Genomic and Functional Studies of SERTAD3, an Oncogenic Protein of the SERTAD Family of Transcription Factors

by

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Preface

This thesis is written in accordance with the manuscript-based thesis preparation guidelines as outlined by the department of Graduate and Postdoctoral Studies. Chapter 1 contains an introduction to the research project as well as a review of the literature pertaining to this field. The submitted manuscript comprises Chapter 2 and conforms to the "Guidelines for Thesis Preparation". A general discussion and summary of results is found in Chapter 3, followed by a thorough bibliography and acknowledgements section.

Papers Included in this Thesis:

Chapter 2 **Darwish H**, Cho J, Loignon M, Alaoui-Jamali M. Overexpression of SERTAD3, a Putative Oncogene Located Within the 19q13 Amplicon, Induces E2F Activity and Promotes Tumor Growth. Oncogene. Accepted for publication.

Contribution of Authors:

The candidate performed much of the work presented in this thesis. Contributions of other authors to this work are described as follows: J Cho performed SERTAD3 overexpression experiments and contributed to the *in vivo* work with nude mice, as well as some of the luciferase assays. Dr M Loignon provided direction for the flow cytometry experiments, assisted with siRNA design, and provided extensive editing to the manuscript in Chapter 2.

Abstract

Gene amplification alters gene expression and can promote oncogenesis. In particular, the amplification of chromosome 19q13.1-13.2 has been found in several cancers and is known to contain the AKT2 oncogene. Two members of the SERTAD gene family of transcription factors, SERTAD1 and SERTAD3, are also located within this region. We report herein the genomic structure, regulation, and functions of SERTAD3. This gene has two transcript variants with short mRNA half-lives, and one of the variants is tightly regulated throughout G1 and S phases of the cell cycle. Overexpression of SERTAD3 induces cell transformation *in vitro* and tumor formation in mice, while inhibition of SERTAD3 by siRNA results in a 2-4 fold reduction in cell growth rate. Furthermore, luciferase assays based on E2F-1 binding indicate that SERTAD3 increases the activity of E2F, which can be strongly reduced by siRNA inhibition of SERTAD3. Together, our data support that SERTAD3 contributes to oncogenesis at least in part via an E2F-dependent mechanism.

Résumé

L'amplification d'un gene altère son expression et peut induire l'oncogenèse. En particulier, l'amplification du chromosome 19q13.1-13.2, qui contient l'oncogène AKT2, a été associé à plusieurs cancers. Par ailleurs, deux members de la famille SERTAD de facteurs de transcription, SERTAD1 et SERTAD3, sont egalement situés dans cette region du chromosome 19. Nous rapportons ici la structure du genome, son mode de régulation ainsi que les functions de SERTAD3. Cette dernière a deux variantes de transcription avec des ARNm ayant des demi-vies de courte durée et dont l'une est étroitement régulée dans les phases de G1 et S du cycle cellulaire. La surexpression de SERTAD3 entraine la transformation de cellules *in vitro* et la formation de tumeurs chez la souris, alors que l'inhibition de SERTAD3 par siRNA résulte en une réduction du taux de croissance des cellules de deux à quatre fois. De plus, des analyses de luciferase basées sur E2F-1 indiquent que SERTAD3 augmente l'activité de E2F et que celle-ci peut être fortement réduite par l'inhibition de SERTAD3 par siRNA. En somme, nos données soutiennent que SERTAD3 contribue à l'oncogenèse au moins en partie par un méchanisme dependent de E2F.

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List of Abbreviations

$p34^{SEI-1}$	SERTAD1
AKT2	Protein kinase B (β), serine/threonine kinase
B-Myb	Myb related protein B transcription factor
BLAST	Basic Local Alignment Search Tool
C-Myb	Myb related protein C transcription factor
C-Myc	Myc related protein C transcription factor
CDCA4	Cell division cycle associated 4 (aka HEPP)
CDK1	Cyclin-dependent kinase 1 (aka cdc2 kinase)
CDK2	Cyclin-dependent kinase 2
CDK3	Cyclin-dependent kinase 3
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
CIP	Cyclin-dependent kinase interacting protein ($p21^{CIP}$), a known CKI
CKI	Cyclin-dependent kinase inhibitor (INK4 and CIP/KIP families)
DM	Double minute
DNA	Deoxyribonucleic acid
DP	Transcription factor DP, E2F transcription factor subunit
E1A	Adenoviral E1A protein
E2F	Transcription factor E2F
EDTA	Ethylenediaminetetraacetic acid
EMS-1	Cortactin
ErbB-2	Human epidermal growth factor receptor 2 (aka HER2/neu)
EST	Expressed sequence tag
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
HEPP	Hematopoietic progenitor protein
HER2	Human epidermal growth factor receptor 2 (aka ErbB-2)
HPV	Human papillomavirus
HSR	Homogeneously-staining region

HTLV-1	Human lymphocytic virus 1
INK4	Inhibitors of CDK4
KIP	Kinase-inhibitory proteins ($p27^{KIP1}$, $p57^{KIP2}$), known CKIs
MAPK	Mitogen-activated protein kinases
MDM-2	Murine double minute 2
N-Myc	Myc related protein N transcription factor
NLS	Nuclear localization signal
p53	Tumor suppressor and transcription factor p53
PBS	Phosphate buffered saline
PHD	Plant homeodomain
PI	Propidium iodide
pRb	Tumor suppressor protein Rb (aka Retinoblastoma protein/pocket protein)
RBT1	Replication protein A binding transactivator 1 (aka SERTAD3)
RNA	Ribonucleic acid
RPA	Replication protein A
RPA32	32 kDa subunit of RPA
SCCHN	Small cell cancer of the head and neck
SERTA	Protein motif common to SEI-1, RBT1, and TARA
SERTAD1	SERTA domain-containing protein 1
SERTAD2	SERTA domain-containing protein 2
SERTAD3	SERTA domain-containing protein 3
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40
TARA	Taranis gene from *D. melanogaster*, similar to SERTAD genes
TRIP-Br1	Transcriptional regulator interacting with PHD-bromodomain 1 (SERTAD1)
TV1	SERTAD3 transcript variant 1
TV2	SERTAD3 transcript variant 2
UTR	Untranslated region

CHAPTER 1

Introduction and Literature Review

1.1 – General Background

Understanding the mechanisms of tumorigenesis and the role of certain genes in cancer development is essential for the discovery of cancer therapies, diagnostics, and prevention. Proteins involved in regulating cell division and proliferation are of particular interest, since a major property of cancer is the acquisition of uncontrolled cell growth. Alterations in cell cycle proteins often lead to increased cell growth, as cell cycle checkpoints are crossed more readily. Thus, the study of cell cycle proteins has great potential to yield important information in the field of cancer research.

This review focuses on cell division and the alteration of cell cycle checkpoints that take place in cancer. Cell cycle progression of normal cells is discussed with emphasis on the proteins necessary for proper G1/S checkpoint control. Common alterations within this checkpoint will be explored in relation to cancer, highlighting the various methods of stimulating the transcriptional activity of E2F complexes such as the inhibition of tumor suppressors and the overexpression of proto-oncogenes. In particular, the relationship between E2F activity and the cyclin-dependent kinase modulator SERTAD1 will be discussed. Gene amplification as a mechanism for proto-oncogene overexpression will also be addressed. The information reviewed herein will lay the foundation for studying SERTAD3, a protein thought to be involved in cell cycle progression and cancer.

1.2 – Cell Division

1.2.1 – Background

The cell cycle is a complex process that is regulated at many levels. Cells need to cross intrinsic checkpoint mechanisms in order to progress through G1, S, G2, and M phases of the cell cycle. Signals are integrated from both inside and outside of the cell to drive cell cycle progression, especially from G1 to S phase when DNA replication occurs. Once a cell enters S phase, it is committed to cell division and will ultimately undergo mitosis and produce two daughter cells. Thus, the progression from G1 to S phase is tightly regulated. The point in late G1 phase where the cell commits to DNA replication is called the restriction point (Weinberg 1995, Herwig and Strauss 1997), since cells can stay arrested in G1 phase if they have not progressed through this point (Figure 1.1).

Several factors that promote progression through the restriction point include growth factors, mitogens, nutrient availability, cell-cell interactions, and DNA integrity (Lukas *et al.* 1994, Sherr 1996, Herwig and Strauss 1997). These factors influence the expression of genes encoding proteins that regulate S phase entry through the activation or repression of transcription factors. If a cell does not receive adequate signals for cell growth, it will be arrested in G1 phase, but once the restriction point has been crossed it will proceed through the cell cycle independently of mitogenic signals (Herwig and Strauss 1997).

Controlled cell growth and DNA integrity rely heavily on properly functioning checkpoint mechanisms. Cell cycle checkpoints ensure that the cell does not progress to the next phase of growth until the previous phase is successfully completed. For example, cells will only enter S phase if there are enough external growth signals in G1 to promote the formation of pre-replication complexes on the DNA (Herwig and Strauss 1997).

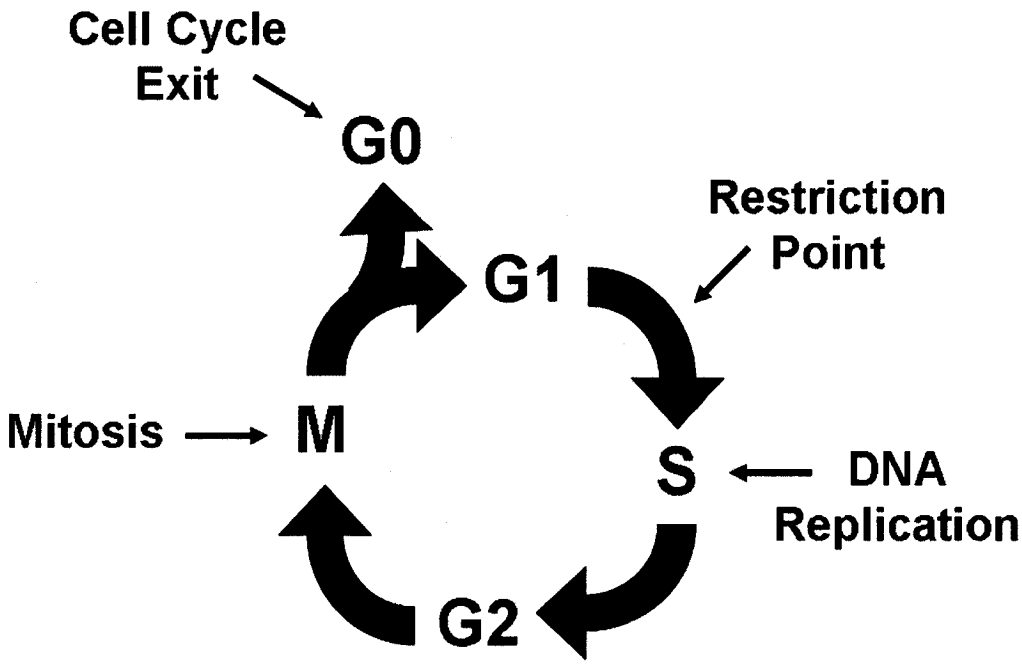


Figure 1.1 – The Cell Cycle. After a cell divides to produce two daughter cells in mitosis (M phase) it either exits the cell cycle (G0) or enters G1 phase. These cells then prepare for S phase entry by transcribing genes necessary for DNA replication. Once a cell crosses the restriction point it is committed to DNA replication and cell division. In contrast, if a cell has not crossed the restriction point it can be arrested in G1 phase.

It is important to stress that regulation of the cell cycle occurs at the level of transcription factors. The activation of transcription factors in early-to-mid G1 phase increases the expression of proteins necessary for DNA replication as well as proteins responsible for activating the next set of transcription factors. The cascade of activating transcription factors will continue until the cell crosses the restriction point and enters S phase.

1.2.2 – Cyclins and Cyclin-Dependent Kinases

The cyclin family of proteins is crucial for cell cycle progression. These proteins integrate growth signals, target transcription factor repressors for phosphorylation, and regulate transcription factors through direct protein-protein binding (Yam *et al.* 2002, Hwang and Clurman 2005, Aleem *et al.* 2005, Baker *et al.* 2005). Cyclins were named for their transient expression throughout the cell cycle, where protein levels increase at specific points in order to drive cells through particular stages of the cell cycle. The cyclins that are responsible for progression through G1 and into S phase are cyclins D, E, and A.

The D-type cyclins (D1, D2, and D3) are stimulated by growth factors, mitogens, and nutrients, and integrate these signals to begin the process of crossing the restriction point (Lukas 1994, Sherr 1996). Cyclin D is the only cyclin that accumulates due to growth signals and is quickly degraded when lacking these factors, thereby preventing entry into S phase (Matsushime *et al.* 1991, Herwig and Strauss 1997). If growth factors, mitogens, and nutrients are present, the expression of cyclin D will increase in mid-G1 phase and trigger the transcription of proteins necessary for S phase entry, including cyclin E.

Increased expression of D-type cyclins can lead to a shorter G1 phase, a reduction in growth factor dependence, and increased cell proliferation (Quelle *et al.* 1993, Resnitzky *et al.* 1994). Conversely, reduced cyclin D expression leads to longer G1 phase, and complete knockout of D-type cyclins prevent entry into S phase (Baldin *et al.* 1993, Quelle *et al.* 1993).

E-type cyclins (E1 and E2) are stimulated by the activation of D-type cyclins. Thus, levels of cyclin E will increase after cyclin D proteins reach their peak in expression. Cyclin E activity is crucial for S phase entry, as it interacts with and subsequently inactivates several transcriptional repressors. The inactivation of these repressors allow for the expression of cyclin A, the final cyclin required for entering S phase and initiating DNA synthesis (Hwang and Clurman 2005).

Cyclin A was the first cyclin to be discovered in mammalian cells (Swenson *et al.* 1986). It has functions in both S phase and mitosis, and it is very important for cell cycle progression. There are two variants of A-type cyclins in humans, cyclin A1 and cyclin A2. Cyclin A1 is only expressed in embryonic cells and in cells that undergo meiosis, while cyclin A2 is the variant that is important for somatic cell division (Yang *et al.* 1997, Murphy *et al.* 1997, Yam *et al.* 2002). Cyclin A2 is necessary for entry into S phase; cells synchronized in G1 phase may have high levels of cyclin D or cyclin E, but will always have low cyclin A2 expression (Lavia and Jansen-Durr 1999). Ectopic expression of cyclin A2 promotes S phase entry, while knockdown of cyclin A2 has been shown to block the progression through S phase (Rosenberg *et al.* 1995, Zindy *et al.* 1992, Yam *et al.* 2002).

All cyclins require the partnership of kinases to phosphorylate protein targets and

promote cell cycle progression. These kinases are constitutively expressed, but can only function when complexed to their respective cyclins. Thus, they are known as cyclin-dependent kinases, or CDKs. There are eight known CDKs (CDK1 through CDK8), each one binding to specific cyclins (Deshpande *et al.* 2005, Santamaria and Ortega 2006). In G1 phase, the D-type cyclins form a complex with either CDK4 or CDK6, while cyclin E and cyclin A can each form a complex with CDK2. There is new evidence that CDK1 may also bind to cyclin E during G1/S phase in CDK2-knockout mice, suggesting a redundancy among several CDKs (Aleem *et al.* 2005, Martin *et al.* 2005).

The cyclin molecules recruit CDKs to target proteins such as the pRb family (see section 1.2.5) that are subsequently phosphorylated and inactivated. Physical interaction between the cyclin and CDK creates a complex that is both active and specific. Through this targeted phosphorylation, CDK/cyclin complexes can regulate the E2F family of transcription factors responsible for S phase entry and cell cycle progression (see section 1.2.4).

1.2.3 – CDK Inhibitors

The activity of CDKs is regulated not only by cyclin binding but also by cyclin-dependent kinase inhibitors (CKIs). These inhibitors bind to and inactivate CDKs, preventing the phosphorylation of target proteins such as the pRb family of proteins. The inhibitory action of CKIs counteracts the stimulatory activity of cyclins, ultimately slowing progression through the cell cycle (Lee and Yang 2001).

One group of CKIs is the INK4 family, consisting of several members: p15 ^{INK4B}, p16

^{INK4A}, p18 ^{INK4C}, and p19 ^{INK4D}. The INK4 proteins specifically bind to CDK4 and CDK6 through their ankyrin-like repeats, acting as allosteric inhibitors of D-type cyclins. The suppression of cyclin D/CDK kinase activity prevents the initial events leading to the crossing of the restriction point (Serrano *et al.* 1993, Hirai *et al.* 1995). p16 ^{INK4A} has been studied extensively and is thought to be the primary cell cycle inhibitory protein of the INK4 family. Abnormalities in p16 expression or function have been linked to deregulated cell growth and tumor formation (Kamb 1995, Cairns *et al.* 1995, Lee and Yang 2001).

The second group of CKIs is the CIP/KIP family of inhibitors. These proteins can bind to and inhibit all types of CDK/cyclin complexes (Xiong *et al.* 1993, Harper *et al.* 1993), although there is recent evidence that the CIP/KIP proteins can actually activate CDK4 by promoting the assembly and stability of CDK4/cyclin D complexes (Cheng *et al.* 1999, Sherr and Roberts 1999). There are three members of the CIP/KIP family: p21 $^{WAF1/CIP1}$, p27 ^{KIP1}, and p57 ^{KIP2}. Although the CIP/KIP family can bind to all CDKs, their effect is primarily seen in G1/S phase when both p21 and p27 inhibit CDK2/cyclin E and CDK2/cyclin A through complexation (Polyak *et al.* 1994, Li *et al.* 1994, Slingerland *et al.* 1994).

The amount of p21 is low in quiescent cells but increases as G1 phase progresses. The expression of p21 can be induced by the p53 tumor suppressor (el-Deiry *et al.* 1993, Li *et al.* 1994) as well as through p53-independent mechanisms. The p53 oncosuppressor may sense DNA damage and translate this into increased p21 expression, arresting cells in G1 phase. Other proteins known to induce p21 expression are STATs, protein kinase C, and MAP kinases (Chin *et al.* 1996, Zeng and el-Deiry 1996, Liu *et al.* 1996). In contrast

to p21 expression, p27 levels are high in quiescent cells but decline throughout G1 in response to extracellular mitogenic signals (Polyak *et al.* 1994, Herwig and Strauss 1997).

1.2.4 – E2F Family of Transcription Factors

E2F is the primary transcription factor involved in G1/S phase progression. The E2F transcription factor actually exists as a heterodimer consisting of one E2F unit and one DP unit. There are five main E2F proteins (E2F-1 through E2F-5) and three DP proteins (DP-1 to DP-3) leading to many possible combinations with varying function (Herwig and Strauss 1997, Johnson and Schneider-Broussard 1998, Lavia and Jansen-Durr 1999). In addition, there are three additional E2F family members (E2F-6 through E2F-8) that lack protein domains responsible for transcriptional activity and repressor protein binding but contain functional DNA binding domains (Morkel *et al.* 1997, de Bruin *et al.* 2003, Logan *et al.* 2005, Zhu *et al.* 2005). Thus, E2F-1 through E2F-5 are transcriptional activators while E2F-6 through E2F-8 are repressors of transcription because they can compete with transcriptionally active E2F proteins for DNA binding sites.

The E2F/DP transcription factor complex (hereafter referred to as E2F) is responsible for the expression of many genes that are important for S phase entry, including DNA synthesis genes such as DNA polymerase α, dihydrofolate reductase (DHFR), thymidine kinase, and histone H2A (Herwig and Strauss 1997, Zhu *et al.* 2005). E2F stimulates the expression of genes necessary for cell cycle progression such as cyclin D, cyclin E, cyclin A, and cdc2. In addition, proto-oncogenes are regulated by E2F such as C-myb, B-myb, C-myc, and N-myc (Johnson and Schneider-Broussard 1998, Herwig and Strauss 1997).

During early G1 phase, E2F activity is repressed via direct protein-protein binding to the pRb family (see section 1.2.5). As G1 phase progresses, the kinase activity of CDK4/cyclin D and CDK6/cyclin D allow E2F to initiate the transcription of cyclin E, which further promotes E2F activity and drives the cell into S phase (Ohtani *et al.* 1996).

Ectopic expression of E2F has been shown to transform mouse fibroblasts (Singh 1994, Yang and Sladek 1995), initiate DNA synthesis in quiescent cells, and prevent cell cycle exit (Johnson *et al.* 1993, Herwig and Strauss 1997). Whereas E2F-1 through E2F-3 overexpression is sufficient to drive cells through the cell cycle, E2F-4 and E2F-5 require co-expression of DP proteins to stimulate cell growth (De Le Luna *et al.* 1996, Lavia and Jansen-Durr 1999). This may be due to the localization of the transcription factors. E2F-1 through E2F-3 all contain a nuclear localization signal (NLS), whereas E2F-4 and E2F-5 do not. The latter E2F proteins may be targeted to the nucleus through the NLS found in proteins such as DP-2 (Magae *et al.* 1996, De La Luna *et al.* 1996, Johnson and Schneider-Broussard 1998). Furthermore, E2F-4 and E2F-5 may enter the nucleus through the binding of repressor proteins of the pRb family (De La Luna *et al.* 1996). The E2F/DP heterodimers will act as transcriptional activators, while the E2F/repressor complexes will act as transcriptional repressors inside the nucleus since they can bind DNA but can no longer promote transcription. Thus, E2F-4 and E2F-5 are considered weak transcriptional activators in contrast to the strong activators E2F-1 through E2F-3.

1.2.5 – The pRb Family of Pocket Proteins

The Rb-1 gene was discovered when studying retinoblastoma. It was found that Rb-1 mRNA was present in retinal cells but was absent in retinoblastoma cells (Friend *et al.*

1986, Friend *et al.* 1987). Further studies confirmed that the RB-1 gene product pRb was a tumor suppressor by demonstrating that the addition of pRb to cells lacking the protein would slow cell growth and suppress tumorigenesis (Goodrich *et al.* 1991, Takahashi *et al.* 1991). Additionally, viral oncoproteins known to increase cell proliferation and tumorigenesis were found to interact with and inactivate pRb. The region of viral oncogene binding was common among the pRb family, and was subsequently named the "pocket" region of these proteins. Thus, the pRb family members are known as pocket proteins (Herwig and Strauss 1997, Zhu *et al.* 2005).

There are three members of in the pRb pocket protein family: pRb, p107, and p130. These three proteins are involved in the G1/S checkpoint, repressing the transcription of proteins necessary for progression through the cell cycle. Each of the pRb family members can bind to E2F when hypophosphorylated, inhibiting the transcription factor complex. The inhibitory action of pRb is due to either the masking of the transactivation domain of E2F or the conversion of E2F into a transcriptional repressor (Johnson and Schneider-Broussard 1998, Lavia and Jansen-Durr 1999). E2F will become active once it is released from its pRb binding partner.

The different E2F proteins will be bound by specific pocket proteins. E2F-1 through E2F-3 are all bound by pRb. E2F-4 interacts with all three proteins (pRb, p107, and p130), while E2F-5 binds to p130 only (Lavia and Jansen-Durr 1999). The differences in binding partners allows for the sequential activation of transcription factors and the expression of proteins necessary for progressing through the cell cycle checkpoints.

E2F activation occurs via phorphorylation of the pRb family by CDK/cyclin complexes. The cyclin-kinase complexes target specific pocket proteins for inactivation.

pRb is primarily phosphorylated by either CDK4/cyclin D or CDK6/cyclin D, but can also be inactivated by CDK2/cyclin E (Herwig and Strauss 1997). The phosphorylation of p107 and p130 occurs later in the G1/S transition and relies on both CDK2/cyclin E and CDK2/cyclin A activity. Once S phase is reached, CDK2/cyclin A and CDK1/cyclin B are responsible for maintaining pRb in a hyperphosphorylated state. pRb remains in this inactive state until the end of mitosis (M phase) when it becomes dephosphorylated and then reverts to the active hypophosphorylated form (Schafer 1998, Weinberg 1995).

1.2.6 – Progression of Cells Through G1/S

In early G1 phase, pocket proteins bind to E2F and prevent the transcription of proteins necessary for DNA replication. As cells receive growth signals, cyclin D expression increases and the resulting protein forms complexes with CDK4/6. However, CKIs from both the INK4 and CIP/KIP families bind to the CDK/cyclin complexes and inhibit their kinase activity. Most of the CKIs can inhibit CDK4 and CDK6, but the $p16^{INK4A}$ protein is the primary inhibitor found complexed to these CDKs.

When the cell receives enough growth signals, the amount of CDK4/cyclin D and CDK6/cyclin D overwhelm the inhibitory proteins. The excess kinase complexes are active and can phosphorylate the pocket protein in the inactive pRb/E2F complex (Herwig and Strauss 1997). Phosphorylated pRb releases E2F, allowing it to direct the transcription of genes such as cyclin E and E2F-1 (thereby creating a positive feedback loop of activation). Since pRb is largely responsible for maintaining control of the G1 checkpoint, the inactivation of pRb by CDK4/cyclin D is the major force driving cells through the restriction point (Schafer 1998). The CDK4/cyclin D complex can also

activate E2F independent of enzymatic activity by sequestering CKIs such as p21 and p27, thereby reducing their inhibitory effect on CDK2/cyclin E and CDK2/cyclin A complexes (Santamaria and Ortega 2006).

Next, cyclin E and CDK2 target p107 and p130 for phosphorylation and permit E2F to activate the transcription of proteins necessary for S phase entry including thymidine kinase, B-myb, and cyclin A (Möröy and Geisen 2004, Chang *et al.* 1995). The cyclin A/CDK2 complexes also target p107 and p130 for phosphorylation and are responsible for the final push into S phase (Johnson and Schneider-Broussard 1998, Lavia and Jansen-Durr 1999). E2F transcription factors that are activated by CDK2/cyclin A will direct the transcription of the remaining proteins necessary for DNA replication (Figure 1.2).

Finally, the DNA binding ability of free E2F is inhibited in S phase by cyclin A through direct protein-protein interactions and the phosphorylation of DP (Mudryj *et al.* 1991, Krek *et al.* 1994, Xu *et al.* 1994). The inactivation of E2F in S phase is important for maintaining DNA integrity and preparing the cell for the next phases of the cell cycle. The presence of viral oncoproteins such as adenoviral E1A can prevent the cyclin A/CDK2 complex from properly regulating E2F, leading to an increase in cell proliferation and genomic instability (Faha *et al.* 1992).

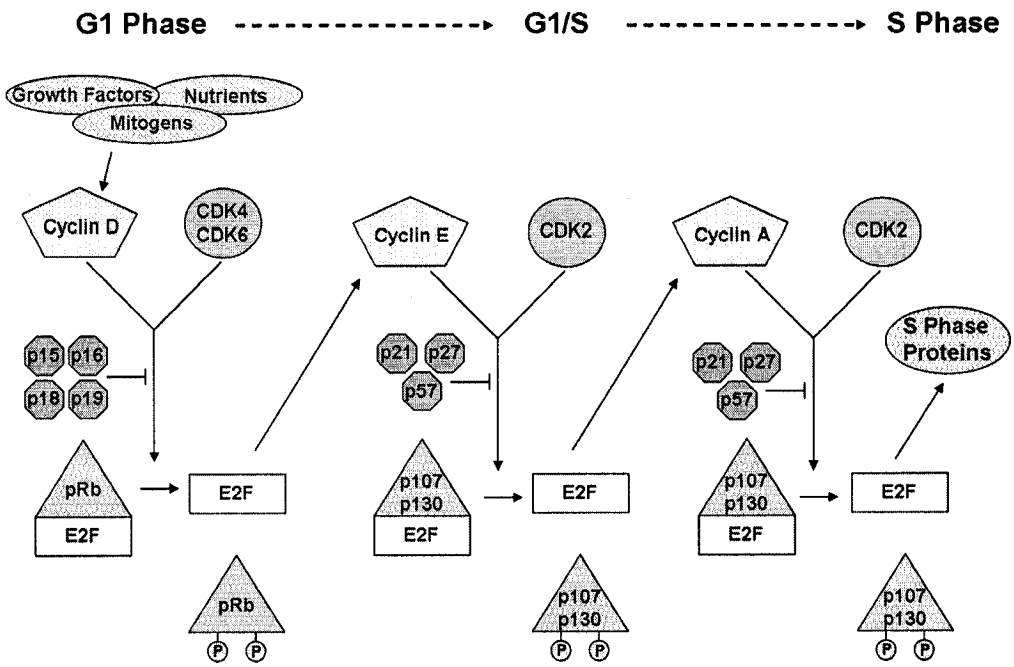


Figure 1.2 – G1 and G1/S Checkpoints. Normal progression of cells through G1 phase and the G1/S transition of the cell cycle. Initially, pocket proteins bind to E2F transcription factor complexes, preventing transcriptional activity. In early-to-mid G1 phase, cyclin D and either CDK4 or CDK6 combine to phosphorylate pRb, allowing E2F to direct the transcription of several genes including cyclin E. INK4 proteins act as inhibitors of CDK kinase activity. Next, cyclin E forms a complex with CDK2 to activate E2F and drive the transcription of cyclin A. Finally, cyclin A combines with CDK2 to activate E2F complexes responsible for promoting the expression of the rest of S phase genes necessary for DNA replication.

1.3 – Cell Cycle Deregulation in Cancer

1.3.1 – Background

Most cells contain functional cell cycle checkpoint mechanisms and are dependent on nutrients and other growth factors. However, when a cell loses proper control of a checkpoint, aberrant cell growth and proliferation may occur. The deregulation of cell growth often leads to genomic instability and tumorigenesis. Cells that become cancerous almost always lose the ability to arrest the cell cycle in G1 phase but often maintain their G2/M checkpoint. Thus, the loss of G1 checkpoint mechanisms is an extremely important event in tumorigenesis.

There are several ways in which cells can lose control of the G1 checkpoint: pRb loss or inactivation, cyclin overexpression, loss of cyclin-dependent kinase inhibitors, or the overexpression of CDK modulators (Hunter and Pines 1994, Chibazakura 2004). Each of these mechanisms leads to increased activation of E2F. The mechanisms of checkpoint loss are not necessarily mutually exclusive, but because they may act in a similar manner the alteration of more than one pathway could be redundant. For example, there have been no reports of a tumor with losses of both pRb and p16, since without functional pRb the CDK4 and CDK6 kinases do not need to be inhibited (Herwig and Strauss 1997).

1.3.2 – pRb Inactivation

The loss of pRb was first observed in retinoblastoma patients who were missing one or both copies of the Rb-1 gene from chromosome 13q14. Soon after this discovery, it was found that pRb was the target of viral oncoproteins such as SV40 Large T antigen,

HPV E7 protein, and the adenoviral E1A protein (Herwig and Strauss 1997, Zhu *et al.* 2005). Since then, deletions or loss-of-function mutations have been found in many tumors, including cancers of the prostate, brain, breast, lung, and esophagus (Phillips *et al.* 1994, He *et al.* 1995, Nielsen *et al.* 1997, Betticher *et al.* 1997, Xing *et al.* 1999).

Inactivation of pRb in cancer is directly related to E2F function, as all know pRb mutants from human cancer have lost the ability to regulate E2F (Johnson and Schneider-Broussard 1998). Like viral oncoproteins, proto-oncogenes found within the cell may inhibit pRb when overexpressed. The oncoprotein MDM-2 is known to bind pRb and prevent binding between the pocket protein and E2F (Xiao *et al.* 1995), leading to increases in cell proliferation. MDM-2 is overexpressed or amplified in several cancers such as sarcomas (Oliner *et al.* 1992), leukemia (Bueso-Ramos *et al.* 1996). The oncoprotein may also contribute to cell proliferation through pRb-independent mechanisms; MDM-2 can inhibit transcriptional activity of the p53 tumor suppressor, likely causing a reduction in p21 levels (Oliner *et al.* 1992, Haines *et al.* 1994).

Mutations or losses of the other pocket proteins, p107 and p130, are very rare in cancer. One possible explanation for this is that the two pocket proteins share a high degree of homology and are therefore partially redundant (Zhu *et al.* 2005). The cell would have to lose both genes to affect cell cycle progression. Losing only one of the two genes would not be advantageous for the cell, so it would not be under selective pressure to retain this mutation. The loss of only one gene, such as pRb, is seen more often in cancer because its loss would promote cell proliferation and lead to clonal expansion.

1.3.3 – Increased Expression of Cyclins

In normal cells, cyclin D expression is dependent on growth factors, mitogens, and nutrient availability. Conversely, cells that overexpress cyclin D do not rely heavily on growth factors and can more readily inactivate the pRb tumor suppressor. Thus, overexpression/amplification of D-type cyclins is observed in many cancers (Baker *et al.* 2005). Cyclin D1 overexpression is associated with 50% breast, 30% esophageal, and 35-64% of head and neck squamous cell carcinomas (Bartek *et al.* 1996, Sherr 1996, Weinberg 1995, Deshpande *et al.* 2005).

Since D-type cyclins promote the G1/S transition by inactivating pRb, tumors will rarely contain both the loss of pRb and the gain of cyclin D. Thus, most tumors with increased levels of cyclin D will contain wild-type pRb, and cancers that have lost pRb function will not begin to overexpress cyclin D at a later stage (Baker *et al.* 2005).

Overexpression of cyclin E1 has also been reported in cancer, particularly in breast carcinomas (Gray-Bablin *et al.* 1996, Yasmeen *et al.* 2003, Möröy and Geisen 2004). However, the overexpression of cyclin E1 is not the only means by which this cyclin can promote cancer progression. Studies have shown that up to five different isoforms of cyclin E may be expressed in breast tumors (Porter and Keyomarsi 2000, Santamaria and Ortega 2006). The shorter variants are generated by proteolysis of their N-terminus, losing the region where p21 and p27 binding occurs. Thus, these cyclin E isoforms are resistant to CIP/KIP inhibition and are considered hyperactive (Akli *et al.* 2004). The presence of shorter cyclin E1 isoforms has been correlated with genomic instability and poor prognosis in breast cancer (Keyomarsi *et al.* 2002).

1.3.4 – Loss of Cyclin-Dependent Kinase Inhibitors

The INK4 family of CKIs antagonizes the function of D-type cyclins and stops cell cycle progression in response to checkpoint activation. Loss of $p16^{INK4A}$ or other INK4 members can lead to uncontrolled G1/S progression and cell proliferation (Lee and Yang 2001). Numerous cancers show losses or mutations of $p16^{INK4A}$ protein (Cordon-Cardo 1995) including melanoma, acute lymphocytic leukemia, osteosarcoma, renal cell carcinoma, and cancers of the esophagus, lung, pancreas, bladder, head and neck, breast, brain, and ovaries (Kamb 1995, Cairns *et al.* 1995, Lee and Yang 2001). Furthermore, the inactivation of p16 transcription due to the methylation of 5' CpG islands has been seen in cancers of the head and neck, breast, prostate, brain, lung, colon, esophagus, and bladder (Herman *et al.* 1995).

Tumorigenesis has been associated with the loss of CIP/KIP family members. Although genetic alterations of p21 and p27 are rare in cancer, reduction of p27 protein levels due to increased degradation has been correlated with poor prognosis in cancer patients (Catzavelos *et al.* 1997, Catzavelos *et al.* 1999). Poor cancer patient survival rates have been associated with low p27 levels in breast, colorectal, and gastric carcinomas (Porter *et al.* 1997, Loda *et al.* 1997, Mori *et al.* 1997).

Small interfering RNA can be used to help determine the function of a protein within the cell, as this siRNA will target specific mRNA for degradation and thus knock down protein levels. Using siRNA, it was shown that p27 knockdown promotes cell cycle progression in mitogen-deprived cells. The expression of p27 normally increases when mitogens are absent, so the knockdown showed that p27 plays an important role in slowing cell growth under conditions of mitogen deprivation (Coats *et al.* 1996). This

example illustrates how knocking down protein expression using siRNA can be a useful tool when determining the function of a protein within the cell cycle.

1.3.5 – CDK Modulators and SERTAD1

The kinase activity of cyclin-dependent kinases is positively regulated by cyclins and negatively regulated by CKIs. In addition, there are other proteins that can modulate the activity of CDKs. Examples include gankyrin (Li and Tsai 2002) and the Tax protein from human lymphocytic virus 1 (HTLV-1) (Suzuki *et al.* 1996). Gankyrin is a protein that can compete with $p16^{INK4A}$ and form a ternary structure with CDK4/cyclin D2, thereby increasing kinase activity (Li *et al.* 2004). The tax protein can either bind to and sequester $p16^{INK4A}$ or form a ternary structure with CDK4/cyclin D2. Both actions of Tax increase CDK4 kinase activity (Li *et al.* 2004).

A recently discovered protein, SERTAD1 ($p34^{SEI-1}$/TRIP-Br1) (Sugimoto *et al.* 1999, Hsu 2001), has also been shown to interact with CDK4 and increase kinase activity (Hsu *et al.* 2001, Li *et al.* 2004, Li *et al.* 2005). Unlike gankyrin and Tax, SERTAD1 forms a quaternary structure with CDK4/cyclin D2 and $p16^{INK4A}$, suggesting different CDK4 binding sites for SERTAD1 and $p16^{INK4A}$. Thus SERTAD1 antagonizes the inhibitory action of $p16^{INK4A}$ but does not compete with the INK4 protein for binding to CDK4.

Preliminary studies have shown that SERTAD1 stimulates E2F transcriptional activity, possibly through direct interaction with DP-1. Co-expression of E2F-1 and DP-1 in the osteosarcoma cell line SaOS-2 increased E2F reporter activity 10-fold, while the expression of E2F-1, DP-1, and SERTAD1 together increased luciferase activity 25-fold (Hsu *et al.* 2001). Further studies have demonstrated that the E2F-stimulatory activity of

SERTAD1 is due partially to its PHD-bromo interacting domain (Sim *et al.* 2004).

The expression of SERTAD1 is increased in various cancers, including ovarian carcinomas (Tang *et al.* 2002, Tang *et al.* 2005) and small cell cancer of the head and neck (SCCHN) (Li *et al.* 2005). In SCCHN samples where overexpression of SERTAD1 was observed, $p16^{INK4A}$ levels were either absent or found at low levels (Li *et al.* 2005). Consistent with the relationship between SERTAD1 and cancer, SKOV-3 cells treated with SERTAD1 siRNA showed decreases in cell growth and colony formation in soft agar (Tang *et al.* 2005).

1.4 – Gene Amplification

1.4.1 – Gene Amplification in Cancer

Cancer often arises from the overexpression of proto-oncogenes or other genes involved in promoting cell cycle progression. One mechanism by which genes are overexpressed is through gene amplification. Abnormalities in cell division can lead to unbalanced translocations of genetic information and the duplication of small chromosomal regions known as amplicons. The extra copies of genes lead to increased expression and, in the case of proto-oncogenes, offer a growth advantage to cells that contain these amplified regions. This enhancement of growth may eventually lead to tumorigenesis. Thus, gene amplification is a potential contributing factor for tumorigenesis and cancer progression (Lengauer *et al.* 1998).

Gene amplification is seen as either homogenously-staining regions (HSRs) or double minutes (DMs). HSRs are found within the chromosome and are composed of several copies of an amplicon. Since they occur within a chromosome, HSRs are

considered stable amplifications. In contrast, double minutes are extrachromosomal pieces of DNA. DMs also contain several copies of amplicons, but because they lack the chromosomal machinery for proper replication and segregation they are considered unstable amplifications. These two types of amplifications are microscopically visible during metaphase (Nielsen *et al.* 1993).

There are many examples of specific gene amplification in cancer. The most common amplification occurs with the ErbB-2 (HER2/neu) tyrosine kinase receptor at chromosome 17q12 (Slamon *et al.* 1989, Borresen *et al.* 1990, Bieche *et al.* 1996, Ross and Fletcher 1999, Luoh 2002, Gunnarsson *et al.* 2003). Extra copies of this proto-oncogene promote the development of aggressive tumors with poor clinical prognosis. ErbB-2 amplification is seen primarily in breast cancer, although there are reports of ErbB-2 amplification within ovarian, bladder, and gastric cancers (Slamon *et al.* 1989, Simon *et al.* 2003, Vidgren *et al.* 1999). Other known amplifications in cancer are chromosome 2p24 containing the N-myc proto-oncogene (Shiloh *et al.* 1986, Schneider *et al.* 1992, Hiemstra *et al.* 1994), chromosome 11q13 containing cyclin D (Zaharieva *et al.* 2003, Ormandy *et al.* 2003), and chromosome 12q13-14 containing both CDK4 and the p53 suppressor MDM2 (Oliner *et al.* 1992, Reifenberger *et al.* 1993).

1.4.2 – The 19q13 Amplicon

A commonly recurring amplicon in cancer is located on the long arm of chromosome 19. Tang *et al.* (2002) observed that 39% of examined ovarian cancers showed gains of 19q13, and that four ovarian cancer cell lines contained many copies of 19q13.1-13.2 in the form of HSRs. The amplified region at 19q13 occurs in a wide variety of solid tumors

including pancreatic carcinomas and adenomas (Miwa *et al.* 1996, Curtis *et al.* 1998, Hoglund *et al.* 1998), ovarian carcinomas (Thompson *et al.* 1996, Bicher *et al.* 1997), breast cancer (Muleris *et al.* 1995), small cell and non-small cell lung cancers (Petersen *et al.* 1997), glioblastomas (Beghini *et al.* 2003), and hepatocellular carcinomas (Marchio *et al.* 1997).

Amplicons that are often found in cancer will likely contain oncogenes that promote cell growth and proliferation. The amplicon at chromosome 19q13.1-13.2 includes the gene coding for AKT2 (PKB-β), a serine/threonine kinase with tumorigenic properties (Testa and Bellacosa 2001). AKT2 has been shown to antagonize p21 and p27, stabilize cyclin D1, and promote invasion and metastasis (Testa and Bellacosa 2001, Bellacosa *et al.* 2004, Arboleda *et al.* 2003). AKT2 overexpression is correlated with a number of cancers, particularly pancreatic and ovarian carcinomas (Cheng *et al.* 1996, Altomare *et al.* 2003, Bellacosa *et al.* 1995).

The 19q13.1-13.2 amplicon also contains the CDK4 modulating protein SERTAD1 and a close family member SERTAD3. The two genes are located in tandem on chromosome 19 in a region several centimorgans from the AKT2 gene. As previously mentioned, SERTAD1 overexpression has been linked to SCCHN and can promote cell cycle progression through both CDK4 binding and E2F activation. SERTAD3 shares a high degree of homology with SERTAD1, suggesting that both of these genes may play a role in tumorigenesis when amplified.

1.5 – SERTAD3

1.5.1 – Previous Findings

SERTAD3 was discovered in our laboratory using the yeast two-hybrid system with the middle subunit of replication protein A (RPA32) as bait (Cho *et al.* 2000). The interactions were confirmed using immunoprecipitation assays on GST-tagged proteins, where GST-SERTAD3 could pull-down RPA32 and vice versa. In the yeast one-hybrid system, where ... , we showed that SERTAD3 has significant transactivation function. Furthermore, deletion mutants of the protein showed that its transactivity is due to a 22-amino acid domain in the C-terminus. Thus, SERTAD3 was initially named RBT1 for Replication Protein A Binding Transactivator 1. Ectopic expression of GFP-tagged protein demonstrated that SERTAD3 is localized in the nucleus. This observation supported the previous findings that SERTAD3 can bind to DNA and modulate transcription.

Analysis of SERTAD3 *h*ESTs in GenBank suggested that the gene was expressed in a wide variety of human tissues, though the level of expression varied among tissue types. Since SERTAD3 *h*ESTs were commonly seen in cancer cell lines, we wanted to explore the relationship between SERTAD3 and cancer. The level of gene expression was examined in a panel of normal and cancer cell lines. It was found that SERTAD3 expression was higher in cancer cells than normal cells, showing a 5- to 10-fold higher expression in cancer cell lines such as MCF-7 and SaOS-2.

1.5.2 – Rationale for Project

Genes that are involved in tumorigenesis and cell proliferation can offer much insight into the understanding of the mechanisms of cancer development and progression. Furthermore, there is increasing emphasis on the discovery of novel targets for both anticancer therapeutics and prognostic markers for detecting and monitoring cancer in patients.

There are several reasons to hypothesize that SERTAD3 behaves like an oncogene. First, the genomic position of SERTAD3 within the 19q13.1-13.2 amplicon suggests a link between protein levels of SERTAD3 and cancer. This is supported by the differential expression of SERTAD3 among normal and cancer cell lines. Second, the effect of SERTAD proteins on CDK4 and E2F activity, coupled with the homology between family members, suggests that SERTAD3 is also involved in cell cycle progression. Taken together, it is highly likely that SERTAD3 may act as an oncogene within the cell.

There is great incentive to study the functions of SERTAD3, as its role in cell cycle progression and tumorigenesis may help us to understand the various mechanisms of cancer. Furthermore, if SERTAD3 plays an important role in carcinogenesis then there is a potential to use it as a therapeutic target for cancer. Thus, the primary focus of this research is to characterize the SERTAD3 gene and to elucidate its function through both the overexpression and knockdown of SERTAD3 protein levels.

CHAPTER 2

Overexpression of SERTAD3, a Putative Oncogene Located Within the 19q13 Amplicon, Induces E2F Activity and Promotes Tumor Growth

Abstract

Gene amplification alters gene expression and can promote oncogenesis. The amplified region of chromosome 19q13.1-13.2 has been associated with several cancers. The well characterized oncogene AKT2 is located in this amplicon. Two members of the same gene family (SERTAD1 and SERTAD3) are also located within this region. We report herein the genomic structure, regulation, and functions of SERTAD3. SERTAD3 has two transcript variants with short mRNA half-lives, and one of the variants is tightly regulated throughout G1 and S phases of the cell cycle. Overexpression of SERTAD3 induces cell transformation *in vitro* and tumor formation in mice, while inhibition of SERTAD3 by siRNA results in a 2-4 fold reduction in cell growth rate. Furthermore, luciferase assays based on E2F-1 binding indicate that SERTAD3 increases the activity of E2F, which can be strongly reduced by siRNA inhibition of SERTAD3. Together, our data support that SERTAD3 contributes to oncogenesis at least in part via an E2F-dependent mechanism.

Introduction

Genetic and chromosomal abnormalities are common features in cancer. Mutations responsible for the inactivation of tumor-suppressor genes or the activation of survival genes are the principal mode of tumor initiation (Sieber *et al.* 2003; Lengauer *et al.* 1998). Gene amplification is the gain of small pieces of chromosomes (0.5-10 Mb). These amplified chromosomal regions, known as amplicons, exist as multiple copies that can be revealed as either homogenously staining regions of chromosomes or as double minutes (Barker 1982; Alitalo *et al.* 1983; Lengauer *et al.* 1998). Most amplicons contain genes that promote cell growth, and hence can lead to a growth advantage to cells that contain these amplified regions (Lengauer *et al.* 1998).

Specific gene amplifications have been implicated in a wide variety of cancers. Examples of known amplicons in cancer are chromosome 17q12 containing ErbB-2 (Ross and Fletcher 1999; Luoh 2002), chr 11q13 containing CCND1 and EMS1 (Zaharieva *et al.* 2003; Ormandy *et al.* 2003), chr 12.13-14 containing the p53 suppressor MDM2 (Oliner *et al.* 1992; Reifenberger *et al.* 1993), and the frequent amplification of N-Myc on chr 2p24 in neuroblastomas (Shiloh *et al.* 1986; Schneider *et al.* 1992; Heimstra *et al.* 1994).

One region of recurrent gene amplification is located on the long arm of chromosome 19. Amplification of chr 19q13 has been linked to different types of cancers including pancreatic carcinomas and adenomas (Miwa *et al.* 1996; Curtis *et al.* 1998; Hoglund *et al.* 1998), ovarian carcinoma (Thompson *et al.* 1996; Bicher *et al.* 1997; Wang *et al.* 1999), breast cancer (Muleris *et al.* 1995), small cell and non-small cell lung cancers (Petersen *et al.* 1997), glioblastoma (Beghini *et al.* 2003), and hepatocellular carcinoma (Marchio *et*

al. 1997), strongly supporting that genes included in this amplicon can participate in tumor formation independent of tissue type.

SERTAD1, a putative oncogene, is found close to AKT2 within the 19q13.1-13.2 amplicon (Tang *et al.* 2002). SERTAD1 is a transcription factor known to inhibit $p16^{INK4a}$ activity, interact with E6 of HPV 16, and interact with PHD-containing proteins such as TIF1β (Sugimoto *et al.* 1999; Hsu *et al.* 2001; Gupta *et al.* 2003). In addition to SERTAD1, we have identified a novel gene that is located on chromosome 19q13.1 in tandem with SERTAD1 (Figure 2.1a). This gene, which we named SERTAD3 (RBT1, or **R**eplication protein A **B**inding **T**ransactivator), is a member of the SERTAD family of transcription factors found to interact with the second subunit of replication protein A (RPA2) (Cho *et al.* 2000). There is significant homology between all of the family members, especially between SERTAD3 and SERTAD1 (31% identity and 46% similarity, Figure 2.1b). The location of SERTAD3, along with its overexpression in several cancers and the high similarity to SERTAD1, suggest an oncogenic role for this protein.

Herein, we demonstrate that members of this family share binding domains and have similar genomic structures. SERTAD3 is shown to have two transcript variants with distinct regulation patterns and conservation. When examining the functions of SERTAD3 overexpression in non-transformed cells, we find that this gene has oncogenic properties both *in vitro* and *in vivo*; cells overexpressing SERTAD3 exhibit a loss of contact inhibition, have the ability to form colonies in soft agar, and can form tumors in mice. Conversely, SERTAD3 knockdown cells exhibit a marked decrease in cell growth that is proportional to the reduction of SERTAD3 expression. Furthermore, using reporter assays we show SERTAD3 stimulates E2F-1 activity. Altogether these studies strongly

support that SERTAD3 promotes oncogenesis through the induction of E2F-1 transcriptional activity.

Results

Homology of SERTA Domain-Containing Proteins

The SERTA domain, a ~47-residue motif named for the first proteins that were discovered with this motif (**SE**I-1, **R**BT1, and **TA**RA) (Calgaro *et al.* 2002), is thought to be important for protein-protein interactions. The SERTAD3 gene is localized at chromosome 19q13.1-13.2 in tandem with SERTAD1, approximately 15 kb apart and in proximity to the AKT2 oncogene. SERTAD3 and SERTAD1 are members of a larger family that includes SERTAD2/TRIP-Br2 and CDCA4/HEPP (Calgaro *et al.* 2002; Abdullah *et al.* 2001).

The SERTAD family shares a similar, unusual genomic structure. All members have intronless coding regions yet contain one intron in their 5' UTRs (Figure 2.1a). Furthermore, the splice acceptor site is always found within 7 base pairs of the ATG start codon. TARA, the Drosophila homologue of mammalian SERTAD genes, also contains one intron in its 5' UTR (Calgaro *et al.* 2002). Members of the SERTAD family show significant homology (Figure 2.1b), especially within protein domains such as the SERTA domain. It has been shown that most of the SERTA domain in SERTAD1 is included in its CDK4 binding site (Sugimoto *et al.* 1999) and heptad repeat region. Other common protein motifs can be identified in SERTAD homologues including the cyclin A binding site, PHD-Bromo interacting domain and C-terminal activation domain. The locations of these protein motifs are summarized in Figure 2.1c.

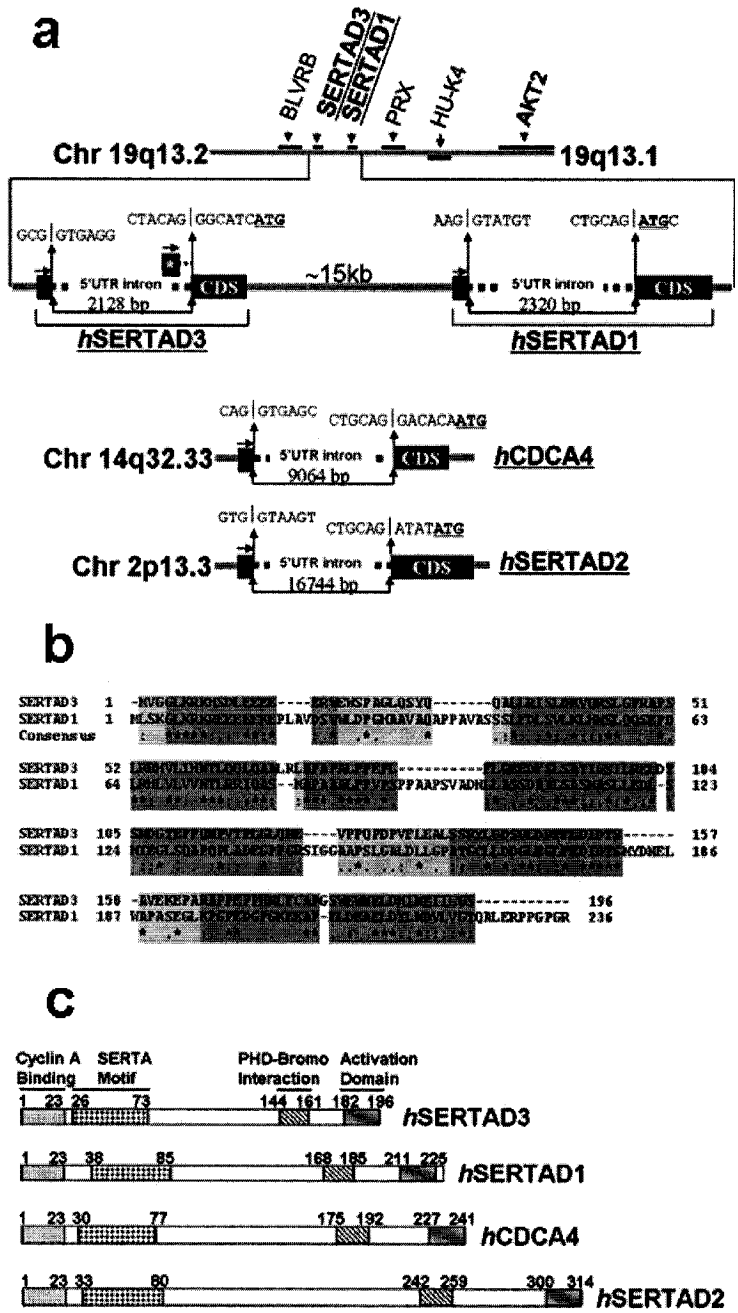


Figure 2.1 - Genomic Organization of SERTAD Genes. (**a**) The SERTAD3 and SERTAD1 genes are located in tandem on chromosome 19 within the 19q13.1-13.2 amplicon in close proximity to AKT2. All genes containing a SERTA domain have an intron in their 5' UTR close to the translational start site (ATG). Splicing donor and acceptor sites are indicated. (**b**) Members of the SERTAD family have a high degree of sequence homology. SERTAD3 and SERTAD1 share the highest homology with 31% identity and 46% similarity. (**c**) All members of the SERTAD family contain the same protein- and DNA-binding motifs including the cyclin A binding motif, SERTA domain, PHD-Bromo interacting domain, and C-terminal transcriptional activation domain.

Characterization of SERTAD3

To determine whether genes homologous to SERTAD3 existed in other species, a similarity search was carried out using the SERTAD3 amino acid sequence. The full-length protein (196 aa) was BLASTed against other species. The sequences of several mammalian SERTAD3 proteins were aligned using ClustalW (Figure 2.2a), revealing a high degree of conservation ranging from 83% identity (rat) to 98% identity (rhesus monkey).

Unlike other genes in the SERTAD family, SERTAD3 has two distinct transcript variants differing only in their 5' UTR. Both variants contain identical coding regions and 3' UTRs that are found in a single exon (exon 3). Transcript variant 1 (TV1, NM_013368) includes a 276 bp 5' UTR from exon 2. There is a 240 bp intron separating exons 2 and 3 that is excised during the transcription of TV1. Transcript variant 2 (TV2, NM_203344) consists of 162 bp 5' UTR from exon 1 spliced with exon 3. The genomic organization of SERTAD3 is illustrated in Figure 2.2b.

We then compared the conservation of the two SERTAD3 transcript variants among these mammalian species. Nucleotide sequences from exons 1 and 2 (TV2 and TV1 5' UTRs, respectively) were BLASTed and the resulting matches aligned using ClustalW. There was a significant similarity between the 5' UTR of TV2 and the SERTAD3 5' UTR of other mammalian species, especially the region directly upstream of the splice site (76-93 % identity, data not shown). No significant similarity to other species was found with the TV1 5' UTR. This suggests that SERTAD3 TV2 has been evolutionarily conserved among mammals, and that the 5' UTR of TV1 has been introduced exclusively into the human genome, possibly through the incorporation of a viral genome.

Cell Cycle Analysis of SERTAD3 Transcript Variants

Genes that play an important role in cell survival and proliferation are often tightly regulated (Alarcon-Vargas and Ronai, 2004). SERTAD1 is differentially expressed throughout the G1 and S phases (Sugimoto *et al.* 1999), so we wanted to explore SERTAD3 gene expression throughout the cell cycle.

Primary human fibroblasts (CRL2097) were synchronized through serum starvation. Total RNA was collected at various time points and transcriptional levels were tested using RT-PCR. Synchronization was confirmed by cell cycle analysis (Figure 2.3a) and by examining levels of cyclin A and CDK4 using RT-PCR (Figure 2.3b). As expected, cyclin A expression increases dramatically as cells enter S phase, while CDK4 expression remains constant throughout the cell cycle.

We then examined the RNA levels of SERTAD3. A significant difference was found when looking at the levels of SERTAD3 transcript variants. TV1 expression is almost undetectable when the fibroblasts are released but then is quickly induced, peaking 2 h post-release (Figure 2.3c). There is another potential increase in TV1 expression as cells enter S phase. However, TV2 expression remains unchanged throughout G1 and S phases (data not shown).

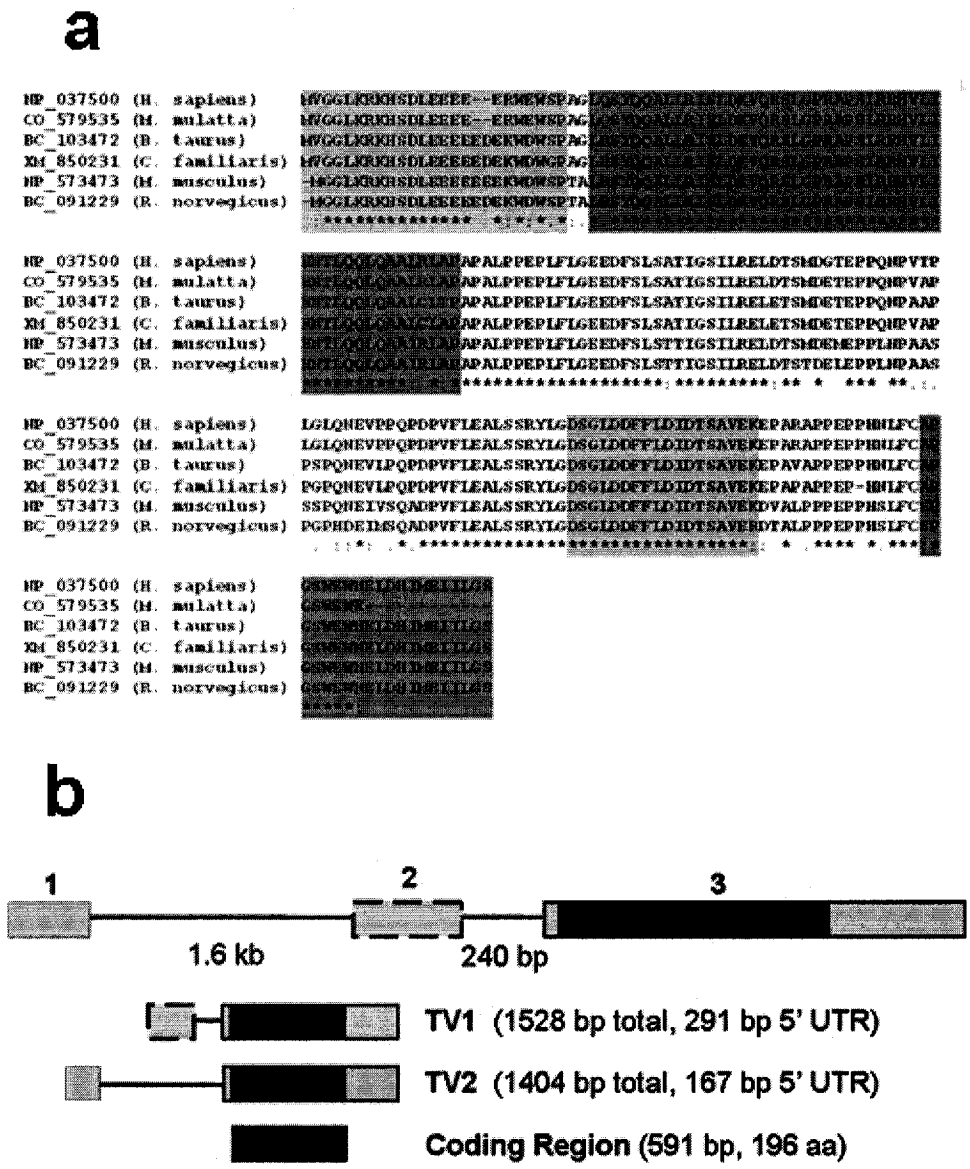


Figure 2.2 - Genomic Structure of SERTAD3. (**a**) Alignment of mammalian SERTAD3 protein. The amino acid sequence is highly conserved (83-98%), especially within the protein- and DNA-binding domains such as the SERTA domain (residues 26-73 in human SERTAD3). (**b**) The coding region (591 bp) is shown in black while untranslated regions (UTRs) are shown in gray. Exon 3 is comprised of the terminal 7 bp of the 5' UTR, the coding region, and the 3' UTR. Transcript variant 1 (TV1, NM_013368) contains exons 2+3, whereas transcript variant 2 (TV2, NM_203344) contains exons 1+3.

The expression pattern of SERTAD3 TV1 throughout the cell cycle is highly similar to its family member SERTAD1 (Sugimoto *et al.* 1999), exhibiting a peak in both RNA and protein levels 2 hours after release from G0/G1. Since these two genes are located in tandem on chromosome 19q13.1-q13.2, there is reason to believe that SERTAD3 TV1 and SERTAD1 share common regulatory mechanisms. SERTAD3 TV2 is constitutively expressed throughout the cell cycle and thus appears to be regulated differently.

Based on the tight regulation of SERTAD3 throughout the cell cycle, we predicted that the mRNA of SERTAD3 would degrade quickly. We examined the stability of both transcript variants in CRL2097 cells as well as MCF-7 cells because these breast cancer cells express a high level of SERTAD3. Cells were treated with actinomycin-D and the RNA was harvested after 2, 4, 8, and 24 hours post-treatment. Semi-quantitative RT-PCR was used to compare mRNA levels of TV1, TV2, and GAPDH. We find that both transcript variants of SERTAD3 have similarly short mRNA half-lives in MCF-7 cells (Figure 2.3d); there was a 50% decrease of initial transcripts after 2 hours and a decrease of over 90% after 8 hours. Similar results were obtained when using CRL2097 cells (data not shown), confirming that the rapid turnover of SERTAD3 is not cell type-specific. The tight regulation of SERTAD3 at the levels of both transcription and mRNA stability suggests that this protein is important within the cell and may have important functions in survival or tumorigenesis.

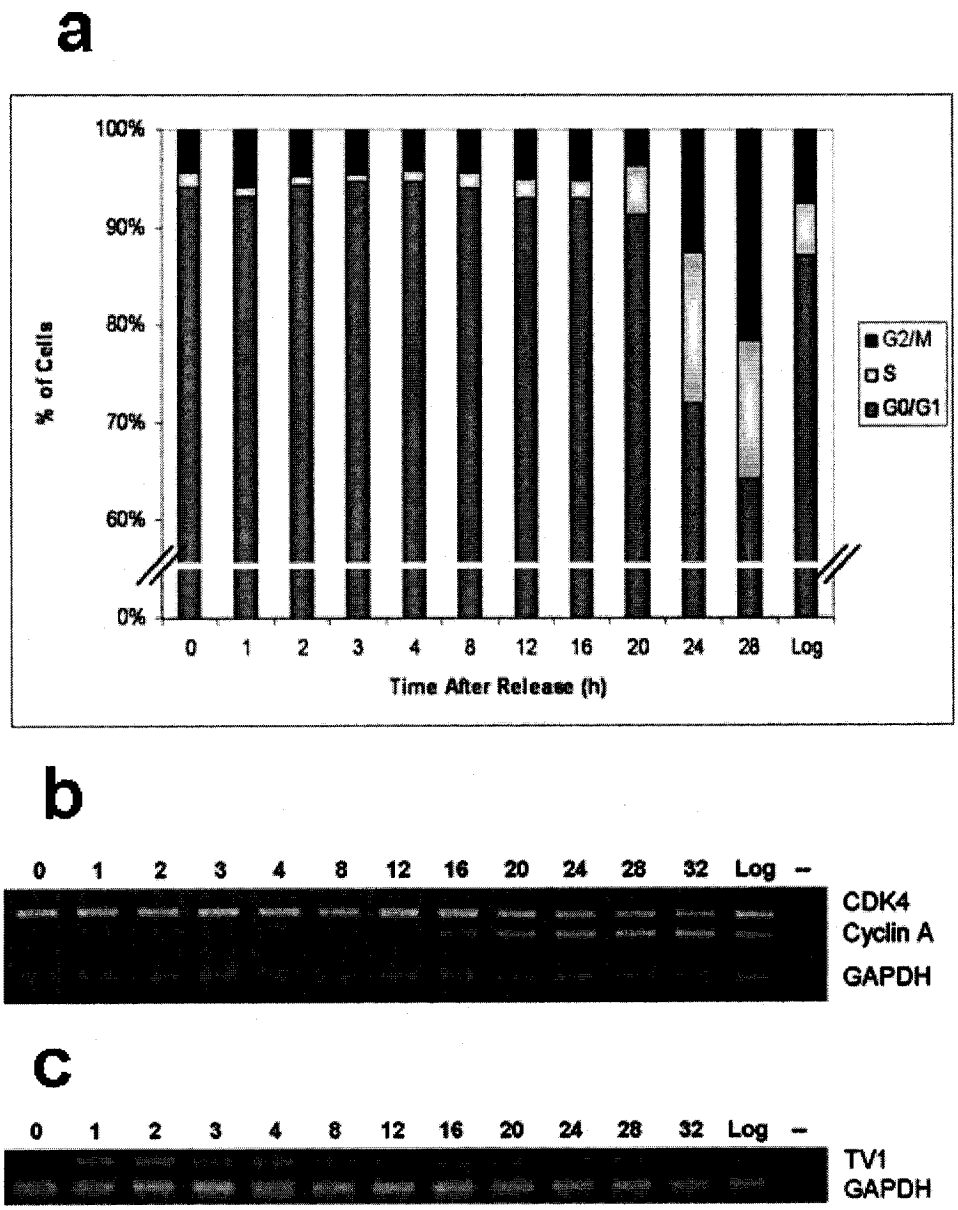


Figure 2.3 – Expression of SERTAD3 Throughout G1/S Phases. Primary human fibroblasts (CRL2097) were synchronized by serum starvation, released with 10% FBS, and collected from 0-32 hours. Exponentially growing fibroblasts were also harvested (Log). (**a**) Synchronization was validated by cell cycle analysis. The number of cells within each phase is plotted as a percentage of the total number. (**b**) The expression of cyclin A and CDK4 were examined using semi-quantitative RT-PCR to validate synchronization. GAPDH was used as an internal control. A negative RT-PCR control (--) is included. (**c**) SERTAD3 TV1 expression is regulated throughout G1 and S phases, peaking at 2 h after release from serum starvation.

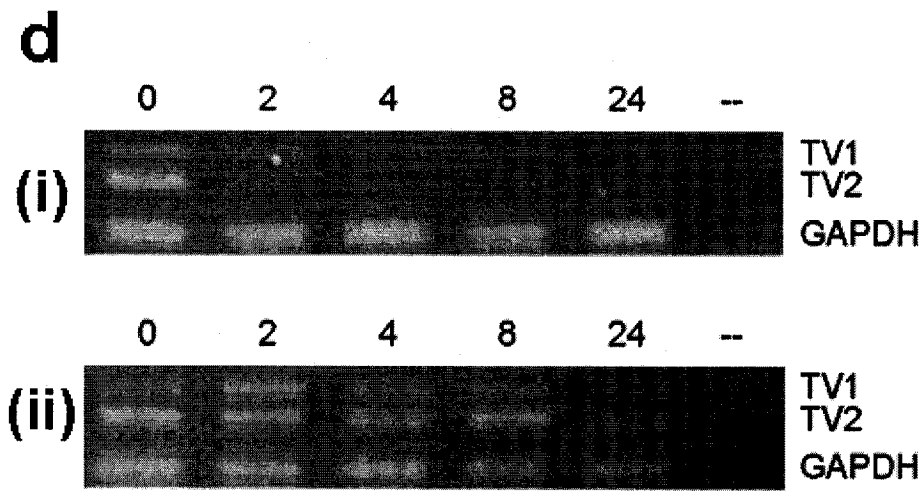


Figure 2.3 – Expression of SERTAD3 Throughout G1/S Phases. (**d**) Stability of SERTAD3 mRNA. MCF-7 cells were treated with either 10 μg/mL actinomycin-D (i) to inhibit mRNA production or ethanol control (ii). RNA was harvested and the abundance of SERTAD3 transcripts was measured using RT-PCR. GAPDH was used as an internal control. Levels of both transcripts decrease significantly after 2 h of actinomycin-D treatment, whereas GAPDH levels are not significantly reduced after 24 h.

Overexpression of SERTAD3 in NIH3T3 Cells Causes Cellular Transformation

Normal (non-transformed) cells express low amounts of SERTAD3 compared to cancer cells (Cho *et al.* 2000). To address the impact of SERTAD3 on oncogenic cell transformation, we overexpressed SERTAD3 in NIH3T3 cells. We established a polyclonal population of SERTAD3-expressing cells (NIH3T3^{SERTAD3}), which showed two- to three-fold higher SERTAD3 protein expression (Figure 2.4a) than the control cells, suggesting that higher levels of SERTAD3 may not be tolerated by these cells. Consistent with this hypothesis, significantly fewer colonies survived puromycin selection than the control-infected cells.

Loss of contact inhibition is a key characteristic of cell transformation. NIH3T3 cells were grown to confluence and serum starved. Upon serum stimulation, the NIH3T3^{vector} cells remain in G0/G1 likely as a result of contact inhibition while NIH3T3^{SERTAD3} resume cell cycle progression (Figure 2.4b) showing that NIH3T3^{SERTAD3} fibroblasts are not contact inhibited. Furthermore, we performed a soft agar colony formation assay and found that greater than 50% of plated cells grew into colonies whereas only 5-10% of parental cells formed colonies (Figure 2.4c).

To assess the potential of NIH3T3^{SERTAD3} to form tumors, control and SERTAD3-overexpressing NIH3T3 were injected subcutaneously into the flanks of nude mice. Interestingly, NIH3T3^{SERTAD3} cells form fast-growing tumors while control mice injected with parental NIH3T3 did not (Figure 2.4d, 2.4e).

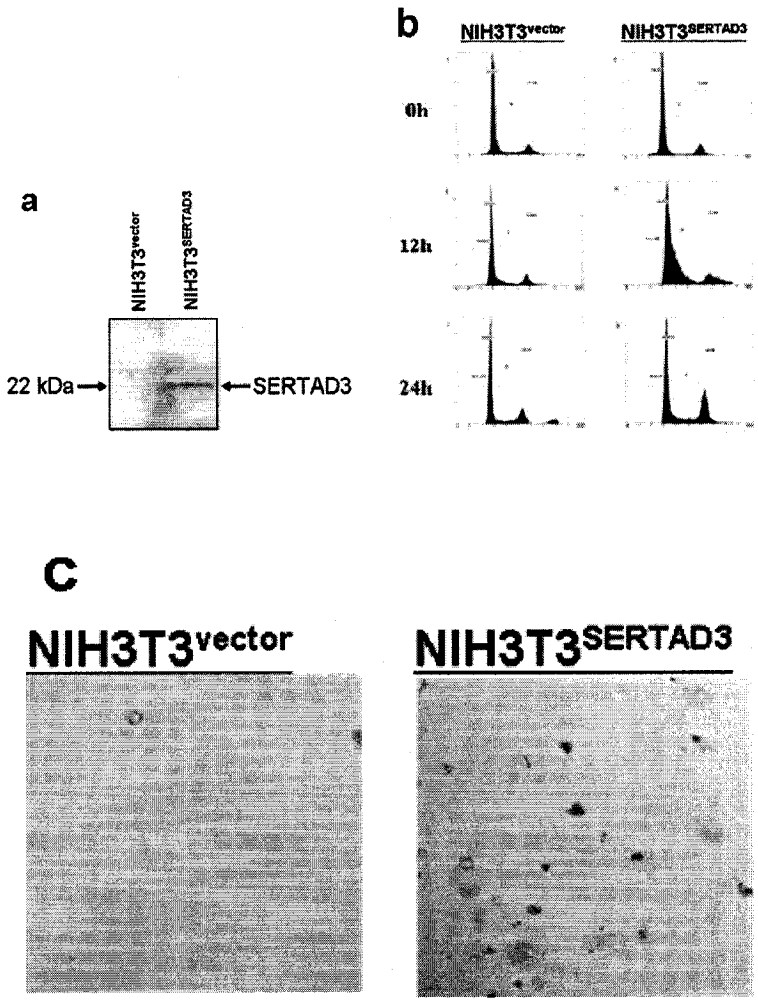


Figure 2.4 – Cellular Transformation in NIH3T3. (**a**) Western blot of protein extracts from cells stably transfected with empty pBabe vector (NIH3T3^{vector}) or pBabe-SERTAD3 (NIH3T3^{SERTAD3}). Protein extracts (100 μg) were loaded in each lane and probed with rabbit anti-SERTAD3 antibody. (**b**) Contact inhibition of confluent NIH3T3 cells. Parental cells and cells overexpressing SERTAD3 were serum starved at 100% confluence for 48 hours prior to release. The cell cycle distribution was determined by flow cytometry. NIH3T3^{SERTAD3} cells were able to continue through the cell cycle. (**c**) Growth in soft agar 9 days subsequent to plating. There was an average 6-fold increase in colony formation when using NIH3T3^{SERTAD3} cells.

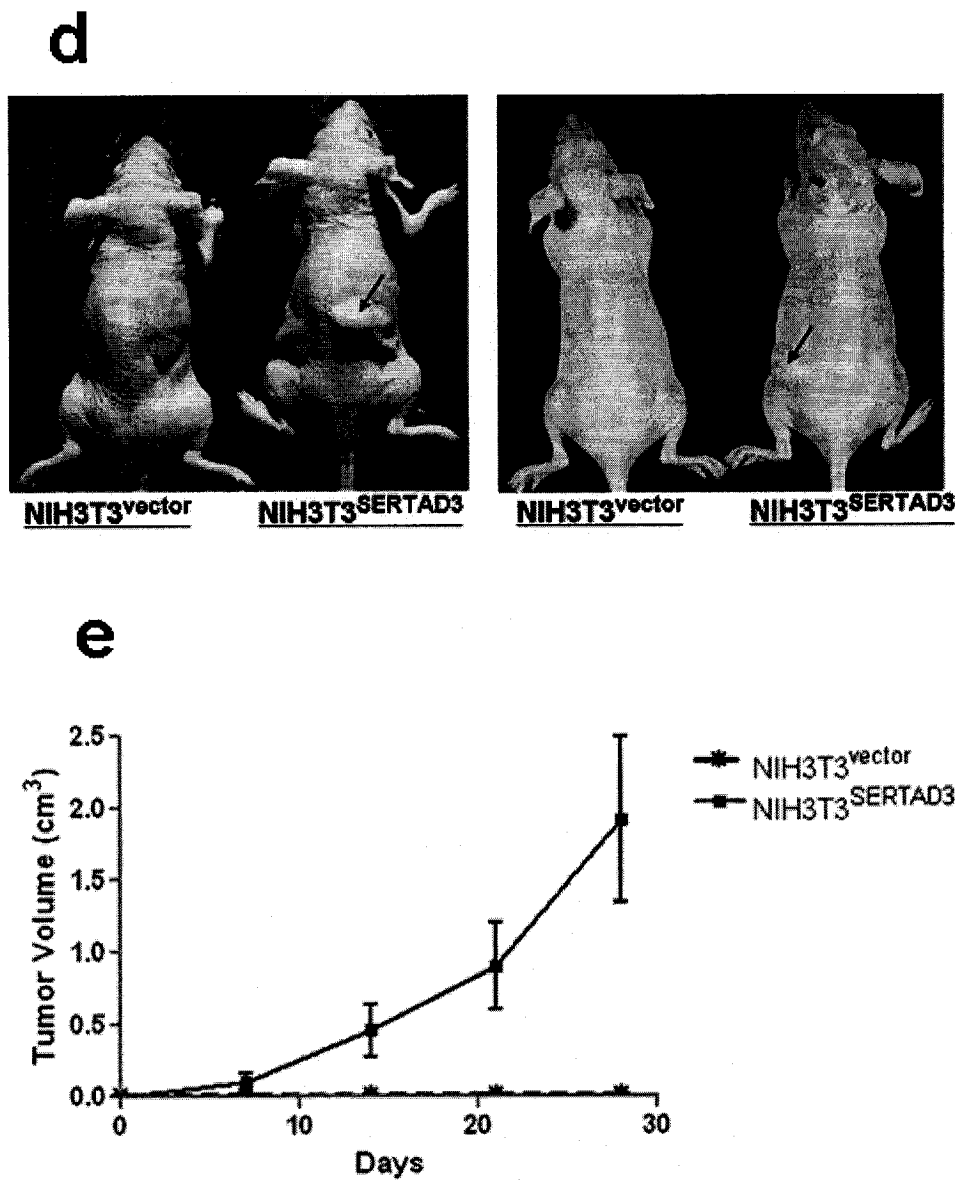


Figure 2.4 – Cellular Transformation in NIH3T3. **(d)** Tumor growth in Balb/c Nu/Nu mice. Eight mice were injected with either NIH3T3^{vector} or NIH3T3^{SERTAD3} exponentially growing fibroblasts of which four representative mice are shown. All mice (4/4) injected with NIH3T3^{SERTAD3} cells formed tumor nodules whereas no tumors were formed with NIH3T3^{vector} cells (0/4). **(e)** Graphical representation of tumor growth over time.

SERTAD3 siRNA Inhibits Cell Proliferation

To further establish the connection between SERTAD3 expression and cell proliferation, endogenous SERTAD3 was knocked down using SERTAD3 siRNA. Both SERTAD3 transcript variants were efficiently knocked down in MCF-7 cells (Figure 2.5a), whereas the expression of other SERTAD family members remained unchanged (Figure 2.5b).

Following siRNA transfection, the effect of SERTAD3 knockdown on cell growth was studied in MCF-7 cells by counting cells every 24 h. The number of cells in both treatments was compared to both non-transfected cells and those transfected with non-targeting siRNA (control). Efficient SERTAD3 knockdown was confirmed by RT-PCR (Figure 2.5a). The growth of MCF-7 cells is directly proportional to the level of SERTAD3 (Figure 2.5c). After 3 days in culture, there is a 2-fold difference in growth between control-treated cells and those transfected with the lower SERTAD3 siRNA concentration, and a 4-fold difference when comparing control-treated cells with those transfected with the highest SERTAD3 siRNA concentration.

SERTAD3 Expression is Correlated with E2F-1 Transcriptional Activity

Members of the SERTAD family share significant homology in their respective cyclin A binding, heptad repeat, PHD-bromo interaction, and C-terminal activation domains (Figure 2.1c). The cyclin A-binding and hydrophobic heptad repeat domains (zipper) of SERTAD proteins are similar to those found in members of the E2F family of transcriptional activators. We reasoned that SERTAD3 regulates the cell cycle, at least in part, via modulation of E2F activity.

To explore this hypothesis, the impact of SERTAD3 on E2F-regulated genes was examined using a luciferase reporter plasmid containing four consecutive E2F-1 binding sites with dyad symmetry. This vector (pLuc-$(E2F)_4$) was co-expressed in NIH3T3 cells with pCMV-E2F1 and either pCMV-SERTAD3 or pCMV-SERTAD1. As shown in Figure 2.6a, expression of exogenous E2F-1 in NIH3T3 increases reporter gene activity. Consistent with a role for SERTAD3 and its homolog SERTAD1 to increase E2F-1 activity, E2F reporter activity is higher in cells overexpressing SERTAD3 or SERTAD1 compared to control cells.

The connection between E2F-1 activity and SERTAD3 expression was further tested using MCF-7 cells and SERTAD3 siRNA. As expected, cells containing the pLuc-$(E2F)_4$ reporter vector show a high level of luciferase activity when expressing exogenous E2F-1. However, when these cells are co-transfected with SERTAD3 siRNA (S2) the luciferase activity decreases 4-fold (Figure 2.6b). In addition, Western blot analysis of synchronized MCF-7 cells treated with either SERTAD3 siRNA or control siRNA demonstrate that SERTAD3 knockdown decreases E2F-1 accumulation in G1 phase (Figure 2.6c). These experiments offer strong evidence that SERTAD3 potentiates E2F-1 transcriptional activity. Thus, SERTAD3 overexpression may induce cell transformation and proliferation through the transcriptional activation of E2F.

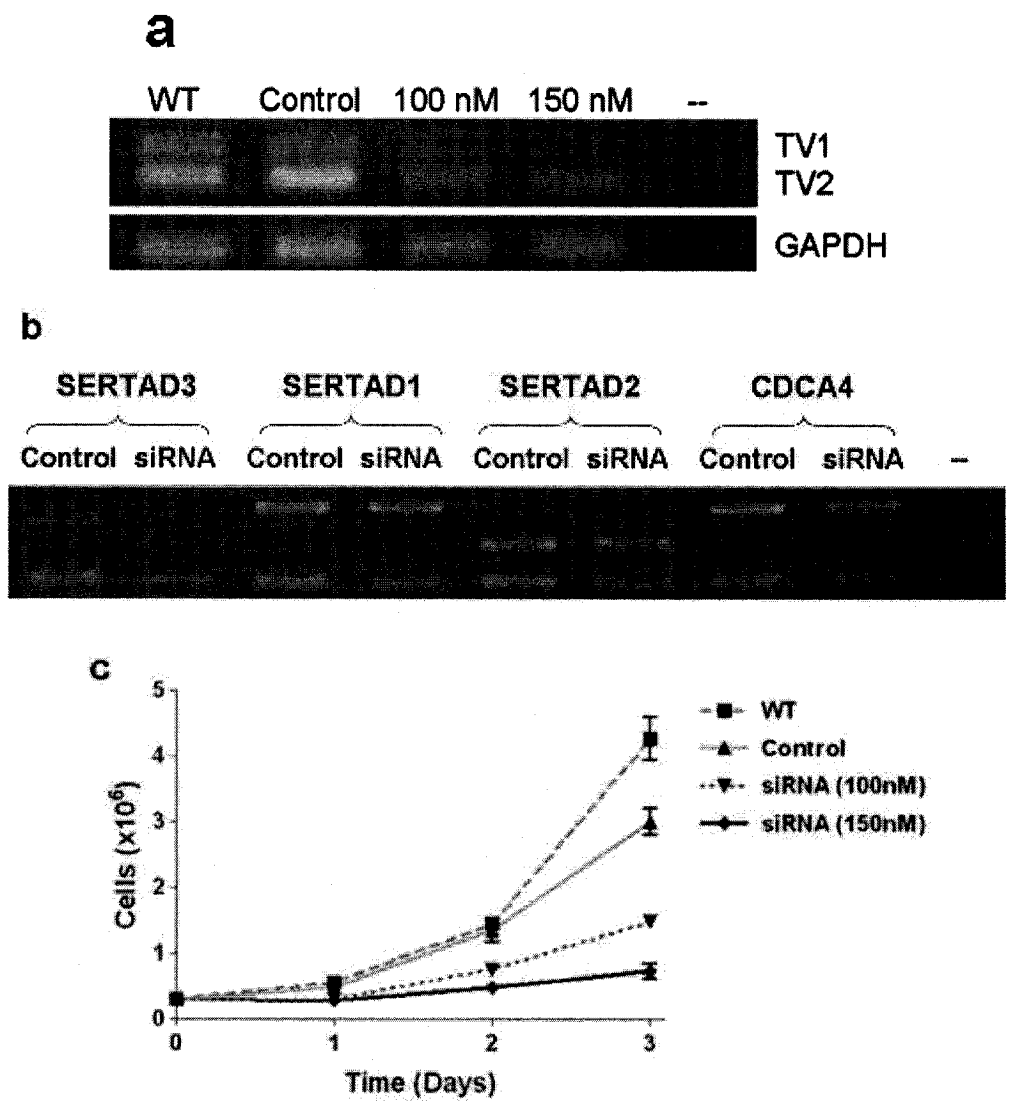
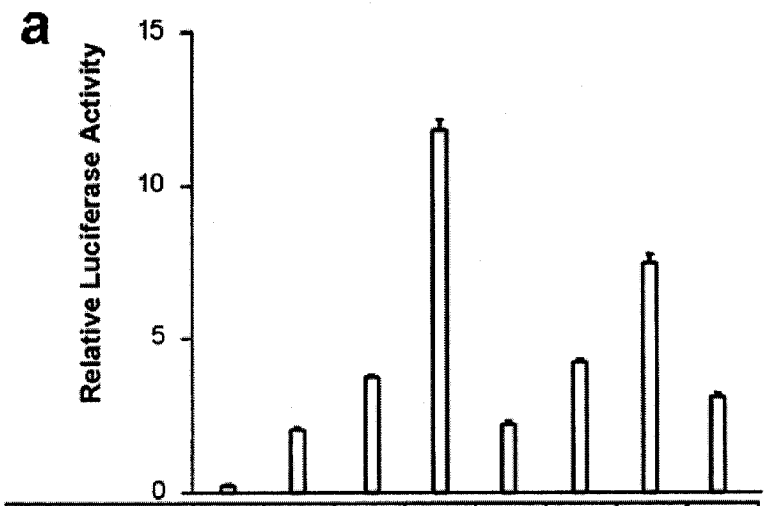


Figure 2.5 – SERTAD3 siRNA Inhibits MCF-7 Growth. (**a**) SERTAD3 siRNA knocks down SERTAD3 transcripts. MCF-7 cells were treated with either non-targeting siRNA (control) or SERTAD3 siRNA in 100 and 150 nM concentrations. SERTAD3 mRNA was harvested after 48 h and the abundance of SERTAD3 transcripts were measured using RT-PCR. GAPDH was used as an internal control. Both transcripts are knocked down significantly when using the two siRNA concentrations. (**b**) Expression of the SERTAD family was determined in SERTAD3 siRNA- and control siRNA-treated cells. No knockdown was observed in other SERTAD members. GAPDH was used as an internal control. (**c**) Effect of SERTAD3 siRNA on MCF-7 cell growth. MCF-7 cells were treated with either non-targeting siRNA (control) or SERTAD3 siRNA in 100 and 150 nM concentrations. Equal amounts of cells were plated in 60 mm dishes and the number of cells was counted every 24 h. Results are shown as the mean ± SEM.



pLuc-(E2F)$_4$	30	30	30	30	30	30	30	30
E2F	-	30	30	30	30	30	30	30
SERTAD3	-	-	120	240	480	-	-	-
SERTAD1	-	-	-	-	-	120	240	480

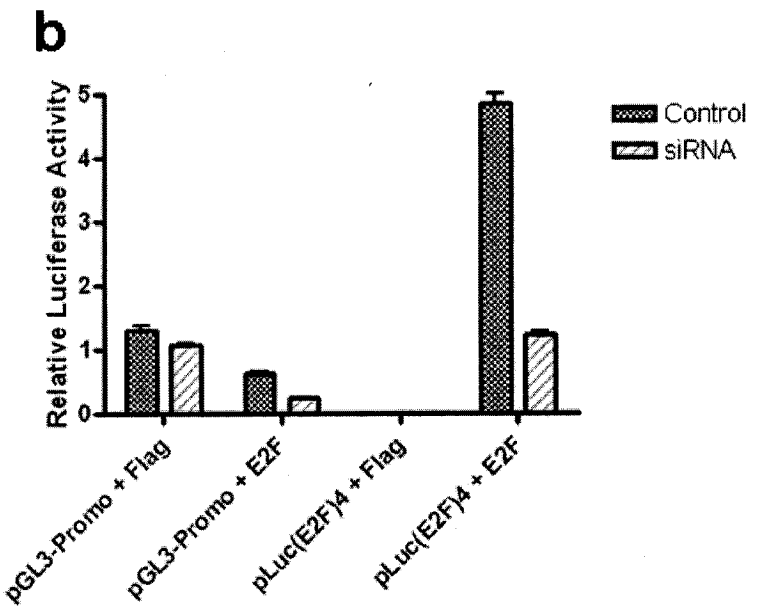


Figure 2.6 – Transcriptional Assays. (**a**) Stimulation of E2F transcriptional activity in NIH3T3 cells. E2F activity was assayed using a luciferase reporter plasmid containing four consensus E2F-1 binding sites (pLuc-(E2F)$_4$). Transfection of plasmid quantities is shown in nanograms. Note that exogenous addition of SERTAD3 or SERTAD1 increases the activity of E2F. (**b**) Effect of SERTAD3 siRNA on E2F activity in MCF-7 cells. Transcriptional activity of E2F is decreased 4-fold in cells co-transfected with 150 nM SERTAD3 siRNA.

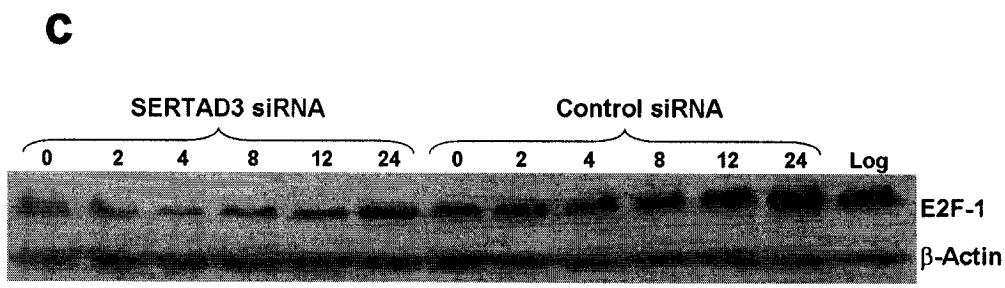


Figure 2.6 - Transcriptional Assays. (c) Synchronized MCF-7 cells were transfected with either SERTAD3 siRNA or control siRNA, and the protein levels of E2F-1 throughout G1 phase were measured using Western blot. SERTAD3 knockdown decreased E2F-1 accumulation in MCF-7 cells compared to control. β-Actin was used as a loading control.

Discussion

Gene amplification often leads to overexpression due to the presence of multiple gene copies in the chromosome. Specific regions of amplification are seen in several cancers and have a significantly higher occurrence than random amplification. It is thought that recurrent amplicons are advantageous to cells because they contain survival genes that promote cell growth. If amplicons contain important oncogenes, they will be found more often in cancer.

We previously reported that SERTAD3 (RBT1) has a higher expression in cancer cells (Cho *et al.* 2000). Here, we describe the localization of SERTAD3 within the 19q13.1-13.2 amplicon located next to its family member SERTAD1. One of the two SERTAD3 transcript variants (TV1) is tightly regulated throughout G1 and S phases of the cell cycle. The pattern of expression is identical to that of SERTAD1 (Sugimoto *et al.* 1999), and the fact that these two genes are located in tandem suggest common mechanisms of gene regulation.

Every member of the SERTAD family contains several motifs responsible for protein-protein interactions and DNA binding. The SERTA domain is the most highly conserved region among these proteins, though little is known about its functions. Of significance to this study, the cyclin A binding domain found in these proteins is similar to the one found in E2F proteins (Cho *et al.* 2000). The PHD-interacting domain could also be an important protein-protein interacting motif, especially when considering the importance of PH domains in protein regulation. For example, AKT2 and several other regulatory proteins contain PHDs (Bienz 2006).

The oncogenic nature of SERTAD3 was demonstrated using both expression vectors

and siRNA. Mouse fibroblasts that stably overexpress SERTAD3 show transformed behavior *in vitro*. Furthermore, these transformed fibroblasts are able to form tumors in nude mice whereas the control cells cannot. The growth rate of MCF-7, a breast cancer cell line, is diminished when knocking down the levels of SERTAD3, lending further evidence that this protein confers a growth advantage to cells. These data support the theory that SERTAD3 may influence cancer development and progression.

Overexpression of E2F-1 has been shown to cause cellular transformation in rodent cells (Singh *et al.* 1994; Yang and Sladek 1995). SERTAD3 overexpression may cause transformation in NIH3T3 fibroblasts in part through modulation of the E2F-1 pathway. While the normal physiological functions of SERTAD3 have not been fully defined, we speculate that one of its roles involves the modulation of the E2F-1 transcription factor. In support of this hypothesis, the results of luciferase assays have clearly shown that NIH3T3 cells transfected with SERTAD3 have significantly greater E2F-1 activity in comparison to parental cells. Furthermore, knocking down SERTAD3 levels in MCF-7 cells with siRNA causes a reduction in both E2F transcriptional activity and accumulation. Ongoing studies will further establish the connections between SERTAD3 and E2F-regulated genes.

In summary, we have shown that the amplified region of chromosome 19q13.1-13.2 contains SERTAD3 and SERTAD1, two highly conserved homologues. Furthermore, both its function and regulation indicate an important role for this protein in cellular growth and proliferation. Taken together, this information suggests that the presence of SERTAD3 stabilizes the 19q13.1-13.2 amplicon in cancer and that overexpression of SERTAD3 due to the amplification of chromosome 19q13.1-13.2 can promote

oncogenesis, likely through E2F activation.

Materials and Methods

Cell Lines

NIH3T3 mouse fibroblasts, primary human fibroblasts (CRL2097), MCF-7 breast carcinoma cells, and HEK293GP cells were obtained from the American Type Culture Collection (Manassas, VA). NIH3T3, CRL2097, and MCF-7 cells were cultured in DMEM, MEM, and RPMI 1640 medium, respectively, supplemented with 10% FBS. All cell lines were maintained in culture at 37°C in an atmosphere of 5% CO_2.

Plasmids and Generation of Cells Stably Expressing SERTAD3

pBabe-puro (Morgenstern and Land 1990), a MuLV-based retroviral vector, was used to transduce the SERTAD3 gene. The cDNA for wild-type SERTAD3 (NM_013368) was derived by PCR using Pfu turbo (Stratagene, La Jolla, CA), cloned into pBabe via *Bam*HI and *Eco*RI restriction sites, and confirmed by DNA sequence analysis. The insert was also excised from SERTAD3-pBabe and cloned into pcDNA3.1zeo (Invitrogen, Carlsbad) and pFLAG-CMV2 (Kodak, New Haven). pBabe or SERTAD3-pBabe vector were transfected into the packaging cell line 293GP using Lipofectamine (GIBCO BRL). Viral supernatants (500 ml each of SERTAD3-pBabe and pBabe) were collected at 24 and 48 h after transfection. Viral titer was determined using PA18G-BHK-21 cells and puromycin selection. The concentrated viral stocks had titers of approximately 10^9 infectious units per ml. NIH3T3 cells were infected with SERTAD3-pBabe or pBabe and then subjected to selection in the same medium containing puromycin (2 μg/ml). Cells were selected in puromycin for 1 week with one change of medium in between. Cells were subsequently pooled, maintained under selection and characterized.

Synchronization of Cells in G0/G1

Synchronization of CRL2097, NIH3T3, and MCF-7 cells was achieved through serum starvation (0.02% FBS) for 60 h. The cells were released with 10% FBS and collected after 0-32 h. Cells in an exponential (log) phase of growth were also collected for comparison.

Flow Cytometry

Synchronization of CRL2097 and NIH3T3 fibroblasts in G0/G1 was achieved as mentioned above and analyzed by flow cytometry as previously described (Loignon *et al.* 2002). Briefly, cells were washed with PBS containing 10 mM EDTA, trypsinized, resuspended in 1 ml of PBS/EDTA and fixed by addition of 3 ml of ice-cold 100% ethanol. Fixed cells were pelleted, washed with 4 ml of PBS/EDTA and stained with a buffer containing 0.05 mg/ml propidium iodide (PI) (Molecular Probes), 0.1% sodium citrate, and 0.2 mg/ml RNase A. The fraction of the population in each phase of the cell cycle was then determined as a function of DNA content using a FACScan flow cytometer equipped with CellFit software (Beckton Dickinson).

RNA Isolation

TRIzol was used to extract total RNA from cells according to the instructions of the manufacturer. RNA pellets were washed twice with 70% ethanol, resuspended in 40 µl RNase-free H_2O, and stored at –80°C. RNA quantity and quality were assessed on an Eppendorf BioPhotometer.

RT-PCR

Semi-quantitative reverse transcriptase-PCR was performed using the QIAGEN One-Step RT-PCR kit and 0.1 μg RNA in a PTC-100 thermal cycler (MJ Research) under the following conditions: 50 °C for 30 min, 94 °C for 15 min, followed by cycling 94 °C for 45 s, 59-61 °C for 45 s, and 72 °C for 45 min, and a final step of 10 min at 72 °C. Primers used in this study are listed in Table 1.

Stability of SERTAD3 mRNA

MCF-7 and CRL2097 cells were plated in 60 mm dishes at a confluency of 25% (1.5 x 10^6 cells) and cultured 24 hours. Cells were treated with either 10 μg/ml actinomycin-D dissolved in 100 % ethanol or ethanol alone as a negative control. RNA was harvested at 0, 2, 4, 8, and 24 hours post-treatment. mRNA stability was measured using semi-quantitative RT-PCR.

siRNA Transfections

SERTAD3 siRNA was designed by Dharmacon using their SMARTselection design algorithm (siRNA sequence = GGC AUG UCC UCA UCC AUA A). Transfections were carried out according to the manufacturer's instructions with final siRNA concentrations of 100-150 nM. The cells were harvested 24-48 hours post-transfection. Non-targeting siRNA #1 was used as a negative control.

Table 2.1: Primers for RT-PCR

Name	Forward Primer	Reverse Primer	# of Cycles	Fragment Size (bp)
SERTAD3	TTC AGA GCT ACC AGC AAG C	GTC CAG AAA GAA GTC ATC CAG G	28	400
SERTAD3-TV1	ATT GTG GAA GAA CAG TTT TAA GGC	TGT TAT GGA TGA GGA CAT GCC	37	297
SERTAD3-TV2	GCT GTG GGC GTT CCA GGA G	TGT TAT GGA TGA GGA CAT GCC	31	233
CDK4	TTA CTG AGG CGA CTG GAG GC	CAG AGA TTC GCT TGT GTG GG	28	600
Cyclin A2	TCC ATG TCA GTG CTG AGA GG	GAA GGT CCA TGA GAC AAG GC	28	451
SERTAD1	CTA GTG AGC AAG ATG CTG AGC	CTG GCC ATG GAG GCT GAA AG	34	362
SERTAD2	GTG GAG CTG CAT GTG ATA TAT G	GGA TCC GCC TCA ACA TGT TG	34	236
CDCA4	TCG CGG GTC AGG ACA CAA TG	TCT CGA GGG CCA TCC ATC TC	31	475
GAPDH	CCG GGA AAC TGT GGC GTG AT	GAA GGC CAT GCC AGT GAG CT	26	125

Western Blotting

Protein analysis by Western blot was performed as previously described (Loignon *et al.* 2002) using rabbit anti-SERTAD3 antibody (Cho *et al.* 2000) and mouse anti-E2F1 SC-250X (Santa Cruz) at dilutions of 1:200, and mouse anti-βActin (Sigma) at a dilution of 1:4000.

Tumor Xenographs in Nude Mice

Exponentially growing cells were suspended in PBS (10^6 cells per 0.1 ml) and injected subcutaneously into the flanks of Balb/c Nu-Nu mice. Tumor volumes were measured every second or third day by external measurement. Tumor volumes were estimated using the equation: volume = $\pi/6$ (length x width^2). Statistical significance in tumor growth between control and SERTAD3 overexpressing tumors was defined at the 0.05 confidence level using a two-tailed T test.

Transfections and Luciferase Assay

Mammalian cell transient transfections were performed using either Lipofectamine or Lipofectamine 2000 reagent according to the manufacturer's recommendations. All transfections were done in triplicate and repeated 3 times. Cells were seeded at 5×10^4 cells/well in 24-well plates and transfected after 24 hours. The pGL3-Basic and pGL3-Promoter plasmids as well as the *Renilla* luciferase plasmid pRL-null were obtained from Promega.

Lipofectamine was used to transfect plasmid DNA into mammalian cells. Cells were transfected with 0.2-1 μg total plasmid DNA, consisting of 30-500 ng of each expression

vector and 30 ng of *Renilla* control (pRL-null). Lipofectamine 2000 was used to co-transfect both plasmid DNA and siRNA into cells. 100 ng each of reporter and expression vectors were added to 40 ng *Renilla* control and then mixed with 30 pmol of siRNA (Dharmacon) and 1.5 µl Lipofectamine 2000. Transfection volumes were increased to 200 µl and placed on cells.

Thirty-six hours after transfection, cells were washed with PBS without Ca^{2+} and Mg^{2+} and harvested with Passive Lysis Buffer (Promega, Madison, WI). Luciferase activity was measured as previously described (Yen *et al.* 2002) using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was calculated and reported as a ratio between firefly luciferase and *Renilla* luciferase activity. Measurements were repeated 3 times and are shown as the mean +/- SEM.

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CHAPTER 3

General Discussion

3.1 – Overview

Genes involved in cell cycle progression and cell proliferation play key roles in carcinogenesis. A large number of genes that regulate cell proliferation have been identified to date, many of which have been associated with cancer development. However, there are still numerous genes involved in cell growth that have yet to be discovered or whose functions within the cell cycle need to be elucidated. Determining the role of genes involved in the cell cycle will lead to a greater understanding of cancer progression and may aid in the development of cancer therapeutics.

One novel gene that may play a significant role in cell cycle progression is SERTAD3. Preliminary studies have shown that SERTAD3 is localized within the nucleus, can interact with RPA32, and contains a C-terminal transactivation domain (Cho *et al.* 2000). Our research has expanded the knowledge of both the structure and function of SERTAD3, further supporting its involvement in cell growth and cell cycle progression. First, the genomic and protein structures of SERTAD3 are similar to other members of the SERTAD family such as the oncogenic SERTAD1. Second, the SERTAD3 gene is found within a commonly amplified chromosomal region in cancer. Third, overexpression of SERTAD3 leads to the loss of contact inhibition, colony growth in soft agar, and tumor formation in nude mice, while the knockdown of SERTAD3 transcripts decreased cell growth *in vitro*. Finally, luciferase experiments show that SERTAD3 promotes E2F-1 transcriptional activity.

3.2 – The SERTAD Family

Several mammalian proteins contain a SERTA domain and have been grouped into the SERTA domain-containing (SERTAD) family. Although the exact functions of the SERTA domain are unknown, it is believed to be important for protein-protein binding. Indeed, the region of SERTAD1 responsible for CDK4 binding encompasses most of the SERTA domain (Li *et al.* 2004). The SERTAD family shares other protein motifs such as the cyclin A-binding domain, the PHD-bromodomain interacting motif, and the C-terminal transactivation domain (Figure 2.1c). There is also a high level of genetic similarity among the SERTAD family. Each gene contains an intron within their 5' UTRs but have an intronless coding region. The high degree of amino acid similarity and common protein domains, coupled with the preservation of an uncommon genetic structure, suggest a high level of conservation among the family members and a similar function within the cell.

3.3 – Comparison of SERTAD3 and SERTAD1

Among the SERTAD family, the two proteins with the highest degree of similarity at the nucleotide and amino acid level are SERTAD3 and SERTAD1. Furthermore, the genomic location of these two genes is identical. Both genes are found in tandem within chromosome 19q13.1-13.2, separated by ~15 Kb. The AKT2 proto-oncogene is also found in this region, located several centimorgans from the two SERTAD genes on chromosome 19. As previously mentioned, the 19q13.1-13.2 region containing these three genes is commonly amplified in various types of cancer. Based on the similar genetic structures of SERTAD3 and SERTAD1, coupled with their location within a known amplicon, it is reasonable to assume that the two genes

have similar functions that may be related to oncogenesis.

The amount of SERTAD3 mRNA throughout the G1 and S phases of the cell cycle showed a distinct pattern of expression. The expression of SERTAD3 transcript variant 1 increased dramatically 2 h post G0/G1-release. TV1 mRNA levels dropped quickly after 2 h, but began to increase again around the G1/S transition. This pattern of differential expression is identical to SERTAD1 expression (Sugimoto *et al.* 1999). Genes that are tightly regulated throughout the cell cycle often have important roles in cell growth and checkpoint mechanisms, suggesting that SERTAD3 and SERTAD1 are involved in cell cycle progression.

Finally, luciferase assays using an E2F-1 expression plasmid and a reporter plasmid containing consensus E2F-1 binding sites demonstrated that SERTAD3 expression promotes the transcriptional activity of E2F-1. Furthermore, SERTAD3 knockdown using siRNA greatly reduced E2F-1 activity. However, it remains unclear whether this occurs via direct or indirect mechanisms. The activation of E2F was also observed when co-expressing SERTAD1, validating earlier experiments (Hsu *et al.* 2001). Our results show that the effect of SERTAD3 is concentration-dependent where E2F-1 activity increases proportionally to SERTAD3 levels up to a certain concentration. Above this level of SERTAD3 expression, E2F-1 activity begins to be inhibited. Similar results were found when expressing SERTAD1. These findings support previous findings of SERTAD1 where CDK4 activation occurs up to a specific concentration before changing to an inhibitory function (Li *et al.* 2004). Based on these results, it is likely that SERTAD3 and SERTAD1 activate E2F in a similar manner, possibly through CDK activation.

3.4 – Oncogenic Properties of SERTAD3

Mouse fibroblasts stably overexpressing SERTAD3 lose cell-cell contact inhibition that is characteristic of non-transformed fibroblasts and are able to form colonies in soft agar. The *in vitro* results were further supported by the *in vivo* experiments where SERTAD3-overexpressing cells, and not parental cells, formed tumors in the flanks of nude mice. Thus, the overexpression of SERTAD3 appears to transform normal fibroblasts and promote tumorigenesis.

Additionally, the SERTAD3 siRNA experiment in the MCF-7 breast cancer cell line demonstrated that cell growth was dependent on SERTAD3 levels. Growth rates were proportional to SERTAD3 expression, where the highest amount of SERTAD3 knockdown showed the greatest reduction in cell growth. These results suggest that normal SERTAD3 levels are required for proper cell division and growth, and that reducing SERTAD3 expression will directly affect cell proliferation.

Our results show convincing evidence that SERTAD3 acts as a proto-oncogene. Overexpression of SERTAD3 causes a phenotype similar to transformed cells, while SERTAD3 knockdown decreases cell growth. Furthermore, SERTAD3 gene expression is tightly regulated during the cell cycle. The gene is differentially expressed through G1 and S phases, and SERTAD3 mRNA has a short half-life. Together, these results strongly suggest that SERTAD3 plays an oncogenic role within the cell.

3.5 – CDK Activation and Cancer

Cell cycle progression is dependent on active cyclin-dependent kinases to turn on transcription factors and inactivate repressors of transcription. Hence, CDK activation

is a common occurrence in cancer (Cordon-Cardo 1995, Kamb 1995, Deshpande 2005). As previously mentioned (see section 1.3), there are several mechanisms of CDK activation including increased cyclin expression, loss of CDK inhibitors (CKIs), and increased CDK modulator expression/activity. Each mechanism promotes the kinase activity of CDKs and pushes cells through the cell cycle, leading to increased proliferation and ultimately tumorigenesis.

CDK modulators other than cyclins have recently been discovered, and their role in CDK activation is increasingly recognized as being important within the cell. One of these modulators, SERTAD1, interacts with CDK4 and antagonizes the inhibitory effect of $p16^{INK4A}$. Li *et al.* (2004) reported a 2-fold increase in CDK4 activity in the presence of SERTAD1. This activation of CDK4 may be partially responsible for the observed activation of E2F-1 transcriptional activity caused by SERTAD1 expression (Hsu *et al.* 2001).

There is reason to believe that SERTAD3 may also act as a CDK modulator. SERTAD3 contains the same protein binding domains as SERTAD1 such as the SERTA domain and PHD-bromo interacting domain. The regulated expression of SERTAD3 is analogous to cyclin expression, suggesting that it works at specific stages of the cell cycle and may interact with protein partners that are constitutively expressed within the cell. Moreover, SERTAD3 can activate E2F-1 transcriptional activity. Given this evidence, it is likely that SERTAD3 promotes cell growth and proliferation through the binding and activation of CDKs.

3.6 – CDK3 and SERTAD3

Since the discovery of human CDK3 (Meyerson *et al.* 1992), researchers have been trying to elucidate its *in vivo* cyclin binding partner and its role within the cell

cycle. Early reports suggested that CDK3 was a regulator of the G1/S transition. A dominant-negative mutant of CDK3 could arrest cells in G1 phase, and this cell cycle arrest could be overcome by wild-type CDK3 but not CDK2 (van den Heuvel and Harlow 1993, Hofmann and Livingston 1996). Using *in vitro* experiments it was shown that recombinant CDK3 could interact with and become activated by cyclin E, E2 and A (Harper *et al.* 1995, Connoll-Crowley *et al.* 1997, Hengstschläger *et al.* 1999). However, the *in vivo* kinase activity of CDK3 does not correspond to the expression of either cyclin E or A in the cell cycle (Braun *et al.* 1998). Kinase activity peaked 2-3 h after release from G0/G1 arrest, much earlier than the presence of cyclin E or A.

The endogenous binding partner of CDK3 remained unclear until 2004, when it was discovered that CDK3 forms a complex with cyclin C in G0 to begin pRb phosphorylation and promote cell cycle entry (Ren and Rollins 2004, Sage 2004). These findings explain why CDK3 kinase activity was seen so early after G0/G1 release, as well as how cells containing dominant-negative CDK3 mutants did not progress to S phase. Given that CDK3/cyclin C can promote cell cycle entry, it is likely that the activation of this complex would shorten G0 and G1 phases and lead to an increase in cell cycle progression. Indeed, it was found that cyclin C is overexpressed in 88% of colon adenocarcinomas and amplified in 27% of these samples (Bondi *et al.* 2005).

It was much more difficult to synchronize cells overexpressing SERTAD3 compared to parental cells. Under normal circumstances, serum starvation will force cells to exit the cell cycle and enter G0 phase. However, SERTAD3-overexpressing cells were less able to be arrested in G0 phase using the same conditions. This observation suggested that SERTAD3 may have a functional role in G0 exit, and since

this protein is thought to interact with CDKs, we hypothesized that SERTAD3 interacts with CDK3 to promote the G0/G1 transition.

Preliminary experiments have shown promising results. Both cyclin C and CDK3 mRNA expression peak at 2-3 h after release from G0/G1 arrest in CRL2097 cells (Figure 3.1a), coinciding with SERTAD3 TV1 expression. The overlap between SERTAD3 expression and CDK3 activity, coupled with the difficulty in synchronizing SERTAD3-overexpressing cells in G0/G1, indicate a possible functional relationship. The interaction between SERTAD3 and CDK3 (or CDK3/cyclin C) may explain why the overexpression of SERTAD3 leads to a loss of contact inhibition and increased growth, and why SERTAD3 knockdown decreased cell proliferation.

3.7 – Proposed Mechanisms of SERTAD3

There is significant evidence supporting the oncogenic role of SERTAD3 within the cell. However, the exact mechanisms have not been elucidated. The data gathered from these experiments offer several potential explanations for the direct relationship between SERTAD3 expression and cell growth.

As described in section 3.6, SERTAD3 may promote CDK3 kinase activity and help push cells into the cell cycle, thereby shortening G1 phase and increasing cell proliferation. Alternatively (or in parallel), SERTAD3 may activate other CDKs within the cell cycle, especially CDKs such as CDK4 and CDK6 that have high kinase activity in early-to-mid G1 phase where SERTAD3 expression is highest. These hypothetical mechanisms are supported by the similar structures and expression of SERTAD3 and SERTAD1, the family member that has been shown to bind to and activate CDK4.

Figure 3.1 – Expression of CDK3 and Cyclin C. The mRNA levels of CDK3 and cyclin C were assessed throughout G1 and S phases using RT-PCR. There is a 2- to 3-fold increase in CDK3 expression and a 1.5- to 2-fold increase in cyclin C expression at 2 h post-release.

CDK activation may not be the only way that SERTAD3 can drive cell cycle progression. Based on the luciferase assays with E2F-1, it is possible that SERTAD3 may directly bind to E2F or DP transcription factors to promote transasctivity. Indeed, SERTAD1 was shown to directly interact with recombinant DP-1 (Hsu *et al.* 2001). Since the two SERTAD proteins contain almost identical binding domains, it is likely that SERTAD3 may be able to function in a similar manner (Figure 3.2).

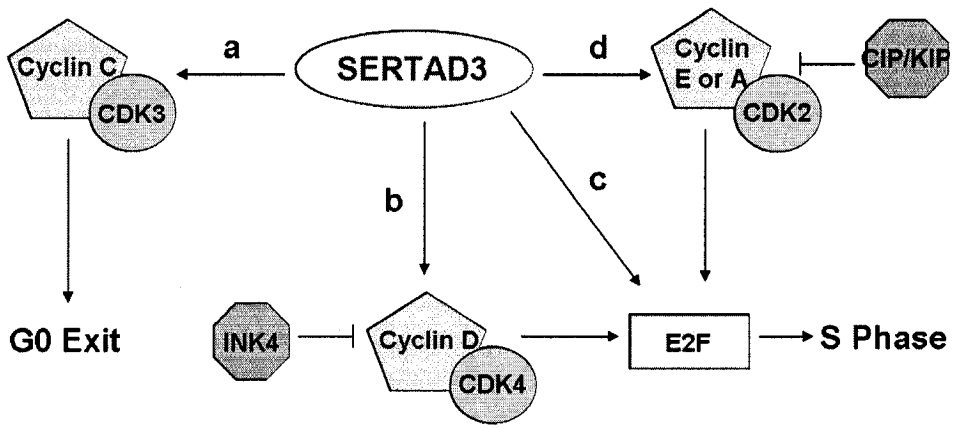

Figure 3.2 – Potential Mechanisms of SERTAD3 Within Cell Cycle. (**a**) CDK3 and cyclin C work together to begin pRb phosphorylation and push cells out of G0 arrest. SERTAD3 may promote CDK3 kinase activity and thus G0 exit. (**b**) SERTAD3 may activate CDK4 and antagonize the inhibitory function of $p16^{INK4A}$, driving E2F activity in G1 phase. (**c**) E2F may be activated by SERTAD3 through a direct protein-protein interaction with either the E2F or DP subunit. (**d**) SERTAD3 may interact with other CDKs such as CDK2 to promote cell cycle progression.

Conclusions

SERTAD3 is a member of the highly conserved family of SERTA domain-containing proteins. The SERTAD3 gene is located in a chromosomal region known to be amplified in various cancers, supporting previous findings of gene overexpression in cancer cell lines. SERTAD3 has two transcript variants with short mRNA half-lives, and one of the variants is tightly regulated throughout G1 and S phases of the cell cycle. Overexpression of SERTAD3 induces cell transformation *in vitro* and tumor formation in mice, while inhibition of SERTAD3 by siRNA results in a 2-4 fold reduction in cell growth rate. Furthermore, luciferase assays based on E2F-1 binding sites indicate that SERTAD3 increases the activity of E2F, which can be strongly reduced by siRNA inhibition of SERTAD3. The mechanism of SERTAD3 activity likely involves CDK activation, suggesting that SERTAD3 may regulate E2F activity through CDK modulation. Together, our data support that SERTAD3 contributes to oncogenesis at least in part via an E2F-dependent mechanism.

Future Directions

The connection between SERTAD3 and E2F is promising and should be explored further. We have shown a correlation between SERTAD3 expression and E2F-1 transcriptional activity using luciferase assays. It would be useful to validate these results using chromatin immunoprecipitation (ChIP) assays. We hypothesize that ChIP assays will demonstrate a higher degree of association between E2F proteins and their E2F-responsive elements within genes. To carry out this experiment, wild-type cells and cells with either SERTAD3 overexpression or inhibition will be treated to covalently bind proteins to DNA. The DNA will be digested and then various E2F proteins will be immunoprecipitated to determine the extent of their DNA binding in the presence or absence of SERTAD3.

To further validate these results, the abovementioned cells will be used to determine whether E2F-dependent gene expression is affected by SERTAD3 expression. Protein extracts will be collected at several timepoints throughout the cell cycle and the expression of genes such as DHFR and B-myb will be measured using both RT-PCR and Western blot.

The preliminary results involving CDK3 should also be examined in more detail. Since our theory is that SERTAD3 helps CDK3 phosphorylate pRb in the beginning of G1 phase, it would be important to look at the phosphorylation status of pRb in the presence or absence of SERTAD3. This can be done using synchronized cells with different levels of SERTAD3 expression. Protein extracts from these cells would be analyzed by Western blot using a phospho-specific pRb antibody. If our theory is correct, the amount of phosphorylated pRb would increase more rapidly in cells that overexpress SERTAD3 than in cells with little SERTAD3 expression.

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