity.

Tail tips cultured for 48 hours in the presence of T_3, T_3 with PD098059 (T_3P), or T_3 with Wortmannin (T_3W) were homogenized and immunoprecipitated ERK complexes were assayed for kinase activity using an MBP substrate. The extent of inhibition is indicated for each kinase.

These kinase assays indicated that both PD098059 and Wortmannin functioned as ERK inhibitors in cultured tail tips; however, the inhibition of these important kinases did not correlate with the inhibition of T_3-induced tail regression.

4. Discussion

In this study, it was shown that Cdk activity is required for T_3-dependent regression of the tadpole tail. Maximal kinase activity of cyclin A-containing complexes coincides with a period of tail growth and does not seem to be directly related to the execution of the tail regression program. It is apparent that there are multiple Cdks that confer activity to this complex, depending upon the developmental stage. Given that roscovitine does not inhibit Cdk4 or Cdk6 activities at the doses used [261] and that both PD098059 and Wortmannin fail to inhibit tail regression, it is unlikely that cyclin D-containing complexes play a significant role in the commitment to tail regression. Cdk1-containing complexes also do not appear to be involved in tail regression due to their lack of measurable activity during metamorphosis. Cdk2 has previously been found to associate with cyclin A and E *in vivo* [420] and its activity has often been correlated with the induction of apoptosis. Cdk2-containing complexes immunoprecipitated from the tail tips of metamorphosing *R.catesbeiana* tadpoles display activity during late premetamorphosis and early prometamorphosis. This activity could account for some of the activity seen for immunoprecipitated cyclin A-containing complexes at those stages; however, no Cdk2 activity was evident during tail regression.

Steady state levels of a 46 kDa cyclin A protein remain constant throughout metamorphosis. However, a 35 kDa protein appears upon induction of tail regression that is strikingly similar to an apoptotic form of cyclin A2 described in *Xenopus* embryos

upon exposure to ionizing radiation [96]. The *Xenopus* protein was generated by caspase cleavage of the 46 kDa cyclin A2 protein. This mechanism of generation is unlikely in *Rana* tail tips as concomitant loss in the 46 kDa protein was not observed. The amount of the 35 kDa protein decreases in the presence of roscovitine suggesting that complexes containing this truncated form of cyclin A may play a role in the T_3-dependent response.

Two temporally distinct cyclin A-associated kinase activities have been identified in the tail. The first peak in kinase activity correlates with Cdk2 activity during prometamorphosis (TK stages XII-XV), while the second peak occurs at the beginning of metamorphic climax (TK stages XVIII-XX) and is associated with an unidentified kinase. This unidentified kinase is inhibited by roscovitine *in vitro* suggesting that its catalytic domain is similar to that of the Cdk family. Cdk3 can interact with cyclin A *in vitro* [420] and is therefore a possible contender; however, it has not been previously shown to interact with cyclin A in cells under normal (non-transfected) conditions and the pattern of activity evident during metamorphosis suggests that it is unlikely to account for the cyclin A-associated activity seen during metamorphic climax.

Based upon the evidence presented, a model for Cdk activity during T_3-dependent apoptosis in the context of both natural and precocious metamorphosis is proposed (**Figure 2.15**). The model predicts that low-level Cdk activity phosphorylates a substrate that is present in very low amounts in the premetamorphic tadpole. Once phosphorylated, this protein is activated and contributes to the establishment of a proapoptotic state. Activation of this limited protein substrate can be prevented by the Cdk inhibitor, roscovitine, as was shown by the significant inhibition of tail regression in both pre- and prometamorphic tails (**Figure 2.3A,B**). Exogenous T_3 treatment results in the synthesis

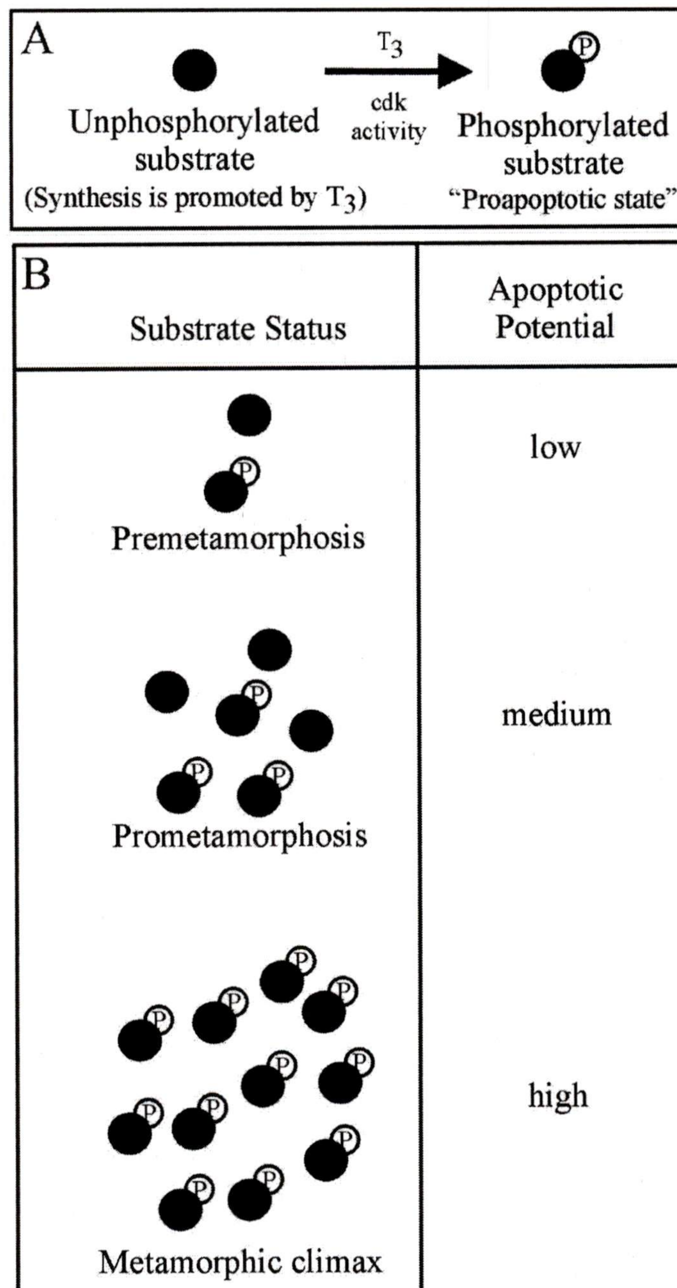


Figure 2.145. Proposed model for the role of Cdks during T_3-dependent tail regression.

(A) T_3 promotes the synthesis of a critical substrate that is the target for phosphorylation by Cdks. (B) The endogenous apoptotic potential in the tadpole tail is a function of the amount of phosphorylated substrate present at each developmental stage.

and phosphorylation of this substrate. This is exemplified by the sensitivity of T_3-induced or accelerated tail regression to inhibition by roscovitine at all developmental stages (**Figure 2.1 and 2.4**). As endogenous T_3 levels increase during prometamorphosis, more substrate is synthesized and available for activation by Cdks. Since Cdk activity also increases (**Figure 2.6 and 2.7**), much of the substrate becomes phosphorylated to the proapoptotic form. At metamorphic climax, all of the substrate (which is now at very high levels) is phosphorylated, thus promoting the execution of apoptotic events and active tail regression (**Figure 2.3**). Since all of the substrate pool has been posttranslationally-modified, roscovitine is incapable of affecting tail regression at that stage because there is no further substrate available for phosphorylation. The tadpole tail is mitotically active in premetamorphic tadpoles; however, cell division is attenuated by increasing TH levels [210]. Immunoblot analyses of the PCNA proliferative marker using tail tip homogenates from tadpoles undergoing natural and precocious metamorphosis show an expected decrease in steady state levels corresponding to this attenuation of cell proliferation (**Figure 2.9 and 2.10**). One would therefore expect that a decrease in proliferation would correlate with the onset of apoptosis. However, roscovitine treatment of cultured tail tips also resulted in a reduction in PCNA steady state levels in the presence and absence of T_3 (**Figure 2.11**), but this did not correlate with tail regression. Since no tail regression was observed under these conditions, this suggests that inhibition of proliferation is independent of the apoptotic process. Thus, the collective data suggest that the Cdk activity necessary for T_3-dependent apoptosis is distinct from that required for the initiation of cell proliferation. This may be manifested by differential substrate specificity and Cdk complex composition.

Chapter 3. Roscovitine inhibits TH-induced gene expression in the tail tip of *R. catesbeiana* tadpoles

1. Introduction

The purine analogue, roscovitine (2-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine) is a potent, but reversible specific competitive inhibitor of Cdks 1, 2, and 5 [8, 67, 124, 130, 262, 343, 401, 413] that arrests cells in the G_1 and the G_2/M phases of the cell cycle [8, 262, 308], inhibits DNA synthesis [339, 418], causes nucleolar fragmentation [62], induces apoptosis [11, 266], and inhibits apoptosis [53, 115, 153, 255, 286] in human cell lines.

We have shown that roscovitine inhibits the regression of cultured *R. catesbeiana* tail tips following T_3 treatment (chapter 2). It also inhibits the natural regression of tail tips obtained from pre- and prometamorphic tadpoles, but has no effect on the regression of tails derived from tadpoles undergoing metamorphic climax. By contrast, roscovitine was able to inhibit T_3-induced tail regression at all stages, suggesting that this compound was able to inhibit the induction of the TH-dependent genetic program.

The TH-dependent genetic program is initiated by the binding of T_3 to the nuclear TRs, recruiting the basal transcription factors and RNAP II to the promoter [206], suggesting that RNAP II may be a possible target for roscovitine inhibition of T_3-induced tail regression. Roscovitine's inhibitory effects have been most commonly attributed to its activity against Cdks 1, 2, and 5; however, there is some evidence that roscovitine can also inhibit Cdks that regulate transcription [130, 401].

Cdk7 has a dual role in regulating cell cycle progression as a component of CAK and as a component of the transcription factor TFIIH. Cdk7/cyclin H/Mat1 (ménage-à-trois

1) constitute CAK which activates several Cdks, including Cdks 1, 2, 4, and 6, through phosphorylation of a conserved threonine residue in their predicted T-loops [95, 230]. CAK also forms part of the RNAP II transcription factor, TFIIH [102]. TFIIH binds to promoter-bound RNAP II and the CAK component phosphorylates the CTD of the polymerase. This event stimulates promoter clearance and the onset of transcript elongation. The Cdk8/cyclin C complex has also been found associated with RNAP II early in the transcription cycle; however, it is biochemically distinct from the Cdk7 complex and shows limited sensitivity to inhibition by purine analogues [320]. The Cdk9/cyclin T complex comprises the RNAP II elongation factor pTEFb [303] which promotes CTD phosphorylation after promoter clearance at a step that occurs later in transcription than either of those regulated by Cdk7 or Cdk8 [311].

The CTD of mammalian RNAP II contains 52 near-perfect heptapeptide repeats of YSPTSPS [57]. Five out of the seven residues in the repeat are phosphate acceptors that are differentially phosphorylated by the CTD kinases. This differential phosphorylation is known to regulate the transition between transcriptional initiation and elongation, but may also function to modulate gene-specific transcription [10, 280, 360]. Roscovitine blocks phosphorylation of the CTD, thereby inhibiting mRNA synthesis, in human fibroblasts and HCT116 colon cancer cells [251], suggesting that its growth suppressive activity may be a combination of Cdk inactivation impacting on cell cycle progression and transcriptional inhibition. Roscovitine transcriptional inhibition is selective [228] which may be due to the presence of additional CTD kinases whose activities are not affected by roscovitine, including CKII, DNA-PK and the tyrosine kinases, c-Abl and Arg [16, 17, 19]. Cdk1 complexes and the ERKs, which are all inhibited by roscovitine,

also phosphorylate the CTD. The abundance and diversity of CTD kinases suggests that the site of CTD phosphorylation may be important in the regulation of transcriptional specificity.

Metamorphic amphibian tail regression is dependent upon changes in gene expression; therefore, the phosphorylation state of RNAP II was examined during natural metamorphosis and T_3-induced regression of cultured tails. Given that roscovitine inhibits both tail regression and the activities of CTD kinases, the effect of roscovitine on the phosphorylation state of RNAP II was evaluated. The data suggest that analysis of overall RNAP II phosphorylation levels does not correlate with induction of the metamorphic program. Given that the relative phosphorylation state of RNAP II affects the specificity of gene transcription [322] and that roscovitine differentially affects the steady state levels of mRNA transcripts [228], analysis of changes in gene expression was therefore necessary to determine the impact of roscovitine treatment on the tail. Real-time quantitative PCR analysis suggests that roscovitine may alter the TH-dependent genetic program in the tail.

2. Materials and Methods

2.1 Animal care.

Taylor and Kollros (TK) [381] stage VI-XXI *Rana catesbeiana* tadpoles were either locally caught or purchased (Wm. A. Lemberger Co). Animals were housed in the University of Victoria aquatics facility and maintained in dechlorinated, aerated tap water at 15°C in a constant flow-through system with exposure to natural daylight. Tadpoles were fed Nutrafin® flakes (Rolf C. Hagen Inc.) daily.

2.2 Tail organ culture.

The procedure used for tail culture was adapted from procedures described previously [144]. Tadpoles were sacrificed in 0.1% tricaine methanesulfonate (MS-222) (Syndel Laboratories). The animals were then immersed in sterile distilled water for 10 seconds, followed by a 5 second immersion in 70% ethanol and two subsequent 10 second rinses in sterile distilled water. Tail tips (2 cm) were removed and placed into Tadpole Minimal Essential Medium (TMEM: 55% strength solution of MEM (Invitrogen) adjusted to pH 7.1 and supplemented with 25 mM HEPES, 3 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 1.2 mM NaH_2PO_4, 20 mM NaCl, 2mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 50 μg/ml neomycin) at a density of 4 tips per 10 ml media. Tips were incubated for 24 hours at 25°C prior to addition of treatment reagents. After 24 hours, cultured tail tips were treated with equal volumes of dimethyl sulfoxide (DMSO) (vehicle), 3,5,3'-triidothyronine (T_3) (Sigma), or T_3 + roscovitine (Sigma). The final concentration of T_3 in the media was 100 nM while that of roscovitine was 60 μM. Media and chemicals were changed daily for the duration of the time course.

2.3 *In vivo* T_3 tadpole treatment.

Prior to treatment with T_3 or the vehicle (DMSO), TK stage VI tadpoles (6 per 8L water) were acclimated at room temperature for 2 days without feeding. After two days, equal volumes of T_3 or the DMSO vehicle were added to the water at a final concentration of 10^{-7} M T_3. Animals were euthanized in 0.1% MS-222 at 24 hour time points over 72 hours and the tail tips were collected for tissue homogenization and biopsy.

2.4 Tissue homogenization.

Tail tips were homogenized on ice in a buffer containing 25 mM HEPES, 10 mM EDTA, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM DTT, 100 μM PMSF, 4 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain and 300 μg/ml benzamidine using a volume of 3 ml buffer per gram tissue [267]. Homogenates were centrifuged at 12,000 x g for 20 min at 4°C and the collected supernatant was stored at –70°C. The concentration of protein within the homogenate was determined using the Bio-Rad Protein Assay (Bio-Rad).

2.5 Immunoprecipitation (IP) and kinase assay.

Tissue homogenates (500 μg) were diluted to a 1 ml volume in IP buffer containing 50 mM HEPES (pH 8.0), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, and 0.1% Tween-20. Homogenates were precleared by rotation with 20 μl/ml of protein G-Sepharose beads (Amersham) for 20 minutes at 4°C. Following preclearing, the mixture was centrifuged at 3,000xg for 10 min at 4°C and the supernatant transferred to microfuge tubes containing 20 μl of fresh beads and 5 μg of polyclonal antibody. Immunoprecipitations were carried out using rabbit polyclonal antibody ERK1 (K-23) (Santa Cruz Biotechnology). The antibody-bead-homogenate solution was mixed by rotation at 4°C for 2 hours and the beads were washed 3 times with 1 ml IP buffer. The complexes were further washed in 1 ml kinase reaction buffer containing 50 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 1 mM EDTA, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM dithiothreitol and 20 μM reduced glutathione. The immunoprecipitates were then incubated in 25 μl kinase reaction buffer containing 1.2 μM cold ATP (Invitrogen), 10 μCi γ^{32}P-ATP (Amersham) and 6.75 pmole of RNAP II CTD (gift from

Michael E. Dahmus, Department of Biochemistry and Biophysics, University of California) for 40 minutes at 30°C. The reactions were terminated by boiling for 3 minutes in sodium dodecyl sulfate (SDS) sample buffer [227]. The protein substrate was then resolved by 6% SDS-PAGE. The resulting gel was dried and exposed to phosphor imaging screens (Amersham) for ~ 16 hours. Radiographic data was obtained using a Storm 820 optical scanner phosphor imaging system at 50 μM resolution (Amersham).

2.6 *In vitro* roscovitine inhibition assay.

Immune complexes were immunoprecipitated from tadpole tail homogenate and incubated in kinase reaction buffer containing equal volumes of either DMSO or 50 μM roscovitine for 40 minutes at 30°C. Kinase reactions were resolved by SDS-PAGE and visualized by exposure to phosphor imaging screens, as described in section 2.5.

2.7 Immunoblotting.

Equal quantities of tail tissue homogenate (30 μg/lane) in SDS sample buffer were electrophoresed through 6% SDS-polyacrylamide gels and transferred to 0.2 μm nitrocellulose (Bio-Rad) [146]. Protein loading was verified by membrane staining with 0.1% Ponceau S (Sigma) in 5% acetic acid. Membranes were blocked with 5% nonfat milk in 0.2M NaCl, 4.2 mM KCl, 12.7 mM Na_2HPO_4, 2.3 mM KH_2PO_4, and 0.15% Tween 20 (PBST) overnight at 4°C and then incubated with primary antibody solutions diluted in 1% nonfat milk/PBST for 1h at room temperature, shaking. The antibodies: RNA polymerase II (N-20); ERK1 (K-23); ERK2 (C-14); and cyclin H (C-18) were obtained from Santa Cruz Biotechnology.

Table 3. 1. Characterization of antibodies used for immunoblotting.

Antibody	Species	Epitope	Specifity
RNA polymerase II (N-20)	Rabbit polyclonal	N-terminus of large subunit of RNA polymerase II of mouse origin.	Mouse, rat and human reactive
ERK1 (K-23)	Rabbit polyclonal	Subdomain XI of ERK1 of rat origin.	Broad interspecies reactivity, including murine, human, chicken and frog.
ERK2 (C-14)	Rabbit polyclonal	Carboxy terminus of ERK2 of rat origin.	Broad interspecies reactivity, including murine, human, chicken and frog.
Cyclin H (C-18)	Rabbit polyclonal	Carboxy terminus of cyclin H of human origin.	Mouse, rat and human reactive.

Antibodies were diluted accordingly: rabbit anti-RNA polymerase II, 1/200; rabbit anti-ERK1, 1/2500; rabbit anti-ERK2, 1/2500; and rabbit anti-cyclin H, 1/200. Following the primary antibody incubation, blots were washed for 1h at room temperature in PBST, shaking. Washed blots were then incubated with secondary antibodies diluted in 1% nonfat milk/PBST for 30 minutes at room temperature, shaking. Goat anti-rabbit and goat anti-mouse HRP-conjugated antibodies were diluted 1/3000 in 1% nonfat milk/PBST. Blots were washed for an additional hour in PBST following incubation with secondary antibodies and then processed using the enhanced chemiluminescence (ECL) method, as described by the manufacturer (Amersham) and exposed to Kodak Biomax film.

2.8 Tissue Biopsy

For RNA isolation, duplicate tissue biopsies were taken from the tadpole dorsal tail fin using a dermal punch that cut out 2mm-diameter tail fin discs (Stevens, Vancouver, BC). These biopsies were immediately immersed in 50 µl of the RNA preservative RNALater (Ambion, Austin, TX) as previously described [395] and stored at 4°C until RNA processing was required.

2.9 Preparation of total RNA

The fin biopsies were ground in 100 µl of Trizol (Invitrogen Canada Inc, Burlington, ON) using a disposable Kostle polypropylene micropestle and 20 µl of sand according to the manufacturer's instructions. After phase separation, either 20 µg of yeast tRNA or glycogen was added as a nucleic acid carrier. Isolated RNA was subsequently resuspended in 10 µl of RNase-free water and stored at –70°C.

2.10 Preparation of total cDNA

The total RNA isolated from each tissue (~1µg/10µl) was used to produce cDNA. The RNA was annealed with 500 ng of random hexamer oligonucleotides (Amersham Biosciences Corp) and 450 nM dNTPs (Invitrogen Canada Inc) in a 10 µl volume, at 65°C for 10 minutes. The RNA was chilled on ice following the annealing process and then heated to 42°C for 2 hours in a 20 µl solution of 1x FSB (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 20 mM DTT, 10 units RNasin (Promega Corp.) and 200 units SUPERSCRIPT II RNase H^- Reverse Transcriptase (Invitrogen Canada Inc). The resulting cDNA was then diluted twenty-fold with DEPC-treated water for use in PCR reactions.

2.11 Primer design

Primers were designed by Dr. N. Veldhoen against the following sequences: *Rana catesbeiana* TRβ (GENBANK: L27344); *Rana duboisi* 16S (GENBANK: U66113); *Xenopus laevis* L8 (GENBANK: U00920); *Xenopus laevis* K-Ras (GENBANK: AF08280); *Xenopus laevis* MPK1 (ERK2) (GENBANK: X59813); *Xenopus laevis* MAPK phosphatase (GENBANK: X83742); and *Xenopus laevis* 18S (GENBANK: X04025) using Primer Premier v4.1 (Premier Biosoft International).

Table 3. 2. Primer sequences used in the isolation of *R.catesbeiana* gene fragments.

Gene target	Sense primer (5'-3')	Antisense primer (5'-3')	Target size (bp)
TRβ	AGCAGCATGTCAGGGTAC	TGAAGGCTTCTAAGTCCA	538
16S	AGAAGGAACTCGGCAAAT	CCAACATCGAGGTCGTAA	533
L8	CAGGGGACAGAGAAAAGGTG	ACGACGAGCAGCAATAAGAC	711
K-Ras	TGGTGGTGGTTGGTGCTA	TGAGCTTTCTTGCCACAG	486
ERK2	TTTTGGATTGGCTCGTGT	TTGGGTCATAATACTGCTC	456
MAPK phos.	GTCAAGAGAGGCGGAAAG	GGCTGTAATGCCAAGGGT	456
18S	AGAAACGGCTACCACAT	TAGCGGCACAATACGA	508

Following isolation of L8, K-Ras, MPK1, and MAPK phosphatase gene fragments from *Rana catesbeiana*, new primers were designed for *Rana catesbeiana*-specific gene sequences for use in QPCR.

Table 3. 3. Primer sequences designed against *R.catesbeiana* gene sequence for amplification of gene fragments by quantitative PCR.

Gene target	Sense primer (5'-3')	Antisense primer (5'-3')	Target size (bp)
L8	CAGGGGACAGAGAAAAGGTG	TGAGCTTTCTTGCCACAG	270
K-Ras	GTTTCCTCTGCGTCTTTG	GCTTGGTGTCTACCGTTC	156
ERK2	GGCTTTGGAGCTGCATTT	TATCTGGTCTGTCGGTTGC	229
MAPK phos.	CGGCTGATCGCTGGATTG	CACTGAGGGGCAGGCTTA	232

2.12 PCR amplification and isolation of gene fragments

The gene fragments were amplified from stage XIX *R.catesbeiana* mixed tissues using 1.25 units of *Taq* DNA polymerase (Invitrogen Canada Inc) in 50 μl PCR reactions containing 2 μl of 1/20 diluted cDNA, 1X PCR buffer (Invitrogen Canada Inc.), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 10 pmoles of each primer (alphaDNA). The PCR reactions were overlayed with 50 μl of mineral oil. The thermocycle program included a 7 minute denaturation step at 94°C followed by 45 cycles of 60s at 94°C, 60s at 52°C, and 60s at 72°C followed by a final 10 minute elongation step at 72°C, using the Perkin Elmer Thermal Cycler. The amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. Agarose gels were prepared with OmniPur molecular grade agarose powder (EM Science) in 1X TAE buffer (40 mM Tris-acetic acid pH 8.0, 1 mM EDTA pH 8.0) containing 0.0015% EtBr (Invitrogen Canada Inc). DNA samples were loaded in STOP buffer (7.0 M Urea, 50% sucrose, 50 mM EDTA, 0.1% bromophenol blue). DNA was visualized and photographed with an AlphaImager 2000 Documentation and Analysis System (Alpha Innotech Corporation). Purified PCR

products were isolated by excising the bands from the agarose gel and the isolated DNA was purified from the agarose using the freeze-squeeze technique [380]. Briefly, gel chunks were spun to the bottom of the microfuge tube by centrifugation at 10,000 x g for 5 minutes at 4°C. DNA was squeezed from the agarose by subjecting the gel chunks to alternating 5 minute immersions in a dry ice/ethanol bath and 5 minute immersions in a 37°C water bath, over a 30 minute period. The DNA solution was then spun at 10,000 x g for 10 minutes at 4°C and cloned into the pCRII-TOPO plasmid vector (Invitrogen Canada Inc).

2.13 Cloning and sequencing

The isolated *Taq* polymerase-amplified PCR products were cloned into the pCRII-TOPO plasmid vector using the TOPO TA Cloning Kit (Invitrogen Canada Inc) according to the manufacturer's instructions. The TOPO cloning reaction was transformed into TOP10 chemically competent *E.coli* cells using the One Shot system (Invitrogen Canada Inc), according to the manufacturer's instructions, plated onto LB agar containing 100 μg/ml ampicillin (Sigma) and 80 μg/ml of molecular biological grade X-gal (US Biologicals) and grown overnight at 37°C. Isolated colonies were then inoculated into LB broth containing 100 μg/ml ampicillin. Broth cultures were grown for 16 hours at 37°C with vigorous shaking. After 16 hours, plasmids were isolated from these cultures using the QIAprep Miniprep kit (Qiagen Inc) according to the manufacturer's instructions. Isolated plasmids (2 μl of a 50 μl mini-prep) were individually digested for 1 hour at 37°C with 4 units of EcoRI (New England Biolabs Ltd.) in NEB Buffer 2 (10mM Tris HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT). Vector and insert were separated by electrophoresis through a 1.5% agarose gel and visualized by ethidium bromide staining.

Clones containing an insert of the correct size were submitted to the Centre for Environmental Health (University of Victoria, Victoria, BC) and automated dideoxynucleotide sequencing was performed using a NEN Global IR2 DNA Sequencer (LI-COR, Inc., Lincoln, NB). Sequence identities were confirmed by alignment with GenBank sequences using BLAST.

2.14 Quantitative PCR Analysis

Quantitative analysis of gene expression in *R.catesbeiana* tissues was conducted using the Mx4000 Multiplex Quantitative PCR System (Stratagene). Gene expression for TRβ and 16S in *R.catesbeiana* tissues was determined by quantitative amplification using 1 unit of Platinum Taq DNA polymerase (Invitrogen Canada Inc) in a 15 μl quantitative polymerase chain reaction (QPCR) initiated by activating the enzyme at 95°C for 9 minutes followed by 40 cycles of 15s at 95°C, 30s at 55°C, and 45s at 72°C. A 15 μl QPCR reaction contained 10 mM Tris, 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween-20, 0.8% glycerol, a 1/40,000 dilution of SYBR Green I (Molecular Probes Inc.), 0.2 mM dNTPs, 10 pmoles of each primer, 83.3 nM ROX reference dye (Stratagene) and 2 μl of 1/20 diluted cDNA. Three fluorescence readings were taken during the annealing step of each cycle for both the SYBR green I (492 nm excitation wavelength/516 nm emission wavelength) and the ROX reference dye (585 nm excitation wavelength/610 nm emission wavelength). Controls lacking either cDNA template or Platinum *Taq* DNA polymerase were included to assess the specificity of target cDNA amplification. Following cDNA amplification, the SYBR Green I fluorescence data was corrected for volume variations using the ROX dye signal. The cycle threshold (Ct) for each experiment was then

optimized using the moving average and adaptive baseline algorithms (Mx4000, Stratagene).

Standard curves were generated for each of the genes analyzed by amplifying the desired gene target from plasmid DNA containing the appropriate insert. Reactions were conducted in triplicate over a range of 400 to 250,000 molecules of DNA per reaction. The Ct values obtained from those reactions were plotted against the log copy number of each plasmid and the resulting linear equation was used to determine the copy number of each gene in *R.catesbeiana* tissues. The invariant 16S control was used as a normalization factor to account for variations in cDNA input.

QPCR reactions were conducted on cDNA obtained from multiple animals for each experiment (natural metamorphosis analysis: n = 4-15 for each stage grouping; precocious metamorphosis analysis: n = 5 for each time point; tail culture analysis: n = 5-10 for each treatment).

2.15 Statistical analysis

Statistical analysis was conducted using In-Stat v3.01 (GraphPad Software Inc., San Diego, CA). The One-way analysis of variance (ANOVA) was conducted to determine the p-value and the Tukey post-test was conducted if $p>0.05$.

3. Results

3.1 Gene expression in the tail is altered during natural metamorphosis.

Amphibian metamorphosis is induced by TH; however, natural progression through metamorphosis is modulated by additional hormones, including adrenal steroids, catecholamines and prolactin [109, 140, 141, 165, 182, 213, 239, 325, 415]. The interplay between these hormones influences the timing and specificity of the

metamorphic program and seems to rely on changes in gene expression [394]. Using array technology, Veldhoen *et al* [394] identified 79 genes whose expression levels were altered in the *Xenopus* tadpole tail during natural metamorphosis. Given that induction of the TH-dependent genetic program depends on the transcriptional activity of RNAP II, attempts were made to clone TH-regulated genes as well as those known to regulate the polymerase itself. The desired targets included components of the cell cycle and the MAPK signal transduction pathway. Only four genes were successfully cloned and found to be suitable for QPCR analysis: K-Ras, MAPK phosphatase, ERK2 and TRβ. TRβ has been extensively characterized in *Xenopus* [35, 86, 394, 406, 424], while changes in the steady state levels of MAPK phosphatase transcripts were recently described by Veldhoen *et al* [394] in *Xenopus*. To determine the extent of genetic modulation in the tail of *Rana catesbeiana* tadpoles undergoing natural metamorphosis, those four genes were analyzed by QPCR and normalized to 16S transcript levels (**Figure 3.1**).

MAPK phosphatase, ERK2, K-Ras, and TRβ all showed increased transcript steady state levels during natural metamorphosis. MAPK phosphatase transcript steady state levels increased 6-fold at prometamorphic TK stages XVIII-XX, relative to the levels in premetamorphic tail ($p<0.01$), while ERK2, K-Ras and TRβ only showed increased steady state levels at metamorphic climax (TK stage XXI-XXII). TRβ steady state levels increased approximately 4-fold at metamorphic climax, relative to the levels in premetamorphic animals ($p<0.001$), while ERK2 steady state transcript levels increased approximately 3-fold ($p<0.05$). K-Ras transcript levels remained relatively constant throughout development, dropping slightly between stages XVIII-XX, and increasing at

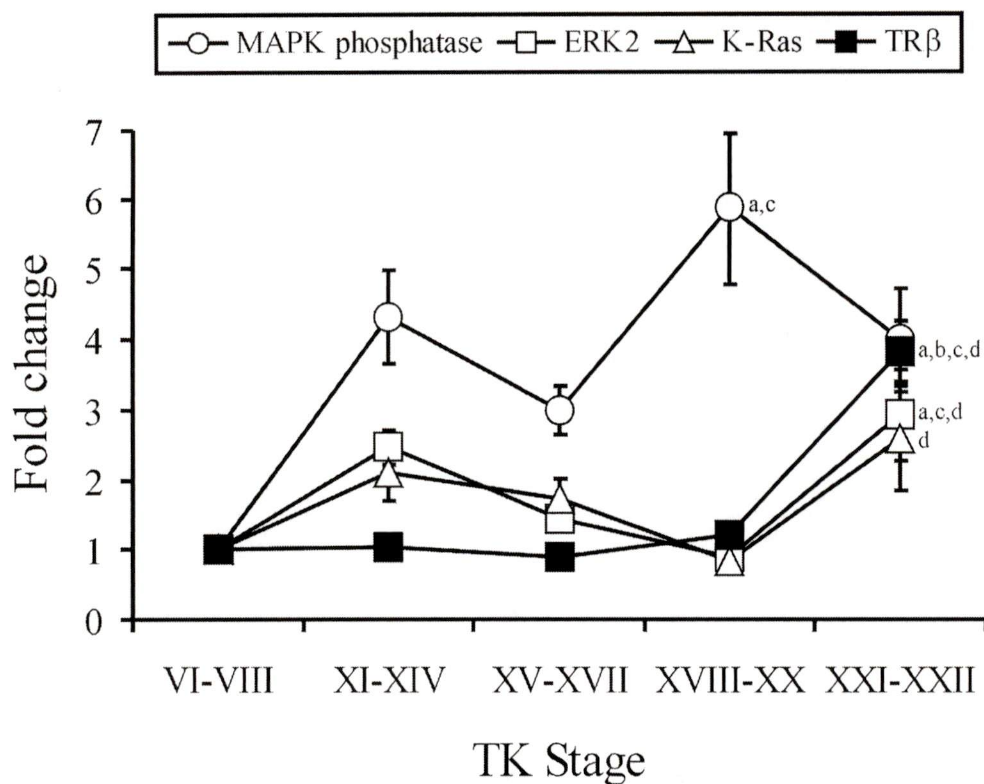


Figure 3.1. Gene expression in the tail changes during natural metamorphosis.

Transcript levels were normalized to 16S and are displayed as fold change relative to stage VI-VIII transcripts. Multiple animals were sampled for each stage grouping (VI-VIII: n=4; XI-XIV: n=8; XIV-XVII: n=12; XVIII-XX: n=9; XXI-XXII: n=8). Error bars represent the SEM. Statistically significant differences are indicated accordingly: a = as compared to VI; b = as compared to XI-XIV; c = as compared to XV-XVII; d = as compared to XVIII-XX.

stages XXI-XXII ($p<0.05$ relative to XVIII-XX). The MAPK phosphatase and TRβ data are consistent with those obtained by Veldhoen *et al* [394] in *Xenopus.*

3.2 RNA polymerase II is phosphorylated during natural metamorphosis.

The synthesis of mRNA in eukaryotes is regulated by RNAP II in a multistage process that requires numerous transcription factors, including the general transcription factor TFIIH. These transcription factors and the polymerase itself are all subject to regulation by phosphorylation. This phosphorylation is mediated by numerous kinases, including Cdks 7, 8, and 9, and the ERKs [322]. Changes in transcription are essential in executing the tail metamorphic program; therefore, the protein steady state levels and phosphorylation state of RNA polymerase was examined by immunoblotting (**Figure 3.2**). The protein steady state levels of several CTD kinase components including, cyclin H, ERK1, and ERK2, were also examined.

RNA polymerase II became increasingly phosphorylated during natural metamorphosis. The hypophosphorylated form (IIa; Mr= 250kDa) was invariant at all stages examined; however, the steady state levels of phosphorylated forms, including IIo, increased during development. This increase in phosphorylation does not correlate with the steady state levels of cyclin H (Mr= 32kDa), ERK1 (Mr= 36kDa) and ERK2 (Mr= 36kDa), which were invariant.

Cyclin A-containing complexes were sensitive to inhibition by roscovitine *in vitro* and showed differential kinase activity during natural metamorphosis; therefore, the ability of those complexes to phosphorylate the RNAP II CTD was examined (data not shown). CTD phosphorylation by ERK1 complexes was examined in conjunction with cyclin A-containing complexes, as a positive control (**Figure 3.3**). ERK1 complexes

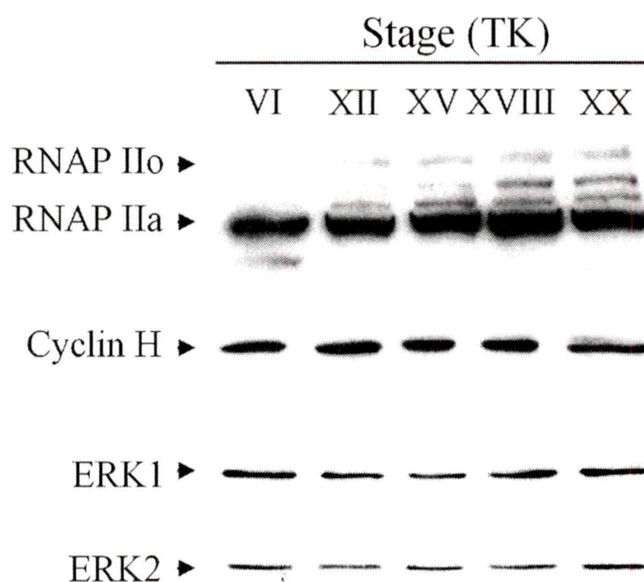


Figure 3.2. RNA polymerase II is phosphorylated during natural metamorphosis.

Tail tip protein homogenates were separated by 6% SDS-PAGE for RNA polymerase II and 12% SDS-PAGE for cyclin H, ERK1 and ERK2 and immunoblotted with rabbit polyclonal antibodies specific for the N-terminus of RNA polymerase, cyclin H, ERK1 or ERK2. The putative hypophosphorylated (IIa) and hyperphosphorylated (IIo) forms of RNA polymerase II are indicated, based upon relative migration.

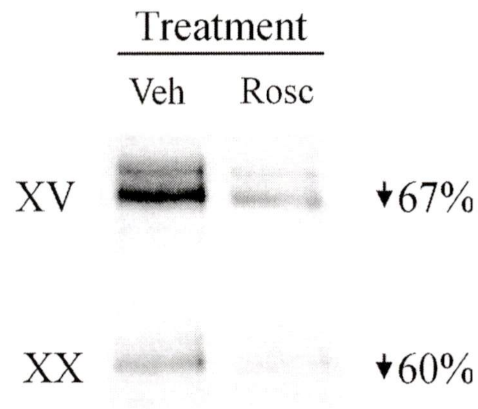


Figure 3.3. CTD of RNAP II is phosphorylated by ERK1.

ERK1 complexes were immunoprecipitated from premetamorphic TK stage XV and metamorphic TK stage XX tail tip homogenates. Immunoprecipitates were incubated in the presence of DMSO vehicle (Veh) or 50 μM roscovitine (Rosc) during the kinase reaction. The extent of inhibition is indicated for each stage. CTD was resolved by 6% SDS-PAGE, migrating at 110 kDa.

phosphorylated the CTD in a stage-dependent manner; however, the cyclin A-containing complexes exhibited no detectable CTD kinase activity. ERK2 complexes were not examined because they demonstrated no differential MBP kinase activity (data not shown), while the ERK1 complexes showed a stage-dependent pattern of phosphorylation similar to that seen for CTD.

3.3 Roscovitine affects protein steady state levels

Given that roscovitine can inhibit CTD-specific ERK1 activity *in vitro* and may inhibit the activity of Cdk7 [130] complexes, it was postulated that roscovitine would inhibit the phosphorylation of RNAP II *in vivo*. This hypothesis was addressed by immunoblot analysis of RNAP II, cyclin H, ERK1 and ERK2 in cultured premetamorphic tail tips treated with roscovitine (**Figure 3.4**).

Tail tips were treated with vehicle (DMSO), T_3, roscovitine, and T_3 + roscovitine for 24 or 48 hours and protein steady state levels were examined by immunoblot analysis. At 24 hours, T_3 treatment induced a reduction in RNAP IIo and cyclin H steady state levels, while ERK1 and ERK2 both showed a modest increase. Roscovitine alone also reduced RNAP IIo steady state levels relative to the vehicle control; however, this reduction was not as substantial as that seen in the T_3 treated tails. By contrast, roscovitine treatment further reduced cyclin H steady state levels and had minimal effect on the levels of the ERK proteins with respect to the vehicle control. Coapplication of T_3 with roscovitine had a dramatic impact of the steady state levels of RNAP II. Both forms of RNAP II were substantially reduced. The steady state levels of cyclin H were reduced upon roscovitine treatment independent of T_3 presence; whereas, ERK steady state levels were not affected by roscovitine.

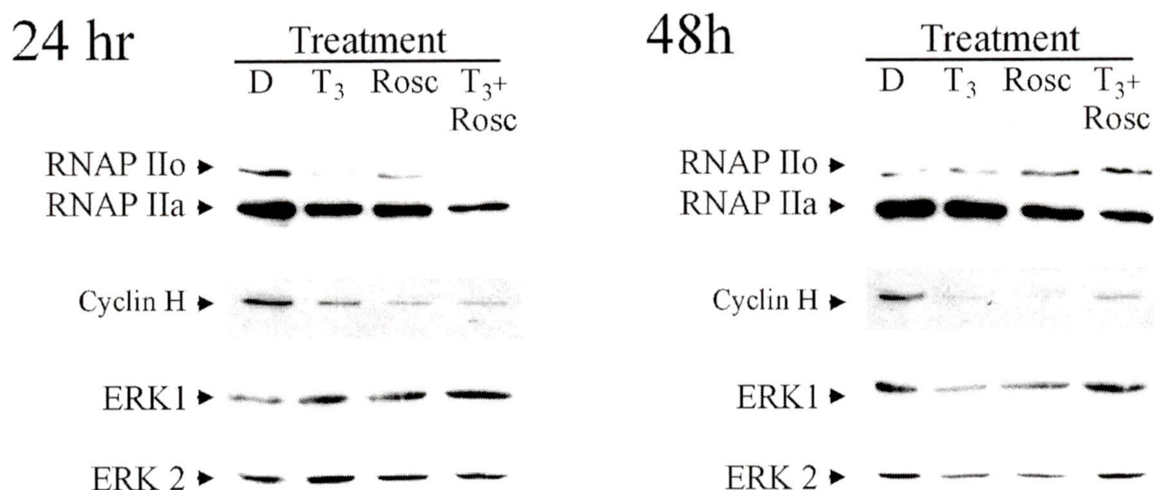


Figure 3.4. Roscovitine affects protein steady state levels.

Tadpole tail tips were cultured at 25°C for 24 or 48 hours in the presence of DMSO vehicle (D), 100 nM T_3, 100nM T_3 + 60 μM roscovitine (T_3 + Rosc) or 60 μM roscovitine alone (Rosc). Multiple animals were sampled for each treatment (24h DMSO: n=6; 24h T_3: n=10; 24h T_3 + Rosc: n=6; 24h Rosc: n=6; 48h DMSO: n=5; 48h T_3: n=10; 48h T_3 + Rosc: n=6; 48h Rosc: n=6). Tail tip protein homogenates were separated by 6% SDS-PAGE for RNA polymerase II and 12% SDS-PAGE for cyclin H, ERK1 and ERK2 and immunoblotted with rabbit polyclonal antibodies specific for the N-terminus of RNA polymerase, cyclin H, ERK1 or ERK2. The putative hypophosphorylated (IIa) and hyperphosphorylated (IIo) forms of RNA polymerase II are indicated, based upon relative migration.

At 48 hours, T_3 treatment had little effect on RNAP II steady state levels; although, it reduced the levels of cyclin H, ERK1 and ERK2 as compared with the DMSO vehicle. Treatment with roscovitine, by contrast, increased the levels of RNAP IIo, while reducing the levels of the IIa form. Cyclin H levels were considerably reduced by roscovitine treatment, while ERK1 and ERK2 levels were moderately reduced. The coapplication of T_3 and roscovitine further reduced the steady state levels of the IIa form, but did not alter the steady state levels of the IIo form as compared to tails treated with roscovitine alone. The coapplication also attenuated the inhibitory effects of treatment with either T_3 or roscovitine alone on cyclin H, ERK1 and ERK2 levels.

3.4 Roscovitine differentially alters the steady state levels of gene transcripts

Roscovitine was previously shown to inhibit T_3-induced tail regression of cultured *R.catesbeiana* tail tips (chapter 2). Given that roscovitine can inhibit the activities of numerous kinases, including Cdks 1, 2, 5, 7, and 9 [130], it was postulated that in addition to inhibiting the activities of kinases associated with cell cycle progression, roscovitine may also influence the activities of kinases involved in transcriptional regulation. Lam *et al* [228] recently assessed the effects of roscovitine on transcription using cDNA array technology and found that roscovitine's effects on transcription included both down- and up-regulation of gene transcripts contrary to the predicted global down-regulation. Roscovitine's effects on gene expression in the tadpole tail were evaluated by QPCR analysis of 4 genes, including the T_3-direct-response gene, TRβ, and components of the MAPK signal transduction pathway, K-Ras, ERK2 and MAPK phosphatase (**Figure 3.5**).

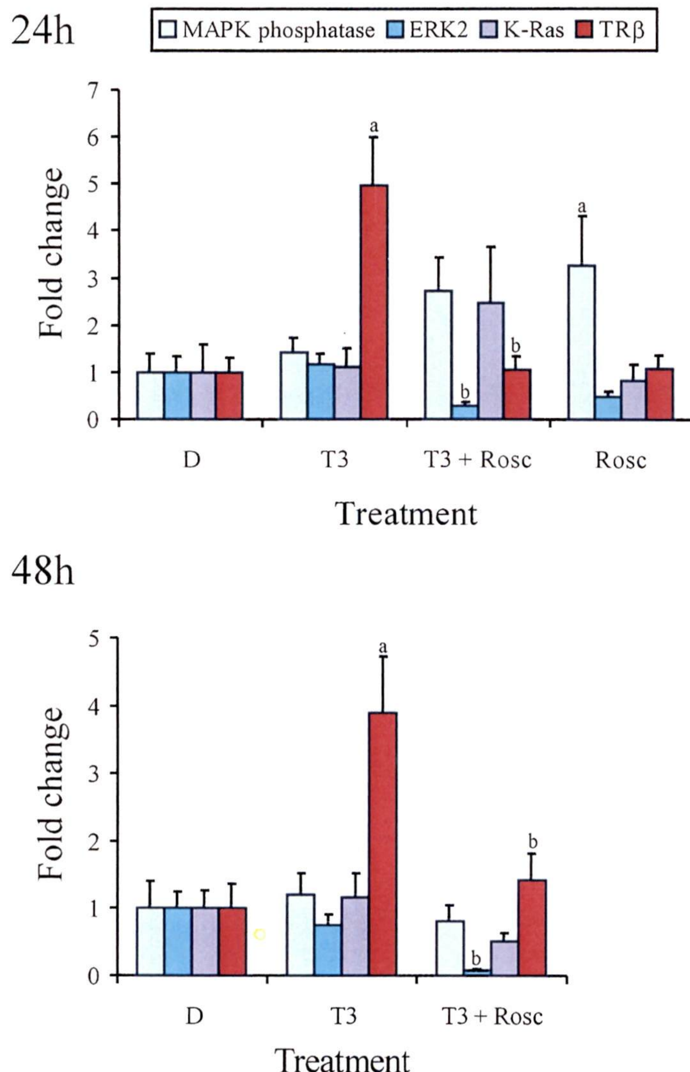


Figure 3.5. Roscovitine affects the steady state levels of TRβ and ERK2.

Transcript levels were normalized to 16S and are displayed as fold change relative to the DMSO transcripts for each time point. Tadpole tail tips were cultured at 25°C for 24 or 48 hours in the presence of DMSO vehicle (D), 100 nM T_3, 100nM T_3 + 60 μM roscovitine (T3 + Rosc) or 60 μM roscovitine alone (Rosc). Multiple animals were sampled for each treatment (24h DMSO: n=6; 24h T_3: n=10; 24h T_3 + Rosc: n=6; 24h Rosc: n=8; 48h DMSO: n=5; 48h T_3: n=10; 48h T_3 + Rosc: n=6). Error bars represent the SEM. Statistically significant differences ($p<0.05$) are indicated accordingly: a=D to T3 comparison; b=T3 toT3+Rosc comparison.

Tail tips were treated with vehicle (DMSO), T_3, roscovitine, and T_3 + roscovitine for 24 or 48 hours and transcript steady state levels were then examined by QPCR analysis and normalized relative to 16S. As expected, T_3 treatment resulted in increased transcript levels for TRβ at both 24 hour and 48 hour time points. TRβ transcripts were increased 5-fold at 24 hours ($p<0.05$) and 4-fold at 48 hours ($p<0.05$) relative to the DMSO control. The transcript levels of MAPK phosphatase, ERK2, and K-Ras were not significantly affected by T_3 treatment. This observation is consistent with previous findings in our lab [147]. Treatment with roscovitine alone (24 hours) had no effect on transcript levels for ERK2, K-Ras, and TRβ; however, MAPK phosphatase levels were significantly increased ($p<0.05$) with respect to the DMSO-treated tails. Coapplication of T_3 and roscovitine resulted in complete attenuation of the T_3-induced increase of TRβ transcripts at both time points ($p<0.05$). ERK2 transcript levels were also significantly reduced at both time points (24 hours: $p<0.05$; 48 hours: $p<0.01$).

3.5 Roscovitine affects the steady state levels of "normalizer" gene transcripts suggesting a lower RNA yield

In order to ensure that the QPCR results for each gene are comparable from one sample to the next, we used the same amount of starting tissue (i.e. one biopsy punch) to convert to cDNA. We then measured the endogenous levels of L8 ribosomal protein transcript and used this as a normalizer for cDNA input. Previous work demonstrated that the levels of L8 transcript were unaffected by T_3 treatment [351], which was shown to be the case in this study. However, we found that roscovitine $\pm T_3$ substantially reduced the levels of L8 transcript. Since this transcript may be a target for roscovitine *via* RNAP II, we decided to examine the steady state levels of 18S and 16S (**Figure 3.6**) rRNA that are synthesized by other chemically-distinct RNAPs [21].

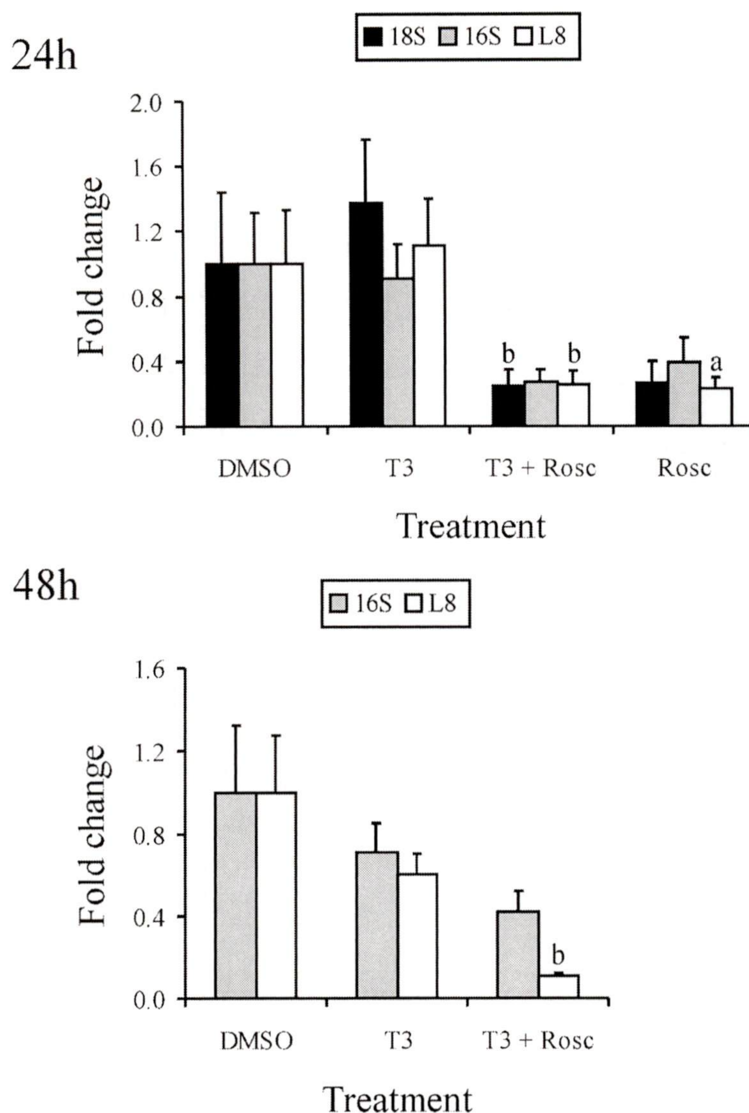


Figure 3.6. Roscovitine may affect total RNA yield obtained from biospies obtained from cultured tail tips.

Transcript levels are displayed as fold change relative to the DMSO stranscripts for each time point. Tadpole tail tips were cultured at 25°C for 24 or 48 hours in the presence of DMSO vehicle (D), 100 nM T_3, 100nM T_3 + 60 μM roscovitine (T3 + Rosc) or 60 μM roscovitine alone (Rosc). Multiple animals were sampled for each treatment (24h DMSO: n=6; 24h T_3: n=10; 24h T_3 + Rosc: n=6; 24h Rosc: n=8; 48h DMSO: n=5; 48h T_3: n=10; 48h T_3 + Rosc: n=6). Error bars represent the SEM. Statistically significant differences ($p<0.05$) are indicated accordingly: a=D to Rosc comparison; b=T3 to T3+Rosc comparison.

Tail treatment with roscovitine $\pm T_3$ resulted in a substantial reduction (2-4 fold) in levels of all 3 gene transcripts. Since 16S rRNA was the least affected, we used it as a normalizer. Given that 18S RNA constitutes approximately 80% of the cellular RNA [160], reduction of that transcript should be a fairly good indicator that RNA yield has been decreased. Roscovitine reduced 18S transcript levels by 4-fold as compared to DMSO-treated tails while the coapplication of T_3 and roscovitine resulted in a 6-fold reduction as compared to T_3-treated tails.

4. Discussion

Amphibian metamorphosis is an excellent model system in which to study the molecular basis of TH action in vertebrates. The change from tadpole to frog is entirely dependent upon endogenous TH, while metamorphosis can be precociously induced by the addition of TH to the tadpole environment. The metamorphic program is dependent upon TH and involves a tissue-specific alteration of gene expression programs. TRβ expression is rapidly and directly affected [192] in a manner that is resistant to protein synthesis inhibitors [316]. TRβ is one transcription factor among many that initiates a second wave of gene expression coinciding with a change in the observed morphology of the tail. These late response genes include several proteases that function to rapidly degrade the tail following induction [35]. These genes fall into several groups as defined by their kinetics of regulation and the cells in which they are expressed [22, 35, 405]. Several genetic programs regulating tail regression appear to be involved. This was shown in metamorphic *Xenopus laevis* tadpoles following inhibition of TH production, resulting in a differential loss of tail tissue [87].

The metamorphic program is further complicated by the involvement of additional hormone modulators that contribute to the genetic expression profile of the tail during natural metamorphosis [109, 140, 141, 165, 182, 213, 239, 325, 415]. Veldhoen *et al.* [394] identified 79 genes whose steady-state expression levels were altered in the tadpole tail during natural metamorphosis. Of those genes, 34 were previously identified as TH responsive in frogs or mammals. They further found that the levels of 28 transcripts were altered during premetamorphosis, 31 during prometamorphosis and 43 at the onset of tail regression.

The original intent of this work was to characterize the effect of roscovitine on the steady state levels of several gene transcripts, including: TRβ; TH-responsive genes downstream of TRβ, such as stromelysin-3; and various genes important in signal transduction and cell cycle regulation. These genes were selected because roscovitine was known to inhibit the kinase activities of various Cdks and was found to inhibit T_3-induced tail regression (chapter 2). Given the correlation of roscovitine activity with the commitment point between 24 and 48 hours (chapter 2), it is reasonable to assume that roscovitine could be affecting transcription. It was therefore postulated that roscovitine could inhibit tail regression by interfering with the induction of the TH-dependent genetic program in the tail. The most likely targets of this inhibition are the Cdks involved in regulating transcriptional activation through phosphorylation of RNAP II.

RNAP II is progressively phosphorylated during natural metamorphosis (**Figure 3.2**); however, without analyzing the activity of potential CTD kinases, it is not possible to identify those responsible for this regulation. The ERKs and the CAK complex are known to phosphorylate the CTD; however, the CTD kinase activity of those complexes

was not fully examined during natural regression and remains an important avenue to explore. It is unlikely that the ERKs play a significant role in tail regression given that treatment of cultured tails with the MEK inhibitor, PD098059, had no effect on T_3-induced tail regression (chapter 2). The CTD of RNAP II is an unlikely substrate for cyclin A-containing complexes since they did not phosphorylate the CTD protein *in vitro*. Therefore, the data suggests that neither the ERKs nor the cyclin A-containing complexes regulate the transcriptional activity of RNAP II, to coordinate events associated with tail regression. Given that both Cdk7 and Cdk9 are also sensitive to inhibition by roscovitine and that both function to regulate transcription, the CTD kinase activity of those proteins should be examined to establish a connection between induction of the TH-dependent genetic program and RNAP II-mediated transcription.

While RNAP II is extensively phosphorylated during natural metamorphosis, this phosphorylation was not inducible by T_3 treatment (**Figure 3.4**), suggesting that a general change in the phosphorylation state of RNAP II is not causitive of the induction of tail regression. The treatment of tadpole tail tips with roscovitine $\pm$ T_3 substantially altered the protein steady state levels of RNAP II, ERK1, ERK2 and cyclin H; however, it is not possible to correlate those differences with meaningful changes in gene expression. Given that the differential phosphorylation state of RNAP II contributes to the specificity of transcription, it is possible that each RNAP II profile observed in the tail following treatment with DMSO, roscovitine and T_3 $\pm$ roscovitine may represent a unique transcriptional state. It is also clear that tissue fate cannot be correlated with the phosphorylation state of RNAP II. Neither DMSO nor T_3 + roscovitine treated tails undergo regression in culture and yet they exhibit different RNAP II profiles.

The complexity of this regulation suggests that the analysis of transcript levels might be more useful in determining the impact of roscovitine treatment on gene expression. Therefore, the effect of roscovitine treatment on the transcript steady state levels of MAPK phosphatase, ERK2, K-Ras and TRβ in tail tips was examined. Roscovitine alone had no effect on ERK2, K-Ras and TRβ transcript levels; however, MAPK phosphatase transcripts were increased. Given that roscovitine inhibits several transcriptional regulators, it was initially thought that a reduction in transcript levels would be the only logical outcome of roscovitine treatment. However, Lam *et al.* [228] found that treatment of the lymphoma cell line OCl-Ly3 with roscovitine resulted in multiple genetic outcomes including both up- and down-regulation of gene transcripts. This contrasts with the global inhibition of transcription observed following cell treatment with the broad-spectrum Cdk inhibitor, flavopiridol [43, 45]. Given these results, roscovitine is expected to affect transcription; however, it may not be purely inhibitory. In fact, roscovitine was only found to reduce the transcript levels of TRβ and ERK2 in the presence of T_3 and had no effect on the transcript levels of those gene transcripts in the absence of T_3. This suggests that roscovitine is specifically affecting the induction of the TH-dependent genetic program. Interpretation of results is complicated by the apparent reduction in total RNA yield following treatment with roscovitine (**Figure 3.6**). Regardless of this effect, it is clear that roscovitine has a differential impact on steady state transcript levels.

This work is preliminary and numerous studies remain to determine the function of Cdks in regression of the tadpole tail during metamorphosis, as well as the impact of roscovitine on the genetic program. Roscovitine influences the TH-dependent genetic

program in the tadpole tail; however, the magnitude of that influence remains uncertain. Roscovitine is known to inhibit mRNA synthesis[251]; however, this inhibition is selective [228]. In the context of TH-induced tail regression, it remains necessary to examine the transcript levels of genes activated downstream of TRβ. To determine the impact of roscovitine treatment on gene expression in the tadpole tail large scale analysis of changes in gene expression using the MAGEX frog cDNA array (www.viagenx.com) will be useful. This approach may provide insight into the mechanisms involved in regulating the TH-dependent program in the tail.

Given that roscovitine inhibits numerous Cdks as well as unrelated kinases, such as the ERKs, it is not entirely useful for elucidation of Cdk function in tail regression. A more specific Cdk inhibitor may be necessary to determine the exact function of each of the Cdks in tail regression. Unfortunately, current research on the development of novel Cdk inhibitors has focused on the development of purine analogues as competitive inhibitors for the ATP-binding pocket [63, 64]. Considering the similarity of this site between the Cdks, this approach will not likely yield inhibitors of the required specificity. It may then be necessary to find another approach to address the issue. The use of cell lines may be more conducive to genetic manipulations, allowing transfections with dominant-negative or antisense constructs. The TH-responsive *Xenopus laevis* XLTC-15 cell line derived from tadpole tail [423] could be used for this purpose or a primary cell line could be established from *Rana catesbeiana* tail epidermis. The results of this study are promising and could serve to further clarify the roles of transcription in tail regression and of the transcriptional Cdks in regulating this process.

Summary and Conclusion

The objective of this study was to determine the function of Cdks in executing the apoptotic process of amphibian tail regression. Results suggest a role for cyclin A-containing complexes during prometamorphosis, and implicate the activation of Cdks that regulate transcription during initiation of the TH-dependent genetic program.

The Cdk inhibitor, roscovitine, was applied to cultured tail tips treated with T_3 and was found to inhibit T_3-induced regression. Given that cyclin A- and cyclin E-containing complexes have been most commonly associated with the onset of apoptosis, they were the initial focus of this work. Cyclin E-containing complexes showed low levels of histone H1 activity that was invariant during natural metamorphosis. Cyclin A-containing complexes exhibited high levels of histone H1 activity that increased during development and peaked during prometamorphosis, at TK stage XV. This activity remained high at stage XVIII before dropping to a moderate level at stages XIX and XX. The kinase activity of those complexes was further reduced at stages XXI and XXII. Cyclin A regulates the kinase activity of Cdk1 and Cdk2 *in vivo*; therefore, those kinases were examined in the same context. Cdk1 complexes exhibited low histone H1 kinase activity, while Cdk2 complexes showed an increase in activity correlating with the activity of the cyclin A-containing complexes from stages VI through XV, inclusive. Cdk2 kinase activity was reduced by stage XVIII and remained low at stage XX. Therefore, it is likely that at least part of the activity of cyclin A-containing complexes is associated with Cdk2 during pre- and early prometamorphosis; however, analysis of ^{35}S-Methionine labelled cyclin A immunoprecipitates did not reveal substantial levels of the Cdk2 as compared with Cdk2 immunoprecipitates, suggesting that additional kinases

may contribute to the activity of those complexes (data not shown). In addition, the kinase associated with cyclin A at stages XVIII, XIX and XX, remains unidentified. This kinase was found to be sensitive to inhibition by roscovitine so is mechanistically similar to the Cdks.

Roscovitine inhibited the regression of cultured tails obtained from animals treated with T_3 for 24 hours prior to culturing with roscovitine. This inhibition was significantly reduced in animals treated with T_3 for 48 hours prior to roscovitine treatment, corresponding to the commitment point. It is evident that this transition does involve distinctive changes in the gene expression profile [147]; therefore, roscovitine may be acting at the transcriptional level.

Recent work showed that roscovitine affects the activities of Cdk7 and Cdk9, which are both involved in the regulation of transcription through phosphorylation of RNAP II. Changes in the phosphorylation state of RNAP II during tail regression and during induction of the TH-dependent genetic program were examined, but were not consistent with tail regression. However, the observation that RNAP II levels are substantially decreased at 24 hours upon roscovitine treatment in T_3 treated tadpole tails suggests that some phosphorylation of RNAP II may be important in transcriptional regulation. This is supported by the observation that TRβ upregulation is attentuated by roscovitine treatment.

This study presents data suggesting that Cdk activity is essential for regression of the tadpole tail during metamorphosis; however, the exact function of those Cdks in mediating this process remains to be determined. A more targeted approach will be necessary to elucidate the roles of each of the Cdks studied herein.

The identification of the putative novel kinase associated with cyclin A may clarify the function of that kinase in the tail, during metamorphosis. Initial attempts at characterization of the unidentified kinase using 2D gel electrophoresis and mass spectrometry (work carried out by Dominik Domanski) have proven inconclusive. Initial work employed a large-scale immunoprecipitation reaction for isolation of cyclin A-containing complexes; however, affinity column chromatography may be more useful for enrichment of protein samples prior to separation on 2D gels.

The involvement of active Cdks in the apoptotic process is a controversial topic given that their exact function in that process remains undetermined. Therefore, the use of in-gel kinase assays and phospho-ICAT may aid in the identification of potential substrates for cyclin A-containing complexes in the tail during natural metamorphosis. The substrate specificity of a cyclin-Cdk complex reflects the preferences of both the cyclin and the Cdk. A comparison between the substrate specificity of cyclin A-containing complexes derived from stage XV and XX tail tips may therefore clarify the function of those complexes during tail regression.

Finally, both Cdk7 and Cdk9 are known to regulate transcription through differential phosphorylation of RNAPII; however, the impact of that phosphorylation on the execution of a developmentally regulated apoptotic program has not been previously examined. Consequently, analysis of Cdk7 and Cdk9 kinase activity during tail regression may serve to clarify their function in the induction of the apoptotic program. Use of the MAGEX cDNA array may also serve to indicate the extent of genetic modification resulting from roscovitine treatment, facilitating a targeted analysis of genes specifically affected by roscovitine.

This work has contributed to the understanding of Cdk involvement in developmentally regulated apoptosis, but considerable work remains to fully elucidate the function of those kinases during this process.

References

1. Adachi, S., H. Ito, M. Tamamori-Adachi, Y. Ono, T. Nozato, S. Abe, M. Ikeda, F. Marumo, and M. Hiroe, *Cyclin A/cdk2 activation is involved in hypoxia-induced apoptosis in cardiomyocytes.* Circulation Research, 2001. **88**(4): p. 408-414.

2. Adams, J.M. and S. Cory, *The Bcl-2 protein family: arbiters of cell survival.* Science, 1998. **281**(5381): p. 1322-6.

3. Agarwal, M.L., A. Agarwal, W.R. Taylor, and G.R. Stark, *p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(18): p. 8493-7.

4. Akoulitchev, S., S. Chuikov, and D. Reinberg, *TFIIH is negatively regulated by cdk8-containing mediator complexes.* Nature, 2000. **407**(6800): p. 102-6.

5. Akoulitchev, S., T.P. Makela, R.A. Weinberg, and D. Reinberg, *Requirement for TFIIH kinase activity in transcription by RNA polymerase II.* Nature, 1995. **377**(6549): p. 557-60.

6. Albanese, C., J. Johnson, G. Watanabe, N. Eklund, D. Vu, A. Arnold, and R.G. Pestell, *Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions.* Journal of Biological Chemistry, 1995. **270**(40): p. 23589-97.

7. Alessi, D.R., A. Cuenda, P. Cohen, D.T. Dudley, and A.R. Saltiel, *PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo.* Journal of Biological Chemistry, 1995. **270**(46): p. 27489-94.

8. Alessi, F., S. Quarta, M. Savio, F. Riva, L. Rossi, L.A. Stivala, A.I. Scovassi, L. Meijer, and E. Prosperi, *The cyclin-dependent kinase inhibitors olomoucine and roscovitine arrest human fibroblasts in G1 phase by specific inhibition of CDK2 kinase activity.* Experimental Eye Research 1998. **245**(1): p. 8-18.

9. Antonsson, B. and J.C. Martinou, *The Bcl-2 protein family.* Experimental Eye Research 2000. **256**(1): p. 50-7.

10. Arias, J.A., S.R. Peterson, and W.S. Dynan, *Promoter-dependent phosphorylation of RNA polymerase II by a template-bound kinase. Association with transcriptional initiation.* Journal of Biological Chemistry, 1991. **266**(13): p. 8055-61.

11. Atienza, C., Jr., M.J. Elliott, Y.B. Dong, H.L. Yang, A. Stilwell, T.J. Liu, and K.M. McMasters, *Adenovirus-mediated E2F-1 gene transfer induces an apoptotic response in human gastric carcinoma cells that is enhanced by cyclin dependent kinase inhibitors.* International Journal of Molecular Medicine, 2000. **6**(1): p. 55-63.

12. Balmanno, K. and S.J. Cook, *Sustained MAP kinase activation is required for the expression of cyclin D1, p21Cip1 and a subset of AP-1 proteins in CCL39 cells.* Oncogene, 1999. **18**(20): p. 3085-97.

13. Baniahmad, A., I. Ha, D. Reinberg, S. Tsai, M.J. Tsai, and B.W. O'Malley, *Interaction of human thyroid hormone receptor beta with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormone.* Proceedings of the National Academy of Science (USA), 1993. **90**(19): p. 8832-6.

14. Barrera-Hernandez, G., K. Park, A. Dace, Q. Zhan, and S.-Y. Cheng, *Thyroid hormone-induced cell proliferation in GC cells is mediated by changes in G1 cyclin/cyclin-dependent kinase levels and activity.* Endocrinology, 1999. **140**: p. 5267-5274.

15. Bartek, J., J. Bartkova, and J. Lukas, *The retinoblastoma protein pathway and the restriction point.* Current Opinion in Cell Biology, 1996. **8**(6): p. 805-14.

16. Baskaran, R., G.G. Chiang, T. Mysliwiec, G.D. Kruh, and J.Y. Wang, *Tyrosine phosphorylation of RNA polymerase II carboxyl-terminal domain by the Abl-related gene product.* Journal of Biological Chemistry, 1997. **272**(30): p. 18905-9.

17. Baskaran, R., M.E. Dahmus, and J.Y. Wang, *Tyrosine phosphorylation of mammalian RNA polymerase II carboxyl-terminal domain.* Proceedings of the National Academy of Science (USA), 1993. **90**(23): p. 11167-71.

18. Becker, K.B., K.C. Stephens, J.C. Davey, M.J. Schneider, and V.A. Galton, *The type 2 and type 3 iodothyronine deiodinases play important roles in coordinating development in Rana catesbeiana tadpoles.* Endocrinology, 1997. **138**: p. 2989-2997.

19. Bensaude, O., F. Bonnet, C. Casse, M.F. Dubois, V.T. Nguyen, and B. Palancade, *Regulated phosphorylation of the RNA polymerase II C-terminal domain (CTD).* Biochemistry and Cell Biology 1999. **77**(4): p. 249-55.

20. Beresford, P.J., D. Zhang, D.Y. Oh, Z. Fan, E.L. Greer, M.L. Russo, M. Jaju, and J. Lieberman, *Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks.* Journal of Biological Chemistry, 2001. **276**(46): p. 43285-93.

21. Berg, J.M., J.L. Tymoczko, and L. Stryer, *Biochemistry*. 5th ed. 2002, New York: WH Freeman and Company.

22. Berry, D.L., R.A. Schwatzman, and D.D. Brown, *The expression pattern of thyroid hormone response genes in the tadpole tail identifies multiple resorption programs.* Developmental Biology, 1998. **203**(1): p. 12-23.

23. Betticher, D.C., N. Thatcher, H.J. Altermatt, P. Hoban, W.D. Ryder, and J. Heighway, *Alternate splicing produces a novel cyclin D1 transcript.* Oncogene, 1995. **11**(5): p. 1005-11.

24. Blanchard, D.A., S. Mouhamad, M.-T. Auffredou, A. Pesty, J. Bertoglio, G. Leca, and A. Vazquez, *Cdk2 associates with MAP kinase in vivo and its nuclear translocation is dependent on MAP kinase activation in IL-2-dependent Kit 225 T lymphyocytes.* Oncogene, 2000. **19**(36): p. 4184-4189.

25. Bokoch, G.M., *Caspase-mediated activation of PAK2 during apoptosis: proteolytic kinase activation as a general mechanism of apoptotic signal transduction?* Cell death and Differentiation 1998. **5**(8): p. 637-45.

26. Bonnet, F., M. Vigneron, O. Bensaude, and M.F. Dubois, *Transcription-independent phosphorylation of the RNA polymerase II C-terminal domain (CTD) involves ERK kinases (MEK1/2).* Nucleic Acids Research, 1999. **27**(22): p. 4399-404.

27. Bortner, D. and M. Rosenberg, *Overexpression of cyclin A in the mammary glands of transgenic mice results in the induction of nuclear abnormalities and increased apoptosis.* Cell Growth and Differentiation, 1995. **6**(12): p. 1579-1589.

28. Bosch, M., J. Gil, O. Bachs, and N. Agell, *Calmodulin inhibitor W13 induces sustained activation of ERK2 and expression of p21(cip1).* Journal of Biological Chemistry, 1998. **273**(34): p. 22145-50.

29. Bottazzi, M.E., X. Zhu, R.M. Bohmer, and R.K. Assoian, *Regulation of p21(cip1) expression by growth factors and the extracellular matrix reveals a role for transient ERK activity in G1 phase.* Journal of Cell Biology, 1999. **146**(6): p. 1255-64.

30. Boulton, T.G., S.H. Nye, D.J. Robbins, N.Y. Ip, E. Radziejewska, S.D. Morgenbesser, R.A. DePinho, N. Panayotatos, M.H. Cobb, and G.D. Yancopoulos, *ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF.* Cell, 1991. **65**(4): p. 663-75.

31. Bratton, S.B., G. Walker, S.M. Srinivasula, X.M. Sun, M. Butterworth, E.S. Alnemri, and G.M. Cohen, *Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes.* EMBO Journal, 2001. **20**(5): p. 998-1009.

32. Bregman, D.B., L. Du, S. van der Zee, and S.L. Warren, *Transcription-dependent redistribution of the large subunit of RNA polymerase II to discrete nuclear domains.* Journal of Cell Biology, 1995. **129**(2): p. 287-98.

33. Bregman, D.B., R.G. Pestell, and V.J. Kidd, *Cell cycle regulation and RNA polymerase II.* Frontiers in bioscience, 2000. **5**: p. D244-57.

34. Brent, G.A., *Thyroid hormone action: down novel paths focus on "thyroid hormone induces activation of mitogen-activated protein kinase in cultured cells".* American Journal of Physiology, 1999. **276**(5 Pt 1): p. C1012-C1013.

35. Brown, D.D., Z. Wang, J.D. Furlow, A. Kanamori, R.A. Schwartzman, B.F. Remo, and A. Pinder, *The thyroid hormone-induced tail resorption program during Xenopus laevis metamorphosis.* Proceedings of the National Academy of Science (USA), 1996. **93**: p. 1924-1929.

36. Brown, D.D., Z. Wang, A. Kanamori, B. Eliceiri, J.D. Furlow, and R. Schwartzman, *Amphibian metamorphosis: a complex program of gene expression changes controlled by the thyroid hormone.* Recent Progress in Hormone Research, 1995. **50**: p. 309-315.

37. Brown, N.R., M.E. Noble, J.A. Endicott, E.F. Garman, S. Wakatsuki, E. Mitchell, B. Rasmussen, T. Hunt, and L.N. Johnson, *The crystal structure of cyclin A.* Structure, 1995. **3**(11): p. 1235-47.

38. Brown, N.R., M.E. Noble, A.M. Lawrie, M.C. Morris, P. Tunnah, G. Divita, L.N. Johnson, and J.A. Endicott, *Effects of phosphorylation of threonine 160 on cyclin-dependent kinase 2 structure and activity.* Journal of Biological Chemistry, 1999. **274**(13): p. 8746-56.

39. Buendia, B., A. Santa-Maria, and J.C. Courvalin, *Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis.* Journal of Cell Science, 1999. **112 (Pt 11)**: p. 1743-53.

40. Buermeyer, A.B., N.E. Thompson, L.A. Strasheim, R.R. Burgess, and P.J. Farnham, *The HIP1 initiator element plays a role in determining the in vitro requirement of the dihydrofolate reductase gene promoter for the C-terminal domain of RNA polymerase II.* Molecular and Cellular Biology, 1992. **12**(5): p. 2250-9.

41. Buolamwini, J.K., *Cell cycle molecular targets in novel anticancer drug discovery.* Current Pharmaceutical Design, 2000. **6**(4): p. 379-92.

42. Buscaglia, M., J. Leloup, and A. deLuze, in *Metamorphosis*, M. Balls and M. Bownes, Editors. 1985, Clarendon: New York. p. 273-293.

43. Carlson, B.A., M.M. Dubay, E.A. Sausville, L. Brizuela, and P.J. Worland, *Flavopiridol induces G1 arrest with inhibition of cyclin-dependent kinase (CDK) 2 and CDK4 in human breast carcinoma cells.* Cancer Research, 1996. **56**(13): p. 2473-8.

44. Castano, E., P. Gross, Z. Wang, R.G. Roeder, and T. Oelgeschlager, *The C-terminal domain-phosphorylated IIO form of RNA polymerase II is associated with the transcription repressor NC2 (Dr1/DRAP1) and is required for transcription activation in human nuclear extracts.* Proceedings of the National Academy of Science (USA), 2000. **97**(13): p. 7184-9.

45. Chao, S.H., K. Fujinaga, J.E. Marion, R. Taube, E.A. Sausville, A.M. Senderowicz, B.M. Peterlin, and D.H. Price, *Flavopiridol inhibits P-TEFb and blocks HIV-1 replication.* Journal of Biological Chemistry, 2000. **275**(37): p. 28345-8.

46. Chau, A.S. and E.K. Shibuya, *Mos-induced p42 mitogen-activated protein kinase activation stabilizes M-phase in Xenopus egg extracts after cyclin destruction.* Biology of the Cell 1998. **90**(8): p. 565-72.

47. Chen, G. and D.V. Goeddel, *TNF-R1 signaling: a beautiful pathway.* Science, 2002. **296**(5573): p. 1634-5.

48. Chen, R.H., C. Abate, and J. Blenis, *Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase.* Proceedings of the National Academy of Science (USA), 1993. **90**(23): p. 10952-6.

49. Chen, R.H., P.C. Juo, T. Curran, and J. Blenis, *Phosphorylation of c-Fos at the C-terminus enhances its transforming activity.* Oncogene, 1996. **12**(7): p. 1493-502.

50. Cheng, M., P. Olivier, J.A. Diehl, M. Fero, M.F. Roussel, J.M. Roberts, and C.J. Sherr, *The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts.* EMBO Journal, 1999. **18**(6): p. 1571-83.

51. Cheng, M., V. Sexl, C.J. Sherr, and M.F. Roussel, *Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1).* Proceedings of the National Academy of Science (USA), 1998. **95**(3): p. 1091-6.

52. Chie, L., S. Amar, H.F. Kung, M.C. Lin, H. Chen, D.L. Chung, V. Adler, Z. Ronai, F.K. Friedman, R.C. Robinson, C. Kovac, P.W. Brandt-Rauf, Z. Yamaizumi, J. Michl, and M.R. Pincus, *Induction of oocyte maturation by jun-N-terminal kinase (JNK) on the oncogenic ras-p21 pathway is dependent on the raf-MEK signal transduction pathway.* Cancer Chemotherapy and Pharmacology, 2000. **45**(6): p. 441-9.

53. Choi, K.S., Y.W. Eom, Y. Kang, M.J. Ha, H. Rhee, J.W. Yoon, and S.J. Kim, *Cdc2 and Cdk2 kinase activated by transforming growth factor-beta1 trigger apoptosis through the phosphorylation of retinoblastoma protein in FaO hepatoma cells.* Journal of Biological Chemistry, 1999. **274**(45): p. 31775-83.

54. Clarke, D.J. and J.F. Gimenez-Abian, *Checkpoints controlling mitosis.* Bioessays, 2000. **22**(4): p. 351-63.

55. Cobb, M.H., *MAP kinase pathways.* Progress in Biophysics and Molecular Biology, 1999. **71**(3-4): p. 479-500.

56. Conaway, R.C., J.N. Bradsher, and J.W. Conaway, *Mechanism of assembly of the RNA polymerase II preinitiation complex. Evidence for a functional interaction between the carboxyl-terminal domain of the largest subunit of RNA polymerase II and a high molecular mass form of the TATA factor.* Journal of Biological Chemistry, 1992. **267**(12): p. 8464-7.

57. Corden, J.L., *Tails of RNA polymerase II.* Trends in Biochemical Sciences, 1990. **15**(10): p. 383-7.

58. Coverley, D., H. Laman, and R.A. Laskey, *Distinct roles for cyclins E and A during DNA replication complex assembly and activation.* Nature Cell Biology, 2002. **4**(7): p. 523-8.

59. Cross, M.J., A. Stewart, M.N. Hodgkin, D.J. Kerr, and M.J. Wakelam, *Wortmannin and its structural analogue demethoxyviridin inhibit stimulated phospholipase A2 activity in Swiss 3T3 cells. Wortmannin is not a specific inhibitor of phosphatidylinositol 3-kinase.* Journal of Biological Chemistry, 1995. **270**(43): p. 25352-5.

60. Crump, D., K. Werry, N. Veldhoen, G. van Aggelen, and C.C. Helbing, *Exposure to the herbicide acetochlor results in increased thyroid hormone-dependent gene expression and accelerated metamorphosis in Xenopus laevis.* Environmental Health Perspectives, 2002. **110**: p. 1199-1205.

61. Davey, J.C., K.B. Becker, M.J. Schneider, D.L. St Germain, and V.A. Galton, *Cloning of a cDNA for the type II iodothyronine deiodinase.* Journal of Biological Chemistry, 1995. **270**: p. 26786-26789.

62. David-Pfeuty, T., *Potent inhibitors of cyclin-dependent kinase 2 induce nuclear accumulation of wild-type p53 and nucleolar fragmentation in human untransformed and tumor-derived cells.* Oncogene, 1999. **18**(52): p. 7409-22.

63. Davies, T.G., J. Bentley, C.E. Arris, F.T. Boyle, N.J. Curtin, J.A. Endicott, A.E. Gibson, B.T. Golding, R.J. Griffin, I.R. Hardcastle, P. Jewsbury, L.N. Johnson, V. Mesguiche, D.R. Newell, M.E. Noble, J.A. Tucker, L. Wang, and H.J. Whitfield, *Structure-based design of a potent purine-based cyclin-dependent kinase inhibitor.* Nature Structural Biology, 2002. **9**(10): p. 745-9.

64. Davies, T.G., D.J. Pratt, J.A. Endicott, L.N. Johnson, and M.E. Noble, *Structure-based design of cyclin-dependent kinase inhibitors.* Pharmacology & Therapeutics, 2002. **93**(2-3): p. 125-33.

65. Davis, P., A. Shih, H. Lin, L. Martino, and F. Davis, *Thyroxine promotes association of mitogen-activated protein kinase and nuclear thyroid hormone receptor (TR) and causes serine phosphorylation of TR.* Journal of Biological Chemistry, 2000. **275**(48): p. 38032-38039.

66. Davis, S.T., B.G. Benson, H.N. Bramson, D.E. Chapman, S.H. Dickerson, K.M. Dold, D.J. Eberwein, M. Edelstein, S.V. Frye, R.T. Gampe Jr, R.J. Griffin, P.A. Harris, A.M. Hassell, W.D. Holmes, R.N. Hunter, V.B. Knick, K. Lackey, B. Lovejoy, M.J. Luzzio, D. Murray, P. Parker, W.J. Rocque, L. Shewchuk, J.M. Veal, D.H. Walker, and L.F. Kuyper, *Prevention of chemotherapy-induced alopecia in rats by CDK inhibitors.* Science, 2001. **291**(5501): p. 134-7.

67. De Azevedo, W.F., Jr., H.J. Mueller-Dieckmann, U. Schulze-Gahmen, P.J. Worland, E. Sausville, and S.H. Kim, *Structural basis for specificity and potency of a flavonoid inhibitor of human CDK2, a cell cycle kinase.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(7): p. 2735-40.

68. De Bondt, H.L., J. Rosenblatt, J. Jancarik, H.D. Jones, D.O. Morgan, and S.H. Kim, *Crystal structure of cyclin-dependent kinase 2.* Nature, 1993. **363**(6430): p. 595-602.

69. Deak, M., A.D. Clifton, L.M. Lucocq, and D.R. Alessi, *Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB.* EMBO Journal, 1998. **17**(15): p. 4426-41.

70. Derijard, B., M. Hibi, I.H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R.J. Davis, *JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain.* Cell, 1994. **76**(6): p. 1025-37.

71. Desdouets, C., C. Ory, G. Matesic, T. Soussi, C. Brechot, and J. Sobczak-Thepot, *ATF/CREB site mediated transcriptional activation and p53 dependent repression of the cyclin A promoter.* FEBS Letters 1996. **385**(1-2): p. 34-8.

72. Diehl, J.A., M. Cheng, M.F. Roussel, and C.J. Sherr, *Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization.* Genes & Development, 1998. **12**(22): p. 3499-511.

73. Dobashi, Y., M. Shojh, E. Kondo, T. Akiyama, and T. Kameya, *CDK4, a possible critical regulator of apoptosis in rat pheochromocytoma PC12 cells.* Biochemistry and Biophysical Research Communications, 1998. **253**(3): p. 609-613.

74. Dodd, M. and J. Dodd, *The biology of metamorphosis*, in *Physiology of the Amphibia*, B. Lofts, Editor. 1976, Academic Press: New York. p. 467-599.

75. Donjerkovic, D. and D.W. Scott, *Regulation of the G1 phase of the mammalian cell cycle.* Cell Research, 2000. **10**(1): p. 1-16.

76. Douville, E. and J. Downward, *EGF induced SOS phosphorylation in PC12 cells involves P90 RSK-2.* Oncogene, 1997. **15**(4): p. 373-83.

77. Dragnev, K.H., S.J. Freemantle, M.J. Spinella, and E. Dmitrovsky, *Cyclin proteolysis as a retinoid cancer prevention mechanism.* Annals of the New York Academy of Sciences, 2001. **952**: p. 13-22.

78. Dubois, M.F., S. Bellier, S.J. Seo, and O. Bensaude, *Phosphorylation of the RNA polymerase II largest subunit during heat shock and inhibition of transcription in HeLa cells.* Journal of Cellular Physiology, 1994. **158**(3): p. 417-26.

79. Dubois, M.F. and O. Bensaude, *Phosphorylation of RNA polymerase II C-terminal domain (CTD): a new control for heat shock gene expression?* Cell Stress Chaperones, 1998. **3**(3): p. 147-51.

80. Dubois, M.F., M. Vincent, M. Vigneron, J. Adamczewski, J.M. Egly, and O. Bensaude, *Heat-shock inactivation of the TFIIH-associated kinase and change in the phosphorylation sites on the C-terminal domain of RNA polymerase II.* Nucleic Acids Research, 1997. **25**(4): p. 694-700.

81. Earnshaw, W.C., L.M. Martins, and S.H. Kaufmann, *Mammalian caspases: structure, activation, substrates, and functions during apoptosis.* Annual Review of Biochemistry, 1999. **68**: p. 383-424.

82. el-Deiry, W., T. Tokino, V. Velculescu, D. Levy, R. Parsons, J. Trent, D. Lin, W. Mercer, K. Kinzler, and B. Vogelstein, *WAF1, a potential mediator of p53 tumor suppression.* Cell, 1993. **75**: p. 817-825.

83. el-Deiry, W.S., *Regulation of p53 downstream genes.* Seminars in Cancer Biology., 1998. **8**(5): p. 345-57.

84. el-Deiry, W.S., J.W. Harper, P.M. O'Connor, V.E. Velculescu, C.E. Canman, J. Jackman, J.A. Pietenpol, M. Burrell, D.E. Hill, Y. Wang, and et al., *WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis.* Cancer Research, 1994. **54**(5): p. 1169-74.

85. Elenbaas, B. and R.A. Weinberg, *Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation.* Experimental Eye Research 2001. **264**(1): p. 169-84.

86. Eliceiri, B. and D. Brown, *Quanititation of endogenous thyroid hormone receptors α and β during embryogenesis and metamorphosis in Xenopus laevis.* Journal of Biological Chemistry, 1994. **269**: p. 24459-24465.

87. Elinson, R.P., B. Remo, and D.D. Brown, *Novel structural elements identified during tail resorption in Xenopus laevis metamorphosis: lessons from tailed frogs.* Developmental Biology, 1999. **215**: p. 243-252.

88. Ellenrieder, C., B. Bartosch, G.Y. Lee, M. Murphy, C. Sweeney, M. Hergersberg, M. Carrington, R. Jaussi, and T. Hunt, *The long form of CDK2 arises via alternative splicing and forms an active protein kinase with cyclins A and E.* DNA and Cell Biology, 2001. **20**(7): p. 413-23.

89. Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata, *A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD.* Nature, 1998. **391**(6662): p. 43-50.

90. Evans, R., *The steroid and thyroid hormone receptor superfamily.* Science, 1988. **240**: p. 889-895.

91. Eymin, B., O. Sordet, N. Droin, B. Munsch, M. Haugg, M. Van de Craen, P. Vandenabeele, and E. Solary, *Caspase-induced proteolysis of the cyclin-dependent kinase inhibitor p27Kip1 mediates its anti-apoptotic activity.* Oncogene, 1999. **18**(34): p. 4839-47.

92. Fadok, V.A., D.R. Voelker, P.A. Campbell, D.L. Bratton, J.J. Cohen, P.W. Noble, D.W. Riches, and P.M. Henson, *The ability to recognize phosphatidylserine on apoptotic cells is an inducible function in murine bone marrow-derived macrophages.* Chest, 1993. **103**(2 Suppl): p. 102S.

93. Fang, F., G. Orend, N. Watanabe, T. Hunter, and E. Ruoslahti, *Dependence of cyclin E-CDK2 kinase activity on cell anchorage.* Science, 1996. **271**(5248): p. 499-502.

94. Ferri, K.F. and G. Kroemer, *Organelle-specific initiation of cell death pathways.* Nature Cell Biology, 2001. **3**(11): p. E255-63.

95. Fesquet, D., N. Morin, M. Doree, and A. Devault, *Is Cdk7/cyclin H/MAT1 the genuine cdk activating kinase in cycling Xenopus egg extracts?* Oncogene, 1997. **15**(11): p. 1303-7.

96. Finkielstein, C., L. Chen, and J. Maller, *A role for G1/S cyclin-dependent protein kinases in the apoptotic response to ionizing radiation.* Journal of Biological Chemistry, 2002. **277**: p. 38476-38485.

97. Fleury, C., M. Pampin, A. Tarze, and B. Mignotte, *Yeast as a model to study apoptosis?* Bioscience Reports, 2002. **22**(1): p. 59-79.

98. Forman, B.M., J. Casanova, B.M. Raaka, J. Ghysdael, and H.H. Samuels, *Half-site spacing and orientation determines whether thyroid hormone and retinoic acid receptors and related factors bind to DNA response elements as monomers, homodimers, or heterodimers.* Molecular Endocrinology, 1992. **6**(3): p. 429-42.

99. Forman, B.M. and H.H. Samuels, *Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model.* Molecular Endocrinology, 1990. **4**(9): p. 1293-301.

100. Franklin, A.E. and W.Z. Cande, *Nuclear organization and chromosome segregation.* The Plant Cell, 1999. **11**(4): p. 523-34.

101. Freeman, R.S., S. Estus, and E.M.J. Johnson, *Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of cyclin D1 during programmed cell death.* Neuron, 1994. **12**: p. 3443-3551.

102. Frit, P., E. Bergmann, and J.M. Egly, *Transcription factor IIH: a key player in the cellular response to DNA damage.* Biochimie, 1999. **81**(1-2): p. 27-38.

103. Frodin, M. and S. Gammeltoft, *Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction.* Molecular and Cellular Endocrinology, 1999. **151**(1-2): p. 65-77.

104. Fu, T.J., J. Peng, G. Lee, D.H. Price, and O. Flores, *Cyclin K functions as a CDK9 regulatory subunit and participates in RNA polymerase II transcription.* Journal of Biological Chemistry, 1999. **274**(49): p. 34527-30.

105. Fukunaga, R. and T. Hunter, *MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates.* EMBO Journal, 1997. **16**: p. 1921-1933.

106. Fung, T.K., W.Y. Siu, C.H. Yam, A. Lau, and R.Y. Poon, *Cyclin F is degraded during G2-M by mechanisms fundamentally different from other cyclins.* Journal of Biological Chemistry, 2002. **277**(38): p. 35140-9.

107. Furstenthal, L., B.K. Kaiser, C. Swanson, and P.K. Jackson, *Cyclin E uses Cdc6 as a chromatin-associated receptor required for DNA replication.* Journal of Cell Biology, 2001. **152**(6): p. 1267-78.

108. Furukawa, Y., S. Iwase, Y. Terui, J. Kikuchi, T. Sakai, M. Nakamura, S. Kitagawa, and M. Kitagawa, *Transcriptional activation of the cdc2 gene is associated with Fas-induced apoptosis of human hematopoietic cells.* Journal of Biological Chemistry, 1996. **271**(45): p. 28469-77.

109. Galton, V., *Mechanisms underlying the acceleration of thyroid hormone-induced tadpole metamorphosis by corticosterone.* Endocrinology, 1990. **127**(6): p. 2997-3002.

110. Garriga, J., J. Peng, M. Parreno, D.H. Price, E.E. Henderson, and X. Grana, *Upregulation of cyclin T1/CDK9 complexes during T cell activation.* Oncogene, 1998. **17**(24): p. 3093-102.

111. Gauthier, F.V., M.A. Qadir, P.A. Merrifield, and B.G. Atkinson, *Postembryonic expression of the myosin heavy chain genes in the limb, tail, and heart muscles of metamorphosing amphibian tadpoles.* Microscopic Research Techniques, 2000. **50**: p. 456-472.

112. Gavin, A.C. and A.R. Nebreda, *A MAP kinase docking site is required for phosphorylation and activation of p90(rsk)/MAPKAP kinase-1.* Current Biology, 1999. **9**(5): p. 281-4.

113. Gervais, J.L., P. Seth, and H. Zhang, *Cleavage of CDK inhibitor p21(Cip1/Waf1) by caspases is an early event during DNA damage-induced apoptosis.* Journal of Biological Chemistry, 1998. **273**(30): p. 19207-12.

114. Ghoda, L., X. Lin, and W.C. Greene, *The 90-kDa ribosomal S6 kinase (pp90rsk) phosphorylates the N-terminal regulatory domain of IkappaBalpha and stimulates its degradation in vitro.* Journal of Biological Chemistry, 1997. **272**(34): p. 21281-8.

115. Giardina, S.F. and P.M. Beart, *Kainate receptor-mediated apoptosis in primary cultures of cerebellar granule cells is attenuated by mitogen-activated protein and cyclin-dependent kinase inhibitors.* British Journal of Pharmacology, 2002. **135**(7): p. 1733-42.

116. Gibson, T.J., J.D. Thompson, A. Blocker, and T. Kouzarides, *Evidence for a protein domain superfamily shared by the cyclins, TFIIB and RB/p107.* Nucleic Acids Research, 1994. **22**(6): p. 946-52.

117. Gilbert, L.I. and E. Frieden, *Metamorphosis: A problem in developmental biology*. 2nd ed, ed. L.I. Gilbert and E. Frieden. 1981, New York: Plenum Press.

118. Gilbert, L.I., J.R. Tata, and B.G. Atkinson, *Metamorphosis: postembryonic reprogramming of gene expression in amphibian and insect cells*. Metamorphosis: postembryonic reprogramming of gene expression in amphibian and insect cells. 1996, San Diego: Academic Press.

119. Gil-Gomez, G., A. Berns, and H. Brady, EMBO Journal, 1998. **17**: p. 7209-7218.

120. Gil-Gomez, G., A. Berns, and H.J. Brady, *A link between cell cycle and cell death: Bax and Bcl-2 modulate Cdk2 activation during thymocyte apoptosis.* EMBO Journal, 1998. **17**(24): p. 7209-18.

121. Gille, H. and J. Downward, *Multiple Ras effector pathways contribute to G1 cell cycle progression.* The Journal of biological chemistry, 1999. **274**(31): p. 22033-22040.

122. Gille, H., M. Kortenjann, O. Thomae, C. Moomaw, C. Slaughter, M.H. Cobb, and P.E. Shaw, *ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation.* EMBO Journal, 1995. **14**(5): p. 951-62.

123. Glotzer, M., A.W. Murray, and M.W. Kirschner, *Cyclin is degraded by the ubiquitin pathway.* Nature, 1991. **349**(6305): p. 132-8.

124. Gray, N.S., L. Wodicka, A.M. Thunnissen, T.C. Norman, S. Kwon, F.H. Espinoza, D.O. Morgan, G. Barnes, S. LeClerc, L. Meijer, S.H. Kim, D.J. Lockhart, and P.G. Schultz, *Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors.* Science, 1998. **281**(5376): p. 533-8.

125. Gupta, S., D. Campbell, B. Derijard, and R.J. Davis, *Transcription factor ATF2 regulation by the JNK signal transduction pathway.* Science, 1995. **267**(5196): p. 389-93.

126. Hadzic, E., V. Desai-Yajnik, E. Helmer, S. Guo, S. Wu, N. Koudinova, J. Casanova, B.M. Raaka, and H.H. Samuels, *A 10-amino-acid sequence in the N-terminal A/B domain of thyroid hormone receptor alpha is essential for transcriptional activation and interaction with the general transcription factor TFIIB.* Molecular and Cellular Biology, 1995. **15**(8): p. 4507-17.

127. Hadzic, E., I. Habeos, B.M. Raaka, and H.H. Samuels, *A novel multifunctional motif in the amino-terminal A/B domain of T3Ralpha modulates DNA binding and receptor dimerization.* Journal of Biological Chemistry, 1998. **273**(17): p. 10270-8.

128. Hagting, A., C. Karlsson, P. Clute, M. Jackman, and J. Pines, *MPF localization is controlled by nuclear export.* EMBO Journal, 1998. **17**(14): p. 4127-38.

129. Hai, T. and T. Curran, *Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity.* Proceedings of the National Academy of Science (USA), 1991. **88**(9): p. 3720-4.

130. Hajduch, M., L. Havlieek, J. Vesely, R. Novotny, V. Mihal, and M. Strnad, *Synthetic cyclin dependent kinase inhibitors. New generation of potent anti-cancer drugs.* Advances in Experimental Medicine and Biology, 1999. **457**: p. 341-53.

131. Hakem, A., T. Sasaki, I. Kozieradzki, and J.M. Penninger, *The cyclin-dependent kinase Cdk2 regulates thymocyte apoptosis.* The Journal of Experimental Medicine 1999. **189**(6): p. 957-68.

132. Hamel, P.A. and J. Hanley-Hyde, *G1 cyclins and control of the cell division cycle in normal and transformed cells.* Cancer Investigation, 1997. **15**(2): p. 143-52.

133. Hampsey, M. and D. Reinberg, *RNA polymerase II as a control panel for multiple coactivator complexes.* Current Opinion in Genetics and Development, 1999. **9**(2): p. 132-9.

134. Handa, K., M. Yamakawa, H. Takeda, S. Kimura, and T. Takahashi, *Expression of cell cycle markers in colorectal carcinoma: superiority of cyclin A as an indicator of poor prognosis.* International Journal of Cancer, 1999. **84**(3): p. 225-33.

135. Harper, J.W. and S.J. Elledge, *Cdk inhibitors in development and cancer.* Current Opinion in Genetics and Development, 1996. **6**(1): p. 56-64.

136. Harvey, C. and G. Williams, *Mechanism of Thyroid Hormone Action.* Thyroid, 2002. **12**: p. 441-446.

137. Harvey, K.J., D. Lukovic, and D.S. Ucker, *Caspase-dependent Cdk activity is a requisite effector of apoptotic death events.* Journal of Cell Biology, 2000. **148**(1): p. 59-72.

138. Harwell, R.M., D.C. Porter, C. Danes, and K. Keyomarsi, *Processing of cyclin E differs between normal and tumor breast cells.* Cancer Research, 2000. **60**(2): p. 481-9.

139. Hashiguchi, M., T. Saito, S.I. Hisanaga, and T. Hashiguchi, *Truncation of CDK5 Activator p35 Induces Intensive Phosphorylation of Ser202/Thr205 of Human Tau.* Journal of Biological Chemistry, 2002. **277**(46): p. 44525-44530.

140. Hayes, T., *Interdependence of corticosterone and thyroid hormones in larval toads (Bufo borealis). I. Thyroid hormone-dependent and independent effects of corticosterone on growth and development.* Journal of Experimental Zoology, 1995. **271**(2): p. 95-102.

141. Hayes, T.B., *Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis.* American Zoologist, 1997. **37**: p. 185-194.

142. Heichman, K.A. and J.M. Roberts, *Rules to replicate by.* Cell, 1994. **79**(4): p. 557-62.

143. Helbing, C., C. Gallimore, and B.G. Atkinson, *Characterization of a Rana catesbeiana Hsp30 gene and its expression in the liver of this amphibian during both spontaneous and thyroid hormone-induced metamorphosis.* Developmental Genetics, 1996. **18**: p. 223-233.

144. Helbing, C.C. and B.G. Atkinson, *3,5,3'-Triiodothyronine-induced carbamyl phosphate synthetase gene expression is stabilized in the liver of Rana catesbeiana tadpoles during heat shock.* Journal of Biological Chemistry, 1994. **269**: p. 11743-11750.

145. Helbing, C.C., G. Gergely, and B.G. Atkinson, *Sequential up-regulation of thyroid hormone β receptor, ornithine transcarbamylase and carbamyl phosphate synthetase mRNAs in the liver of Rana catesbeiana tadpoles during spontaneous and thyroid hormone-induced metamorphosis.* Developmental Genetics, 1992. **13**: p. 289-301.

146. Helbing, C.C., C.L. Wellington, M. Gogela-Spehar, T. Cheng, G. Pinchbeck, and R.N. Johnston, *Quiescence versus apoptosis: Myc abundance determines pathway of exit from the cell cycle.* Oncogene, 1998. **17**: p. 1491-1501.

147. Helbing, C.C., K. Werry, D. Crump, D. Domanski, and N. Veldhoen, *Expression profiles of novel thyroid hormone-responsive genes and proteins in the tail of Xenopus laevis tadpoles undergoing precocious metamorphosis.* Molecular Endocrinology, 2003. **(Submitted)**.

148. Hengartner, C.J., V.E. Myer, S.M. Liao, C.J. Wilson, S.S. Koh, and R.A. Young, *Temporal regulation of RNA polymerase II by Srb10 and Kin28 cyclin-dependent kinases.* Molecular Cell, 1998. **2**(1): p. 43-53.

149. Hengartner, M.O., *The biochemistry of apoptosis.* Nature, 2000. **407**(6805): p. 770-6.

150. Herber, B., M. Truss, M. Beato, and R. Muller, *Inducible regulatory elements in the human cyclin D1 promoter.* Oncogene, 1994. **9**(7): p. 2105-7.

151. Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin, *Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain.* Genes and Development, 1993. **7**(11): p. 2135-48.

152. Hiromura, K., J.W. Pippin, M.J. Blonski, J.M. Roberts, and S.J. Shankland, *The subcellular localization of cyclin dependent kinase 2 determines the fate of mesangial cells: role in apoptosis and proliferation.* Oncogene, 2002. **21**(11): p. 1750-8.

153. Hiromura, K., J.W. Pippin, M.L. Fero, J.M. Roberts, and S.J. Shankland, *Modulation of apoptosis by the cyclin-dependent kinase inhibitor p27(Kip1).* The Journal of Clinical Investigation, 1999. **103**(5): p. 597-604.

154. Hirose, Y. and J.L. Manley, *RNA polymerase II is an essential mRNA polyadenylation factor.* Nature, 1998. **395**(6697): p. 93-6.

155. Hirose, Y., R. Tacke, and J.L. Manley, *Phosphorylated RNA polymerase II stimulates pre-mRNA splicing.* Genes and Development, 1999. **13**(10): p. 1234-9.

156. Ho, C.K. and S. Shuman, *Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme.* Molecular Cell, 1999. **3**(3): p. 405-11.

157. Hoang, A.T., K.J. Cohen, J.F. Barrett, D.A. Bergstrom, and C.V. Dang, *Participation of cyclin A in Myc-induced apoptosis.* Proceedings of the National Academy of Sciences (USA), 1994. **91**: p. 6875-6879.

158. Hollenberg, S.M., C. Weinberger, E.S. Ong, G. Cerelli, A. Oro, R. Lebo, E.B. Thompson, M.G. Rosenfeld, and R.M. Evans, *Primary structure and expression of a functional human glucocorticoid receptor cDNA.* Nature, 1985. **318**(6047): p. 635-41.

159. Horlein, A.J., A.M. Naar, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamel, M. Soderstrom, C.K. Glass, and e. al, *Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptro corepressor.* Nature, 1995. **377**: p. 397-404.

160. Horton, H.R., L.A. Moran, R.S. Ochs, J.D. Rawn, and K.G. Scrimgeour, *Principles of Biochemistry.* 3rd ed. 2002, Upper Saddle River, NJ: Prentice Hall.

161. Horton, L.E. and D.J. Templeton, *The cyclin box and C-terminus of cyclins A and E specify CDK activation and substrate specificity.* Oncogene, 1997. **14**(4): p. 491-8.

162. Hsiao, K.M., S.Y. Chou, S.J. Shih, and J.E. Ferrell, Jr., *Evidence that inactive p42 mitogen-activated protein kinase and inactive Rsk exist as a heterodimer in vivo.* Proceedings of the National Academy of Science (USA), 1994. **91**(12): p. 5480-4.

163. Hsu, S.L., M.C. Chen, Y.H. Chou, G.Y. Hwang, and S.C. Yin, *Induction of p21(CIP1/Waf1) and activation of p34(cdc2) involved in retinoic acid-induced apoptosis in human hepatoma Hep3B cells.* Experimental Eye Research 1999. **248**(1): p. 87-96.

164. Hsu, S.L., S.C. Yin, M.C. Liu, U. Reichert, and W.L. Ho, *Involvement of cyclin-dependent kinase activities in CD437-induced apoptosis.* Experimental Eye Research 1999. **252**(2): p. 332-41.

165. Huang, H. and D.D. Brown, *Prolactin is not a juvenile hormone in Xenopus laevis metamorphosis.* Proceedings of the National Academy of Science (USA), 2000. **97**: p. 195-199.

166. Huang, H., N. Marsh-Armstrong, and D. Brown, *Metamorphosis is inhibited in transgenic Xenopus laevis tadpoles that over express type III deiodinase.* Proceedings of the National Academy of Sciences (USA), 1999. **96**: p. 962-967.

167. Hunt, T., *Cyclins and their partners: from a simple idea to complicated reality.* Seminars in Cell Biology, 1991. **2**(4): p. 213-22.

168. Hunter, T. and M. Karin, *The regulation of transcription by phosphorylation.* Cell, 1992. **70**(3): p. 375-87.

169. Huuhtanen, R.L., C.P. Blomqvist, T.O. Bohling, T.A. Wiklund, E.J. Tukiainen, M. Virolainen, B. Tribukait, and L.C. Andersson, *Expression of cyclin A in soft tissue sarcomas correlates with tumor aggressiveness.* Cancer Research, 1999. **59**(12): p. 2885-90.

170. Ishizuya-Oka, A. and A. Shimozawa, *Programmed cell death and heterolysis of larval epithelial cells by macrophage-like cells in the anuran small intestine in vivo and in vitro.* Journal of Morphology, 1992. **213**(2): p. 185-95.

171. Iwamuro, S. and J.R. Tata, *Contrasting patterns of expression of thyroid hromone and retinoid X receptor genes during hormonal manipulation of Xenopus tadpole tail regression in culture.* Molecular and Cellular Endocrinology, 1995. **113**: p. 235-243.

172. Izutsu, Y., M. Kaiho, and K. Yoshizato, *Different distribution of epidermal basal cells in the anuran larval skin correlated with the skin's region specific fate at metamorphosis.* Journal of Experimental Zoology, 1993. **267**: p. 605-615.

173. Jacobs, H.W., E. Keidel, and C.F. Lehner, *A complex degradation signal in Cyclin A required for G1 arrest, and a C-terminal region for mitosis.* EMBO Journal, 2001. **20**(10): p. 2376-86.

174. Jacotot, E., K.F. Ferri, and G. Kroemer, *Apoptosis and cell cycle: distinct checkpoints with overlapping upstream control.* Pathologie Biologie, 2000. **48**(3): p. 271-9.

175. Janicke, R.U., X.Y. Lin, F.H. Lee, and A.G. Porter, *Cyclin D3 sensitizes tumor cells to tumor necrosis factor-induced, c-Myc-dependent apoptosis.* Molecular and Cellular Biology, 1996. **16**(10): p. 5245-53.

176. Janknecht, R., W.H. Ernst, and A. Nordheim, *SAP1a is a nuclear target of signaling cascades involving ERKs.* Oncogene, 1995. **10**(6): p. 1209-16.

177. Janknecht, R., W.H. Ernst, V. Pingoud, and A. Nordheim, *Activation of ternary complex factor Elk-1 by MAP kinases.* EMBO Journal, 1993. **12**(13): p. 5097-104.

178. Jeffrey, P., S. Gorina, and N. Pavletich, *Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms.* Science, 1995. **267**: p. 1498-1502.

179. Jin, Y.H., K.J. Yoo, Y.H. Lee, and S.K. Lee, *Caspase 3-mediated cleavage of p21WAF1/CIP1 associated with the cyclin A-cyclin-dependent kinase 2 complex is a prerequisite for apoptosis in SK-HEP-1 cells.* Journal of Biological Chemistry, 2000. **275**(39): p. 30256-63.

180. Joel, P.B., J. Smith, T.W. Sturgill, T.L. Fisher, J. Blenis, and D.A. Lannigan, *pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167.* Molecular and Cellular Biology, 1998. **18**(4): p. 1978-84.

181. Johnson, G.L. and R.R. Vaillancourt, *Sequential protein kinase reactions controlling cell growth and differentiation.* Current Opinion in Cell Biology, 1994. **6**(2): p. 230-8.

182. Jolivet-Jaudet, G. and J. Leloup-Hatey, *Variations in aldosterone and corticosterone plasma levels during metamorphosis in Xenopus laevis tadpoles.* General and Comparative Endocrinology, 1984. **56**(1): p. 59-65.

183. Jones, C. and C. Smythe, *Activation of the Xenopus cyclin degradation machinery by full-length cyclin A.* Journal of Cell Science, 1996. **109 (Pt 5)**: p. 1071-9.

184. Jones, K.E., J.H. Brubaker, and W.W. Chin, *Evidence that phosphorylation events participate in thyroid hormone action.* Endocrinology, 1994. **134**(2): p. 543-8.

185. Judelson, C. and M.L. Privalsky, *DNA recognition by normal and oncogenic thyroid hormone receptors. Unexpected diversity in half-site specificity controlled by non-zinc-finger determinants.* Journal of Biological Chemistry, 1996. **271**(18): p. 10800-5.

186. Kagedal, K., U. Johansson, and K. Ollinger, *The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress.* FASEB Journal, 2001. **15**(9): p. 1592-4.

187. Kagedal, K., M. Zhao, I. Svensson, and U.T. Brunk, *Sphingosine-induced apoptosis is dependent on lysosomal proteases.* The Biochemical Journal, 2001. **359**(Pt 2): p. 335-43.

188. Kaldis, P., A.A. Russo, H.S. Chou, N.P. Pavletich, and M.J. Solomon, *Human and yeast cdk-activating kinases (CAKs) display distinct substrate specificities.* Molecular Biology of the Cell, 1998. **9**(9): p. 2545-60.

189. Kaltenbach, J.C., *Endocrinology of amphibian metamorphosis*, in *Metamorphosis: postembryonic reprogramming of gene expression in amphibian and insect cells*, L.I. Gilbert, J.R. Tata, and B.G. Atkinson, Editors. 1996, Academic Press: San Diego. p. 403-431.

190. Kamb, A., *Cell-cycle regulators and cancer.* Trends in Genetics, 1995. **11**(4): p. 136-40.

191. Kanamori, A. and D. Brown, *Cultured cells as a model for amphibian metamorphosis.* Proceedings of the National Academy of Sciences (USA), 1993. **90**: p. 6013-6017.

192. Kanamori, A. and D.D. Brown, *The regulation of thyroid hormone receptor beta genes by thyroid hormone in Xenopus laevis.* Journal of Biological Chemistry, 1992. **267**: p. 739-745.

193. Kang, M.E. and M.E. Dahmus, *RNA polymerases IIA and IIO have distinct roles during transcription from the TATA-less murine dihydrofolate reductase promoter.* Journal of Biological Chemistry, 1993. **268**(33): p. 25033-40.

194. Kang, M.E. and M.E. Dahmus, *The unique C-terminal domain of RNA polymerase II and its role in transcription.* Advances in Enzymology and Related Areas of Molecular Biology 1995. **71**: p. 41-77.

195. Katz, D., M.J. Reginato, and M.A. Lazar, *Functional regulation of thyroid hormone receptor variant TR alpha 2 by phosphorylation.* Molecular and Cellular Biology, 1995. **15**(5): p. 2341-8.

196. Kaufmann, H., R. Marone, M.A. Olayioye, J.E. Bailey, and M. Fussenegger, *Characterization of an N-terminally truncated cyclin A isoform in mammalian cells.* Journal of Biological Chemistry, 2001. **276**(32): p. 29987-93.

197. Kawada, M., S. Yamagoe, Y. Murakami, K. Suzuki, S. Mizuno, and Y. Uehara, *Induction of p27Kip1 degradation and anchorage independence by Ras through the MAP kinase signaling pathway.* Oncogene, 1997. **15**(6): p. 629-37.

198. Kawahara, A., B. Baker, and J. Tata, *Developmental and regional expression of thyroid hormone receptor genes during Xenopus metamorphosis.* Development, 1991. **112**: p. 933-943.

199. Kawahara, A., Y. Gohda, and A. Hikosaka, *Role of type III iodothyronine 5-deiodinase gene expression in temporal regulation of Xenopus metamorphosis.* Development Growth and Differentiation, 1999. **41**(3): p. 365-373.

200. Kerr, J.F., B. Harmon, and J. Searle, *An electron-microscope study of cell deletion in the anuran tadpole tail during spontaneous metamorphosis with special reference to apoptosis of striated muscle fibers.* Journal of Cell Science, 1974. **14**(3): p. 571-85.

201. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* The British Journal of Cancer, 1972. **26**(4): p. 239-57.

202. Keyomarsi, K., D. Conte, Jr., W. Toyofuku, and M.P. Fox, *Deregulation of cyclin E in breast cancer.* Oncogene, 1995. **11**(5): p. 941-50.

203. Kikuyama, S., K. Kawamura, S. Tanaka, and K. Yamamoto, *Aspects of amphibian metamorphosis. Hormonal control.* International Reviews in Cytology, 1993. **145**: p. 105-148.

204. Kim, E., L. Du, D.B. Bregman, and S.L. Warren, *Splicing factors associate with hyperphosphorylated RNA polymerase II in the absence of pre-mRNA.* Journal of Cell Biology, 1997. **136**(1): p. 19-28.

205. Kim, H.Y. and Y. Cho, *Structural similarity between the pocket region of retinoblastoma tumour suppressor and the cyclin-box.* Nature Structural Biology, 1997. **4**(5): p. 390-5.

206. Kim, M.K., J.S. Lee, and J.H. Chung, *In vivo transcription factor recruitment during thyroid hormone receptor-mediated activation.* Proceedings of the National Academy of Science (USA), 1999. **96**(18): p. 10092-7.

207. Kim, Y., M. Hamburgh, H. Frankfort, and W. Etkin, *Reduction in the latent period of the response to thyroxin by tadpole tail discs fused to discs pretreated with thyroxin.* Developmental Biology, 1977. **55**(2): p. 387-91.

208. King, A.J., H. Sun, B. Diaz, D. Barnard, W. Miao, S. Bagrodia, and M.S. Marshall, *The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338.* Nature, 1998. **396**(6707): p. 180-3.

209. King, R.W., M. Glotzer, and M.W. Kirschner, *Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates.* Molecular Biology of the Cell, 1996. **7**(9): p. 1343-57.

210. Kinoshita, T. and F. Sasaki, *Body-specific proliferation of adult precursor cells in Xenopus larval epidermis.* Histochemistry, 1994. **101**: p. 397-404.

211. Kitagawa, M., H. Higashi, H.K. Jung, I. Suzuki-Takahashi, M. Ikeda, K. Tamai, J. Kato, K. Segawa, E. Yoshida, S. Nishimura, and Y. Taya, *The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2.* EMBO Journal, 1996. **15**(24): p. 7060-9.

212. Kliewer, S.A., K. Umesono, D.J. Mangelsdorf, and R.M. Evans, *Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling.* Nature, 1992. **355**(6359): p. 446-9.

213. Kloas, W., M. Reinecke, and W. Hanke, *Stage-dependent changes in adrenal steroids and catecholamines during development in Xenopus laevis.* General and Comparative Endocrinology, 1997. **108**(3): p. 416-426.

214. Knockaert, M., P. Greengard, and L. Meijer, *Pharmacological inhibitors of cyclin-dependent kinases.* Trends in Pharmacological Sciences, 2002. **23**(9): p. 417-25.

215. Kobayashi, H., E. Stewart, R. Poon, J.P. Adamczewski, J. Gannon, and T. Hunt, *Identification of the domains in cyclin A required for binding to, and activation of, p34cdc2 and p32cdk2 protein kinase subunits.* Molecular Biology of the Cell, 1992. **3**(11): p. 1279-94.

216. Koenig, R.J., R.L. Warne, G.A. Brent, J.W. Harney, P.R. Larsen, and D.D. Moore, *Isolation of a cDNA clone encoding a biologically active thyroid hormone receptor.* Proceedings of the National Academy of Science (USA), 1988. **85**(14): p. 5031-5.

217. Koepp, D.M., J.W. Harper, and S.J. Elledge, *How the cyclin became a cyclin: regulated proteolysis in the cell cycle.* Cell, 1999. **97**(4): p. 431-4.

218. Koff, A., A. Giordano, D. Desai, K. Yamashita, J.W. Harper, S. Elledge, T. Nishimoto, D.O. Morgan, B.R. Franza, and J.M. Roberts, *Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle.* Science, 1992. **257**(5077): p. 1689-94.

219. Kolch, W., *Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions.* The Biochemical Journal, 2000. **351**(Pt 2): p. 289-305.

220. Kong, M., E.A. Barnes, V. Ollendorff, and D.J. Donoghue, *Cyclin F regulates the nuclear localization of cyclin B1 through a cyclin-cyclin interaction.* EMBO Journal, 2000. **19**(6): p. 1378-88.

221. Kothakota, S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T.J. McGarry, M.W. Kirschner, K. Koths, D.J. Kwiatkowski, and L.T. Williams, *Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis.* Science, 1997. **278**(5336): p. 294-8.

222. Krammer, P.H., *CD95's deadly mission in the immune system.* Nature, 2000. **407**(6805): p. 789-95.

223. Krug, E.C., *Ph.D. dissertation.* Department of Physiology-Anatomy, University of Calfiornia, Berkeley., 1980.

224. Kuchin, S., P. Yeghiayan, and M. Carlson, *Cyclin-dependent protein kinase and cyclin homologs SSN3 and SSN8 contribute to transcriptional control in yeast.* Proceedings of the National Academy of Science (USA), 1995. **92**(9): p. 4006-10.

225. Kuiper, G.G. and A.O. Brinkmann, *Steroid hormone receptor phosphorylation: is there a physiological role?* Molecular and Cellular Endocrinology, 1994. **100**(1-2): p. 103-7.

226. LaBaer, J., M.D. Garrett, L.F. Stevenson, J.M. Slingerland, C. Sandhu, H.S. Chou, A. Fattaey, and E. Harlow, *New functional activities for the p21 family of CDK inhibitors.* Genes and Development, 1997. **11**(7): p. 847-62.

227. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(259): p. 680-685.

228. Lam, L.T., O.K. Pickeral, A.C. Peng, A. Rosenwald, E.M. Hurt, J.M. Giltnane, L.M. Averett, H. Zhao, R.E. Davis, M. Sathyamoorthy, L.M. Wahl, E.D. Harris, J.A. Mikovits, A.P. Monks, M.G. Hollingshead, E.A. Sausville, and L.M. Staudt, *Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol.* Genome Biology 2001. **2**(10): p. RESEARCH0041.

229. Larochelle, S., J. Chen, R. Knights, J. Pandur, P. Morcillo, H. Erdjument-Bromage, P. Tempst, B. Suter, and R.P. Fisher, *T-loop phosphorylation stabilizes*

the CDK7-cyclin H-MAT1 complex in vivo and regulates its CTD kinase activity. EMBO Journal, 2001. **20**(14): p. 3749-59.

230. Larochelle, S., J. Pandur, R.P. Fisher, H.K. Salz, and B. Suter, *Cdk7 is essential for mitosis and for in vivo Cdk-activating kinase activity.* Genes and Development, 1998. **12**(3): p. 370-81.

231. Lavoie, J.N., G. L'Allemain, A. Brunet, R. Muller, and J. Pouyssegur, *Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway.* Journal of Biological Chemistry, 1996. **271**(34): p. 20608-16.

232. Lazar, M., *Thyroid hormone receptors: multiple forms, multiple possibilities.* Endocrinology Reviews, 1993. **14**: p. 184-193.

233. Lazar, M.A., *Steroid and thyroid hormone receptors.* Endocrinology and Metabolism Clinics of North America, 1991. **20**(4): p. 681-95.

234. Lee, H. and P.M. Yen, *Recent advances in understanding thyroid hormone receptor coregulators.* Journal of Biomedical Science, 1999. **6**(2): p. 71-8.

235. Lees, E., B. Faha, V. Dulic, S.I. Reed, and E. Harlow, *Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner.* Genes and Development, 1992. **6**(10): p. 1874-85.

236. Lees, E.M. and E. Harlow, *Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase.* Molecular & Cellular Biology, 1993. **13**(2): p. 1194-201.

237. Lees, J.A., K.J. Buchkovich, D.R. Marshak, C.W. Anderson, and E. Harlow, *The retinoblastoma protein is phosphorylated on multiple sites by human cdc2.* EMBO Journal, 1991. **10**(13): p. 4279-90.

238. Leitman, D.C., C.H. Costa, H. Graf, J.D. Baxter, and R.C. Ribeiro, *Thyroid hormone activation of transcription is potentiated by activators of cAMP-dependent protein kinase.* Journal of Biological Chemistry, 1996. **271**(36): p. 21950-5.

239. Leloup, J. and M. Buscaglia, *La triiodothyronine, hormone de la metamorphose des amphibiens.* Comptes Rendues de l'Academie de les Sciences Paris Serie D, 1977. **284**: p. 2261-2263.

240. Levkau, B., H. Koyama, E. Rains, B. Clurman, B. Herren, K. Orth, J. Roberts, and R. Ross, *Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade.* Molecular Cell, 1998. **1**: p. 553-563.

241. Lewis, T.S., P.S. Shapiro, and N.G. Ahn, *Signal transduction through MAP kinase cascades.* Advances in cancer research, 1998. **74**: p. 49-139.

242. Li, H., J.M. Lahti, and V.J. Kidd, *Alternatively spliced cyclin C mRNA is widely expressed, cell cycle regulated, and encodes a truncated cyclin box.* Oncogene, 1996. **13**(4): p. 705-12.

243. Li, H., H. Zhu, C.J. Xu, and J. Yuan, *Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis.* Cell, 1998. **94**(4): p. 491-501.

244. Li, L.Y., X. Luo, and X. Wang, *Endonuclease G is an apoptotic DNase when released from mitochondria.* Nature, 2001. **412**(6842): p. 95-9.

245. Li, P., D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, E.S. Alnemri, and X. Wang, *Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade.* Cell, 1997. **91**(4): p. 479-89.

246. Li, Y., C.W. Jenkins, M.A. Nichols, and Y. Xiong, *Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21.* Oncogene, 1994. **9**(8): p. 2261-8.

247. Lin, H.-Y., F.B. Davis, J.K. Gordinier, L.J. Martino, and P.J. Davis, *Thyroid hormone induces activation of mitogen-activated protein kinase in cultured cells.* American Journal of Physiology, 1999. **276**(5 Pt 1): p. C1014-C1024.

248. Lin, H.Y., L.J. Martino, B.D. Wilcox, F.B. Davis, J.K. Gordinier, and P.J. Davis, *Potentiation by thyroid hormone of human IFN-gamma-induced HLA-DR expression.* The Journal of Immunology, 1998. **161**(2): p. 843-9.

249. Lin, H.-Y., A. Shih, F.B. Davis, and P.J. Davis, *Thyroid hormone promotes the phosphorylation of STAT3 and potientiates the action of epidermal growth factor in cultured cells.* Biochem Journal, 1999. **338**(Pt 2): p. 427-432.

250. Liu, Y., J.L. Martindale, M. Gorospe, and N.J. Holbrook, *Regulation of p21WAF1/CIP1 expression through mitogen-activated protein kinase signaling pathway.* Cancer Research, 1996. **56**(1): p. 31-5.

251. Ljungman, M. and M.T. Paulsen, *The cyclin-dependent kinase inhibitor roscovitine inhibits RNA synthesis and triggers nuclear accumulation of p53 that is unmodified at Ser15 and Lys382.* Molecular Pharmacology, 2001. **60**(4): p. 785-9.

252. Lloyd, A.C., F. Obermuller, S. Staddon, C.F. Barth, M. McMahon, and H. Land, *Cooperating oncogenes converge to regulate cyclin/cdk complexes.* Genes and Development, 1997. **11**(5): p. 663-77.

253. Long, J.J., A. Leresche, R.W. Kriwacki, and J.M. Gottesfeld, *Repression of TFIIH transcriptional activity and TFIIH-associated cdk7 kinase activity at mitosis.* Molecular and Cellular Biology, 1998. **18**(3): p. 1467-76.

254. Lozano, J.C., P. Schatt, F. Marques, G. Peaucellier, P. Fort, J.P. Feral, A.M. Geneviere, and A. Picard, *A presumptive developmental role for a sea urchin cyclin B splice variant.* Journal of Cell Biology, 1998. **140**(2): p. 283-93.

255. Maas, J.W., Jr., S. Horstmann, G.D. Borasio, J.M. Anneser, E.M. Shooter, and P.J. Kahle, *Apoptosis of central and peripheral neurons can be prevented with cyclin-dependent kinase/mitogen-activated protein kinase inhibitors.* Journal of Neurochemistry 1998. **70**(4): p. 1401-10.

256. Macleod, K.F., N. Sherry, G. Hannon, D. Beach, T. Tokino, K. Kinzler, B. Vogelstein, and T. Jacks, *p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage.* Genes and Development, 1995. **9**(8): p. 935-44.

257. Mangelsdorf, D.J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and et al., *The nuclear receptor superfamily: the second decade.* Cell, 1995. **83**(6): p. 835-9.

258. Markowitz, R.B., A.S. Hermann, D.F. Taylor, L. He, S. Anthony-Cahill, N.G. Ahn, and W.S. Dynan, *Phosphorylation of the C-terminal domain of RNA polymerase II by the extracellular-signal-regulated protein kinase ERK2.* Biochemical and Biophysical Research Communications, 1995. **207**(3): p. 1051-7.

259. Marshak, D.R. and G.L. Russo, *Regulation of protein kinase CKII during the cell division cycle.* Cellular and Molecular Biology Research, 1994. **40**(5-6): p. 513-7.

260. Mazumder, S., B. Gong, Q. Chen, J.A. Drazba, J.C. Buchsbaum, and A. Almasan, *Proteolytic cleavage of cyclin E leads to inactivation of associated kinase activity and amplification of apoptosis in hematopoietic cells.* Molecular and Cellular Biology, 2002. **22**(7): p. 2398-409.

261. Meijer, L., A. Borgne, O. Mulner, J. Chong, J. Blow, N. Inagki, M. Inagaki, J. Delcros, and J. Moulinoux, European Journal of Biochemistry, 1997. **243**: p. 527-536.

262. Meijer, L., A. Borgne, O. Mulner, J.P. Chong, J.J. Blow, N. Inagaki, M. Inagaki, J.G. Delcros, and J.P. Moulinoux, *Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5.* European Journal of Biochemistry, 1997. **243**(1-2): p. 527-36.

263. Meikrantz, W., S. Gisselbrecht, S.W. Tam, and R. Schlegel, *Activation of cyclin A-dependent protein kinases during apoptosis.* Proceedings of the National Academy of Sciences (USA), 1994. **91**: p. 3754-3758.

264. Meikrantz, W. and R. Schlegel, *Suppression of apoptosis by dominant negative mutants of cyclin-dependent protein kinases.* Journal of Biological Chemistry, 1996. **271**(17): p. 10205-10209.

265. Menon, J. and M. Wahrman, *Differential response of the tail and body epidermis of Rana catesbeiana tadpoles in vitro to the anticancer drug, cisplatin.* In Vitro Cellular and Developmental Biology. Animal, 1999. **35**(1): p. 8-11.

266. Mgbonyebi, O.P., J. Russo, and I.H. Russo, *Roscovitine induces cell death and morphological changes indicative of apoptosis in MDA-MB-231 breast cancer cells.* Cancer Research, 1999. **59**(8): p. 1903-10.

267. Minshull, J., H. Sun, N.K. Tonks, and A.W. Murray, *A MAP kinase-dependent spindle assembly checkpoint in Xenopus egg extracts.* Cell, 1994. **79**(3): p. 475-86.

268. Morgan, D.O., *The dynamics of cyclin dependent kinase structure.* Current Opinion in Cell Biology, 1996. **8**(6): p. 767-72.

269. Morgan, D.O., *Cyclin-dependent kinases: engines, clocks, and microprocessors.* Annual Review of Cell and Developmental Biology, 1997. **13**: p. 261-91.

270. Muise-Helmericks, R.C., H.L. Grimes, A. Bellacosa, S.E. Malstrom, P.N. Tsichlis, and N. Rosen, *Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway.* The journal of biological chemistry, 1998. **273**(45): p. 29864-72.

271. Nagano, M., K. Ui-Tei, H. Suzuki, Z. Piao, and Y. Miyata, *CDK inhibitors suppress apoptosis induced by chemicals and by excessive expression of a cell death gene, reaper, in Drosophila cells.* Apoptosis, 2000. **5**(6): p. 543-50.

272. Nagata, S., *Apoptotic DNA fragmentation.* Experimental Eye Research 2000. **256**(1): p. 12-8.

273. Nakajima, T., A. Fukamizu, J. Takahashi, F.H. Gage, T. Fisher, J. Blenis, and M.R. Montminy, *The signal-dependent coactivator CBP is a nuclear target for pp90RSK.* Cell, 1996. **86**(3): p. 465-74.

274. Niculescu, A.B., 3rd, X. Chen, M. Smeets, L. Hengst, C. Prives, and S.I. Reed, *Effects of p21(Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication.* Molecular and Cellular Biology, 1998. **18**(1): p. 629-43.

275. Nigg, E.A., *Targets of cyclin-dependent protein kinases.* Current Opinion in Cell Biology, 1993. **5**(2): p. 187-93.

276. Nishikawa, A., M. Kaiho, and K. Yoshizato, *Cell death in the anuran tadpole tail: thyroid hormone induces keratinization and tail-specific growth inhibition of epidermal cells.* Developmental Biology, 1989. **131**(2): p. 337-44.

277. Noble, M.E., J.A. Endicott, N.R. Brown, and L.N. Johnson, *The cyclin box fold: protein recognition in cell-cycle and transcription control.* Trends in Biochemical Sciences, 1997. **22**(12): p. 482-7.

278. North, S. and P. Hainaut, *p53 and cell-cycle control: a finger in every pie.* Pathologie Biologie, 2000. **48**(3): p. 255-70.

279. Nugent, J.H., C.E. Alfa, T. Young, and J.S. Hyams, *Conserved structural motifs in cyclins identified by sequence analysis.* Journal of Cell Science, 1991. **99 (Pt 3)**: p. 669-74.

280. O'Brien, T., S. Hardin, A. Greenleaf, and J.T. Lis, *Phosphorylation of RNA polymerase II C-terminal domain and transcriptional elongation.* Nature, 1994. **370**(6484): p. 75-7.

281. Oelgeschlager, T., *Regulation of RNA polymerase II activity by CTD phosphorylation and cell cycle control.* Journal of Cellular Physiology, 2002. **190**(2): p. 160-9.

282. Okada, R., T. Iwata, T. Kato, M. Kikuchi, K. Yamamoto, and S. Kikuyama, *Cloning of bullfrog thyroid-stimulating hormone (TSH) beta subunit cDNA: expression of TSHbeta mRNA during metamorphosis.* General and Comparative Endocrinology, 2000. **119**(2): p. 224-31.

283. Okuda, M., *The role of nucleophosmin in centrosome duplication.* Oncogene, 2002. **21**(40): p. 6170-4.

284. Oofusa, K., O. Tooi, A. Kashiwagi, K. Kashiwagi, Y. Kondo, Y. Watanabe, T. Sawada, K. Fujikawa, and K. Yoshizato, *Expression of thyroid hormone receptor betaA gene assayed by transgenic Xenopus laevis carrying its promoter sequences.* Molecular and Cellular Endocrinology, 2001. **181**: p. 97-110.

285. Orti, E., J.E. Bodwell, and A. Munck, *Phosphorylation of steroid hormone receptors.* Endocrine Review, 1992. **13**(1): p. 105-28.

286. Padmanabhan, J., D.S. Park, L.A. Greene, and M.L. Shelanski, *Role of cell cycle regulatory proteins in cerebellar granule neuron apoptosis.* The Journal of Neuroscience, 1999. **19**(20): p. 8747-56.

287. Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta, *Cyclin A is required at two points in the human cell cycle.* EMBO Journal, 1992. **11**: p. 961-971.

288. Page, K., J. Li, Y. Wang, S. Kartha, R. Pestell, and M. Hershenson, *Regulation of cyclin D(1) expression and DNA synthesis by phosphatidylinositol 3-kinase in airway smooth muscle cells.* American Journal of Respiratory Cell and Molecular Biology, 2000. **23**: p. 436-443.

289. Park, D.S., S.E. Farinelli, and L.A. Greene, *Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons.* Journal of Biological Chemistry, 1996. **271**(14): p. 8161-9.

290. Park, D.S., B. Levine, G. Ferrari, and L.A. Greene, *Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons.* The Journal of Neuroscience, 1997. **17**(23): p. 8975-83.

291. Park, D.S., E.J. Morris, L.A. Greene, and H.M. Geller, *G1/S cell cycle blockers and inhibitors of cyclin-dependent kinases suppress camptothecin-induced neuronal apoptosis.* The Journal of Neuroscience, 1997. **17**(4): p. 1256-70.

292. Park, D.S., E.J. Morris, J. Padmanabhan, M.L. Shelanski, H.M. Geller, and L.A. Greene, Journal of Cell Biology, 1998. **143**: p. 457-467.

293. Park, D.S., E.J. Morris, J. Padmanabhan, M.L. Shelanski, H.M. Geller, and L.A. Greene, *Cyclin-dependent kinases participate in death of neurons evoked by DNA-damaging agents.* Journal of Cell Biology, 1998. **143**(2): p. 457-67.

294. Parry, D.H. and P.H. O'Farrell, *The schedule of destruction of three mitotic cyclins can dictate the timing of events during exit from mitosis.* Current Biology, 2001. **11**(9): p. 671-83.

295. Patra, D. and W.G. Dunphy, *Xe-p9, a Xenopus Suc1/Cks homolog, has multiple essential roles in cell cycle control.* Genes and Development, 1996. **10**(12): p. 1503-1515.

296. Patra, D. and W.G. Dunphy, *Xe-p9, a Xenopus Suc1/Cks protein, is essential for the Cdc2-dependent phosphorylation of the anaphase- promoting complex at mitosis.* Genes and Development, 1998. **12**(16): p. 2549-59.

297. Patturajan, M., X. Wei, R. Berezney, and J.L. Corden, *A nuclear matrix protein interacts with the phosphorylated C-terminal domain of RNA polymerase II.* Molecular and Cellular Biology, 1998. **18**(4): p. 2406-15.

298. Pavletich, N.P., *Mechanisms of cyclin-dependent kinase regulation: structures of Cdks, their cyclin activators, and Cip and INK4 inhibitors.* Journal of Molecular Biology, 1999. **287**(5): p. 821-8.

299. Payne, D.M., A.J. Rossomando, P. Martino, A.K. Erickson, J.H. Her, J. Shabanowitz, D.F. Hunt, M.J. Weber, and T.W. Sturgill, *Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase).* EMBO Journal, 1991. **10**(4): p. 885-92.

300. Payton, M. and S. Coats, *Cyclin E2, the cycle continues.* The International Journal of Biochemistry and Cell Biology 2002. **34**(4): p. 315-20.

301. Pearson, G., F. Robinson, T.B. Gibson, B.-E. Xu, M. Karandikar, K. Berman, and M.H. Cobb, *Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions.* Endocrine Reviews, 2001. **22**(2): p. 153-183.

302. Peeper, D.S. and R. Bernards, *Communication between the extracellular environment, cytoplasmic signalling cascades and the nuclear cell-cycle machinery.* FEBS Letters 1997. **410**(1): p. 11-6.

303. Peng, J., Y. Zhu, J.T. Milton, and D.H. Price, *Identification of multiple cyclin subunits of human P-TEFb.* Genes and Development, 1998. **12**(5): p. 755-62.

304. Pepperkok, R., P. Lorenz, W. Ansorge, and W. Pyerin, *Casein kinase II is required for transition of G0/G1, early G1, and G1/S phases of the cell cycle.* Journal of Biological Chemistry, 1994. **269**(9): p. 6986-91.

305. Petersen, B.O., J. Lukas, C.S. Sorensen, J. Bartek, and K. Helin, *Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization.* EMBO Journal, 1999. **18**(2): p. 396-410.

306. Pines, J., *Cyclins and cyclin-dependent kinases: take your partners.* Trends in Biochemical Sciences, 1993. **18**(6): p. 195-7.

307. Pines, J. and T. Hunter, *The differential localization of human cyclins A and B is due to a cytoplasmic retention signal in cyclin B.* EMBO Journal, 1994. **13**(16): p. 3772-81.

308. Pippin, J.W., Q. Qu, L. Meijer, and S.J. Shankland, *Direct in vivo inhibition of the nuclear cell cycle cascade in experimental mesangial proliferative glomerulonephritis with Roscovitine, a novel cyclin-dependent kinase antagonist.* The Journal of Clinical Investigation, 1997. **100**(10): p. 2512-20.

309. Porter, D.C. and K. Keyomarsi, *Novel splice variants of cyclin E with altered substrate specificity.* Nucleic Acids Research, 2000. **28**(23): p. E101.

310. Porter, D.C., N. Zhang, C. Danes, M.J. McGahren, R.M. Harwell, S. Faruki, and K. Keyomarsi, *Tumor-specific proteolytic processing of cyclin E generates hyperactive lower-molecular-weight forms.* Molecular and Cellular Biology, 2001. **21**(18): p. 6254-69.

311. Price, D.H., *P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II.* Molecular and Cellular Biology, 2000. **20**(8): p. 2629-34.

312. Price, M.A., A.E. Rogers, and R. Treisman, *Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET).* EMBO Journal, 1995. **14**(11): p. 2589-601.

313. Pumiglia, K.M. and S.J. Decker, *Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway.* Proceedings of the National Academy of Science (USA), 1997. **94**(2): p. 448-52.

314. Puzianowska-Kuznicka, M., S. Damjanovski, and Y.B. Shi, *Both thyroid hormone and 9-cis retinoic acid receptors are required to efficiently mediate the effects of thyroid hormone on embryonic development and specific gene regulation in Xenopus laevis.* Molecular and Cellular Biology, 1997. **17**: p. 4738-4749.

315. Raff, J.W., K. Jeffers, and J.Y. Huang, *The roles of Fzy/Cdc20 and Fzr/Cdh1 in regulating the destruction of cyclin B in space and time.* Journal of Cell Biology, 2002. **157**(7): p. 1139-49.

316. Ranjan, M., J. Wong, and Y. Shi, *Transcriptional repression of Xenopus TR beta gene is mediated by a thyroid hormone response element located near the start site.* Journal of Biological Chemistry, 1994. **269**: p. 24699-24705.

317. Rao, L., D. Perez, and E. White, *Lamin proteolysis facilitates nuclear events during apoptosis.* Journal of Cell Biology, 1996. **135**(6 Pt 1): p. 1441-55.

318. Ravagnan, L., T. Roumier, and G. Kroemer, *Mitochondria, the killer organelles and their weapons.* Journal of Cellular Physiology, 2002. **192**(2): p. 131-7.

319. Regard, E., A. Taurog, and T. Nakashima, *Plasma thyroxine and triiodothyronine levels in spontaneously metamorphosing Rana catesbeiana tadpoles and in adult anuran amphibia.* Endocrinology, 1978. **102**: p. 674-684.

320. Rickert, P., J.L. Corden, and E. Lees, *Cyclin C/CDK8 and cyclin H/CDK7/p36 are biochemically distinct CTD kinases.* Oncogene, 1999. **18**(4): p. 1093-102.

321. Rickert, P., W. Seghezzi, F. Shanahan, H. Cho, and E. Lees, *Cyclin C/CDK8 is a novel CTD kinase associated with RNA polymerase II.* Oncogene, 1996. **12**(12): p. 2631-40.

322. Riedl, T. and J.M. Egly, *Phosphorylation in transcription: the CTD and more.* Gene Expression, 2000. **9**(1-2): p. 3-13.

323. Robinson, D.H. and M.B. Heintzelman, *Morphology of ventral epidermis of Rana catesbeiana during metamorphosis.* The Anatomical Record, 1987. **217**(3): p. 305-17.

324. Rodriguez, J. and Y. Lazebnik, *Caspase-9 and APAF-1 form an active holoenzyme.* Genes and Development, 1999. **13**(24): p. 3179-84.

325. Rollins-Smith, L., K. Barker, and A. Davis, *Involvement of glucocorticoids in the reorganization of the amphibian immune system at metamorphosis.* Developmental Immunolgy, 1997. **5**(2): p. 145-152.

326. Roovers, K. and R.K. Assoian, *Integrating the MAP kinase signal into the G1 phase cell cycle machinery.* Bioessays, 2000. **22**(9): p. 818-826.

327. Roovers, K., G. Davey, X. Zhu, M.E. Bottazzi, and R.K. Assoian, *Alpha5beta1 integrin controls cyclin D1 expression by sustaining mitogen-activated protein kinase activity in growth factor-treated cells.* Molecular Biology of the Cell, 1999. **10**(10): p. 3197-204.

328. Rosenblatt, J., Y. Gu, and D.O. Morgan, *Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A.* Proceedings of the National Academy of Science (USA), 1992. **89**(7): p. 2824-8.

329. Rowan, B.G., N.L. Weigel, and B.W. O'Malley, *Phosphorylation of steroid receptor coactivator-1. Identification of the phosphorylation sites and phosphorylation through the mitogen-activated protein kinase pathway.* Journal of Biological Chemistry, 2000. **275**(6): p. 4475-83.

330. Roy, L.M., K.I. Swenson, D.H. Walker, B.G. Gabrielli, R.S. Li, H. Piwnica-Worms, and J.L. Maller, *Activation of p34cdc2 kinase by cyclin A.* Journal of Cell Biology, 1991. **113**(3): p. 507-14.

331. Rudel, T. and G.M. Bokoch, *Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2.* Science, 1997. **276**(5318): p. 1571-4.

332. Sachs, L.M., S. Damjanovski, P.L. Jones, Q. Li, T. Amano, S. Ueda, Y.-B. Shi, and A. Ishizuya-Oka, *Dual functions of thyroid hormone receptors during Xenopus development.* Comparative Biochemistry and Physiology Part B, 2000. **126**: p. 199-211.

333. Sakahira, H., M. Enari, and S. Nagata, *Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis.* Nature, 1998. **391**(6662): p. 96-9.

334. Sakurai, T., M. Suzuki, T. Sawazaki, S. Ishii, and S. Yoshida, *Anti-oncogene product p53 binds DNA helicase.* Experimental Eye Research 1994. **215**(1): p. 57-62.

335. Sandrock, B. and J.M. Egly, *A yeast four-hybrid system identifies Cdk-activating kinase as a regulator of the XPD helicase, a subunit of transcription factor IIH.* Journal of Biological Chemistry, 2001. **276**(38): p. 35328-33.

336. Sauer, K. and C.F. Lehner, *The role of cyclin E in the regulation of entry into S phase.* Progress in Cell Cycle Research 1995. **1**: p. 125-39.

337. Sausville, E., *Complexities in the development of cyclin-dependent kinase inhibitor drugs.* Trends in Molecular Medicine, 2002. **8**: p. S32-S37.

338. Schaeffer, H.J. and M.J. Weber, *Mitogen-activated protein kinases: specific messages from ubiquitous messengers.* Molecular and Cellular Biology, 1999. **19**(4): p. 2435-2444.

339. Schang, L.M., A. Rosenberg, and P.A. Schaffer, *Roscovitine, a specific inhibitor of cellular cyclin-dependent kinases, inhibits herpes simplex virus DNA synthesis in the presence of viral early proteins.* Journal of Virology, 2000. **74**(5): p. 2107-20.

340. Schreiber, A.M., B. Das, H. Huang, N. Marsh-Armstrong, and D.D. Brown, *Diverse developmental programs of Xenopus laevis metamorphosis are inhibited by a dominant negative thyroid hormone receptor.* Proceedings of the National Academy of Science (USA), 2001. **98**: p. 10739-10744.

341. Schulman, B.A., D.L. Lindstrom, and E. Harlow, *Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A.* Proceedings of the National Academy of Science (USA), 1998. **95**(18): p. 10453-8.

342. Schuringa, J.J., L.J. Jonk, W.H. Dokter, E. Vellenga, and W. Kruijer, *Interleukin-6-induced STAT3 transactivation and Ser727 phosphorylation involves Vav, Rac-1 and the kinase SEK-1/MKK-4 as signal transduction components.* The Biochemical Journal, 2000. **347 Pt 1**: p. 89-96.

343. Senderowicz, A.M. and E.A. Sausville, *Preclinical and clinical development of cyclin-dependent kinase modulators.* Journal of the National Cancer Institute, 2000. **92**(5): p. 376-87.

344. Serizawa, H., T.P. Makela, J.W. Conaway, R.C. Conaway, R.A. Weinberg, and R.A. Young, *Association of Cdk-activating kinase subunits with transcription factor TFIIH.* Nature, 1995. **374**(6519): p. 280-2.

345. Sewing, A., B. Wiseman, A.C. Lloyd, and H. Land, *High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1.* Molecular and Cellular Biology, 1997. **17**(9): p. 5588-5597.

346. Sherr, C. and J. Roberts, *CDK inhibitors: positive and negative regulators of G1-phase progression.* Genes & Development, 1999. **13**(12): p. 1501-1512.

347. Sherr, C.J., *Mammalian G1 cyclins.* Cell, 1993. **73**(6): p. 1059-65.

348. Sherr, C.J., *G1 phase progression: cycling on cue.* Cell, 1994. **79**: p. 551-555.

349. Shi, L., G. Chen, D. He, D.G. Bosc, D.W. Litchfield, and A.H. Greenberg, *Granzyme B induces apoptosis and cyclin A-associated cyclin-dependent kinase activity in all stages of the cell cycle.* The Journal of Immunology, 1996. **157**(6): p. 2381-5.

350. Shi, L., W.K. Nishioka, J. Th'ng, E.M. Bradbury, D.W. Litchfield, and A.H. Greenberg, *Premature p34cdc2 activation required for apoptosis.* Science, 1994. **263**: p. 1143-1145.

351. Shi, Y. and V. Liang, *Cloning and characterization of the ribosomal protein L8 gene from Xenopus laevis.* Biochimica et Biophysica Acta, 1994. **1217**: p. 227-228.

352. Shi, Y.-B., *Amphibian Metamorphosis: From morphology to molecular biology.* 2000, New York: Wiley-Liss.

353. Shi, Y.B. and A. Ishizuya-Oka, *Biphasic intestinal development in amphibians: embryogenesis and remodeling during metamorphosis.* Current Topics in Developmental Biology, 1996. **32**: p. 205-35.

354. Shi, Y.-B. and A. Ishizuya-Oka, *Thyroid hormone regulation of apoptotic tissue remodeling: implications from molecular analysis of amphibian metamorphosis.* Progress in Nucleic Acid Research and Molecular Biology, 2001. **65**: p. 53-100.

355. Shi, Y.-B., J. Wong, M. Puzianowska-Kuznicka, and M.A. Stolow, *Tadpole competence and tissue-specific temporal regulation of amphibian metamorphosis: roles of thyroid hormone and its receptors.* Bioessays, 1996. **18**(5): p. 391-399.

356. Shiekhattar, R., F. Mermelstein, R.P. Fisher, R. Drapkin, B. Dynlacht, H.C. Wessling, D.O. Morgan, and D. Reinberg, *Cdk-activating kinase complex is a component of human transcription factor TFIIH.* Nature, 1995. **374**(6519): p. 283-7.

357. Shih, A., H.Y. Lin, F.B. Davis, and P.J. Davis, *Thyroid hormone promotes serine phosphorylation of p53 by mitogen-activated protein kinase.* Biochemistry, 2001. **40**: p. 2870-2878.

358. Shimizu, T., P.M. O'Connor, K.W. Kohn, and Y. Pommier, *Unscheduled activation of cyclin B1/Cdc2 kinase in human promyelocytic leukemia cell line HL60 cells undergoing apoptosis induced by DNA damage.* Cancer Research, 1995. **55**(2): p. 228-31.

359. Solomon, M.J. and P. Kaldis, *Regulation of CDKs by phosphorylation.* Results and Problems in Cell Differentiation, 1998. **22**: p. 79-109.

360. Song, C.Z., *Requirement for phosphorylation of RNA polymerase II C-terminal domain in transcription is both transcript length and promoter dependent.* Biochemical and Biophysical Research Communications, 1996. **229**(3): p. 810-6.

361. Stewart, N., G.G. Hicks, F. Paraskevas, and M. Mowat, *Evidence for a second cell cycle block at G2/M by p53.* Oncogene, 1995. **10**(1): p. 109-15.

362. Stokoe, D., D.G. Campbell, S. Nakielny, H. Hidaka, S.J. Leevers, C. Marshall, and P. Cohen, *MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase.* EMBO Journal, 1992. **11**(11): p. 3985-94.

363. Strahl, T., H. Gille, and P.E. Shaw, *Selective response of ternary complex factor Sap1a to different mitogen-activated protein kinase subgroups.* Proceedings of the National Academy of Science (USA), 1996. **93**(21): p. 11563-8.

364. Sturgill, T.W., L.B. Ray, E. Erikson, and J.L. Maller, *Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II.* Nature, 1988. **334**(6184): p. 715-8.

365. Sun, H., A.J. King, H.B. Diaz, and M.S. Marshall, *Regulation of the protein kinase Raf-1 by oncogenic Ras through phosphatidylinositol 3-kinase, Cdc42/Rac and Pak.* Current Biology, 2000. **10**(5): p. 281-4.

366. Sun, X., Y. Zhang, H. Cho, P. Rickert, E. Lees, W. Lane, and D. Reinberg, *NAT, a human complex containing Srb polypeptides that functions as a negative regulator of activated transcription.* Molecular Cell, 1998. **2**(2): p. 213-22.

367. Surosky, R.T., R. Strich, and R.E. Esposito, *The yeast UME5 gene regulates the stability of meiotic mRNAs in response to glucose.* Molecular and Cellular Biology, 1994. **14**(5): p. 3446-58.

368. Susin, S.A., N. Zamzami, M. Castedo, E. Daugas, H.G. Wang, S. Geley, F. Fassy, J.C. Reed, and G. Kroemer, *The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis.* The Journal of Experimental Medicine 1997. **186**(1): p. 25-37.

369. Susin, S.A., N. Zamzami, M. Castedo, T. Hirsch, P. Marchetti, A. Macho, E. Daugas, M. Geuskens, and G. Kroemer, *Bcl-2 inhibits the mitochondrial release of an apoptogenic protease.* The Journal of Experimental Medicine 1996. **184**(4): p. 1331-41.

370. Sutherland, C., I.A. Leighton, and P. Cohen, *Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling.* The Biochemical Journal, 1993. **296 (Pt 1)**: p. 15-9.

371. Suzuki, K., K. Sato, K. Katsu, H. Hayashita, D.B. Kristensen, and K. Yoshizato, *Novel Rana keratin genes and their expression during larval to adult epidermal conversion in bullfrog tadpoles.* Differentiation, 2001. **68**(1): p. 44-54.

372. Suzuki, Y., Y. Imai, H. Nakayama, K. Takahashi, K. Takio, and R. Takahashi, *A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death.* Molecular Cell, 2001. **8**(3): p. 613-21.

373. Takuwa, N., Y. Fukui, and Y. Takuwa, *Cyclin D1 expression mediated by phosphatidylinositol 3-kinase through mTOR-p70(S6K)-independent signaling in growth factor-stimulated NIH 3T3 fibroblasts.* Molecular and Cellular Biology, 1999. **19**(2): p. 1346-1358.

374. Talanian, R.V., X. Yang, J. Turbov, P. Seth, T. Ghayur, C.A. Casiano, K. Orth, and C.J. Froelich, *Granule-mediated killing: pathways for granzyme B-initiated apoptosis.* The Journal of Experimental Medicine 1997. **186**(8): p. 1323-31.

375. Tata, J.R., *Early metamorphic competence of Xenopus larvae.* Developmental Biology, 1968. **18**(5): p. 415-40.

376. Tata, J.R., *Gene expression during metamorphosis: an ideal model for post-embryonic development.* Bioessays, 1993. **15**(4): p. 239-248.

377. Tata, J.R., *Hormonal regulation of programmed cell death during amphibian metamorphosis.* Biochemistry and Cell Biology 1994. **72**(11-12): p. 581-8.

378. Tata, J.R., *Hormonal interplay and thyroid hormone receptor expression during amphibian metamorphosis*, in *Metamorphosis: postembryonic reprogramming of gene expression in amphibian and insect cells*, L.I. Gilbert, J.R. Tata, and B.G. Atkinson, Editors. 1996, Academic Press: San Diego. p. 465-503.

379. Tata, J.R., A. Kawahara, and B.S. Baker, *Prolactin inhibits both thyroid hormone-induced morphogenesis and cell death in cultured amphibian larval tissues.* Developmental Biology, 1991. **146**: p. 72-80.

380. Tautz, D. and M. Renz, *An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels.* Analytical Biochemistry., 1983. **132**(1): p. 14-9.

381. Taylor, A.C. and J.J. Kollros, *Stages in the normal development of Rana pipiens larvae.* Anatomical Record, 1946. **94**: p. 7-24.

382. Thornberry, N.A. and Y. Lazebnik, *Caspases: enemies within.* Science, 1998. **281**(5381): p. 1312-6.

383. Thornberry, N.A., T.A. Rano, E.P. Peterson, D.M. Rasper, T. Timkey, M. Garcia-Calvo, V.M. Houtzager, P.A. Nordstrom, S. Roy, J.P. Vaillancourt, K.T. Chapman, and D.W. Nicholson, *A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis.* Journal of Biological Chemistry, 1997. **272**(29): p. 17907-11.

384. Tian, B., T. Takasu, and C. Henke, *Functional role of cyclin A on induction of fibroblast apoptosis due to ligation of CD44 matrix receptor by anti-CD44 antibody.* Experimental Eye Research 2000. **257**(1): p. 135-44.

385. Ting, Y.-T., M.K. Bhat, R. Wong, and S.-y. Cheng, *Tissue-specific stabilization of the thyroid hormone beta1 nuclear receptor by phosphorylation.* Journal of Biological Chemistry, 1997. **272**(7): p. 4129-34.

386. Ting, Y.T. and S.Y. Cheng, *Hormone-activated phosphorylation of human beta1 thyroid hormone nuclear receptor.* Thyroid, 1997. **7**(3): p. 463-9.

387. Toyoshima-Morimoto, F., E. Taniguchi, N. Shinya, A. Iwamatsu, and E. Nishida, *Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase.* Nature, 2001. **410**(6825): p. 215-20.

388. Trembley, J.H., D. Hu, L.C. Hsu, C.Y. Yeung, C. Slaughter, J.M. Lahti, and V.J. Kidd, *PITSLRE p110 protein kinases associate with transcription complexes and affect their activity.* Journal of Biological Chemistry, 2002. **277**(4): p. 2589-96.

389. Trigon, S., H. Serizawa, J.W. Conaway, R.C. Conaway, S.P. Jackson, and M. Morange, *Characterization of the residues phosphorylated in vitro by different C-terminal domain kinases.* Journal of Biological Chemistry, 1998. **273**(12): p. 6769-75.

390. Tsai, L.-H., E. Lees, B. Faha, E. Harlow, and K. Riabowol, *The cdk2 kinase is required for the G1-to S transition in mammalian cells.* Oncogene, 1993. **8**: p. 1593-1602.

391. Tsai, M.J. and B.W. O'Malley, *Molecular mechanisms of action of steroid/thyroid receptor superfamily members.* Annual Review of Biochemistry, 1994. **63**: p. 451-86.

392. Tzagarakis-Foster, C. and M.L. Privalsky, *Phosphorylation of thyroid hormone receptors by protein kinase A regulates DNA recognition by specific inhibition of receptor monomer binding.* The journal of biological chemistry, 1998. **273**(18): p. 10926-32.

393. Ucker, D.S., P.S. Obermiller, W. Eckhart, J.R. Apgar, N.A. Berger, and J. Meyers, *Genome digestion is a dispensable consequence of physiological cell death mediated by cytotoxic T lymphocytes.* Molecular and Cellular Biology, 1992. **12**(7): p. 3060-9.

394. Veldhoen, N., D. Crump, K. Werry, and C. Helbing, *Distinctive gene profiles occur at key points during natural metamorphosis in the Xenopus laevis tadpole tail.* Developmental Dynamics, 2002. **in press**.

395. Veldhoen, N. and C.C. Helbing, *Detection of environmental endocrine-disruptor effects on gene expression in live Rana catesbeiana tadpoles using a tail fin biopsy technique.* Environmental Toxicology and Chemistry, 2001. **20**(12): p. 2704-2708.

396. Vitali, L., J.S. Yakisich, M.F. Vita, A. Fernandez, L. Settembrini, A. Siden, M. Cruz, H. Carminatti, O. Casas, and V. Idoyaga Vargas, *Roscovitine inhibits ongoing DNA synthesis in human cervical cancer.* Cancer Letters 2002. **180**(1): p. 7-12.

397. Wagner, M.J., M. Gogela-Spehar, R.C. Skirrow, R.N. Johnston, K. Riabowol, and C.C. Helbing, *Expression of novel ING variants is regulated by thyroid hormone in the Xenopus laevis tadpole.* Journal of Biological Chemistry, 2001. **276**: p. 47013-47020.

398. Wagner, R., J. Apriletti, M. McGrath, B. West, J. Baxter, and R. Fletterick, *A structural role for hormone in the thyroid hormone receptor.* Nature, 1995. **378**: p. 690-697.

399. Wahi, M. and A.D. Johnson, *Identification of genes required for alpha 2 repression in Saccharomyces cerevisiae.* Genetics, 1995. **140**(1): p. 79-90.

400. Wajant, H., *The Fas signaling pathway: more than a paradigm.* Science, 2002. **296**(5573): p. 1635-6.

401. Wang, D., C. de la Fuente, L. Deng, L. Wang, I. Zilberman, C. Eadie, M. Healey, D. Stein, T. Denny, L.E. Harrison, L. Meijer, and F. Kashanchi, *Inhibition of human immunodeficiency virus type 1 transcription by chemical cyclin-dependent kinase inhibitors.* Journal of Virology, 2001. **75**(16): p. 7266-79.

402. Wang, J., X. Chenivesse, B. Henglein, and C. Brechot, *Hepatitis B virus integration in a cyclin A gene in a hepatocellular carcinoma.* Nature, 1990. **343**(6258): p. 555-7.

403. Wang, J.Y., *Abl tyrosine kinase in signal transduction and cell-cycle regulation.* Current Opinion in Genetics and Development, 1993. **3**(1): p. 35-43.

404. Wang, X., W. Vermeulen, J. Coursen, M. Gibson, S. Lupold, K. Forrester, G. Xu, L. Elmore, H. Yeh, J. Hoeijmakers, and C. Harris, *The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway.* Genes and Development, 1996. **10**: p. 1219-1232.

405. Wang, Z. and D. Brown, *A gene expression screen.* Proceedings of the National Academy of Sciences (USA), 1991. **88**: p. 11505-11509.

406. Wang, Z. and D.D. Brown, *Thyroid hormone-induced gene expression program for amphibian tail resorption.* Journal of Biological Chemistry, 1993. **268**: p. 16270-16278.

407. Waskiewicz, A.J., A. Flynn, C.G. Proud, and J.A. Cooper, *Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2.* EMBO Journal, 1997. **16**(8): p. 1909-20.

408. Weber, J.D., D.M. Raben, P.J. Phillips, and J.J. Baldassare, *Sustained activation of extracellular-signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase.* The Biochemical Journal, 1997. **326**(Pt 1): p. 61-8.

409. Weber, R., *The isolate tadpole tail as a model system for studies on th emechanism of hormone-dependent tissue involution.* General and Comparative Endocrinology, 1969. **S2**: p. 408-416.

410. Weigel, N.L., *Steroid hormone receptors and their regulation by phosphorylation.* The Biochemical Journal, 1996. **319 (Pt 3)**: p. 657-67.

411. Welm, A.L., N.A. Timchenko, Y. Ono, H. Sorimachi, H.S. Radomska, D.G. Tenen, J. Lekstrom-Himes, and G.J. Darlington, *C/EBPalpha is required for proteolytic cleavage of cyclin A by calpain 3 in myeloid precursor cells.* Journal of Biological Chemistry, 2002. **277**(37): p. 33848-56.

412. Woods, D., D. Parry, H. Cherwinski, E. Bosch, E. Lees, and M. McMahon, *Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1.* Molecular and Cellular Biology, 1997. **17**(9): p. 5598-611.

413. Worland, P.J., G. Kaur, M. Stetler-Stevenson, S. Sebers, O. Sartor, and E.A. Sausville, *Alteration of the phosphorylation state of p34cdc2 kinase by the flavone L86-8275 in breast carcinoma cells. Correlation with decreased H1 kinase activity.* Biochemical Pharmacology, 1993. **46**(10): p. 1831-40.

414. Woychik, N.A., *Fractions to functions: RNA polymerase II thirty years later.* Cold Spring Harbor Symposia on Quantitative Biology, 1998. **63**: p. 311-7.

415. Wright, M.L., L.J. Cykowski, L. Lundrigan, K.L. Hemond, D.M. Kochan, E.E. Faszewski, and C.M. Anuszewski, *Anterior pituitary and adrenal cortical hormones accelerate or inhibit tadpole hindlimb gtowth and development depending on stage of spontaneous development or thyroxine concentration in induced metamorphosis.* Journal of Experimental Zoology, 1994. **270**: p. 175-188.

416. Wyllie, A.H., J.F. Kerr, and A.R. Currie, *Cell death: the significance of apoptosis.* International Review of Cytology, 1980. **68**: p. 251-306.

417. Xing, J., D.D. Ginty, and M.E. Greenberg, *Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase.* Science, 1996. **273**(5277): p. 959-63.

418. Yakisich, J.S., J. Boethius, I.O. Lindblom, L. Wallstedt, V.I. Vargas, A. Siden, and M.H. Cruz, *Inhibition of DNA synthesis in human gliomas by roscovitine.* Neuroreport, 1999. **10**(12): p. 2563-7.

419. Yamauchi, K., H. Takeuchi, M. Overall, M. Dziadek, S.L. Munro, and G. Schreiber, *Structural characteristics of bullfrog (Rana catesbeiana) transthyretin and its cDNA--comparison of its pattern of expression during metamorphosis with that of lipocalin.* European Journal of Biochemistry, 1998. **256**(2): p. 287-96.

420. Yamochi, T., K. Semba, K. Tsuji, K. Mizumoto, H. Sato, Y. Matsuura, I. Nishimoto, and M. Matsuoka, *ik3-1/Cables is a substrate for cyclin-dependent kinase 3 (cdk3).* European Journal of Biochemistry, 2001. **268**: p. 6076-6082.

421. Yang, J., X. Liu, K. Bhalla, C.N. Kim, A.M. Ibrado, J. Cai, T.I. Peng, D.P. Jones, and X. Wang, *Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked.* Science, 1997. **275**(5303): p. 1129-32.

422. Yao, S., K. McKenna, S. Sharkis, and A. Bedi, *Requirement of p34cdc2 kinase for apoptosis mediated by the Fas/APO-1 receptor and interleukin 1beta-converting enzyme-related proteases.* Cancer Research, 1996. **56**: p. 4551-4555.

423. Yaoita, Y. and K. Nakajima, *Induction of apoptosis and CPP32 expression by thyroid hormone in a myoblasic cell line derived from tadpole tail.* Journal of Biological Chemistry, 1997. **272**: p. 5122-5127.

424. Yaoita, Y., Y. Shi, and D. Brown, *Xenopus laevis alpha and beta thyroid hormone receptors.* Proceedings of the National Academy of Science (USA), 1990. **87**: p. 7090-7094.

425. Yen, P., *Physiological and molecular basis of thyroid hormone action.* Physiological Reviews, 2001. **81**(3): p. 1097-1142.

426. Yen, P. and W. Chin, *New advances in understanding the molecular mechanism of thyroid hormone action.* Trends in Endocrinology and Metabolism, 1994. **5**: p. 65-72.

427. Yoshizato, K., *Biochemistry and cell biology of amphibian metamorphosis with a special emphasis on the mechanism of removal of larval organs.* International Review of Cytology, 1989. **119**: p. 97-149.

428. Zachariae, W., M. Schwab, K. Nasmyth, and W. Seufert, *Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex.* Science, 1998. **282**(5394): p. 1721-4.

429. Zamzami, N., S.A. Susin, P. Marchetti, T. Hirsch, I. Gomez-Monterrey, M. Castedo, and G. Kroemer, *Mitochondrial control of nuclear apoptosis.* The Journal of Experimental Medicine 1996. **183**(4): p. 1533-44.

430. Zariwala, M., J. Liu, and Y. Xiong, *Cyclin E2, a novel human G1 cyclin and activating partner of CDK2 and CDK3, is induced by viral oncoproteins.* Oncogene, 1998. **17**(21): p. 2787-98.

431. Zhai, Y.L., T. Nikaido, T. Shiozawa, A. Orii, and S. Fujii, *Expression of cyclins and cyclin-dependent kinases in smooth muscle tumors of the uterus.* International Journal of Cancer, 1999. **84**(3): p. 244-50.

432. Zhang, J. and M. Lazar, *The mechanism of action of thyroid hormones.* Annual Review of Physiology, 2000. **62**: p. 439-466.

433. Zhang, X., M. Jeyakumar, and M.K. Bagchi, *Ligand-dependent cross-talk between steroid and thyroid hormone receptors. Evidence for common transcriptional coactivator(s).* Journal of Biological Chemistry, 1996. **271**(25): p. 14825-33.

434. Zhao, Y., C. Bjorbaek, and D.E. Moller, *Regulation and interaction of pp90(rsk) isoforms with mitogen-activated protein kinases.* Journal of Biological Chemistry, 1996. **271**(47): p. 29773-9.

435. Zhou, B.B., H. Li, J. Yuan, and M.W. Kirschner, *Caspase-dependent activation of cyclin-dependent kinases during Fas-induced apoptosis in Jurkat cells.* Proceedings of the National Academy of Science (USA), 1998. **95**(12): p. 6785-90.

436. Zhu, X., M. Ohtsubo, R.M. Bohmer, J.M. Roberts, and R.K. Assoian, *Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein.* Journal of Cell Biology, 1996. **133**(2): p. 391-403.

437. Zimmermann, S. and K. Moelling, *Phosphorylation and regulation of Raf by Akt (protein kinase B).* Science, 1999. **286**(5445): p. 1741-4.

Appendix I. Abbreviations

2D	Two dimensional
AP-1	Activating protein-1
Apaf-1	Apoptotic protease activating factor-1
APC	Anaphase-promoting complex
APC/C	Anaphase-promoting complex/cyclosome
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma
bp	Base pair
°C	Degree Celcius
CAD	caspase-activated Dnase
CAK	Cdk-activating kinase
CBP	CREB-binding protein
Cdk	Cyclin-dependent-kinase
cDNA	Complementary deoxyribonucleic acid
Ci	Curie
CKI	Cdk inhibitory subunit
CKII	Casein kinase II
CRE	cyclic adenosine monophosphate-response element
CREB	cyclic adenosine monophosphate-response element-binding protein
CTD	Carboxy-terminal domain
d	Day
Da	Dalton
dATP	Deoxyadenosinetriphosphate
DBD	DNA-binding domain
DEPC	Diethyl pyrocarbonate
DIABLO	Direct IAP binding protein with low pI
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotidetriphosphate
DTT	Dithiothreitol

EDTA	Ethylenediamintetraacetic acid
Endo G	Endonuclease G
ERα	Estrogen receptor alpha
ERK	Extracellular regulated protein kinase
EtBr	Ethidium bromide
EtOH	Ethanol
GPCR	G protein–coupled receptor
G-Rh2	Ginsenoside-Rh2
GSK-3	Glycogen synthase kinase 3
GTF	General transcription factor
h	hour
HBV	Hepatitis B virus
HDAC	Histone deacetylase
ICAD	Inhibitor of caspase-activated DNase
IIa	Hypophosphorylated form of RNAP II
IIo	Hyperphosphorylated form of RNAP II
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LB medium	Luria-Bertani medium
LBD	Ligand-binding-domain
m	milli
μ	micro
M	molarity
MAPK	Mitogen-activated protein kinase
μCi	microCurie
MDM2	mouse double minute-2
mg	milligram
ml	millitre
μl	microlitre
mM	millimolar
Mnk	MAP kinase-interacting kinase

Mr	Relative migration
MRNA	messenger RNA
MSK	Mitogen- and stress-activated protein kinase
NLK	Nemo-like kinase
NLS	Nuclear localization signal
P	Proline
PAK	p21-activated kinase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C
PTK	Protein tyrosine kinase
pRB	Retinoblastoma protein
QPCR	Quantitative PCR
RNA	Ribonucleic acid
RNAP II	RNA polymerase II
RNAP II-LS	RNA polymerase II large subunit
RSK	p90 kDa ribosomal S6 kinase
rT_3	Reverse T_3
RXR	Retinoid X receptor
S	Serine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Smac	Second mitochondria-derived activator of caspases
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
Sos	Son of Sevenless
SRC-1	Steroid receptor coactivator-1
STAT	Signal transducers and activators of transcription
T	Threonine
T_3	3,5,3'-triiodothyronine
T_4	Thyroxine
TAE buffer	Tris-acetate/EDTA buffer

$TAF_{II}s$	TBP associated factors
Taq DNA	*Thermus aquaticus* DNA polymerase
TBP	TATA box binding protein
TCF	Ternary complex factors
TNFR-1	Tumour necrosis factor receptor-1
TR	Thyroid hormone receptor
TRE	Thryoid hormone response element
Tris	tris(hydroxymethyl)aminomethane
Y	Tyrosine

Vita

Surname: Skirrow Given Names: Rachel Carrie

Place of Birth: Fairview, Alberta, Canada

Educational Institutions Attended:

University of Victoria 1993 to 1999

Degrees Awarded

B.Sc. (Major) 1999

Honours and Awards:

UVic Fellowship for Graduate Studies 1999-2001
Graduate Teaching Research Fellowship (UVic) 1999-2002
Graduate Student Award for Excellence in Teaching (UVic) 2002

Publications:

Wagner MJ, Gogela-Spehar M, Skirrow RC, Johnston RN, Riabowol K, Helbing CC. 2001. Expression of novel ING variants is regulated by thyroid hormone in the Xenopus laevis tadpole. *J. Biol. Chem* **276:** 47013-47020.

Saravolac, E.G., Ludkovski, O., Skirrow, R., Ossanlou, M., Zhang, Y.P., Giesbrecht, C., Thompson, J., Thomas, S., Stark, H., Cullis, P.R., and P. Scherrer. 2000. Encapsulation of plasmid DNA in stabilized plasmid-lipid particles composed of different cationic lipid concentration for optimal transfection activity. *J. Drug Target.* **7**(6): 423-37.

Research Presentations:

Skirrow, R.C. and C.C. Helbing. 2002. Cell cycle regulators are involved in mediating developmentally-regulated apoptosis. 42nd Annual Meeting for the American Society for Cell Biology. San Francisco, CA. *Mol. Biol. Cell. Suppl.* **13**: 164a.

Skirrow, R.C., and C.C. Helbing. 2001. The eternal tail: Inhibition of tadpole tail regression during amphibian metamorphosis. 7th Annual Graduate Student Symposium, University of Victoria, Victoria, BC.

Skirrow, R.C., and C.C. Helbing. 2000. Cyclin A and cyclin E complexes: a role in amphibian metamorphosis. 6th Annual Graduate Student Symposium, University of Victoria, Victoria, BC.

Skirrow, R.C., Velhoen, N., and C.C. Helbing. 2000. The role of cyclins in amphibian tadpole tail regression. Experimental Biology 2001 Conference, Orlando, Florida.

Skirrow, R.C., and C.C. Helbing. 1999. Cyclin A and cyclin E complexes: a role in amphibian metamorphosis. 5th Annual Graduate Student Symposium, University of Victoria, Victoria, BC.

Helbing, C.C., Skirrow, R.C., Wagner, M.J., and Tessaro, M.O. 1999. The role of a novel tumor suppressor candidate from *Xenopus laevis* (XING) in promoting apoptosis: revisiting a classic model system to gain new insights into cancer. Bridging the Straits of Georgia Conference, Cowichan Bay, BC.

University of Victoria Partial Copyright License

I hereby grant the right to lend my thesis to users of the University of Victoria Library, and to make single copies only for such users or in response to a request from the Library of any other university, or similar institution, on its behalf or for one of its users. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by me or a member of the University designated by me. It is understood that copying or publication of this thesis for financial gain by the University of Victoria shall not be allowed without my written permission.

Title of Thesis:

Cyclin dependent kinase activity is necessary for thyroid hormone induced tail regression in the *Rana catesbeiana* tadpole.

Author:

Rachel Carrie Skirrow

Date: February 14, 2003