Büşra AYDOĞDU

INVESTIGATION OF THE CODING SEQUENCE REGION(S) INVOLVED IN G1 PHASE REGULATION OF STEM-LOOP BINDING PROTEIN (SLBP) EXPRESSION

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in

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APPROVAL PAGE

This is to certify that I have read this thesis written by Büşra AYDOĞDU and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Genetics and Bioengineering.

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Thesis Supervisor

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science in Genetics and Bioengineering.

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January 2013

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Supervisor: Assist. Prof. Dr. M. Murat KÖSEOĞLU

ABSTRACT

In the S phase of human cell cycle, the genetic material, DNA, is replicated and the newly synthesized DNA is wrapped around a protein core, called histone octamer. Production of large quantities of histones in a limited time is due to a large increase in the amount of the histone mRNA. Increased histone mRNA levels are primarily due to an increase in the processing of the histone mRNAs. Stem-Loop Binding Protein (SLBP) is a crucial protein for histone mRNA processing and translation. It is cell cycle regulated and its level is highest during S phase. SLBP protein is increased 10 to 20 fold in the late G1 and then degraded in the S/G2 border. Previously, translation efficiency is tested in order to clarify the regulation of SLBP in G1. Although the level of SLBP translation increases to S phase level in mid-G1, the amount of the SLBP protein remains low until late G1 or G1/S border. This suggests that there is another regulation that keeps the SLBP level low. Further it is shown that the SLBP protein is less stable in G1 in comparison to S phase, so the possible mechanism that keeps the level of SLBP low is rapid degradation of newly synthesized SLBP in mid-G1. In this study, in order to find out the coding sequence region(s) that are responsible for this degradation, I generated cell lines that are stably expressing different deletion mutants of SLBP. Further, since APC/Cdh1 is known as the active E3 ligase in G1, I mutated a putative APC target site in SLBP as a tool to investigate its role in G1 regulation of SLBP expression.

Keywords: SLBP, cell cycle, histone, histone mRNA, G1, S phase

STEM LOOP BINDING PROTEİN'İN (SLBP) KODLAMA DİZİSİNDEKİ G1 REGÜLASYONUNDAN SORUMLU BÖLGELERİN ARAŞTIRILMASI

Büşra AYDOĞDU

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ÖZ

İnsan hücrelerinde, hücre döngüsünün S fazında, genetik materyal olan DNA replike edilir ve yeni sentezlenen DNA histon oktamerleri adı verilen protein kompleksleri etrafına sarmalanır. Kısıtlı bir zaman içerisinde çok fazla miktarda histon protein üretilmesi, histon mRNA seviyesindeki artıştan, histon mRNA seviyesinde ki artış ise mRNA işlenmesinin artmasından kaynaklanmaktadır. SLBP, histon mRNA işlenmesi ve translasyonu için gerekli bir proteindir. Hücre döngüsüne bağlı olarak düzenlenir ve S fazında en yüksek seviyeye ulaşır. G1 fazının sonlarına doğru SLBP protein seviyesi 10-20 kat artar, S fazı sonunda ise yıkılır. Önceki çalışmalarda, SLBP'nin G1 fazındaki regülasyonunu açıklığa kavuşturmak amacı ile translasyon etkinliğine bakılmıştır. Translasyon seviyesi G1'in ortalarında S faz seviyesine ulaşmasına rağmen, G1'in geç safhalarına kadar SLBP protein seviyesinin düşük olduğu gözlemlenmiştir. Bu da SLBP proteinini G1 fazında düşük seviyede tutan ekstra bir regülasyona işaret etmektedir. İlaveten, G1 fazındaki SLBP proteinin stabilitesinin S fazı ile karşılaştırıldığında daha düşük olduğu gösterilmiştir. Bu nedenle, SLBP'yi G1'de düşük seviyede tutan muhtemel mekanizma ; yeni sentezlenen SLBP proteinlerinin hızlı bir şekilde degrade olmasıdır. Bu çalışmada, bahsi geçen protein yıkımından sorumlu olan kısımlarının aydınlatılması için, SLBP proteininin kodlanmasından sorumlu bölgelerde farklı delesyonlara sahip konstraktlar kullanarak stabil hücre hatlarını oluşturdum. Ek olarak, SLBP'nin G1 fazındaki ekspresyonundaki rolünü anlamak için yapılan çalışmalarda kullanılmak üzere, G1 fazında aktif olduğu bilinen E3 ligaz APC/Cdh1'ın hedef motifi olan kodlayıcı dizi bölgesini mutasyon ile değişime uğratarak yeni bir konstrakt hazırladım.

Anahtar Kelimeler: SLBP, hücre döngüsü, histon, histon mRNA, G1- S fazı .

To my parents..

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SYMBOL/ABBREVIATION

APC/C	Anaphase promoting complex/cyclosome
APS	Ammonium PerSulphate
CaCl2	Calcium Chloride
CAK	Cdk activating kinase
Cdk	Cyclin dependent kinases
CFU	Colony Forming Unit
CKI	Cdk inhibitory protein
D box	Destruction box
DMSO	Dimethyl Sulphoxide
ECL	Enhanced Chemiluminescence
ELISA	Enzyme Linked Ġmmunosorbant Assay
FACS	Flourescence Activated Cell Sorter
FBS	Fetal Bovine Serum
HCl	Hydrochloric acid
HDE	Histone downstream element
KCL	Potassium Chloride
KH2PO	Potassium Pyrophosphate
LB broth	Luria Betani Broth
MnCl2	Manganase Chloride
MOPS	3-(N-morpholino)propanesulfonic acid
Na2HPO4	Sodium pyrophosphate
NaCl	Sodium Chloride
NaF	Sodium Floride
NaOH	Sodium hydroxide
NPAT	Nuclear protein ataxia-telangiectasia

OD	Optical Density
ON	Over Night
PBS	Phosphate Buffer Saline
Pen/strep	Pennicilin/streptomycin
PI	Propodium iodide
pRb	Retino blastoma protein
pre-RC	Pre-replicative complex
PVDF	Polyvinilidine Fluoride
RbCl2	Rubidium Chloride
RT	Room Temperature
SCF	Skp1-Cul1-F-box protein complex
SDS	Sodium Dedocyl Sulfate
SDS PAGE	Sodium Dedocyl Sulfate Polyacrilamide Gel Electrophoresis
SLBP	Stem-loop binding protein
TEMED	Tetramethylethylenediamine
Ub	Ubiquitin
UPS	Ubiquitin-proteosome system
β –TRCP	β- transducin repeat containing protein

CHAPTER I

INTRODUCTION

1.1 CELL CYCLE AND ITS REGULATION

All cells in living organisms are generated from an ancestral cell by cell division. Cell reproduction is fundamental to development and maintenance of life. While in unicellular organisms, cell division generates a new organism; in multicellular organisms, cell division provides cells for the development of tissues or organs and replacement of those that are damaged or die. The deregulation of the cell proliferation causes the diseases like cancer (Hanahan and Weinberg, 2000).

Highly regulated sequencial events between two cell division is called the cell cycle. There are two important processes that should be well-organized during the cell cycle: one is DNA replication and the other is segregation of the replicated DNA into two daughter cells. These processes give the names to two major phases of the cell cycle. DNA replication, which is also called DNA synthesis, occurs in the S phase (S for the synthesis) and chromosome segregation and cell division occur in M phase (M for the mitosis). S and M phases are seperated by two gap phases called G1 and G2. These gap phases provide time to cells for growth, doubling their proteins and organelles and monitoring the internal and external conditions to ensure that they are suitable to maintain the cycle (Alberts at all., 2008). Thus, the eucaryotic cell cycle is comprised of four sequencial phases: G1, S, G2, M (see Fig. 1.1). Whereas G1, S and G2 phases all together are called interphase M phase contains a series of phases: prophase, metaphase, anaphase, telophase and cytokinesis;.

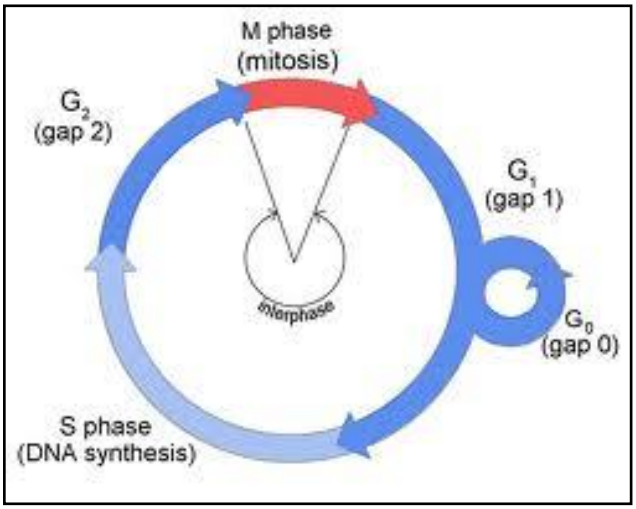

Figure 1.1 The phases of the cell cycle (Harvard University online webpage).

In addition to these phases, if the cells go under unfavorable conditions like lack of nutrients or growth factors, cells enter the G0 phase, known as resting phase or quiescent state. It is a non-dividing phase. The cells can re-enter the cycle if the favorable conditions are provided.

1.1.1 Major Players: Cyclin/Cdk Complexes

The cellular components, especially the genetic material, must be duplicated and divided with extreme precision and reliability over the countless generations. The cell cycle events are ordered in the dependent pathways in which initiation of an event is dependent on completion of the previous events, for example, mitosis is dependent on completion of the DNA replication (Hartwell and Weinert, 1989). Therefore, the events of the cell cycle are ensured to occur in the correct order by the regulatory mechanisms called the cell-cycle control system (Morgan, 2007). The sequencial stages of the cell cycle reflect phosphorylation state of key proteins that mediate the different cell cycle events (Twyman,1998). These proteins are phosphorylated by Cyclin Dependent Kinases (Cdks) which are major components of the cell cycle control system (Morgan,1997). Cdks are Ser/Thr kinases that phosphorylate their target proteins on their serine or threonin residues by transferring a phosphate from ATP to target protein.

Cdks can only be active after the binding of certain Cyclins (Morgan, 1995) whose expression tends to be cell cycle regulated so that different Cdks are active at

different phases of the cell cycle (Garrett, 2001). While CyclinD-Cdk 4/6 is responsible for the G1 progression, CyclinE-Cdk2 triggers the S phase entry, CyclinA-Cdk2 is important for maintenance of the S phase, progression from G2 into mitosis requires the activity of the Cdk1 activated by Cyclin A and Cyclin B.

The expression of Cdks doesn't change significantly throughout the cell cycle and their activity is changed depending the oscillation of cyclins during the cycle. Cyclin binding are necessary for not only kinase activity, but also substrate recognition. Cdks recognize their target motif from their [S/T]PX[K/R] aminoacid sequence and cyclins have a region called hydrophobic patch that accelerate the affinity to substrate which contain a complementary sequence known as RXL motif (Brown *et al..*, 1999). Cyclin binding is not enough to fully activate Cdks. Full activation of a Cdk requires a phosphorylation of a threonin residue on the kinase active site. It is achieved by specific enzymes called Cdk-activating kinases (CAKs). However, cyclin binding is the rate limiting step, because CAK activity always at high level (Harper and Elledge, 1998).

In addition to cyclin binding, Cdks are regulated by posttranslational modifications like activating/inactivating phosphorylation by Wee1 kinase family & Cdc25 phosphatase family (Dunphy, 1994; Nurse, 1990) and association with Cdk inhibitory proteins, CKIs (Sherr and Roberts, 1999; Russo, 1998). There are two groups of CKI proteins and they are primarily employed in the control of G1 and S phase but some of them have relatively little effect on M-Cdk CyclinB/Cdk1. CIP/KIP family that includes p21, p27, p57 inhibit Cyclin/Cdk complexes by interacting with both the cyclin and Cdk. In contrast, members of the INK4/ARF (p15, p16, p18, p19) bind to Cdk4 and Cdk 6 specificly and inhibit them by reducing cyclin binding affinity (Morgan, 2007).

1.1.2 Ubiquitin-Dependent Protein Degradation in Cell Cycle

Transition from one stage of the cell cycle to next stage unidirectionally is achieved by Cdks that needs to its cyclin partner to be active. Cyclin expression level are periodic throughout the cell cycle (Evans *et al..*, 1983). This is the result of regulated proteolysis of cyclins at certain cell cycle stages which is driven by Ubiquitin – Proteosome System, UPS (Glotzer *et al..*, 1991). Moreover, many other critical components of cell cycle are degraded by ubiquitin dependent pathway. For instance CKIs, negative regulators of Cdks, are also targeted by UPS for degradation. Therefore,

there are two important types of post-translational modification that regulates the cell cycle predominantly: phosphorylation and ubiquitylation.

The UPS contains two steps: the covalent attachment of ubiquitin molecules to target protein in an ATP-dependent manner and degradation of the protein that is marked by polyubiquitin chain by 26S proteosome complex (Hershko, 1983; Ciechanover, 1994). Ubiquitin (Ub) is a 76 amino-acid polypeptide which is bound to the target protein with an isopeptide linkage between carboxy terminus of ubiquitin and amino group of lysine of target protein (Pickart 2001; Weissmann 2001). The first step needs at least three enzymes: E1, E2 and E3. The ubiquitin is activated by E1 enzyme in an ATP dependent manner. E2, which is often called ubiquitin-conjugating or ubiquitin-carrier enzyme, takes role in transfer of the activated ubiquitin from E1 to the substrate. This transfer is depends on the ubiquitin ligase (E3). E3 ligases are responsible for the specific recognition and final selection of the target protein (Nandi, 2006). After the target protein is ubiquitylated, it is directed to degradation by 26S proteosome (Goulet and Nepveu, 2004). Before the target protein enter the proteolytic core of the proteosome, ubiquitins are removed by deubiquitinating enzymes, which is identified as ATP-dependent metalloprotease (Berndt *et al..*, 2002; Kim *et al..*, 2003).

E3 ligases are categorized into four major classes: HECT type, RING-finger type, U-box type, PHD-finger type (Nakayama, 2006). RING-finger type E3s are also divided into subfamilies including the cullin-based E3 subfamily which is one of the largest classes of E3s. SCF complex and APC/C are the members of the cullin-based E3 ligase subfamily (Jin *et al.*, 2004; Castro *et al..*, 2005) and important component of the cell cycle machinery (Qiao, *et al..*, 2010).

Skp1-Cul1-Fbox protein (SCF) complex consists of variable and invariable components. Whereas Rbx1 (RING finger protein), Cul1 (scaffold protein) and Skp1 (adaptor protein) are invariable regions, F-box protein is variable component of the SCFs. F-box protein, which binds to Skp1 subunit of the enzyme through its F-box motif, determines the specific substrate recognition of the SCF (Zheng *et al..*, 2002). In humans, there are about 70 different F-box proteins and they are mainly categorized into three groups according to their substrate binding domains (Cenciarelli *et al..*, 1999): those contains WD40 repeats (FBXW), leucine-rich repeats (FBXL) or other domains (FBXO) (Jin *et al..*, 2004) but three of them are thought to be related with the cell cycle

control: Skp2 (FBXL1), FBW7 (FBXW7), β-transducin repeat-containing protein – β-TRCP - (FBXW1/11). With Skp2, SCF targets CKIs (p21, p27, p57) as well as some other cell cycle regulators like Cyclin A/ D, E2F1, FOXO (Nakayama, 2006; Nakayama, 2005). Since it targets Cdk inhibitors for degradation, it is also named as oncogenic protein (Carrano *et al.*,1999; Bornstein *et al.*, 2003; Kamura *et al.*, 2003). On the contrary, FBW7 targets several oncoproteins such as Cyclin E, Myc, Notch 1 / 4, so it is thought to have tumor supressor function (Mao *et al.*., 2004). In addition to Skp2 and FBW7, B-TRCP targets various crucial cell cycle regulators including Wee1A, Cdc25A/B, Emi1/2 (Guardavaccaro *et al.*, 2003).

SCF activity remains constant throughout the cell cycle, so that, the control of substrate ubiquitination rate doesn't depend mainly on SCF activity. It is controlled by changing the affinity between the substrates and F-box protein corresponding them. SCF recognize its target protein via its F-box subunit if the target has been phosphorylated at a specific site or multiple sites (Cardozo and Pagano, 2004). These phosphorylations which SCF targets typically have, are achieved by Cdks, thus the ubiquitination and degradation ocur in specific times during the cell cycle (Morgan, 2007).

Another important E3 ligase for the cell cycle process is the Anaphase-Promoting Complex/ Cyclosome (APC/C). APC, like SCF, consists of invariable core components including APC11 (RBX1-related RING finger protein) and APC2 (CUL1 related scaffold protein) as well as a variable component known as activator- Cdc20 (Cell Division Cycle 20) and Cdh1 (HCT1) corresponding to F-box protein of SCF complex (Castro *et al.*., 2005). Cdc20 and Cdh1 are responsible for activation of the APC and specific substrate recognition (Pfleger, 2001). In contrast to ubiquitination by SCF which requires phosphorylation by Cdks, APC^{Cdc20} and APC^{Cdh1} don't need to phosphorylated substrate. They recognize proteins containing destruction box (D box) with the consensus RxxLxxxxN sequence, KEN box with the KENxxxN sequence (Burton and Solomon, 2001; Pfleger and Kirschner, 2000), A box or O box motifs (Peters, 2006). Cdc20 and Cdh1 activates APC/C in different periods; the former activates APC from mid- mitosis (anaphase) to late mitosis while the latter plays an essential role from late mitosis to the G1/S transition (Visintin, 1997) (see Fig. 1.2).

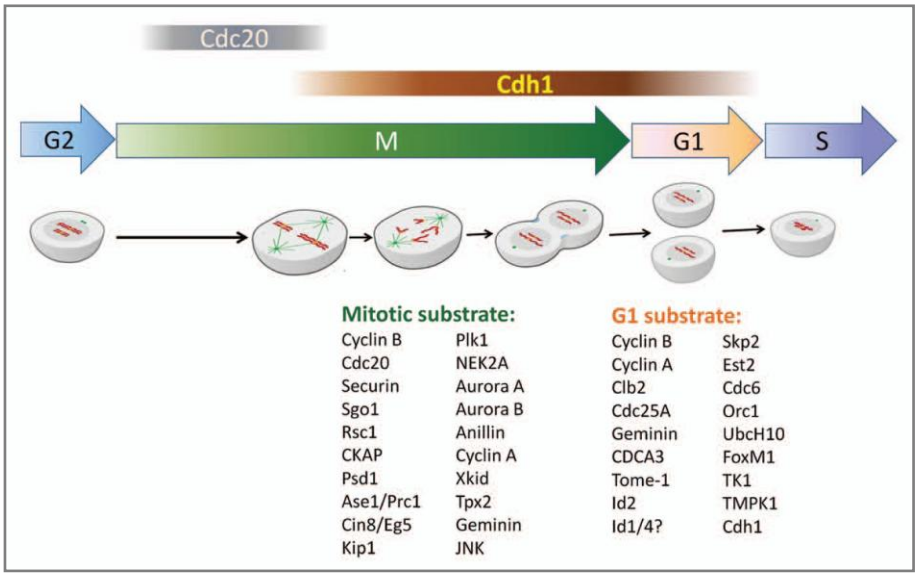

Figure 1.2 Cdc20 and Cdh1 activate APC/C sequencially (Qiao *et al.,* 2010).

When cells reach metaphase, M-Cdks phosphorylate the APC core subunit to enhance Cdc20 binding. Cdc20 activates APC at the metaphase-to-anaphase transition. The active APC^{Cdc20} contributes to the proteolysis of securin, which is inhibitor protein of separase, in order to initiate the sister chromatid segragation. From replication to anaphase, sister chromatids are kept together by cohesin protein complex. The degradation of securin activates the separase that cleaves the cohesin resulting the seperation of sister chromatids (Uhlmann *et al.*., 1999; Yanagida, 2000). APC^{Cdc20} targets M cyclins at metaphase-to-anaphase transition, as well. This cyclin destruction cause the inactivation of the M-Cdks. Unlike Cdc20, Cdh1 need to be dephosphorylated to bind to the APC and its level is relatively constant throughout the cycle (Prinz *et al.*., 1998). M-Cdks phosphorylate the Cdh1 to prevent from binding to the APC from S phase to anaphase. When Cdk is inactivated, it can't phosphorylate Cdh1 anymore, therefore it allows the Cdh1 to access the APC (Jaspersen *et al.*., 1999; Zacharie *et al.*., 1998). Cdh1 function is more extensive that that of Cdc20 (Li and Zhang, 2009). Whereas Cdc20 recognize securin and M cyclins, Cdh1 targets various proteins in late M and G1 phases like mitotic cylins, Aurora A/B, Plk1, geminin,Skp2 (Wasch and Engelbert, 2005).

Despite the SCF complex and the APC are similar in terms of their structure and biochemistry, there are differences between their cellular functions and timing of actions (Nakayama, 2006). Whereas SCF helps the G1/S transition control (Kossatz *et al.*, 2004; Hara *et al..*, 2001), APC is required for the M phase progression (Harper, 2002). SCF complex is active from late G1 to early M phase while the APC ubiquitylates their target proteins from mid-M phase to G1/S border. Emi1 and Emi2 are inhibitors of APC^{Cdc20} and also targets of $SCF^{\beta\text{-}TRCP}$ (Machida and Dutta, 2006). In early M phase, $SCF^{\beta\text{-}TRCP}$ recognize them, cause their degradation and activation of APC. In late M phase, APC^{Cdh1} and APC^{Cdc20} itself cause the ubiquitylation and degradation of Cdc20, resulting the transition from APC^{Cdc20} to APC^{Cdh1} (Foe *et al..*, 2011; Nakayama, 2006). Cdh1 targets the Skp2 F-box protein of SCF complex; the decrease in the Skp2 protein level leads to an increase in level of CKIs like p27. On the other hand, Skp2 expression is increased at the G1/S border resulting p27 degradation and activation of S-Cdks which phosphorylate Cdh1 to induce dissociation of Cdh1 from the APC. Therefore, APC activity is reduced at the G1/S boundary and SCF remains active until early M phase (Lu and Hunter, 2009; Nakayama, 2006).

1.1.3 Events Throughout The Eukaryotic Cell Cycle

The eukaryotic cell cycle comprises of four nonoverlapping phases. The transition from one phase to another are regulated by different Cyclin/ Cdk complexes that are active at certain times. The role of the Cdks is to control cell cycle progression via phosphorylation of specific downstream molecules. The positions in which cells monitor the cellular environment and the completion of previous phase in order to pass through to the next phase are called checkpoints (Garrett, 2001). The first of these is known as Start or Restriction point (R) that occurs at somewhere between mid and late G1. It is an essential checkpoint because it restrains the proliferation if the cell does not receive appropriate cues. According to environmental conditions including nutrients or external signals such as growth factor, activating/inhibitory signals, cells decide to whether pass the restriction point or enter G0, non-dividing phase.

At the molecular level, when the cells in resting phase receive mitogenic stimuli, Cyclin D expression is induced through Ras and PI3K pathways in response to mitogens (Takuwa *et al.*, 1999; Albanese, 1995). Then, Cyclin D binds and activates Cdk 4/6 whose major target is the product of the retinoblastoma tumour supressor gene, pRb.

pRb is the key regulator of G1 progression and is found in hypophosphorylated form in early G1. In this form, pRb interacts with E2F transcription factor family to supress them. Active form of E2F is required for the transcription of genes that are necessary for S phase (Harbour and Dean, 2000). During G1, pRb is phosphorylated by Cdk 4/6, thereby the interaction between pRb and E2F is disrupted. Moreover, the phosphorylated Rb indirectly lead to increasing in Cylin E and Cyclin A expression (Harbour, 1999; Rubin *et al..*, 2005). Cyclin E/ Cdk2 (also Cyclin A/Cdk2) active complexes further phosphorylate pRb resulting in a potential positive feedback loop that enhance the stimulation G1/S gene expression via increased production of E2F (Morgan, 2007; Takahashi *et al..*, 2000; Attwooll *et al..*, 2004).

During entry the cell cycle, the activity of G1/S and S Cdks, CyclinE/Cdk2 and CyclinA/Cdk2, is controlled by specific CKIs: p27 in mammalian cells. Both CyclinE/Cdk2 and CyclinA/Cdk2 are activated by the removal of these inhibitors. After the cells receive mitogenic stimulation, increased level of CyclinD/Cdk4-6 inactivates p27. Cip/Kip family of Cdk inhibitors (including p27) don't inhibit CyclinD/Cdk complexes; on the contrary, they are required for the activation of these complexes. Thus, p27 molecules binds to CyclinD/Cdk complexes, which block the binding of them to CyclinE-A/Cdk2 complexes to inhibit (Larrea *et al..*, 2008; Cheng *et al..*, 1999). p27 is also removed by proteolytic degradation. When cell progress into G1/s and early S phase,by the rising CyclinE/Cdk2 and CyclinA/Cdk2 activity, p27 is phosphorylated, recognized by SCF^{Skp2} E3 ligase and degraded by proteosomes (Hao *et al..*, 2005). In early S phase, the active S-Cdks phosphorylate downstream substrates to initiate the DNA replication and promote the degradation or inhibition of some factors in order to halt the reassembly of prereplicative complex- preRC (Nguyen *et al..*, 2001).

At the end of the S phase, cells enter the G2 phase in which the synthesis of Cyclin A and Cyclin B is increased, resulting in a gradual accumulation of M-Cdks complexes, CyclinA/Cdk1 and CyclinB/Cdk1. By the time the cell reaches to end of the G2, Cyclin/Cdk1 complexes are ready to act but their activity is repressed by inhibitory phosphorylations by the Wee1 protein kinases (Russell and Nurse, 1987). At the end of the G2, Cdc25 phosphotases remove the inhibitory phosphorylation and also, Wee1 kinases are repressed to further enhance the M-Cdk activity (Lindqvist *et al..*, 2005; Mailand *et al..*, 2002). After nuclear envelop breakdown, sister chromatid condensation

and mitotic spindle formation, cells exit from G2 into M phase. The checkpoint at G2/M transition controls the completion and fidelity of DNA replication. If it detects unreplicated or damaged DNA, it sends negative signals to effector mechanisms of the cell to arrest the entry into mitosis by blocking the activation of M-Cdks (Kimura *et al.*., 2001; Stark and Taylor, 2004).

CyclinB/Cdk1 and CyclinA/Cdk1 drives the cell to the metaphase-to-anaphase transition where another checkpoint controls the spindle assembly and proper attachment of sister chromatids to the spindle. For correct mitosis, this step is essential (Gadde and Heald, 2004). APC becomes active when the last chromatid attaches to the spindle (Wassmann and Benezra, 2001). APC initiates the degradation of securin as well as Cyclin A and B, resulting in the seperation of sister chromatids and exit from mitosis.

1.2 CHROMOSOME DUPLICATION AND ITS CONTROL

1.2.1 Replication of DNA

The eukaryotic chromosome is an huge, durable and compact structure which carries the genetic information of the organism. The accurate and effective transfer of this information from generation to generation is achieved by replication of entire genome in each cell division. Chromosome is duplicated in the S phase of the cell cycle and DNA replication is at the heart of this process that should be once per cell cycle (Scalafani and Holzen, 2007).

DNA replication preparations begin between late M and early G1. Before the replication, double stranded DNA must be unwound by helicases. In early G1, the prereplicative complex (pre-RC) that is formed at the replication origin recruits the Mcm helicase complex (Bell and Dutta, 2002; Teer and Dutta, 2006). In late G1, G1/S and early S phase, S-Cdks are activated and promotes the activation of pre-RC complex. In addition to S-Cdks, Cdc7 activity rises in late G1 and remains high until the exit from mitosis. Cdc7, a protein kinase, is also essential regulators of DNA replication (Donaldson *et al.*., 1998). They phosphorylate the Mcm to activate and lead to

recruitment of DNA polymerases with the aim of primer synthesis, initiation and maintenance of DNA synthesis (Waga and Stillman, 1998).

Additionally, pre-RC complex is assembled once per cycle. The reassembly of pre-RC is prevented by two mechanism: S and M Cdks promote the destruction of individual pre-RC components (Ikui *et al.*., 2007) and inactivation of APC activates geminin that inhibits pre-RC assembly (Zielke *et al.*., 2008). Thereby, DNA replication occurs only once and is limited to S phase with a simple mechanism which is important in terms of the inheritance of constant amount of DNA throughout generations.

1.2.2 Histone Synthesis

Chromosome duplication in the S phase consists of the genomic DNA replication and the histone synthesis. In eukaryotic cells, the newly synthesized DNA is assembled with histone and non-histone proteins into a higher-order structure called chromatin fiber (Osley, 1991). Because the histones are the primary components of chromatin structure, they are mainly involved in DNA packaging, . Besides, its is revealed that they have an important role in regulating gene expression. They can be reshaped, shifted or evicted to change the accessibility of the DNA in case of gene expression, DNA repair or DNA replication (Becker, 2002; Henikoff and Ahmad, 2005).

Histone proteins are encoded by two types of genes: replication-dependent and replication-independent genes. In metezoans the vast amount of histones, called canonical histone proteins, are encoded by replication-dependent genes and they are expressed at high level in the S phase (Marzluff, 2005). Despite that, replication-independent genes encode the variant histones whose synthesis retains outside of S phase; they are not cell cycle regulated. Positioning of both histones on chromatin is important for defining heterochromatin boundaries, marking the active genes, regulating the transcription properly and the epigenetic inheritance (Marzluff, 2008).

1.2.2.1 Cell Cycle Regulated Replication-Dependent Histone Proteins

The fundamental unit of DNA packaging is the nucleosome, in which naked DNA is wrapped around a protein core, called the histone octamer. Because they have a huge genome, eukaryotic cells demand for large amount of histones to package the newly synthesized DNA in the S phase. To meet this demand, histones that will form

nucleosomes are synthesized in the S phase to conincide with the DNA synthesis. These histones are core histones H2A, H2B, H3, H4 as well as linker histone H1 (see Fig. 1.3) and encoded by replication-dependent histone genes.

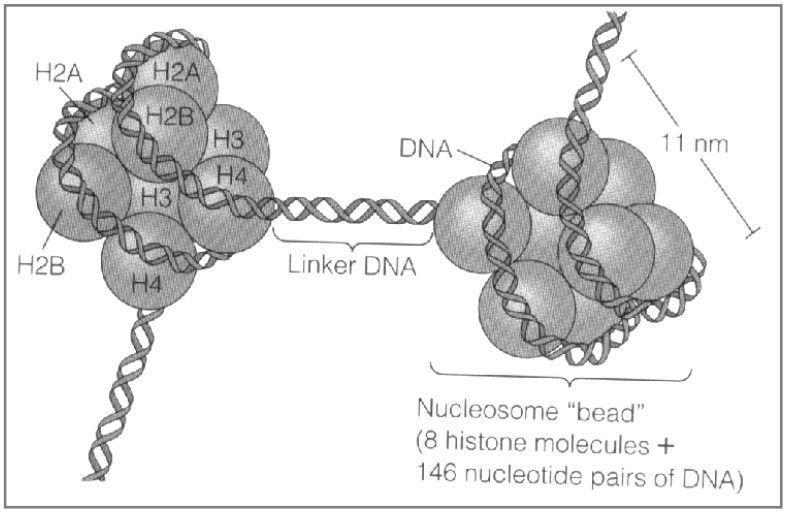

Figure 1.3 The structure of nucleosome (Winona State University webpage).

In order to produce large quantities of histones in a limited time, metezoans have multiple copies of tightly linked histone genes in large clusters which allows the rapid expression in a short time (Marzluff *et al.*., 2002). In early S phase, the amount of histone proteins is increased about 30-35 fold. This is the result of increasing in the amount of histone mRNA which is due primarily to an increasing in the mRNA processing. In early S phase, transcription of histones is increased just ~ 2-3 fold whereas the processing of mRNA is increased ~ 10 fold. So, the histone level is increased ~30 fold. At the end of the S phase, histone mRNA processing is repressed and histone mRNAs are degraded (Harris *et al.*., 1991).

Transcription : When cells enter the S phase, transcription rate of genes encoding canonical histones is increased. At the beginning of the S phase, Cyclin E/Cdk2 phosphorylates NPAT (nuclear protein ataxia-telangiectasia) resulting in enhanced expression of histone genes (Zhao *et al.*., 2000). NPAT is an essential protein to enter to S phase and has a transcription stimulating domain (Ma *et al.*., 2000). But, entry into S

phase is not prevented when this domain is deleted, suggesting that NPAT may have any other role in histone mRNA synthesis (Ye *et al.*., 2003).

Processing: In metezoans, histone mRNAs are unique because of their specific structure. They have 7-methyl-guanosine cap at the 5' end like polyadenylated like polyadenylated mRNAs but they lack introns and polyA tail. Their 3' end contain highly conserved 25-26 nucleotides sequence with a stem-loop structure (see Fig. 1.4) (Dominski and Marzluff, 2007).

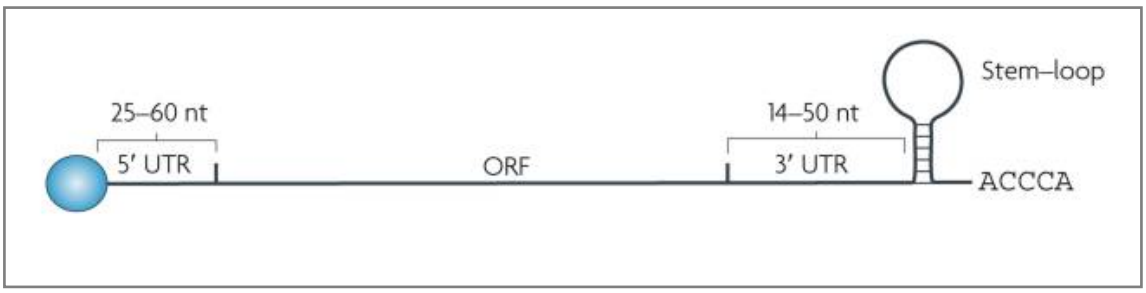

Figure 1.4 The structure of replication-dependent histone mRNA (Marzluff *et al.*., 2008).

Since the replication-dependent histone mRNAs have no intron, the only process for maturation of mRNA is endonucleoytic cleveage of a short piece after stem-loop structure. The cleavage occurs somewhere between stem-loop and histone-downstream elemen(HDE), a purine-rich sequence (Mandel *et al.*., 2007).

Histone mRNA processing starts with the binding of a 31 kD protein called stem-loop binding protein (SLBP) to stem loop structure and then, HDE binds to U7snRNA which has both the HDE complemantary sequence in its 5' end and Sm protein binding site (Scharl and Steitz, 1994). U7snRNA is a component of U7snRNP that contains five Sm protein and two Sm-like protein, LSM11 and LSM10. LSM11 interacts with ZFP100 protein which also contacts SLBP-stem-loop complex (Mowry and Steitz, 1987). Lastly, a cleavage complex containing CPSF73, CPSF100 and several other components that are responsible for the processing of 3' end of the mRNA are recruited by both SLBP and U7snRNP (Marzluff *et al.*., 2008).

Translation: After the pre-mRNAs become mature mRNAs, they are exported from nucleus to cytoplasm for translation. 3' end of the replication-dependent histone mRNAs are required for translation like polyadenylated mRNAs (Gallie *et al.*., 1996). SLBP is also required for the translation of these mRNAs (Sanchez and Marzluff, 2002; Gorgoni, 2005); it binds to histone mRNA and contacts with the SLIP1,which also interacts EIF4G protein (Cakmakci, 2008). These protein-protein interactions circularize the histone mRNA, which means 3' end is brought into proximity of the 5' cap, therefore histone mRNAs are translated efficiently.

Degradation: At the end of the S phase, the demand for histone proteins decreases, for this reason, the level of replication dependent histone mRNAs is reduced by a rapid decreasing in mRNA half-life. In addition to completion of DNA replication, the histone mRNA is degraded when DNA replication is blocked as well (Marzluff 2008). SLBP is required for the degradation of histone mRNAs; it binds to stem-loop which is the cis- element of this degradation. It recruits the proteins that are involved in addition of a short oligo(U) tail to the histone mRNAs (Mullen and Marzluff, 2008). The LSM1-7 heptamer binds to this oligo(U) tail for recruitment of the decapping complex and the exosome in order to degrade the replication-dependent histone mRNAs (Parker and Song, 2004).

As a summary; replication-dependent histone mRNAs are highly cell cycle regulated mRNAs in mammalian cells. There are multiple mechanisms that keep the level of histone mRNAs proper throughout the cell cycle. First of them is enhanced transcription of histone genes just before entry into S phase. Second, the increase in the histone mRNA processing efficiency. And, the third one is change in mRNA half-life and rapid degradation of histone mRNA at the end of the S phase. Moreover, the 3' end of mRNA and SLBP are essential for each proccessing, translation and degradation in histone mRNA metabolism (see Fig. 1.5).

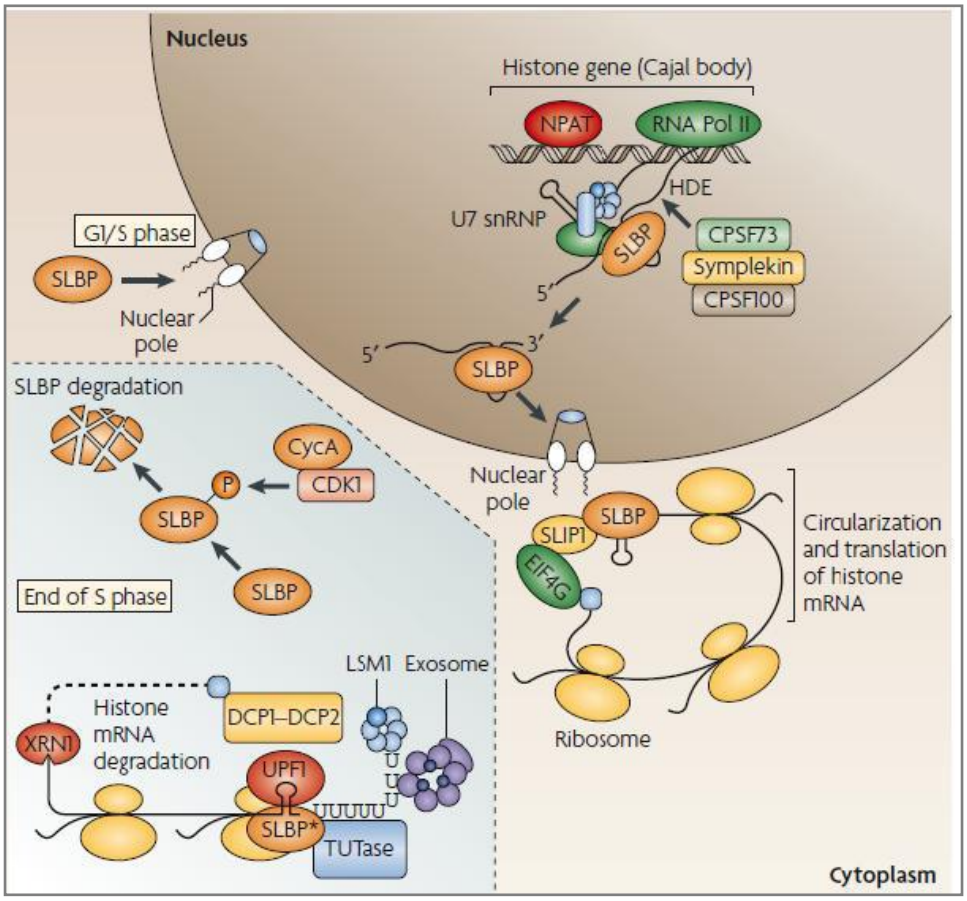

Figure 1.5 The schematic representation of the roles of SLBP in replication dependent histone mRNA metabolism (Marzluff, 2008).

1.2.2.2 SLBP and Its Cell Cycle Regulation

In cycling mammalian cells, SLBP is a cell-cycle regulated protein and involved in multiple steps of histone mRNA metabolism. The level of replication-dependent histone mRNA and SLBP paralel each other throughout the cell cycle but different molecular signals regulate them. Whereas the inhibition of DNA synthesis in the middle of S phase because of any reason, like DNA damage, cause the rapid reduction in the level of histone mRNA; the level of SLBP doesn't affected (Withfield *et al.*., 2004). Moreover, it is shown that the degradation of replication-dependent histone mRNAs doesn't prevented when the SLBP is stabilized at the end of the S phase (Zheng *et al.*., 2003).

The SLBP level reaches the highest in S phase by means of increasing in the late G1 and decreasing at S/G2 border. The SLBP mRNA level vary slightly throughout the cell cycle; the SLBP is regulated by translational and posttranslational mechanisms (Whitfield *et al.*, 2000). At the end of the S phase, the proteosome mediated degradation of SLBP requires two threonin residues of SFTTP motif in the N-terminal and KRKL motif that is a cyclin binding site (Zheng *et al.*, 2003). CyclinA/Cdk1 and CKII initiates the degradation of SLBP at the end of S phase by phosphorylating these threonin residues (Koseoglu *et al.*, 2008). This degradation is an important mechanism that stops the mRNA biosyhthesis, that is, the accumulation of histone mRNAs is prevented until the next S phase (Koseoglu *et al.*, 2010).

The SLBP level is low in G1 due to the low translation efficiency of SLBP mRNA which is increased as cells enter to S phase (Whitfield *et al.*, 2000). In order to clarify the regulation of SLBP in G1, translation efficiency is primarily tested. It is low in early G1 but it becomes similar to S phase level somewhere between early and mid G1 (Koseoglu, 2007). Although it seems that the level of SLBP translation increases to S phase level in mid-G1, the amount of SLBP protein remains low until late G1 or G1/S boundary. Further, it is shown that the SLBP protein is significantly less stable in G1 in comparison to S phase (Koseoglu, 2007). So, these suggest that there is another regulation that keeps the SLBP level low in G1. The rapid degradation of newly synthesized SLBPs in mid-G1 is likely to be the mechanism that keeps the level of SLBP low in this phase. In this dissertation, I developed several tools for investigation of the coding sequence region(s) that are responsible for this regulated degradation. I used constructs which have a series of deletions on coding sequence of SLBP (Figure3.3). By introducing these constructs to HeLa cells, I generated stable cell lines which express different mutants of SLBP. Additionally, I prepared a new construct by mutating the candidate coding sequence region. Since APC/Cdh1 is known as the active E3 ligase in G1, I mutated a putative APC target site (RxxL) in SLBP by using Site-Directed Mutagenesis method as a tool to investigate its role in G1 regulation of SLBP expression.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 List of equipment

Table 2.1 List of equipment.

Orbital Shaker	Hangzhou allsheng instruments	OS-100
Magnetic stirrer and heater	Daihan	Wisestir
Sensitive Balance	PRECISA	
Highly sensitive balance	Sartorius	
Centrifuge 15ml TC	NÜVE	NF800
Centrifuge 1.5 ml	Thermo Scientific	
Western blot apparatus	Hoeffer	
Laminar Flow	ESCO	Type A2
CO2 incubator	Thermo Scientific	
Invert Microscope	Leica	
Ph meter	Thermo Scientific	
-80 C freezer	Daihan	
Refrigerator	BOSCH	

Table 2.1 (continued)

Ice machine	Scotsmann	
Incubator Shaker	Thermo Scientific	
Pipette set	Denville	X3000i
Pipette gun	Drummond	
KODAK film casette	KODAK	
Refriderated centrifuge	Thermo Scientific	
ELISA reader	BIOTEK	
Spectrophotometer	Shimadzu	UV-1700
Distilled water machine	Millipore	
Autoclave	ALP	
Vortex	BIOSAN	
Benchtop mini centrifuge	Wisd	
Flow Cytometry	FACS Calibur	
Nanodrop	Thermo Scientific	

2.1.2 List of Chemicals

Table 2.2 List of Western Blot Chemicals.

Acrylamide/Bis solution (30%)	Bio-Rad	161-0156
Tris	Wisent INC	600-127-LG
Glycine	Merck	5.00190.1000
TEMED	BIOMATIK	A4008-100ml
Ammonium per sulfate	Sigma-Aldrich	31117-1kg
SDS loading buffer	Fermentas	R0891
Protein Ladder	Pierce	26619
SDS	Merck	
NP-40	Applichem	A1694,0250
Tween-20	Sigma-Aldrich	P1376-100ml
Phenylmethanesulfonyl fluoride (PMSF)	Sigma	P7626-1G
P-coumeric acid	Sigma	C9008-10G
Luminol	Sigma	A8511-5G
Ponceu-S	Sigma-Aldrich	P3504-10G
Coomassie Brilliant Blue R-250	Bio-Rad	161-04000
Sodium Hydroxide pellets	Merck	1.06482.1000
Sodium Chloride	Merck	1.06404.1000
KODAK X ray film		
ROCHE PVDF membrane		03010040001
Complete mini EDTA-free protease inhibitor cocktail	Roche	04693159001
Isopropanol	EMBOY	
Bio-Rad Protein Assay	Bio-Rad	500-0006
Gel Blot Papers	Whatman	GB003
Fixer	KONIX	

Table 2.2 (continued)

Developer	KONIX	
Hydrogen Peroxide	Sigma	H1009
Acetic acid	Merck	1,00063,2511
Methanol, HPLC grade	Merck	1.06009.2500
Lumi-Light plus western blotting substrate	Roche	12 015 196 001
Lumi-Light western blotting substrate	Roche	12 015 200 001
NaF	Sigma-Aldrich	SK.S.30105-250G
KCl	Sigma-Aldrich	SK.R.12636-1 KG
NaCl	Sigma-Aldrich	SK.R.13423-1 KG
Na_2HPO_4	Sigma-Aldrich	S3264
KH_2PO	Sigma	P9791
Nitrocellulose membrane	Amersham	RPN303D
Not fat dry milk	Bought from grocery	

Table 2.3 List of Tissue Culture Chemicals.

DMEM/high Glucose	Hyclone	SH30243.01
PBS	Hyclone	SH30256.01
FBS	LONZA	DE14-802F
Penicillin/Streptomycin	HyClone	SV30010
Trypsin	LONZA	CC-5012
Lipofectamine2000 reagent	Invitrogen	11668-027
Dimethyl Sulphoxide	Sigma	D2650
Thymidine	Sigma	T1895-5G
Nocodazole	Sigma	M1404-10MG
Geneticin,liquid	Invitrogen	10130 035
Geneticin, powder	Invitrogen	11811 031
RNAase	Sigma	R6513
PI Stain	Abcam	Ab14083

Table 2.4 List of Chemicals for Bacteria Culture and Cloning.

LB agar	Merck	1.10283.0500
LB broth	Merck	1.10285.0500
Ampicillin	Sigma	A0166-25G
Gene-jet miniprep	Fermentas	K0503
$RbCl_2$	Sigma	83-979-25 G
Potassium Acetate	Sigma	SK.S.25059-1KG
$CaCl_2$	Sigma	SK.R.12022-1KG
$MnCl_2$	Sigma	M3634
Glycerol	Sigma	15524-IL
MOPS	Sigma	M1254
NaOH	Sigma	SK.S.38215-1EA
HCl	Sigma	SK.R.30721-2,5L

Table 2.5 List of constructs.

His SLBP in pcDNA3.1(-) (SFTTP)	Koseoglu LAB
His SLBP in pcDNA3 Δ55N (SFTAP)	Koseoglu LAB
His SLBP in pcDNA3 Δ55-125N	Koseoglu LAB
His SLBP in pcDNA3.1(-) (SFTAP)	Constructed by Umida Djakbarova

Table 2.6 List of Antibodies.

SLBP	Serum (Rabbit)
Goat anti rabbit IgG HRP	Millipore (AP132P)
SLBP (H-3)	Santa-Cruz (sc-376310)
Cdk2 (M-2)	Santa-Cruz (sc-163)

2.2. METHODS

2.2.1 Cell Culture

2.2.1.1 Subculture of HeLa Cells

Hela cells were grown in DMEM/High glucose media containing 10% FBS and 1% penicilin/streptomycin. They were subcultured in 2-3 days when their confluency reach the 80-90% to prevent overconfluency. The old media of cells were removed and cells were washed with PBS. The cells were detached by trypsinization with enough amount of trypsin and incubated at 37 °C for 4-5 minutes. To neutralize the activity of trypsin, media containing FBS was added and cells were seeded into a new plate in proper ratio. The number of passage did not exceed 40.

2.2.1.2 Cell Collection

Hela cells were detached from the plate by trypsinization and transferred to a tube after neutralization for centrifugation. Cells were pelleted at 200g for 5 minutes and washed with PBS by spinning again. All the supernatant was removed. The pellets were used immediately or stored at -80 °C until usage.

2.2.1.3 Cell Lysis

Cells were collected by following the cell collection protocol. The cell pellet was dissolved in Lysis Buffer that comprises of 150mM NaCl, 50mM Tris, Ph: 7.5 and 1% NP-40. The protease inhibitor cocktail (1X) and PMSF (1mM) were added freshly. The pellet was lysed at cold for 30 minutes by shaking at 220 rpm and then, the lysate was cetrifuged at maximum for 10 min at 4 °C in order to eliminate the insoluble materials. The supernatant was collected and stored at -80 °C.

2.2.1.4 Cell Freezing

Hela cells were detached by trypsinization and collected by spinning them at 200g for 5 minutes. The pellet was washed with PBS and spinned again. The pellet was dissolved in 1 ml of DMEM media with 5% DMSO, 20% FBS in specific cryovial tubes. They were frozen by gradual temperature decrease and stored at -80 ºC.

2.2.1.5 Cell Thawing

The Hela cells at -80 ºC were thawed at 37 ºC water bath and immediately transferred to plate containing 10ml of DMEM/High Glucose with 20% FBS and 1% pen/strep. The media was refreshed after cells were attached to the surface of the plate. Hela cells have been passaged for 5-6 times and considered as ready for experiments. Namely, they have been given enough time for recovery from freezing.

2.2.2 Transfection

Cells were seeded that would be at 90% confluency at the day of transfection in 24-well plate. 0,4 µg of DNA was diluted in 50 µl OPTIMEM for each well. –DNA (No DNA in OPTIMEM) was used as a negative control. 1 µl of Lipofectamine2000 reagent was also diluted in 50 µl OPTIMEM for per well. They were incubated for 6-7 minutes at room-temperature(RT) and then, the equal amount of DNA and Lipofectamine2000 solutions were mixed. The mixture was incubated for 20-25 minutes at RT. During this incubation, the media of plates was changed with the media without antibiotic (DMEM+ 10% FBS). The mixture was added to the plates drop by drop. After 4-6 hours, the media of plates was changed with the normal media including pen/strep. The cell were subcultured after 24 hours and collected after 48 hours of transfection to observe the transgene expression.

2.2.3 Generation of Stable Cell Lines

Hela cells were transfected with 0,4 µg of constructs containing wild-type and deleted forms of hSLBP. After 48 hours of transfection, 1/3 of cells were collected for testing the SLBP expression and 2/3 of cells was seeded again for selection of transfected cells and incubated at 37 °C. After a day, when cells were attached to the surface of the plate, 600 mg/ml of selective antibiotic geneticin (G-418) was added to both transfected and –DNA cells. The media was refreshed 1 in 3 days or cells were splitted if needed until the all cells in –DNA plate were died. As a result of selection, cell colonies are observed in plates of transfected cells under invert microscopy. These colonies of cell were pooled again and tested for SLBP expression.

2.2.4 Bacterial Culture

2.2.4.1 Generation of Competent Cell

DH5α cells were strored by freezing in 1 ml autoclaved 15% glycerol at –80 °C. All materials used for generation of compotent cell were kept cold. Small piece of DH5α cells were grown in 3 ml LB broth overnight at 37 °C. 1 ml from this culture is subcultured into 100 ml of LB broth and grown until OD reaches 0.4-0.6 at 600 nm. Cells are pelleted at 4 ºC centrifuge at maximum speed for 10 minutes and TFbI buffer (see Table 2.7) is added. Solution is spinned and TFbII buffer (see Table 2.8) is added. It is aliquited and stored -80 °C.

Table 2.7 Content of TFbI buffer.

30Mm Potassium Acetate	1.465 gr
100Mm RbCl2	6.045 gr
10Mm CaCl2	0.735 g
50Mm MnCl2	4.954 g
15% Glycerol	75 ml

*400 ml of dH2O was added. The pH was adjusted to 5.8 with acetic acid. The volume was brought to 500 ml. Sterilized with 0.2 μm filter.

Table 2.8 Content of TFbII buffer.

10Mm MOPS	0.210 g
10Mm RbCl2	0.121 gr
75Mm CaCl2	1.100 g
15% Glycerol	75 ml

*75ml of dH2O was added and pH was adjusted to 6.5. The solution was sterilized with 0.2 μm filter.

In order to estimate the efficiency of compotent cells, they were transformed different concentration of DNA as it is mentioned in section 2.2.4.2. And the colonies on the plates containing ampicillin are counted and efficiency of competent cells was calculated in terms of CFU/µg.

2.2.4.2 Transformation

50 µl competent cells was thawed on ice and 1 µl DNA was added. After 30 minutes incubation on ice, the mixture was heat shocked at 42 °C for 45 seconds followed by 2 minutes incubation on ice. 950 µl of LB broth was added to competent cells and incubated at 37 °C with 225 rpm agitation for 45 minutes. 50 µl of culture was spreaded on LB agar plates containing 100 µg/ml of Ampicillin. After 16 hours, the colonies were observed and the plates were stored at 4 °C.

2.2.4.3 Miniprep

A single colonis after transformation was chosen and grown in 3 ml of LB broth for no more than 16 hours at 37 °C. After the incubation, Gene-jet miniprep kit (Fermentas) was used and the manufacturer's protocol was followed to isolate the plasmid DNA. The concentration of the DNA was measured by Nanodrop.

2.2.5 Generation of AWSA from RWSL by Site Directed Mutagenesis Kit

In order to generate two amino acid mutations, Site Directed Mutagenesis kit (Stratagene) was used. The procedure is summarized in Figure 2.2. Primers (see Table 2.9) were designed for Arg26 ; Leu29 → Ala26 ; Ala 29 conversion from his-SFTAP SLBP in pcDNA3.1 template.

Table 2.9 Primers for AWSA construct generation.

	Tm	Primer Length	Sequence
Forward Primer	>75	36	CCGTCCCCCGCGGCATGGAGCGCGGGACGGAAGCGC
Reverse Primer	>75	36	GCGCTTCCGTCCCGCGCTCCATGCCGCGGGGGACGG

The primers were diluted and aliquited. The PCR reaction was set according to the Table 2.10. The PCR conditions are also shown in Table 2.11

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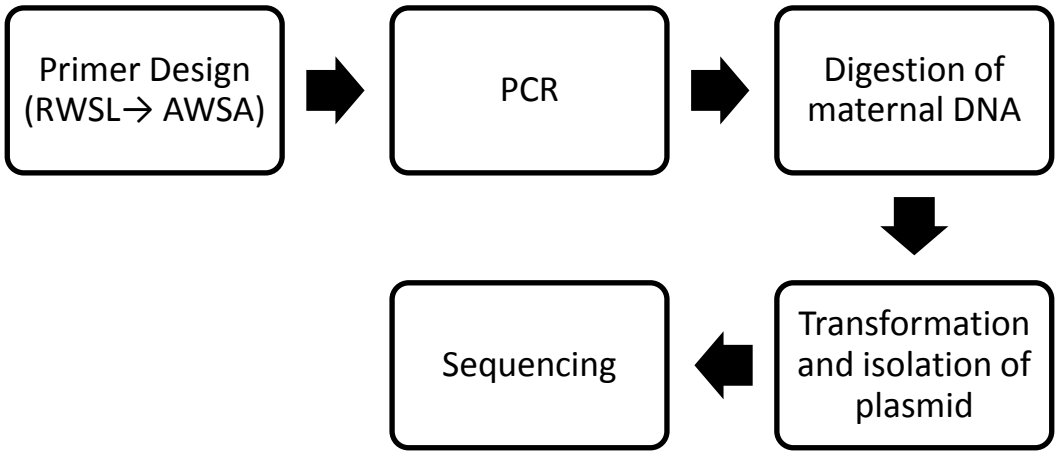

Figure 2.1 The schematic illustration of site directed mutagenesis reaction.

Table 2.10 PCR reaction components.

	Control	RWSL→ AWSA
10X Reaction Buffer	5 µl	5 µl
dsDNA Template	2 µl	2 µl
125 ng Forward Primer	-	5 µl
125 ng Reverse Primer	-	2.5 µl
dNTP	1 µl	1 µl
ddH_2O	42 µl	34.5 µl
Pfu ultra polymerase (2.5 U/ µl)	1 µl	1 µl
Total	51 µl	51 µl

Table 2.11 PCR conditions.

Cycles	Temperature	Time
1	95 ºC	30 sec
22	95 ºC	30 sec
	52 °C	1 min
	68 °C	10 min
1	68 °C	10 min
1	4 °C	Infinite

In order to digest parental DNA in amplification reaction, 1 µl of DpnI was added and incubated for 1.5 hour at 37 ºC. 10 µl of each reaction was run in a 1% agarose gel at 80 V. The bands were visualized under UV.

2.2.5.1 Transformation of AWSA Construct

4 µl of amplification reaction was used to transform 100µl of homemade competent cells as mentioned in 2.2.4.2 section and the cells were pelleted and spreaded on antibiotics containing plates. Several colonies were chosen and plasmids were isolated for sequencing.

2.2.6 Western Blotting

2.2.6.1 Optimization of The Measurement of Protein Concentration

Protein concentrations were measured by Bradford Assay. Either Thermo Coomassie Plus Reagent or BIORAD Protein Assay solution were used. Bio-rad solution was 4 times diluted with dH_2O before use. Standarts were prepared according to manufacturer's protocol. Different dilution series (ratio of samples to bradford reagent) were tried as showed in Table 2.12. In order to measure the OD values of samples, ELISA reader, Nanodrop and Spectrophotometer were used.

Table 2.12 Dilution series, reagents and devices used for Bradford Assay.

Reagent	Dilution (Sample/Reagent)	Device
Thermo CPR	1:50	Nanodrop
Thermo CPR	1:30	Nanodrop
Thermo CPR	10:300	ELISA reader
Thermo CPR	3:300	ELISA reader
Thermo CPR	10:990	Spectrophotometer
Thermo CPR	5:995	Spectrophotometer
Bio-rad	5:995	Spectrophotometer

2.2.6.2 Preparation of 12% SDS Gel

The two glass plates had washed with warn water carefully until they had any blurrines and rinsed with distilled water followed by 70% ethanol. According to the size of the glasses, proper volume of 12% SDS-Polyacrylamide gel were prepared by mixing the solutions on Table 2.13.

Table 2.13 Solutions for Seperating Gel.

12%SDS-PAGE gel	8 ml (ml)	15 ml (ml)	30 ml (ml)
Distilled H_2O	2.7	5	9.9
Acrylamide/Bis	3.2	6	12
1.5 M Tris, pH: 8.8	2	3.8	7.5
10% APS	0.08	0.15	0.3
10% SDS	0.08	0.15	0.3
TEMED	0.0035	0.006	0.012

After pouring the seperating gel, 1-2 ml isoproponal was added to smothen the surface and the gel was left to polymerize at least one hour. After the polymerization of seperating gel, the isopropanol was removed and washed with dH_2O and 70% ethanol. The appropriate amount of stacking gel (see Table 2.14) was prepared, combs were placed and the gel was left for polymerization one hour more.

Table 2.14 Solutions for Stacking Gel.

	2 ml (ml)	3 ml (ml)	8 ml (ml)
Distilled H_2O	1.4	2.1	5.5
Acrylamide/Bis	0.33	0.5	1.3
1 M Tris, pH: 6.8	0.25	0.38	1.0
10% APS	0.02	0.03	0.08
10% SDS	0.02	0.03	0.08
TEMED	0.0002	0.0003	0.0008

2.2.6.3 Preparation of Samples

It was calculated that how much volume of samples must be loaded. They were aliquited and proper amount of 5X Loading Buffer was added. The mixture was boiled for 5 minutes at 100 °C, spinned for 1 minute and brought the room temperature.

2.2.6.4 Running The SDS-PAGE

When the loading of marker and samples was completed, the gel running system was assembled. 1X running buffer (see Table 2.15) was added to upper chamber and no leaking was ensured. This system was placed into the big chamber and running buffer was added till maximum label. The protein had run at constant amper until the loading dye was left the gel.

Table 2.15 The composition of Running Buffer.

	1X
Tris	6.04 g
Glycine	28.8 g
SDS	2 g
dH_2O	Fill to 2 L

*pH should be ~8.3

2.2.6.5 Semi-dry Transfer

According to the gel area that would be transferred, equal size of membrane and Watmann papers were cut. The membrane was activated by leaving it in HPLC grade methanol (this step is just for PVDF); the gel, membrane and Watmann papers were soaked in Towbin transfer buffer (see Table 2.16) for equilibration at least 5 minutes.

Table 2.16 Recipe for Towbin Buffer

25 mM Tris	3 g
192 mM Glycine	14.4 g
SDS	1 g
dH_2O	Fil up to 800 ml

*pH should be around 8.2-8.4. 20% methanol was added freshly.

The system was assembled as seen in the Figure 2.2 and run at proper mA that was calculated by multiplying the surface area of gel and 0,8 at least 1 hour.

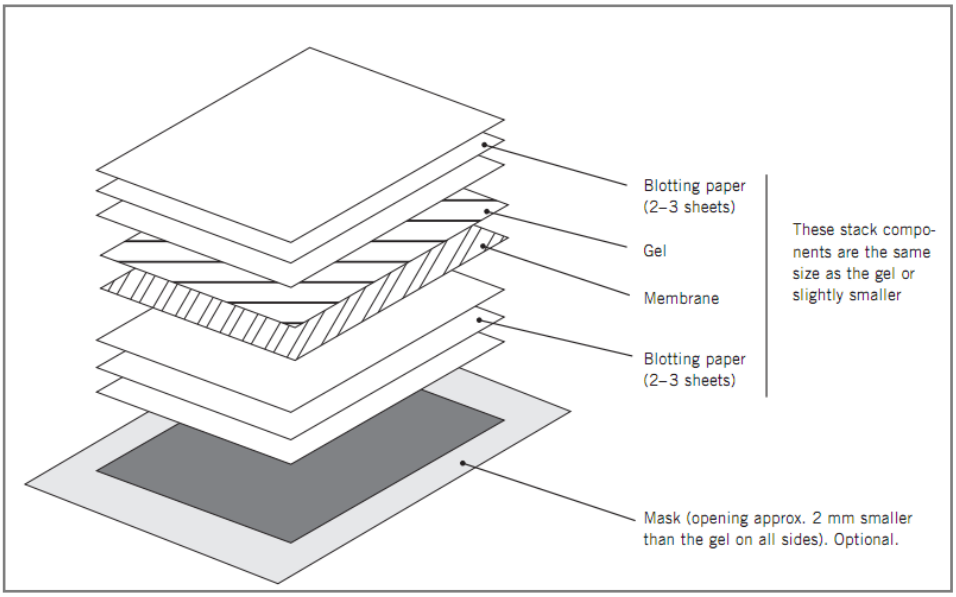

Figure 2.2 The placement of semidry component during transfer (Hoefer TE70X Semidry Blotters instruction manual)

2.2.6.6 Wet Transfer

The membrane, gel and papers were prepared as it is explained in semidry transfer (see section 2.2.6.5). The wet transfer system has a sandwich-like casette that carry the transfer stack. After the casette was loaded, it was placed into the casette tank and fill with the transfer buffer, the outer chamber as well. The system was run at 340 mA for 1 hour or overnight at 90 mA.

2.2.6.7 Staining/ Destaining of Membrane and Gel

After transfer, the membrane was taken into Ponceu-S immediately. It was stained at least 6-7 minutes by shaking and destained with dH_2O. In the same time, the gel was stained with Coomassie Brilliant Blue (CBB) for 20-25 minutes by shaking to check complete transfer. It was destained with destaining solution containing 10% acetic acid and 20% methanol.

2.2.6.8 Blocking and Antibody Incubations

The membrane was blocked with 5% milk in PBS-t solution for 2 hours at RT or ON at 4 °C. The PBS-t was prepared by adding of 0.1% Tween-20 to PBS. Blocking solution was also used for primary and secondary antibody incubations. The membrane was incubated with primary antibody in blocking solution for 2 hours at RT or ON at 4 °C by shaking. After primary incubation, the membrane was washed with PBS-t for 10 minutes (3 times) in order to eliminiate non-specific binding of primary antibodies. Secondary antibody was diluted in blocking solution as well and incubated with membrane for 45 minutes at RT. The washing steps were repeated as described for primary antibody.

2.2.6.9 ECL and Development of Film

2.2.6.9.1 Homemade and Commercial ECL

The membrane was incubated with commercial ECL for between 5-30 minutes, exposed to film for short period of time and immediately used for developing. Homemade ECL (Solution A: 250 mM Luminol, 90 mM p-coumaric acid, 100 mM Tris pH: 8.6, Solution B: 30% H2O2 and 100 mM Tris ph: 8.6)) was prepared freshly. The A and B solutions were mixed 1:1 ratio and the mixture was incubated with the membrane for 1 minute before film exposure for at least 15 minutes.

2.2.6.9.2 Film Development

Developer and Fixer solutions were prepared as 1X from 4X stock solutions freshly and warmed to 30 °C before use. After the ECL incubation, the membrane was placed into film casette in which film was exposed. And then, the film was incubated in developer (3-4 min.) - water (1 min.) – fixer (3-4 min.) – water respectively.

2.2.7 Synchronization

2.2.7.2 Nocodazole Synchronization

In order to optimize the appropriate nocodazole concentration, Hela cells at confluency of 60% were treated with 100 ng/ml, 75 ng/ml, 50 ng/ml of nocodazole for

16 hours. After treatment cells were collected and seeded again as mentioned previously.

2.2.7.3 Thymidine – Nocodazole Synchronization

Hela cells at confluency at 30-40% were treated with 2 mM thymidine for 22 hours. After 1 hour of release, they were treated with 75 ng/ml nocodazole for 12 hours. The mitotic cells were removed from plates and reseeded to release from mitosis.

2.2.8 Flow Cytometry

After cells have been collected they were mixed very gently in order to prevent clusters. They were fixed in 4.5 ml of cold 70% Ethanol and stored at -20 ºC. Cells were pelleted and washed with PBS. Cells were incubated at 37 ºC for 30 minutes with staining solution, PI/RNAase (25 μg/ml PI and 200 μg/ml RNAase A). FACS analysis was carried out by FACS CALIBUR from BOUN AKIL LAB. The data was evaluated by CellQuest software program.

CHAPTER 3

RESULTS

3.1 OPTIMIZATION STUDIES

In the first part of my thesis, I optimized some methods that were going to be used in the investigation of G1 regulation of SLBP. Firstly, in order to verify transient transfections, stable cell lines and several studies, I optimized Western Blotting (WB). In addition, in order to study the changes in protein level throughout the cell cycle, cells should be synchronized at the same phase of the cell cycle. Therefore, I tried different synchronization methods to optimize the protocol and confirmed by FACS.

3.1.1 Optimization of Western Blotting Technique

The Western Blotting technique had been optimized before the main experiments were performed. Firstly, I had problems in equal loading of samples. Since I investigate the degradation of hSLBP, equal loading of samples is a crucial step for my experiments. So, I decided to find out the reason of this and optimize the measurement of total protein concentration. I tried to use different dilution series (sample/reagent), bradford reagents and also devices as listed in Table 3.1. According to the results, I decided to measure the protein concentration of lysates by using Bio-rad reagent at 5:995 (s:r) dilution with spectrophotometer.

The western blotting is a multistep technique and each step is as important as the others. In addition to equal loading, another important step is the transfer of protein from gel to membrane. For this step, I used transfer buffer with no SDS and observed higher transfer efficiency when the membrane was dyed with Ponceu-S after transfer of the proteins from gel to membrane.

Table 3.1 Results of optimization experiments for Bradford Assay.

Reagent	Dilution (Sample/Reagent)	Device	Results
Thermo CPR	1:50	Nanodrop	Negative OD values
Thermo CPR	1:30	Nanodrop	Not equal in WB
Thermo CPR	10:300	ELISA reader	Negative OD values
Thermo CPR	3:300	ELISA reader	Not equal in WB
Thermo CPR	10:990	Spectrophotometer	~ Equal in WB
Thermo CPR	5:995	Spectrophotometer	~ Equal in WB
Bio-rad	5:995	Spectrophotometer	Equal in WB

Furthermore, dilution and incubation times of antibodies were optimized. I tried to find the proper dilution for the primary and secondary antibodies I have. Figure 3.1 is an example result of optimized WB for hSLBP and Cdk2. The nonspecific bands of SLBP serum was used as a loading control, since their expression do not change throughout the cell cycle.

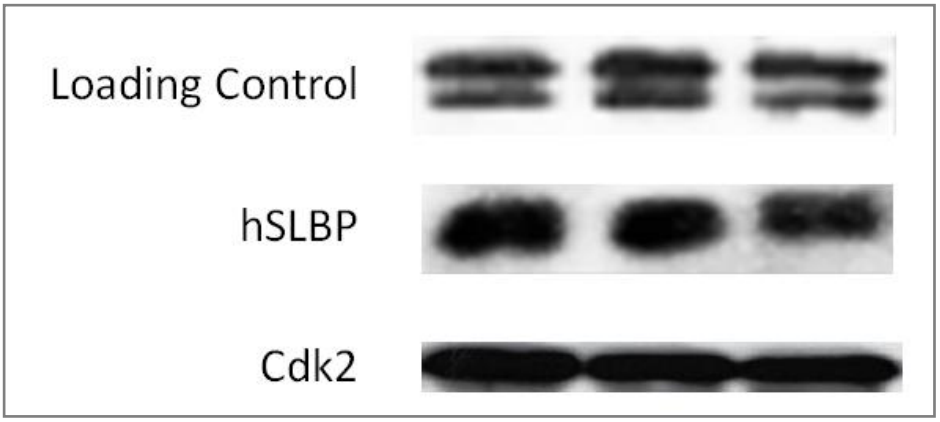

Figure 3.1 WB analysis of three different Hela lysates for hSLBP and Cdk2. Loading control corresponds to a nonspecific band that is recognized by hSLBP serum.

3.1.2 Optimization of Cell Synchronization

Since I investigated the regulation of SLBP in G1, I arrested the cells at M phase and release them to G1. Nocodazole is used to arrest the cells at M phase by interfering with polymerization of microtubules that are responsible for formation of metaphase spindle and cell shape. Therefore, nocodazole-treated cells can not form metaphase spindles but can be observed with their characteristic rounded shape (indicating the cells are in M phase) under invert microscopy. So as to find the appropriate concentration of nocodazole, I incubated the cells with 100 ng/ml, 75 ng/ml and 50 ng/ml of nocodazole for 16 hours and observed. The results (see Table 3.2) were evaluated according to the ratio of cells in mitosis and dead cells. 75 ng/ml was decided to be optimum concentration for synchronization with nocodazole.

Table 3.2 The effect of different concentrations of nocodazole on the ratio of cells in mitosis and dead cells.

Nocodazole Concentration	Cells in mitosis	Dead cells
100 ng/ml	60 %	30 %
75 ng/ml	50-60 %	20 %
50 ng/ml	50-60 %	15-20 %

To release the cells into G1 after nocodazole treatment, cells were washed and re-plated with fresh media. The mitotic cells are round in shape and can collected easily by hiting on the edge of plate. Initially, I washed the cells with PBS to remove the all nocodazole. Then, I collected the cells into a tube with fresh media by knocking and spinned them to re-plate. But this method leads to massive cell death. To solve this problem, I washed the cells with PBS before I detached them from the plate, collected them into e tube with fresh media and re-plate them without need any centrifugation

step. As a result, washing with no spin prevented cells from death after releasing from nocodazole.

In addition to nocodazole alone synchronization, I tried to use the thymidine-nocodazole method to reduce the exposure time of nocodazole. Following the thymidine treatment and release, cells were treated with nocodazole for 12 hours. The FACS results (see Figure 3.2) showed that the cell cycle distribution of synchronized and asynchronized (AS) Hela cells.

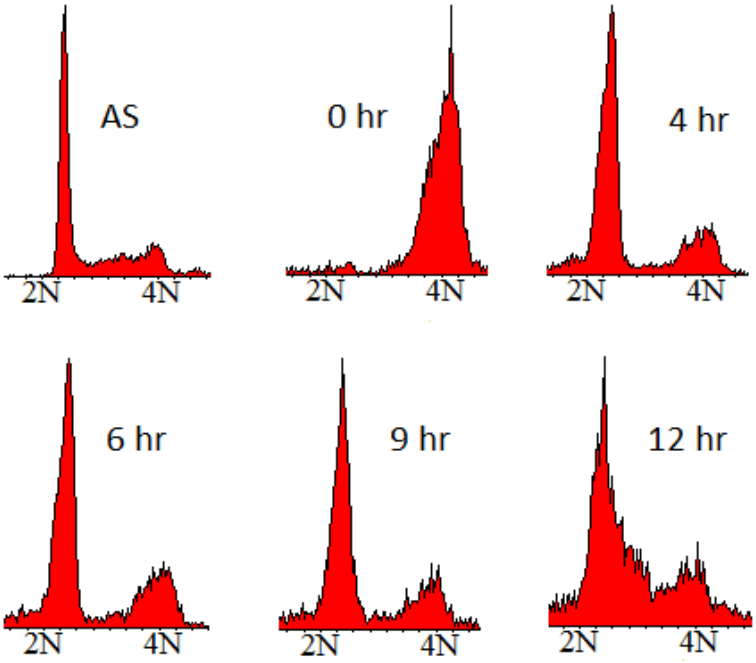

Figure 3.2 FACS analysis of Thymidine-Nocodazole synchronized cells. Cells are synchronized at G2/M border and released. AS indicates asynchrynous cells. At 0 hr, almost all cells seem to be at G2/M and begin to seem in G1 4 hrs after release.

3.2 ESTABLISHMENT OF STABLE CELL LINES

In order to find out coding sequence regions regulating the SLBP degradation in G1, I established the stable cell lines which express wild-type or deletion-mutant form of SLBP.

3.2.1 Verification of The Constructs

To determine the possible region which is associated with the regulated degradation of SLBP in G1, I used several constructs that have different deletions on coding sequence of SLBP as represented in Figure 3.3. Wild-type SLBP has SFTTP aminoacid sequence that is required for its degradation in S/G2 border. By mutating the second threonin (Thr61) residue in SFTTP motif into SFTAP, S/G2 degradation was blocked. So, studying G1 phase degradation independent of S/G2 degradation becomes possible. Before the constructs were used, they had been sequenced to verify their DNA sequence (by IONTEK Company, Turkey). They were sequenced from 5' and 3' by using Sp6 and T7 primers. In Figure 3.4, the results of sequence analysis; in Figure 3.5, protein alignments of deleted and WT SLBP are shown.

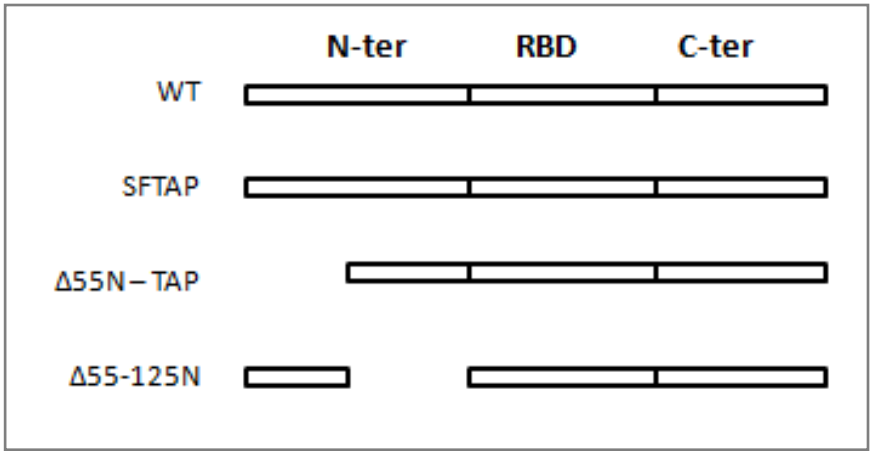

Figure 3.3 Schematic representation of the constructs.

```
                                   1                                         81
SLBP ORF       -------------------ATGGCCTGCCGCCCGCGAAGCCCGCCGAGGCATCAGAGCCG
Delta55N-TAP   ------------------------------------------------------------
Delta55-125N   -------------------ATGGCCTGCCGCCCGCGAAGCCCGCCGAGGCATCAGAGCCG
SFTAP          -------------------ATGGCCTGCCGCCCGCGAAGCCCGCCGAGGCATCAGAGCCG

               82                                                       121
SLBP ORF       CTGCGATGGTGACGCCAGCCCGCCGTCCCCCGCGCGATGGAGCCTGGGACGGAAGCGCAG
Delta55N-TAP   ------------------------------------------------------------
Delta55-125N   CTGCGATGGTGACGCCAGCCCGCCGTCCCCCGCGCGATGGAGCCTGGGACGGAAGCGCAG
SFTAP          CTGCGATGGTGACGCCAGCCCGCCGTCCCCCGCGCGATGGAGCCTGGGACGGAAGCGCAG

               122                                                      161
SLBP ORF       AGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCACCGCGGCGCCGA
Delta55N-TAP   --------------------------ATGGCTCCCATCACCATCACGCAAGCTTGGTACC
Delta55-125N   AGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCACCGCGGCGCCGA
SFTAP          AGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCACCGCGGCGCCGA

               162                                                      221
SLBP ORF       GCGCAGACCCGAGAGCTTTACCACTCCTGAAGGCCCTAAACCCCGTTCCAGATGCTCTGA
Delta55N-TAP   GCGCAGACCCGAGAGCTTTACCGCTCCTGAAGGCCCTAAACCCCGTTCCAGATGCTCTGA
Delta55-125N   GCGC--------------------------------------------------------
SFTAP          GCGCAGACCCGAGAGCTTTACCGCTCCTGAAGGCCCTAAACCCCGTTCCAGATGCTCTGA

               222                                                      281
SLBP ORF       CTGGGCAAGTGCAGTTGAAGAAGATGAAATGAGGACCAGAGTTAACAAAGAAATGGCAAG
Delta55N-TAP   CTGGGCAAGTGCAGTTGAAGAAGATGAAATGAGGACCAGAGTTAACAAAGAAATGGCAAG
Delta55-125N   ------------------------------------------------------------
SFTAP          CTGGGCAAGTGCAGTTGAAGAAGATGAAATGAGGACCAGAGTTAACAAAGAAATGGCAAG

               282                                                       341
SLBP ORF       ATATAAAAGGAAACTCCTCATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAG
Delta55N-TAP   ATATAAAAGGAAACTCCTCATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAG
Delta55-125N   ------------------------------------------------------------
SFTAP          ATATAAAAGGAAACTCCTCATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAG

               342                                                      401
SLBP ORF       TTCTGATTCAAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTGT
Delta55N-TAP   TTCTGATTCAAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTGT
Delta55-125N   -------------------------------CCGGCTGACTTTGAGACAGATGAAAGTGT
SFTAP          TTCTGATTCAAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTGT

               402                                                      461
SLBP ORF       CCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCCTACGATCGTTA
Delta55N-TAP   CCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCCTACGATCGTTA
Delta55-125N   CCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCCTACGATCGTTA
SFTAP          CCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCCTACGATCGTTA

                462                                                     521
SLBP ORF       TATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCATTCATCCCAAGACCCCTAATAA
Delta55N-TAP   TATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCATTCATCCCAAGACCCCTAATAA
Delta55-125N   TATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCATTCATCCCAAGACCCCTAATAA
SFTAP          TATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCATTCATCCCAAGACCCCTAATAA

               522                                                      581
SLBP ORF       ATTTAAGAAGTATAGTCGACGTTCATGGGACCAGCAAATCAAACTCTGGAAGGTGGCTCT
Delta55N-TAP   ATTTAAGAAGTATAGTCGACGTTCATGGGACCAGCAAATCAAACTCTGGAAGGTGGCTCT
Delta55-125N   ATTTAAGAAGTATAGTCGACGTTCATGGGACCAGCAAATCAAACTCTGGAAGGTGGCTCT
SFTAP          ATTTAAGAAGTATAGTCGACGTTCATGGGACCAGCAAATCAAACTCTGGAAGGTGGCTCT

               582                                                       641
SLBP ORF       GCATTTTTGGGATCCTCCAGCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGA
Delta55N-TAP   GCATTTTTGGGATCCTCCAGCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGA
Delta55-125N   GCATTTTTGGGATCCTCCAGCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGA
SFTAP          GCATTTTTGGGATCCTCCAGCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGA
```

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                642                                                     701
SLBP ORF        CCTTGAATCTGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATGT
Delta55N-TAP    CCTTGAATCTGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATGT
Delta55-125N    CCTTGAATCTGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATGT
SFTAP           CCTTGAATCTGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATGT

                702                                                     761
SLBP ORF        GTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAGGATGAGTTTGA
Delta55N-TAP    GTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAGGATGAGTTTGA
Delta55-125N    GTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAGGATGAGTTTGA
SFTAP           GTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAGGATGAGTTTGA

                762                                             813
SLBP ORF        TTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTCAGCCATGAGCTAA
Delta55N-TAP    TTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTCAGCCATGAGCTAA
Delta55-125N    TTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTCAGCCATGAGCTAA
SFTAP           TTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTCAGCCATGAGCTAA
```

Figure 3.4 Nucleotide alignment of constructs.

```
SLBP              (1)  MACRPRSPPRHQSRCDGDASPPSPARWSLGRKRRADGRRWRPEDAEEAEH
Delta55N-TAP           ----------------------------------------MGSHHHHA
Delta55-125N           MACRPRSPPRHQSRCDGDASPPSPARWSLGRKRRADGRRWRPEDAEEAEH
SFTAP                  MACRPRSPPRHQSRCDGDASPPSPARWSLGRKRRADGRRWRPEDAEEAEH

SLBP              (51) RGAERRPESFTTPEGPKPRSRCSDWASAVEEDEMRTRVNKEMARYKRKLL
Delta55N-TAP           SLVPRRPESFTAPEGPKPRSRCSDWASAVEEDEMRTRVNKEMARYKRKLL
Delta55-125N           RGAER-------------------------------------------
SFTAP                  RGAERRPESFTAPEGPKPRSRCSDWASAVEEDEMRTRVNKEMARYKRKLL

SLBP             (101) INDFGRERKSSSGSSDSKESMSTVPADFETDESVLMRRQKQINYGKNTIA
Delta55N-TAP           INDFGRERKSSSGSSDSKESMSTVPADFETDESVLMRRQKQINYGKNTIA
Delta55-125N           ------------------------PADFETDESVLMRRQKQINYGKNTIA
SFTAP                  INDFGRERKSSSGSSDSKESMSTVPADFETDESVLMRRQKQINYGKNTIA

SLBP             (151) YDRYIKEVPRHLRQPGIHPKTPNKFKKYSRRSWDQQIKLWKVALHFWDPP
Delta55N-TAP           YDRYIKEVPRHLRQPGIHPKTPNKFKKYSRRSWDQQIKLWKVALHFWDPP
Delta55-125N           YDRYIKEVPRHLRQPGIHPKTPNKFKKYSRRSWDQQIKLWKVALHFWDPP
SFTAP                  YDRYIKEVPRHLRQPGIHPKTPNKFKKYSRRSWDQQIKLWKVALHFWDPP

SLBP             (201) AEEGCDLQEIHPVDLESAESSSEPQTSSQDDFDVYSGTPTKVRHMDSQVE
Delta55N-TAP           AEEGCDLQEIHPVDLESAESSSEPQTSSQDDFDVYSGTPTKVRHMDSQVE
Delta55-125N           AEEGCDLQEIHPVDLESAESSSEPQTSSQDDFDVYSGTPTKVRHMDSQVE
SFTAP                  AEEGCDLQEIHPVDLESAESSSEPQTSSQDDFDVYSGTPTKVRHMDSQVE

SLBP             (251) DEFDLEACLTEPLRDFSAMS-
Delta55N-TAP           DEFDLEACLTEPLRDFSAMS-
Delta55-125N           DEFDLEACLTEPLRDFSAMS-
SFTAP                  DEFDLEACLTEPLRDFSAMS-
```

Figure 3.5 Protein alignment of constructs.

3.2.2 Transient Cells

48 hours after transfection of Hela cells, they transiently express the proteins encoded from constructs. I collected 1/3 of the cells for WB at 48 hours of transfection to show the transient expression of cells and seeded 2/3 of the cells in order to establish stable cell lines. WB results (see Figure 3.6) show that cells were transfected successfully and express the proteins of interest. I used two kinds of SLBP antibodies; one of them is serum SLBP, the other is commercial SLBP (Santa-Cruz). The commercial SLBP antibody recognizes the aminoacids 56-215 in N terminal of SLBP, so it doesn't recognize Δ55-125N SLBP protein.

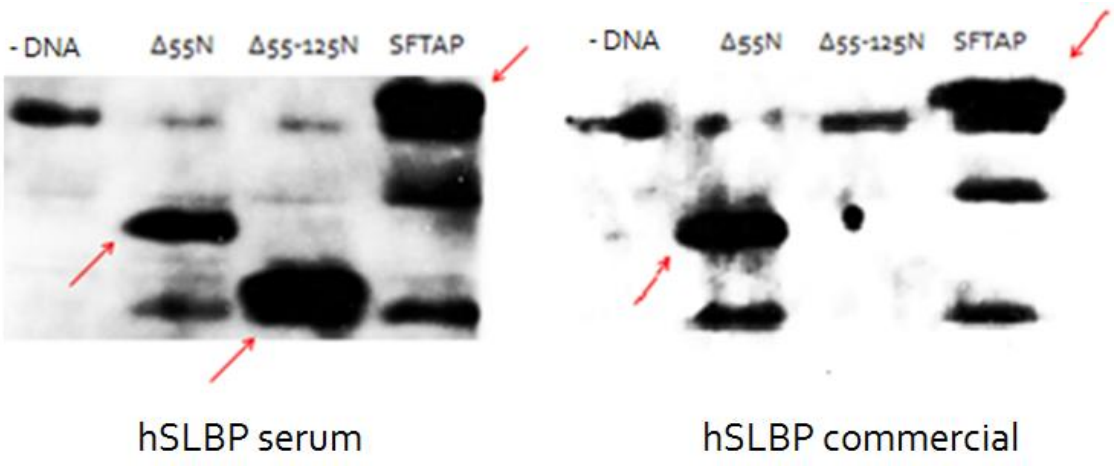

Figure 3.6 Western Blot analysis of transfected and untransfected Hela cells. The arrows show that the transiently expressed proteins. For both panel , -DNA is used as negative control.

3.2.3 Selection of Stable Cells

In order to establish stable cell lines, transfected cells should be selected. All my constructs are in pcDNA3 vector which has geniticin resistance gene. Therefore, I used geniticin as selective antibiotic. To determine the optimum geniticin concentration for selection, cells were seeded on 24-well plate at equal confluency, treated with indicated geniticin concentration (see Table 3.3). I recorded remaining cell percentage per well in indicated days. According to my data, 0.4 mg/ml geniticin cause the death of all cells in the well 14 days after the first treatment .

Table 3.3 Results of the experiment that shows the effect of geniticin concentrations on Hela cells. (Percentages indicate the confluency of living cells in plate; OC: Over-Confluent)

	3rd day	6th day	9th day	12th day	14th day
1 mg/ml	50 %	20-30%	0%	0%	0%
0,8 mg/ml	50-60 %	30-40 %	10%	0%	0%
0,6mg/ml	60 %	40%	20 %	0%	0%
0,4 mg/ml	60-70%	40-50 %	30%	10-15 %	0%
0,2 mg/ml	80-90 %	70 %	60 %	50%	40-45%
0 mg/ml	OC	OC	OC	OC	OC

In addition to geniticin concentration, I tested the effect of plating density on geniticin efficiency. For this aim, Hela cells were seeded in 96-well plate at different plating densities and treated with geniticin. The days when all cells in the well are died are recorded (see Table 3.4). For both geniticin concentration experiments, the literature recommends that choosing the geniticin concentration which is above the one which shows complete cell death in 14 days. According to results of two experiments, 0.6 mg/ml is determined as optimum concentration for selection procedure.

Table 3.4 The effect of both geniticin concentration and plating density on geniticin efficiency. The day number when all cells in the well died was recorded, OC: overconfluent.

Plating Density (cell number per well) →

Geniticin Concentration (mg/ml) ↓

	2000	1000	500	250	125	64	32
1	8. day	8. day	6. day	6. day	4. day	4. day	4. day
0.8	12. day	10. day	8. day	6. day	6. day	4. day	4. day
0.8	10. day	10. day	8. day	8. day	6. day	4. day	4. day
0.6	12. day	12. day	10. day	10. day	10. day	8. day	6. day
0.6	12. day	12. day	10. day	10. day	8. day	8. day	6. day
0.4	14. day	12. day	10. day	10. day	10. day	10. day	8. day
0.4	14. day	12. day	12. day	10. day	10. day	10. day	8. day
0.2	OC	OC	OC	OC	OC	OC	10. day

Following the transfection, the transfected cells were selected with optimum amount of geniticin which is 0.6 mg/ml according the datas above. Untransfected cells are also treated with the geniticin as control. The selection process went on until the all control cells died. But as that point the exogenous SLBP expression was very low. So, I maintained the selection for ~ 2 months until enough protein expression of SLBP was obtained. Then, 0.2 mg/ml geniticin was added to the media of stable cells. At the end of 2 months, the exogenous protein expression was shown with Western Blot (see Figure 3.7).

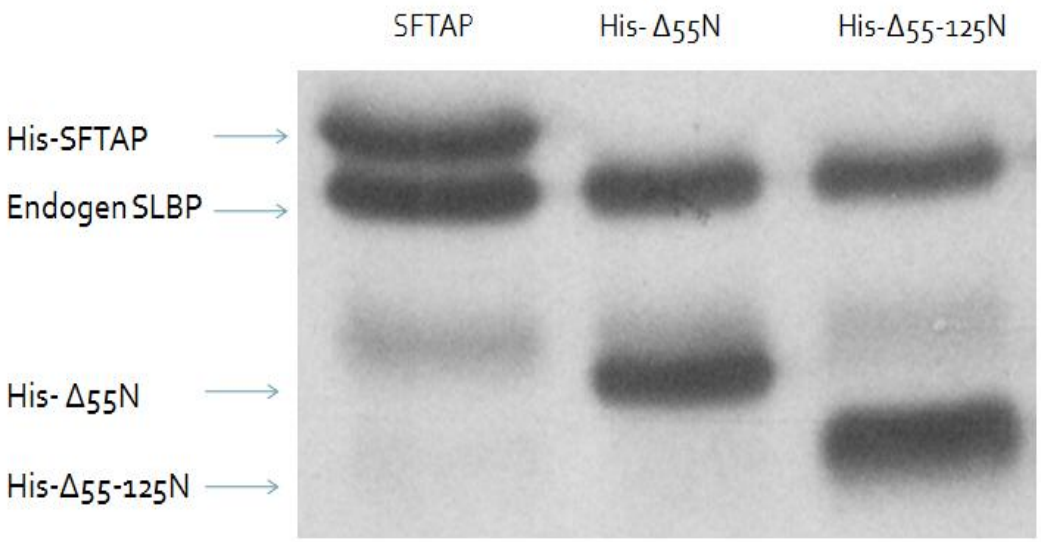

Figure 3.7 The Western Blot analysis of stable cell lines.

3.3 GENERATION OF RWSL→AWSA CONSTRUCT BY SITE DIRECTED MUTAGENESIS

To find out the coding sequence region(s) that are responsible for the degradation of newly synthesized SLBP in G1, I used the constructs which express deletion mutants of SLBP. In addition, I generated a new contruct in order to find the exact region that plays role in this degradation. I mutated a putative target motif (RWSL) of APC/Cdh1 which is active E3 ligase from M phase to end of the G1. To mutate the RWSL motif of SLBP into AWSA motif, I designed primers for four nucleotide change. I used hisSLBP SFTAP (Thr61 → Ala 61) in pcDNA3.1(-) as template dsDNA. The steps of site directed mutagenesis were described in Methods section 2.2.5.

To check the amplification by electrophoresis, I run the PCR products on 1% agarose gel, but couldn't detect any band. The manufacturer's protocol suggests to continue even if no band is visualized at this stage. Therefore, I transformed my PCR samples following DpnI digestion. There were no colonies in both transformation control and site-directed control (-primer) but 22 colonies in sample plate. I chose 3 colonies, sent to sequencing; 2 of them were unsuccessful but one of them had successful mutations. I aligned the nucleotide and protein sequence of wild type and mutated SLBP that I generated (see Figure 3.8; Figure 3.9).

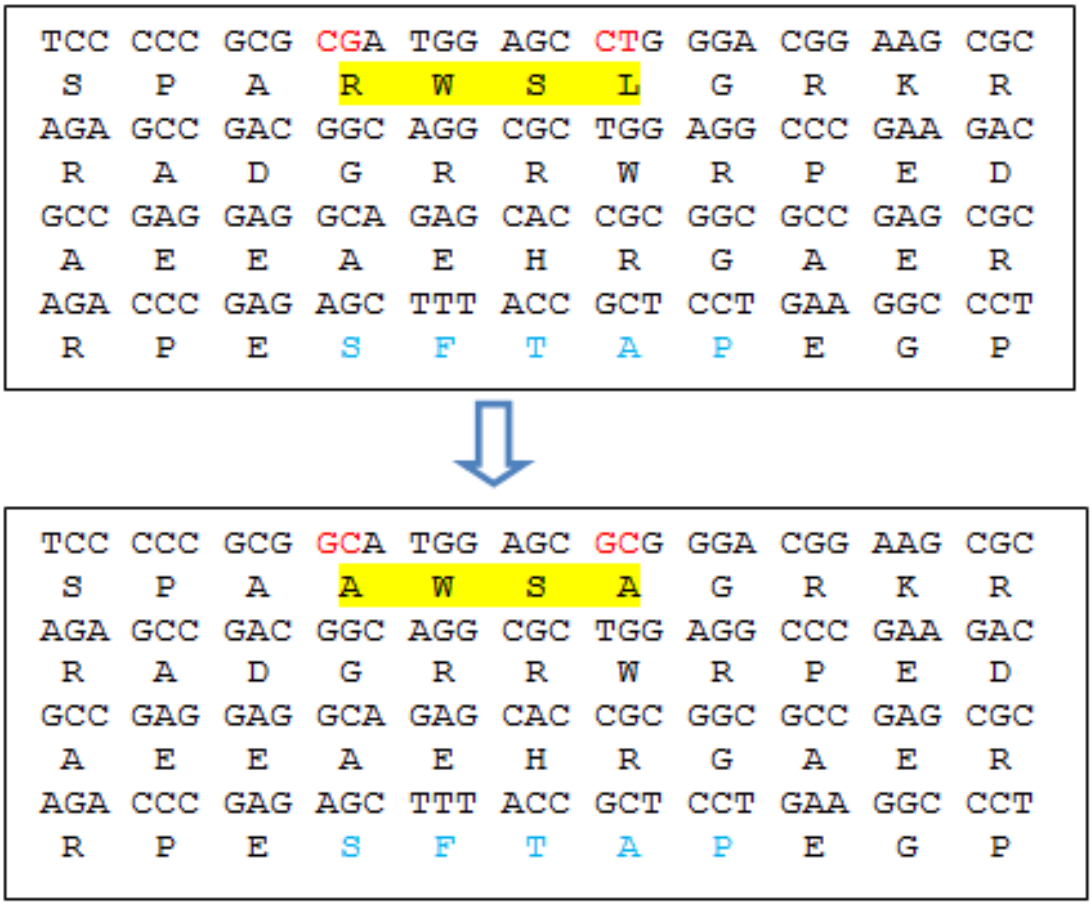

Figure 3.8 Nucleotide and protein aligment of template (above) and mutated (below) region.

```
                                  1                                                  81
SLBP ORF          ----------------------ATGGCCTGCCGCCCGCGAAGCCCGCCGAGGCATCAGAGCCG
SLBP RWSL         ----------------------ATGGCCTGCCGCCCGCGAAGCCCGCCGAGGCATCAGAGCCG
SLBP AWSA         ----------------------ATGGCCTGCCGCCCGCGAAGCCCGCCGAGGCATCAGAGCCG

                  82                                                               121
SLBP ORF          CTGCGATGGTGACGCCAGCCCGCCGTCCCCCGCGCGATGGAGCCTGGGACGGAAGCGCAG
SLBP RWSL         CTGCGATGGTGACGCCAGCCCGCCGTCCCCCGCGCGATGGAGCCTGGGACGGAAGCGCAG
SLBP AWSA         CTGCGATGGTGACGCCAGCCCGCCGTCCCCCGCGGCATGGAGCGCGGACGGAAGCGCAG

                  122                                                              161
SLBP ORF          AGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCACCGCGGCGCCGA
SLBP RWSL         AGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCACCGCGGCGCCGA
SLBP AWSA         AGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCACCGCGGCGCCGA

                  162                                                              221
SLBP ORF          GCGCAGACCCGAGAGCTTTACCACTCCTGAAGGCCCTAAACCCCGTTCCAGATGCTCTGA
SLBP RWSL         GCGCAGACCCGAGAGCTTTACCGCTCCTGAAGGCCCTAAACCCCGTTCCAGATGCTCTGA
SLBP AWSA         GCGCAGACCCGAGAGCTTTACCGCTCCTGAAGGCCCTAAACCCCGTTCCAGATGCTCTGA

                  222                                                              281
SLBP ORF          CTGGGCAAGTGCAGTTGAAGAAGATGAAATGAGGACCAGAGTTAACAAAGAAATGGCAAG
SLBP RWSL         CTGGGCAAGTGCAGTTGAAGAAGATGAAATGAGGACCAGAGTTAACAAAGAAATGGCAAG
SLBP AWSA         CTGGGCAAGTGCAGTTGAAGAAGATGAAATGAGGACCAGAGTTAACAAAGAAATGGCAAG
```

```
                282                                                     341
SLBP ORF        ATATAAAAGGAAACTCCTCATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAG
SLBP RWSL       ATATAAAAGGAAACTCCTCATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAG
SLBP AWSA       ATATAAAAGGAAACTCCTCATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAG

                342                                                     401
SLBP ORF        TTCTGATTCAAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTGT
SLBP RWSL       TTCTGATTCAAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTGT
SLBP AWSA       TTCTGATTCAAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTGT

                402                                                     461
SLBP ORF        CCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCCTACGATCGTTA
SLBP RWSL       CCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCCTACGATCGTTA
SLBP AWSA       CCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCCTACGATCGTTA

                462                                                     521
SLBP ORF        TATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCATTCATCCCAAGACCCCTAATAA
SLBP RWSL       TATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCATTCATCCCAAGACCCCTAATAA
SLBP AWSA       TATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCATTCATCCCAAGACCCCTAATAA

                522                                                     581
SLBP ORF        ATTTAAGAAGTATAGTCGACGTTCATGGGACCAGCAAATCAAACTCTGGAAGGTGGCTCT
SLBP RWSL       ATTTAAGAAGTATAGTCGACGTTCATGGGACCAGCAAATCAAACTCTGGAAGGTGGCTCT
SLBP AWSA       ATTTAAGAAGTATAGTCGACGTTCATGGGACCAGCAAATCAAACTCTGGAAGGTGGCTCT

                582                                                     641
SLBP ORF        GCATTTTTGGGATCCTCCAGCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGA
SLBP RWSL       GCATTTTTGGGATCCTCCAGCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGA
SLBP AWSA       GCATTTTTGGGATCCTCCAGCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGA

                642                                                     701
SLBP ORF        CCTTGAATCTGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATGT
SLBP RWSL       CCTTGAATCTGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATGT
SLBP AWSA       CCTTGAATCTGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATGT

                702                                                     761
SLBP ORF        GTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAGGATGAGTTTGA
SLBP RWSL       GTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAGGATGAGTTTGA
SLBP AWSA       GTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAGGATGAGTTTGA

                762                                                 813
SLBP ORF        TTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTCAGCCATGAGCTAA
SLBP RWSL       TTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTCAGCCATGAGCTAA
SLBP AWSA       TTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTCAGCCATGAGCTAA
```

Figure 3.9 Nucleotide aligment of template and mutated constructs.

In order to check the SLBP protein expression of the newly generated AWSA mutant SLBP construct, the cells were transfected with the construct and protein expression is analyzed with Western Blot by using SLBP antibody (Figure 3.9). Because of the his tag, the protein encoded by construct runs slower in the gel. The result of the WB indicates the successful protein expression.

Figure 3.10 Western Blot analysis of transient protein expression of his-SLBP AWSA construct.

CHAPTER 4

DISCUSSION & CONCLUSION

SLBP, which is involved in processing, translation and degradation of canonical replication-dependent histone mRNAs, is a cell cycle regulated protein. It reaches its highest level in S phase via a rapid increasing at G1/S and decreasing at S/G2 border. The SLBP level is kept low until the next S phase and recovers back to S phase level at G1 or G1/S border. It is shown that the SLBP is phosphorylated on their Thr61 by CycA/Cdk1 and degraded at the end of S phase, but the regulation in G1 is not clear.

Previous studies have shown that translation efficiency of SLBP was low in early G1 but it becomes similar to S phase level in early and mid-G1 (Koseoglu, 2007). Although the level of SBLP translation increases to S phase level in mid-G1, the amount of SLBP protein remains low until late G1 or G1/S border. This suggest that there is another regulation that keeps the SLBP level low in G1. Further, it was shown that the SLBP protein is significantly less stable in G1 in comparison to S phase. According to these data, the mechanism that keeps the SLBP level low may be the rapid degradation of newly synthesized SLBP in mid-G1. It is known that there is a similar regulation mechanism for CycA. In my thesis, I tried to improve the tools that are necessary for the investigation of the responsible region for this degradation.

In the beginning of my studies, I optimized the methods that I used. Firstly, I tried to optimize the Western Blotting to show the protein expression of SLBP. Since I had problems almost in each steps, the optimization of WB was time-consuming. Firstly, I began with optmizing the equal loading of samples which was very crucial for my experiments. Even a slight difference between protein concentrations may misguide me

about the conclusion. I had problems in this step and I used different approaches to optimize. Fortunately, I achieved to load the samples equally as shown in Figure 3.1. Also, I optimized the antibody dilution and incubation time to obtain the most sharp protein bands for the indicated antibodies in Figure 3.1. Furthermore, I tried to optimize the cell cycle synchronization protocol. I used thymidine - nocodazole and only nocodazole methods to synchronize the cells. According to optimization studies that were confirmed by FACs analysis, I achieved to synchronize the cells efficiently with thymidine-nocodazole method.

In order to determine the coding sequence region(s) that are responsible for this regulated degradation, I transfected Hela cells with wild type and deletion-mutant form of SLBP succesfully. Since all the known regulation of SLBP are controlled with its first 125 aminoacid, I focused in this region. I used 3 constructs: his-SLBP SFTAP, his-SLBP Δ55N and his-SLBP Δ55-125N. Two treonine residues in SFTTP sequence of SLBP are required for its degradation at S/G2. The mutation on SFTTP (SFTTP→ SFTAP) blocks this degradation. Therefore, it facilitates to examine the regulation of SLBP in G1 independent of S/G2 regulation. In addition, there will be greater amount of SLBP in G1, since it is not degraded at S/G2. It also helps the observation of SLBP level by Western Blot. His-SLBP Δ55N construct has also mutation on its SFTTP sequence and deletion in its N terminal from aminoacid 1 to 55. The last construct has just a deletion from aminoacids 55 to 125.

In order to stably express the protein encoded by constructs from generation to generation, cells need to incorporate the constructs into their genomic DNA. However, all transfected cells can't achieve this. To select the stable cells, the transfected cells are treated with selective antibotic. The constructs I used are in pcDNA3.1(-) vector containing geniticin resistance gene, so I used geniticin to generate the stable cell lines. Firstly, I designed experiments to find the proper geniticin concentration for the selection. These experiments based on the treatment of HeLa cells with different concentration of geniticin for 14 days. In addition to geniticin concentration, I tested the effect of plating density on geniticin efficiency. According to results of the experiments, 0.6 mg/ml is determined as optimum geniticin concentration for the selection process. Then, I tried to select the transiently transfected cells with optimum geniticin concentration for two months. In the same time, I tried to synchronize the transient

transfected cells acoording to protocols in the literature but I couldn't success. Fortunately, I achieved to generate the stable cell lines.

In this thesis, I concentrated on finding out the coding sequence region(s) that are important to keep the SLBP level low in G1. If SLBP isn't degraded in G1 when the first 55 aminoacid in N terminus is deleted,we can say that the coding sequence region is in the first 55 aminoacid . Similarly, if the SLBP isn't degraded in G1 when the region from aminoacid 55 to 125 is deleted, then the coding sequence region is in somewhere between aminoacid 55-125. Although, we don't know where the coding sequence region is, our potential candidate is the first 55 aminoacid. Because, there is RWSL aminoacid sequence in the first 55 aminoacid in N terminal of SLBP. This sequence (RXXL) is a putative target motif of APC/Cdh1 that is active from late M to end of the G1 phase. So, I mutated this region by Site-Directed Mutagenesis (RWSL→AWSA) to use for further studies. I confirmed the sequence and expression of mutant SLBP that I generated.

As a result, in this dissertation, the optimization studies were completed, the stable cell lines were generated and the construct are prepared but the region that has the coding sequence region(s) should be found with further experiments.

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