FUNCTION OF STRUCTURE-SPECIFIC RECOGNITION PROTEIN-1 IN GENE REGULATION AND MITOSIS

by

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Ap-1	activator protein 1 element
BC100	Buffer C 100
CD	circular dichroism
CDC 68	cell division-cycle mutant #68 (same as yeast Spt16 gene)
ChIP	chromatin immuboprecipitation
cisplatin	cis-diamminedichloroplatinum(II)
CK2	casein kinase 2
CPC	chromosomal passenger complex
CRE	calcium/cAmp responsive element
CTD	carboxyl-terminal domain
C-	carboxy-terminus
dusp5	dual specificity phosphatase 5
DUF	DNA unwinding factor (*Xenopus* heterodimer of Spt16 and SSRP1)
DSIF	DRB sensitivity-inducing factor
DRB	5,6-di-chloro-1-b-D-ribofuranosyl-benzimidazole
EGR1	early growth response gene 1
ERE	EGR1 response element
EM	electron microscopy
EMSA	Electrophoresis Gel Mobility Shift Assay
FACT	facilitating chromatin transcription (human heterodimer of Spt16 and SSRP1)
GST	glutathione-S-transferase
GTFs	general transcription factors
H1299	human non-small cell lung carcinoma
His	6x histidine tag
HMG	high mobility group
id2	inhibitor of differentiation
IEG	immediate early genes
IF	immunofluorescence staining
IgG	immunogloblin type G
IgM	immunogloblin type M
INCENP	inner centromeric protein
IP	immunoprecipitation
IR	ionizing radiation
Krox-24	gene containing sequences homologous to the *Drosophila* Kr finger probe
MAP4	MT-associated protein 4
MT	microtubule
NE	nuclear extract
NEB	nuclear envelope breakdown
NGFI-A	nerve growth factor induced gene A
N-	amino terminus
P11	phosphocellulose column

plau	plasminogen activator and urokinase
PIC	preassembled initiation complex
Pol1	polymerase one
Pob3	polymerase one binding protein 3
RNAPII	RNA polymerase II
RSS	recombination signal sequences
SDS-PAGE	sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
SPT	suppressor of TY
Sp1	specificity protein 1 element
SRE	serum response element
SRF	serum response factor
SSRP1	structure-specific recognition protein-1
TBP	TATA binding protein
TCF	ternary complex factor
TIS8	tetradecanoyl phorbol acetate-induced sequence 8
UV	ultraviolet
WB	Western blot
WCL	whole cell lysates
WT	with wild type
zif268	zinc finger binding protein clone 268

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ABSTRACT

The evolutionarily conserved facilitating chromatin transcription (FACT) complex is a heterodimer of structure-specific recognition protein-1 (SSRP1) and Spt16. FACT has been shown to regulate transcription elongation through a chromatin template and has histone chaperone activity *in vitro*. *In vivo*, it is recruited to several actively transcribed genes similar to RNA polymerase II (RNAPII) in yeast and *Drosophila*. However, its global role in transcription regulation in human cells remains largely elusive. In Chapter Two, I conducted spotted microarray analysis using arrays harboring 8308 human genes to assess the gene expression profile after knocking down SSRP1 or Spt16 levels in human non-small cell lung carcinoma (H1299) cells. I found SSRP1 and Spt16 shared common and individual targets. A subset of genes was regulated by SSRP1 independent of Spt16. Further analyses of some of these genes not only verified these observations but also identified the serum-responsive gene, *early growth response gene 1* (*egr1*), as a novel target for both SSRP1 and Spt16. Using chromatin immunoprecipitation (ChIP), I showed that SSRP1 and Spt16 were recruited to the coding region of *egr1* after serum stimulation and important for the progression of elongation RNAPII on the *egr1* gene.

In addition to the role in transcription, FACT has been purified in a UV-responsive p53 Ser 392 kinase complex in our laboratory. The kinase in the complex is casein kinase 2 (CK2). The association of FACT with CK2 changes the CK2 substrate specificity toward p53 over other tested substrates. CK2, SSRP1, and Spt16 bind to each other via non-overlapping regions *in vitro* and in cells. In addition, SSRP1 is an efficient substrate for CK2 *in vitro.* In Chapter Three, we investigated the regulation of SSRP1

function by CK2. Phosphorylation of SSRP1 by CK2 inhibited the nonspecific DNA-binding activity of SSRP1 and the FACT complex in gel mobility shift assays. Using *in vitro* kinase assay with synthetic peptides as substrates, we identified serines 510, 657, and 688 as phosphorylation targets of CK2 *in vitro*. Mutagenesis of the three serines revealed that serine 510 was more important for the regulation of SSRP1's DNA-binding activity.

Much of our knowledge about the *in vivo* roles of the mammalian FACT subunits regards human SSRP1 and its mouse homolog T160. SSRP1 has fundamental roles in cell growth control. SSRP1 is abundant in rapidly dividing cells and down-regulated during cell differentiation. Mice homozygous for the *ssrp1* deletion mutant were lethal at "embryonic day 3.5". However, it is unclear how SSRP1 is involved in cell growth control. In Chapter Four, we reported the identification of SSRP1 as a novel regulator of microtubule (MT) dynamics. SSRP1 co-localizes with the mitotic spindle and midbody in human cells and associates with MTs *in vitro*. Purified SSRP1 facilitates tubulin polymerization and MT bundling *in vitro*. Depletion of SSRP1 leads to disorganized spindles, and midbodies in cells. Hence, SSRP1 plays a crucial role in MT growth and regulation during mitosis and contributes to the cell growth control.

In summary, my thesis identified the Spt16-dependent and -independent roles of SSRP1 in regulating gene transcription in human cells, characterized the regulation of SSRP1 by CK2, and uncovered the novel role of SSRP1 during mitosis. These findings will benefit our understanding of SSRP1 function.

CHAPTER ONE

INTRODUCTION

My thesis project investigates the regulation of structure-specific recognition protein-1 (SSRP1) and its functional role during transcription and mitosis. In order to accomplish these aims, I have examed the role of SSRP1 and Spt16 in transcriptional regulation, the regulation of SSRP1 by casein kinase 2 (CK2) and the novel role of SSRP1 during mitosis. SSRP1 and Spt16 form a complex called facilitating chromatin transcription (FACT). I will give an introduction about the FACT complex, early growth response gene 1 (EGR1) (a novel FACT target I identified), CK2 and mitosis.

1.1 FACT

1.1.1 Overview of FACT

FACT is a heterodimeric complex consisting of Spt16 and SSRP1 (Orphanides, LeRoy et al. 1998; Orphanides, Wu et al. 1999). SSRP1 binds to H3/H4 tetramers, while Spt16 binds to H2A/H2B dimers and to mononucleosomes (Belotserkovskaya, Oh et al. 2003). Both Spt16 and SSRP1 are highly conserved in all eukaryotes and the stable heterodimer they form is also conserved (Formosa 2003). Human Spt16 is a 120 kD protein and contains a highly acidic and serine-rich carboxyl terminus (Belotserkovskaya, Oh et al. 2003). It shares 35% identity and 54% similarity with its *Saccharomyces cerevisiae* ortholog Spt16/Cdc68 (Formosa 2003). Spt16/Cdc68 is essential for yeast cell growth because *spt16* null mutants are nonviable (Malone, Clark et al. 1991). The partner of Spt16 in the FACT complex is SSRP1, which is a high mobility group (HMG) domain containing protein. This 81 kD protein contains a highly conserved N-terminal region (aa 1–439), an acidic domain (aa 440–496), and an HMG box domain (aa 539–614), with

two flanking basic domains (aa 512–534 and aa 623–640), and a mixed charge domain at the extreme C-terminal region (aa 661–709) (Fig. 1.1) (Dyer, Hayes et al. 1998). These domains are crucial for the functions of SSRP1. For example, the HMG domain mediates SSRP1's DNA binding activity (Shirakata, Huppi et al. 1991; Bruhn, Pil et al. 1992; Gariglio, Foresta et al. 1997) and the highly conserved N-terminal region of SSRP1 has been shown to directly interact with its partner, Spt16, forming a tight heterodimer complex (Orphanides, Wu et al. 1999; Keller and Lu 2002). In yeast, polymerase one binding protein 3 (Pob3) (63kD) and Nhp6 (11kD) form a bipartite SSRP1 analog (Fig.1.1) (Brewster, Johnston et al. 2001). Pob3 is highly homologous to the N terminal region of SSRP1 with 31% identity and 53% similarity (Formosa 2003), whereas Nhp6 is an HMG protein resembling the C terminus of SSRP1 (Brewster, Johnston et al. 2001). The human SSRP1 counterparts of the yeast Pob3 and Nhp6 proteins were detected during apoptosis as the products of both caspases 3- and 7-mediated cleavage of SSRP1 (Landais, Lee et al. 2006), indicating the importance of SSRP1 for cell survival. Indeed, both yeast Pob3 and mammalian SSRP1 are essential for cell and animal viability (Wittmeyer, Joss et al. 1999; Cao, Bendall et al. 2003). Before SSRP1 and Spt16 were identified as the subunits of FACT, they were discovered in different species separately.

1.1.2 The discovery of Spt16

About two decades ago, a group of *suppressor of TY* (*SPT*) genes was identified by selection for mutations that suppress transcription defect caused by insertion of the long terminal repeat of the Ty retrotransposon into the 5' regions of the *HIS4* and *LYS2* genes of *Saccharomyces cerevisiae* (Winston, DT et al. 1984). Genetic analysis places *SPT* genes into two major groups (Formosa 2003). The first group of *SPT* genes changes the

way that initiation sites are selected (Formosa 2003). One of such *SPT* gene is *spt15*, which encodes TATA binding protein (TBP) (Eisenmann, Dollard et al. 1989; Hahn, Buratowski et al. 1989). TBP is the principle regulation signal for choosing where RNA polymerase II (RNAPII) initiates transcription. The second group of *SPT* genes encodes histones and functionally related proteins (Formosa 2003). *spt16* is in the histone group (Malone, Clark et al. 1991), which also includes *spt4*, *spt5*, *spt6*, *spt11* and *spt12* (Formosa 2003). *spt11* and *spt12* encode the histones H2A and H2B (Clark, Norris et al. 1988). Spt4 and Spt5 form a complex homologous to human DRB sensitivity-inducing factor (DSIF), which has both positive and negative roles in regulating transcription elongation by RNAPII (Wada, Takagi et al. 1998) and interacts directly with RNAPII's largest subunit (Yamaguchi, Wada et al. 1999). Spt6 interacts physically and genetically with Spt4 and Spt5 (Hartzog, Wada et al. 1998). Spt6 can also bind directly to the histone H3 (Bortvin and Winston 1996) and serine 2 phosphorylated RNAPII (Yoh, Cho et al. 2007). The involvement of Spt16 in transcription elongation was suggested by the sensitivity of certain *spt16* alleles to 6-azauracil (Orphanides, Wu et al. 1999) and by the genetic interactions of Spt16 with the known elongation factors TFIIS, Spt4/5, and the Paf complex (Krogan, Kim et al. 2002; Lindstrom, Squazzo et al. 2003). All of these observations support the original idea that the regulation of transcription by this group involves interactions between RNAPII and nucleosomes. Interestingly, this group of proteins displays stoichiometric effects: either increasing or decreasing their dosage affects transcriptional regulation (Hirschhorn, Brown et al. 1992). Spt16 was also identified as a transcription factor in two additional yeast genetic screens. In one screen, it was identified as cell division-cycle mutant #68 (CDC68) and found to be required for

cell to pass cell cycle START and enter S phase, since it controlled the expression of G1 cyclins (Rowley, Singer et al. 1991). In the other screen, the *spt16* mutant bypassed the requirement for Swi4 and Swi6 transcriptional activators at an HO:lacZ reporter (Lycan, Mikesell et al. 1994). Spt16 can function as either an activator or repressor depending on the context. But it is mostly required for transcriptional activation of targets, such as the H2A-H2B genes HTA1/SPT11 and HTB1/SPT12, as well as its own transcript (Xu, Johnston et al. 1993).

1.1.3 The discovery of SSRP1

Mouse SSRP1, also named T160, was identified by its ability to bind DNA that contains recombination signal sequences (RSS) (Shirakata, Huppi et al. 1991). Similerly, human SSRP1 was also found to be a protein which binds selectively to DNA modified by the anticancer drug cis-diamminedichloroplatinum(II) (cisplatin) (Bruhn, Pil et al. 1992). Interestingly, SSRP1 possesses a C-terminus of ~80 amino acids which shares a significant sequence homology with a high mobility group protein HMG-1 (Shirakata, Huppi et al. 1991; Bruhn, Pil et al. 1992). The HMG proteins have been defined by their high electrophoretic mobility in polyacrylamide gels and their solubility in 2-5% trichloroacetic acid (Grosschedl, Giese et al. 1994). They have been classified into three families based on their molecular masses, DNA-binding characteristics and amino acid sequence motifs: HMG-1/2, HMG-I(Y) and HMG-14/17 families (Grosschedl, Giese et al. 1994). The HMG-1/2 members have single or multiple HMG domains (Grosschedl, Giese et al. 1994). The HMG-1/2 family is further divided into two subfamilies according to the number of HMG domains present in the protein, their specificity of sequence recognition, and their evolutionary relatedness. The first subfamily has multiple HMG

domains and preferentially binds to non-B-DNA conformations, such as B-Z junctions, stem-loops, cruciforms, four-way junctions, and cisplatin-modified DNA with no obvious, or low, sequence specificity (Grosschedl, Giese et al. 1994). The second subfamily has single HMG domain and interacts with specific nucleotide sequences (A/TA/TCAAAG), and has restricted cell type distribution (Grosschedl, Giese et al. 1994). When hydropathy profiles were performed for the HMG domains of mouse SSRP1 and HMG-1, the two patterns were very similar and contained two hydrophobic peaks in the hydrophilic regions (Shirakata, Huppi et al. 1991). This analysis suggests that the HMG domain in SSRP1 could be a DNA-binding domain as that in HMG-1. Deletion studies showed that indeed the HMG domain of SSRP1 is responsible for DNA binding (Gariglio, Ying et al. 1997). Although it only contains a single HMG domain, SSRP1 binds to cruciform DNA as well as to linear double-stranded DNA with no sequence specificity, similar to the first HMG-1/2 subfamily (Gariglio, Ying et al. 1997). Also, SSRP1 bends DNA and mediates the ligase-catalyzed cyclization of DNA fragments longer than 100bp (Gariglio, Ying et al. 1997). Also similar to the first HMG-1/2 subfamily, SSRP1 is abundant in rapidly dividing cells and down-regulated during cell differentiation (Hertel, De Andrea et al. 1999). When the mouse *ssrp1* expression was restricted by antisense RNA strategy in NIH3T3 cells, the cell proliferation was inhibited and apoptosis was observed (Hertel, Foresta et al. 1997). *ssrp1* homozygous mutant mice died soon after implantation, and preimplantation blastocysts were defective for cell outgrowth and survival *in vitro* (Cao, Bendall et al. 2003). These data suggest that SSRP1 is essential for cell viability and proliferation, as well as for embryonic development.

1.1.4 The discovery of FACT

The knowledge that SSRP1 and Spt16 function together as a heterodimer later came from several reports using yeast, *Xenopus*, and mammalian systems (Wittmeyer and Formosa 1997; Orphanides, LeRoy et al. 1998; Okuhara, Ohta et al. 1999; Orphanides, Wu et al. 1999; Wittmeyer, Joss et al. 1999; Formosa, Eriksson et al. 2001). In yeast, Spt16 and Pob3 were isolated in a screen for proteins which bound to the DNA polymerase α catalytic subunit, polymerase one (Pol1) (Wittmeyer and Formosa 1997). This study identified the yeast ortholog of mammalian SSRP1, Pob3 (Wittmeyer and Formosa 1997). Athough at that time they were not fully recognized as an independently-acting heterodimer, subsequent physical and genetic data indicate that a Spt16-Pob3 heterodimer is the functional form of each protein. Further, it is a highly abundant, stable, nuclear complex that has the ability to associate with chromatin through Nhp6, an HMG-motif DNA-binding protein, in yeast (Wittmeyer, Joss et al. 1999; Formosa, Eriksson et al. 2001). In *Xenopus*, SSRP1 and Spt16 were purified as a heterodimer that could unwind closed-circular duplex DNA in the presence of topoisomerase I and was termed DUF, for DNA unwinding factor (Okuhara, Ohta et al. 1999). Immunodepletion of DUF from *Xenopus* egg extracts drastically impaired DNA replication from exogenously added sperm chromatin or plasmid DNA (Okuhara, Ohta et al. 1999). While a link between Spt16-SSRP1 and DNA replication was established, the first indication of the activity of these proteins came from studies of transcription using factors purified from human cells (Orphanides, LeRoy et al. 1998). Highly purified RNAPII can initiate transcription on a chromatin template with the help of general transcription factors (GTFs), activator, and energy-dependent chromatin remodeling on the promoter. Additional accessory factors are required to promote transcription elongation on the chromatin template. One of these

factors was purified and named FACT (Orphanides, LeRoy et al. 1998). Later, the components of FACT were identified as SSRP1 and Spt16 (Orphanides, Wu et al. 1999).

1.1.5 The function of FACT during transcription

It was found that FACT bound to core histones H2A and H2B, and that covalently cross-linked histone polypeptides within nucleosomes disrupted FACT-dependent transcription (Orphanides, Wu et al. 1999). Thus, FACT may promote disruption of the histone octamer by binding to and removing either one or both of the histone H2A/H2B dimers. This action leaves only the histone H3/H4 tetramer bound to DNA, thus RNAPII could proceed (Orphanides, Wu et al. 1999). Later, this model was verified by experiments showing that FACT-mediated transcription through the nucleosome results in the loss of a single H2A/H2B dimer *in vitro* without ATP hydrolysis (Belotserkovskaya, Oh et al. 2003). Specific loss of H2A/H2B dimers has been observed during active transcription mediated by RNAPII *in vivo* and the removal of H2A/H2B dimers enhances transcription *in vitro* (Reinberg and Sims 2006). Genetic analysis in yeast supports the role of FACT in destabilization of the histone dimer-tetramer interactions during transcription and also suggests a role for yeast FACT in nucleosome reassembly after RNAPII passage, since mutations in *spt16* and *pob3* resulted in the dependence of yeast cells on the Hir/Hpc nucleosome assembly pathway (Formosa, Ruone et al. 2002). Indeed, the histone chaperone activity of FACT (FACT's ability to promote core histone deposition onto DNA) was shown *in vitro*. Also, the highly acidic C terminal of Spt16 is critical for this function of FACT (Belotserkovskaya, Oh et al. 2003).

There is substantial evidence supporting a dual role of FACT in not only relieving nucleosomal inhibition, but also promoting nucleosome assembly (Fig.1.2). On one hand,

inactivation of FACT results in transcription initiation from cryptic initiation sites within the coding region of genes in yeast, implying that FACT is required for reforming normal chromatin after passage of RNAPII (Kaplan, Laprade et al. 2003; Mason and Struhl 2003). On the other hand, yeast FACT physically associates with several transcription elongation factors, such as Spt4/Spt5, Spt6, Chd1 and the Paf1 complex (Kelley, Stokes et al. 1999; Krogan, Kim et al. 2002; Lindstrom, Squazzo et al. 2003). Moreover, yeast FACT specifically associates with active RNAPII genes and travels with elongating RNAPII throughout the mRNA coding region (Mason and Struhl 2003). Immunofluorescence (IF) staining of *Drosophila* polytene chromosomes and high resolution ChIP analysis using antibodies specific to the *Drosophila* homologues of Spt16 and SSRP1 have also shown that FACT is recruited to the actively transcribed loci upon heat shock induction (Saunders, Werner et al. 2003). The kinetics of FACT recruitment and of chromosome tracking *in vivo* resembles that of RNAPII and elongation factors Spt5 and Spt6 (Saunders, Werner et al. 2003). These results support the previous *in vitro* observation: FACT facilitates RNAPII elongation through a chromatin template. Indeed, a *Pob3* mutant strain showed a defect in RNAPII occupation of the *GAL1-YLR454w* allele and the induction of *YLR454w* mRNA from the *GAL1* promoter (Biswas, Dutta-Biswas et al. 2006).

1.1.6 Some unresolved questions

In addition to its general role in transcription elongation, SSRP1 has been shown to play a specific role as well. For example, SSRP1 was found to be a sequence-specific transcription factor of the embryonic ε globin gene where it might play a role as an architectural factor helping to coordinate the assembly of multiprotein complexes

required for stage-specific globin gene expression (Dyer, Hayes et al. 1998). SSRP1 was also identified as a coactivator for serum response factor (SRF) (Spencer, Baron et al. 1999) and a p63 transcriptional activator (Zeng, Dai et al. 2002). Other proteins reported to interact with SSRP1 in mammalian cells include the PU.1 (Nagulapalli, Pongubala et al. 1995) and CHD1 (Kelley, Stokes et al. 1999), a chromodomain, ATPase/helicase containing protein implicated in chromatin remodeling. Yet it is not clear whether SSRP1 can function independently of the FACT complex in these regulations.

Another unresolved question is whether FACT plays a global or gene-specific role in transcriptional regulation in cells. It has been shown that FACT is only recruited to one actively transcribed gene out of three tested genes (Kouskouti and Talianidis 2005) and is dispensable for p21 expression in response to 5,6-di-chloro-1-b-D-ribofuranosyl-benzimidazole (DRB) treatment even though it is recruited to the p21 coding region in response to doxorubicin treatment (Gomes, Bjerke et al. 2006). Moreover, FACT can be recruited to the entire coding region (Mason and Struhl 2003; Saunders, Werner et al. 2003), the 5' portion of the coding region (Kouskouti and Talianidis 2005), or promoter and coding region (Gomes, Bjerke et al. 2006; Pavri, Zhu et al. 2006) depending on which gene is tested in the assay. So it seems that the requirement of FACT during transcription could be gene specific. Therefore, one part of my thesis projects is to determine whether FACT plays a general or specific role in gene transcription. To do so, I have performed spotted microarray analysis and found that expression of many genes are either up-regulated or down-regulated after ablation of the endogenous SSRP1 or Spt16 levels by siRNA in human cells. Also, I have confirmed a subset of genes which are regulated by SSRP1 independent of Spt16 and identified the serum responsive gene,

egr1, as a novel target for both SSRP1 and Spt16. These studies are detailed in Chapter Two of this thesis.

1.2 EGR1

The transcription factor EGR1, which I identify as a novel target for FACT, is also called nerve growth factor induced gene A (NGFI-A), zinc finger binding protein clone 268 (zif268), gene containing sequences homologous to the *Drosophila* Kr finger probe (Krox-24) and tetradecanoyl phorbol acetate-induced sequence 8 (TIS8). These different nomenclatures for the same gene encoding EGR1 are due to the fact that this gene was independently identified by different laboratories. zif268 was initially identified in mouse fibroblasts in 1987 (Lau and Nathans 1987). It was one of the ten genes which were quickly induced by serum or a platelet-derived growth factor. Simultaneously, NGFI-A was identified in a screening strategy that aimed at detecting genes induced by nerve growth factor (NGF) in rat PC12 cells (Milbrandt 1987). Thereafter this gene was also described by several other laboratories (Knapska and Kaczmarek 2004).

EGR1 belongs to the immediate early gene family (IEG) since it is transcriptionally activated within a few minutes of exposure to stimulation in the absence of *de novo* protein synthesis (Milbrandt 1987). It is one of the four members of the EGR family which includes EGR1, EGR2, EGR3 and EGR4 (O'Donovan, Tourtellotte et al. 1999). In mice, *egr1* knockout produces no obvious defects, except for infertility (Lee, Tourtellotte et al. 1995; Lee, Sadovsky et al. 1996; Lee, Wang et al. 1996). The lack of additional phenotypes in *egr1* null mice may be due to the redundancy of EGR family members (Lee, Sadovsky et al. 1996). EGR1 has a highly conserved DNA-binding domain composed of three zinc-finger motifs located in its C-terminal domain (Fig.1.3A)

(O'Donovan, Tourtellotte et al. 1999). These zinc-fingers recognize a 9-bp segment of DNA (5'-GCGC/GGGGCG-3'), with each finger spanning three nucleotides (Fig.1.3B) (Christy and Nathans 1989; Pavletich and Pabo 1991). It also has an activation domain and a repression domain (Fig.1.3A) (Gashler, Swaminathan et al. 1993). The negative regulators of EGR1, NAB1 and NAB2, bind to EGR1 through the repression domain (Russo, Sevetson et al. 1995; Svaren, Sevetson et al. 1996). The predicted molecular weight of EGR1 is 59 kDa, while it appears at a size of 80 kDa on sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Baron, Duss et al. 2003). This shift is probably due post-translational modification of EGR1, such as phosphorylation or glycosylation (Cao, Koski et al. 1990; Lemaire, Vesque et al. 1990).

The expression of EGR1 is ubiquitous, but steady-state levels are low in most tissues, except in brain where high basal expression levels were observed (Baron, Duss et al. 2003). It is induced by many different stimuli ranging from growth factors and cytokines to signals such as ultraviolet (UV) and ionizing radiation, apoptosis-promoting factors and injury (Baron, Duss et al. 2003). The induction of EGR1 is immediate and transient at both mRNA and protein level in a well-defined time dependent fashion (Knapska and Kaczmarek 2004). It has been described that EGR1 mRNA appears within 20 min, reaches the maximum level in about 60 min, and declines within a few hours following serum, fibroblast growth factor or platelet-derived growth factor stimulation (Knapska and Kaczmarek 2004). It was also found that *egr1* mRNA reached the maximal level 30 min after light stimulation (Zangenehpour and Chaudhuri 2002). The mRNA of *egr1* has a half-life of 10-20 min (Cao, Mahendran et al. 1992). Interestingly, okadaic acid and calyculin A, which are specific inhibitors of protein serine/threonine

phosphatases 1 and 2A, stimulated a sustained induction of EGR1 expression in the mouse fibroblasts (Cao, Mahendran et al. 1992). The half-life of okadaic acid-induced EGR1 mRNA is estimated to be 2 h (Cao, Mahendran et al. 1992), suggesting that stability of EGR1 mRNA is regulated through phosphorylation dependent mechanism.

EGR1 regulates several biological functions, such as neurite outgrowth, wound repair, growth control and apoptosis (Yu, de Belle et al. 2004). Growth factors stimulate rapid induction of EGR1 that activates the downstream growth pathways. Yet the role of EGR1 in apoptosis is duplicitous. EGR1 can induce apoptosis by directly stimulating p53 (Nair, Muthukkumar et al. 1997), PTEN (Virolle, Adamson et al. 2001) and Gadd45 (Thyss, Virolle et al. 2005) expression. However, EGR1 can also promote cell survival in human fibrosarcoma cells and mouse NIH3T3 cells in response to UV irradiation (Huang and Adamson 1995; Huang, Fan et al. 1998; de Belle, Huang et al. 1999). The role of EGR1 in human cancer is also paradoxical. In many cases EGR1 appears to act as a tumor suppressor. The expression of EGR1 is consistently down-regulated in several types of cancer cell lines and primary human tumors such as glioblastoma, fibrosarcoma, mammary carcinoma, uterine leiomyomas and lung tumors (Baron, Duss et al. 2003). Re-expression of EGR1 suppresses cell proliferation and reverses the transformed phenotype (Baron, Duss et al. 2003). The tumor suppressor function of EGR1 is linked to its direct control on several tumor suppressors, such as TGFβ1, PTEN, p53, fibronectin and PAI-1 (Baron, Adamson et al. 2006). However, several observations indicate that EGR1 could also have an oncogenic activity. For example, increased expression of EGR1 is consistently observed in prostate cancer (Thigpen, Cala et al. 1996; Eid, Kumar et al. 1998). NAB2, which repressed the transcriptional activity of EGR1, is down-regulated in

primary prostate carcinomas (Abdulkadir, Carbone et al. 2001). Thus, both upregulation of EGR1 and loss of its repressor, NAB2, may contribute to the increased EGR1 activity in human prostate cancer. Moreover, EGR1 is required for prostate cancer progression, not initiation (Baron, Adamson et al. 2006). In one study, a number of growth factors, such as IGF-II, PDGF-A and TGFβ1, were identified as EGR1 target genes in prostate cancer cells (Svaren, Ehrig et al. 2000). In another study, several genes important for cell proliferation or apoptosis, such as cyclin D2, p19INK, Fas and TGFβ1, were identified as EGR1 target genes in mouse prostate cancer cells (Virolle, Krones-Herzig et al. 2003). Thus EGR1 executes its function through its transcriptional activity.

EGR1 itself is also regulated at the transcriptional level. The promoter of the *egr1* gene contains a various of regulatory elements including serum response element (SRE), specificity protein 1 element (Sp1), activator protein 1 element (Ap-1), calcium/cAmp responsive element (CRE), EGR1 response element (ERE) and NFκB-similar element (nuclear factor kappa B) (Knapska and Kaczmarek 2004). The presence of EGR1 binding sites (ERE) in the *egr1* promoter suggests that it can auto-regulate its own expression. Indeed, this has been proved in the luciferase reporter assay (Yu, de Belle et al. 2004). In response to most stimuli, serum response factor (SRF) plays a major role in up-regulating the expression of *egr1* by binding to SRE (Knapska and Kaczmarek 2004). There are a series of serum response elements (SREs) within 0.6 kb of mammalian *egr1* gene 5'-flanking sequence. This region is sufficient to maintain full inducibility in transient transfection assays (Christy and Nathans 1989). It has been well established that SRF is required for immediate-early gene activation (Schratt, Weinhold et al. 2001). SRF and ternary complex factor (TCF) bind cooperatively to SREs to mediate transcriptional

induction in response to serum stimulation (Shaw and Saxton 2003; Buchwalter, Gross et al. 2004).

However, it still remains unclear how exactly *egr1* transcription is regulated immediately in response to serum stimulation. In order to address this issue, one part of my thesis work will identify new regulators for *egr1* transcription. In my microarray analysis, as noted above, I identified *egr1* as a novel target gene for the FACT complex. Functional analyses demonstrated that the FACT complex is essential for the expression of *egr1* in response to serum stimulation and plays an important role for the progression of elongation RNAPII on the *egr1* gene. This work will be described in Chapter Two.

1.3 CK2

In addition to the roles in transcription, FACT is also involved in DNA replication (Okuhara, Ohta et al. 1999; Tan, Chien et al. 2006) and the DNA damage response (Keller, Zeng et al. 2001; Keller and Lu 2002) (Fig.1.4). The involvement of FACT in DNA replication can be explained by the need to overcome the inhibitory effects of nucleosomes at many steps during chromatin-based processes. But the interaction of FACT with different proteins or complexes also defines the specific role of FACT during different processes. For example, FACT associates with transcription elongation factors during transcription (Kelley, Stokes et al. 1999; Krogan, Kim et al. 2002; Lindstrom, Squazzo et al. 2003), with the replicative helicase complex, MCM, during replication (Okuhara, Ohta et al. 1999; Tan, Chien et al. 2006) and with CK2 during the DNA damage response, which was demonstrated in our previous studies (Keller, Zeng et al. 2001; Keller and Lu 2002). Interestingly, the interaction between FACT and CK2 is evolutionarily conserved, as CK2-Pob3-Spt16 complexes have also

been isolated from yeast cells (Krogan, Kim et al. 2002). In human cells, CK2 associates with FACT upon UV irradiation and the substrate specificity of the protein kinase CK2 is altered. The FACT-CK2 complex specifically phosphorylates p53 serine 392 (Keller, Zeng et al. 2001; Keller and Lu 2002). Since there is no p53 homolog in yeast, it is possible that the FACT complex itself is regulated by CK2. This idea has been tested in my thesis work, as shown in Chapter Three.

CK2 is a highly conserved serine/threonine protein kinase that is ubiquitous in the nucleus and cytoplasm of all eukaryotic cells that have been studied (Allende and Allende 1995). Although CK2 was initially identified as a casein kinase, casein does not seem to be the target *in vivo* (Allende and Allende 1995). The CK2 holoenzyme is a heterotetramer with a molecular mass of approximately 130 kD (Allende and Allende 1995). This tetramer is composed of two types of subunits: catalytic α (42 kD) or α' (38 kD) subunits and regulatory β (28 kD) subunits (Allende and Allende 1995). The stoichiometry of the holoenzyme is either $\alpha_2\beta_2$, $\alpha'_2\beta_2$, or $\alpha\alpha'\beta_2$ (Allende and Allende 1995). Moreover, increasing evidence shows that isolated subunits can exist *in vivo* under certain circumstances and possibly have specific functions (Pinna and Meggio 1997). The α and α' subunits are structurally analogous but coded by two different genes (Wirkner, Voss et al. 1994; Yang-Feng, Naiman et al. 1994). Their catalytic domains are ~ 90% identical (Litchfield and Luscher 1993). In contrast, their C-terminal domains are completely unrelated (Litchfield and Luscher 1993), but highly conserved among different species (Litchfield 2003). The regulatory β subunit is a unique protein encoded by a single gene in mammals (Boldyreff and Issinger 1995) and does not belong to a known protein family (Buchou, Vernet et al. 2003). The formation of the CK2

holoenzyme from individual subunits is spontaneous and mediated by the dimerization of the two β subunits, as shown *in vitro* (Graham and Litchfield 2000). The β subunits confer stability to the holoenzyme complex and contribute to the substrate specificity (Guerra, Boldyreff et al. 1999). For example, β subunits stimulate phosphorylation of most CK2 substrates (Guerra, Boldyreff et al. 1999), but inhibit α subunit catalytic phosphorylation of calmodulin (Meggio, Boldyreff et al. 1992).

CK2 has been reported to phosphorylate over 300 substrates (Meggio and Pinna 2003) and to play a role in gene expression, protein synthesis, cell cycle, proliferation and pathological processes such as carcinogenesis and viral tumorigenesis (Pinna and Meggio 1997; Guerra and Issinger 1999). CK2 activity is increased in transformed cell lines (Prowald, Fischer et al. 1984), in solid tumors (Munstermann, Fritz et al. 1990), in rapidly proliferating tissue (Munstermann, Fritz et al. 1990), and also during embryogenesis (Dominguez, Mizuno et al. 2005). Genetic studies in yeast (Padmanabha, Chen-Wu et al. 1990) and in mice (Buchou, Vernet et al. 2003) demonstrate that CK2 is essential for viability and animal embryogenesis. Yeast also harbors two catalytic CK2 isoenzymes, designated as CKA1 and CKA2 (Padmanabha, Chen-Wu et al. 1990). Disruption of both CKA1 and CKA2 is lethal although disruption of either CKA1 or CKA2 remain viable in yeast (Padmanabha, Chen-Wu et al. 1990). This data suggests the functional redundancy of CK2 α and α' subunits. In mice, while knockout of CK2 α' results in viable offspring (Xu, Toselli et al. 1999), knockout of the β subunit leads to a defect in cell-autonomy and early embryonic lethality (Buchou, Vernet et al. 2003).

CK2 has been described as "a protein kinase in need of control" (Guerra, Boldyreff et al. 1999) because it is not regulated by known secondary messengers and has

constitutive activity toward most substrates *in vitro*. Interestingly, it uses either ATP or GTP as phosphor donors with almost the same effectiveness (Niefind, Putter et al. 1999), and also Mg^{2+}, Mn^{2+} or Co^{2+} as a cofactor (Gatica, Hinrichs et al. 1993). It phosphorylates serine or threonine residues that are proximal to acidic amino acids (Pinna 1990). The minimal CK2 target consensus sequence is S/T-X-X-D/E or S/T-D/E, although CK2 also efficiently phosphorylates some non-consensus sequences (Litchfield 2003). In addition to phosphorylation of serine and threonine, CK2 can phosphorylate tyrosine *in vitro* to a lesser extent (Marin, Meggio et al. 1999). It has been reported that yeast nucleolar immunophilin FPR3 is phosphorylated at tyrosine 184 by CK2 (Wilson, Dhillon et al. 1997). So, at least in yeast, CK2 is a dual kinase that targets both serine/threonine and tyrosine.

CK2 not only displays a unique feature in its biochemical activity, but also is regulated through different mechanisms. Classical small molecules, such as cyclic nucleotides, lipid and calcium, which act as typical secondary messengers for other serine/threonine kinases, do not affect CK2 activity (Litchfield 2003). However, some small molecules participate in CK2 regulation. For example, CK2 is inhibited by negatively charged compounds such as heparin, and activated by positively charged compounds, including polyamines, such as spermine and spermidines, and by polylysine (Litchfield 2003). CK2 is also regulated through phosphorylation. A number of physiological phosphorylation sites on both CK2 α and CK2 β have been identified (Litchfield 2003). These sites do not appear directly to effect a significant change in the catalytic activity of CK2. However, they may regulate the interaction of CK2 with other proteins (Litchfield 2003).

Another mechanism for CK2 regulation is protein-protein interaction, shown by the assembly of Spt16-SSRP1-CK2 complex, a kinase complex previously purified by our lab (Keller, Zeng et al. 2001). When Spt16 and SSRP1 are complex with CK2, CK2 preferentially phosphorylates p53 at serine 392 over all other tested substrates, including casein (Keller, Zeng et al. 2001; Keller and Lu 2002). This evidience is the first suggesting that the substrate selectivity of CK2 can be influenced by other bound proteins. The formation of the Spt16-SSRP1-CK2 kinase complex appears to be induced after UV irradiation (Keller, Zeng et al. 2001; Keller and Lu 2002). These proteins interact *in vitro* and in cells via non-overlapping domains (Keller and Lu 2002). Thus, CK2's activity and substrate specificity are regulated through protein-protein interaction. Interestingly, our initial work showed that CK2 efficiently phosphorylated SSRP1 *in vitro* (Keller, Zeng et al. 2001; Keller and Lu 2002). However, the functional consequence of the phosphorylation remains unclear. In this thesis, I have extended this work and explored the potential regulation of SSRP1 function by CK2 through phosphorylation, as detailed in Chapter Three.

1.4 Mitosis and microtubules

In our study on the role of SSRP1 in cell growth, we surprisingly uncovered a novel function of SSRP1 in mitosis. Mitosis is a fundamental biological process that has captured the hearts of many cell biologists for more than a century. During this process, two daughter cells receive one copy of each chromosome. Mitosis consists of two distinct processes: division of the nucleus, or karyokinesis, and division of the cytoplasm, or cytokinesis (Maiato, Sampaio et al. 2004). The division of the nucleus occurs in five stages: prophase, prometaphase, metaphase, anaphase, and telophase (Maiato, Sampaio et

al. 2004). In prophase, chromatin starts to condense and form clearly defined chromosomes. This condensation is due in part to the work of the newly assembled mitotic spindle, a bipolar microtubule-based structure. In vertebrates, nuclear envelope breakdown (NEB) marks the end of prophase and the beginning of prometaphase. During prometaphase, the attachment of microtubules (MTs) to the kinetochore mediates the congression of the chromosome toward the center of the cell. At metaphase, the chromosome is bi-oriented and aligned at the spindle equator. Later, sister chromatids separate and migrate toward opposite poles of the spindle during anaphase. Finally, each set of chromatids decondenses to form two daughter nuclei and a cleavage furrow forms between them during telophase. This cleavage furrow then contracts and eventually forms the midbody. This midbody structure participates in the division of the cytoplasm and the formation of the two daughter cells during cytokinesis. (Maiato, Sampaio et al. 2004)

Both the mitotic spindle and midbody are microtubule-based structures associated with a large variety of microtubule-associated proteins (Maiato, Sampaio et al. 2004). MTs are hollow, cylindrical polymers assembled from α/β-tubulin heterodimers (Desai and Mitchison 1997). In cells, MT subunits are normally organized into 13 linear protofilaments which associate laterally to make up the MT lattice (Desai and Mitchison 1997). The α/β-tubulin heterodimers orient in a uniform head-to-tail fashion within the polymer lattice (Desai and Mitchison 1997). α-tubulin subunits are exposed at the slower polymerizing “minus” end of the MT. β-tubulin subunits are exposed at the faster polymerizing “plus” end and hydrolyze the bound GTP shortly after addition of the tubulin heterodimer. The energy input from GTP hydrolysis allows for nonequilibrium polymerization dynamics, including dynamic instability (Desai and Mitchison 1997).

Dynamic instability is the concept which describes how a single microtubule never reaches a steady-state length, but instead interconverts between states of polymerization and depolymerization (Kline-Smith and Walczak 2004). Thus, populations of MTs will contain both growing and shinking polymers, and transitions between the two states are sudden and unpredicatable. Transitions from polymerization to depolymerization are called catastrophes, while transitions from depolymerization to polymerization are called rescue. Both ends of MTs are dynamic. The less dynamic "minus" ends of MTs are located at the spindle poles, while the more dynamic "plus" ends extend away from the poles (Kline-Smith and Walczak 2004). A portion of MT plus ends attach to sister chromatids at the kinetochore and form morphologically distinct bundles, called K fibers, which allow the chromosomes to be aligned and segregated (Kline-Smith and Walczak 2004). K fibers make up the major part of the spindle. In addition to the K fibers, other populations of microtubules also contribute to the bipolar structure, including interpolar microtubles that overlap to form an antiparallel array, and astral microtubules, that extend from each centrosome away from the spindle where they can interact with the cell cortex (Gadde and Heald 2004).

Proper organization and dynamics of spindle pole MTs are required for mitosis. Various classes of cellular proteins mediate the nucleation (γ-tubulin ring complexes), stabilization (lattice-binding and end-binding MAPs), capture (end-binding MAPs and their partners), depolymerization (including kinl kinesins and Op18), and severing (katanin) of microtubules (Gadde and Heald 2004). Interestingly, several nuclear proteins play roles in the regulation of the MT dynamics. For example, NuMA and TPX2 are sequestered in the nucleus during interphase and then are transported to spindle poles by

dynein during mitosis (Merdes, Heald et al. 2000; Wittmann, Wilm et al. 2000). NuMA has the ability to self-associate into complexes that crosslink MTs (Harborth, Wang et al. 1999) and is required to focus and stabilize MTs at poles during mitosis (Gordon, Howard et al. 2001). TPX2 is also a MT-stabilizing protein and helps to stabilize the organization of spindle poles (Wittmann, Boleti et al. 1998).

The success of mitosis also depends on the integration of chromosomal and cytoskeletal behaviour. A class of proteins known as chromosomal passengers is involved in coordinating the chromosomal and cytoskeletal events of mitosis (Earnshaw and Bernat 1991). These proteins associate with chromosomes during prophase, and become concentrated at the inner centromeres during prometaphase and metaphase. Upon the transition to anaphase, passenger proteins abruptly transfer to the central region of the mitotic spindle, and finally concentrate in the midbody at cytokinesis. The dynamic localization of passenger proteins correlates with their diverse functions during mitosis including chromatin modification (phosphorylation of histone H3), correction of kinetochore attachment errors, aspects of the spindle assembly checkpoint, assembly of a stable bipolar spindle and the completion of cytokinesis (Vagnarelli and Earnshaw 2004). These proteins are present in cells as the chromosomal passenger complex (CPC). This complex has four members: Aurora B, inner centromeric protein (INCENP), Survivin and Borealin (Vader, Medema et al. 2006). Disregulation of or knockdown of function of any member of the passenger complex produces defects in chromosome alignment and cytokinesis (Vagnarelli and Earnshaw 2004). Recent evidence suggests that two distinct passenger complexes exist during mitosis: a holocompex of Aurora B, INCENP, Survivin and Borealin, and a subcomplex of Aurora B plus INCENP (Gassmann, Carvalho et al.

2004). The holocomplex functions during chromosome alignment and cytokinesis, whereas the subcomplex is thought to be responsible for modifying histone H3 (Gassmann, Carvalho et al. 2004). The enzymatic heart of the CPC is the Aurora B serine/threonine protein kinase which executes the various functions of the CPC by activating key substates at precise locations and specific times during mitosis (Vader, Medema et al. 2006). The three nonenzymatic subunits of the CPC are essential for the function of Aurora B. They determine activity, localization, stability, and also possibly substrate specificity of Aurora B (Vader, Medema et al. 2006). INCENP is the major Aurora B activator in human cells (Honda, Korner et al. 2003). Aurora B interacts with INCENP through its conserved C-teriminal IN-box (Kaitna, Mendoza et al. 2000) (Adams, Wheatley et al. 2000). Survivin interacts with INCENP via its first 47 amino acids, a region required for localization of the CPC (Ainsztein, Kandels-Lewis et al. 1998). Borealin promotes the interaction between Survivin and INCENP (Lens, Vader et al. 2006). Interestingly, a fusion protein consisting of Survivin and INCENP lacking its first 47 amino acids is sufficient to target a functional CPC to centromeres and midbodies in the absence of endogenous Borealin (Vader, Kauw et al. 2006). However, Borealin is essential in the localization of the endogenous proteins to the centromere (Vader, Medema et al. 2006). Moreover, recent data showed that Borealin also interacts with the N-terminal of INCENP and can interact with double-stranded DNA *in vitro* (Klein, Nigg et al. 2006), suggesting that Survivin and Borealin cooperate to localize INCENP and Aurora B. Furthermore, INCENP and Survivin interact directly with polymerized microtubules (Mackay, Eckley et al. 1993; Li, Ambrosini et al. 1998), suggesting a dual interaction of the CPC with microtubules via INCENP and Survivin. However, it is

unclear whether this interaction is crucial for proper CPC localization to central spindle and midbody.

Although our understanding of mitosis has greatly advanced during the last two decades, new microtubule-associated proteins are still emerging and their essential roles during mitosis will continually contribute to our knowledge. In this thesis, I show that the transcription elongation factor SSRP1 also plays a novel role during mitosis. This finding stemed from our surprising observation that SSRP1 co-localizes with mitotic MT and depletion of SSRP1 caused abnormal MT structure in cells, as detailed in Chapter Four.

1.5 Summary

In summary, my thesis work is split into three parts: the role of FACT in gene specific transcription regulation, CK2 regulation of SSRP1, and the role of SSRP1 in mitosis. These studies provide critical information and new discoveries for our better understanding of the Spt16-dependent and independent- functions of SSRP1 in cell proliferation and in the growth signal response.

Fig. 1.1. Schematic diagrams of the functional domains of human SSRP1 and its yeast homologs. Numbers above and below the schematic correspond to amino acid positions. The acidic domain is shown in purple. The HMG domain is shown in blue. The two basic domains are shown in green. This figure is modified from (Dyer, Hayes et al. 1998) and (Landais, Lee et al. 2006).

Fig.1.1

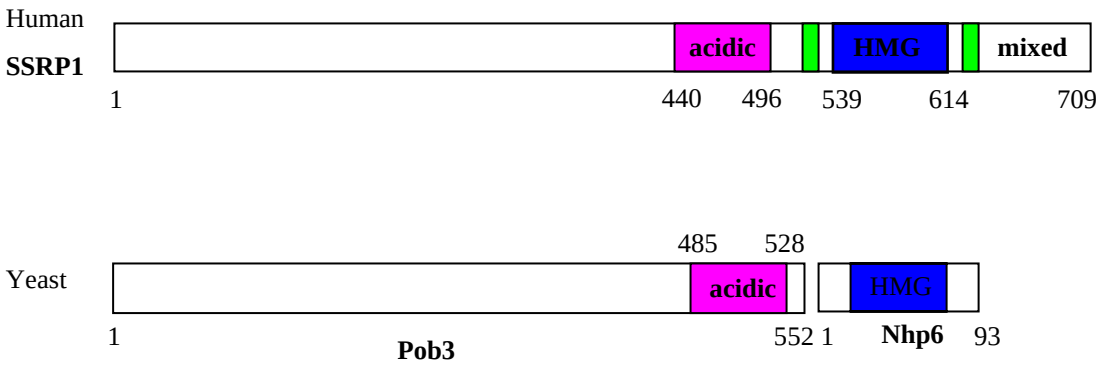

Human
SSRP1
acidic
HMG
mixed
1
440
496
539
614
709
485
528
Yeast
acidic
HMG
1
552
1
93
Pob3
Nhp6

Fig. 1.2. The dual role of FACT during transcription. FACT displaces the histone H2A/H2B dimer from the nucleosome octamer and allows RNAPII to pass through it. After passage of RNAPII, FACT deposits the H2A/H2B dimer back to DNA to reset the chromatin structure.

Fig.1.2

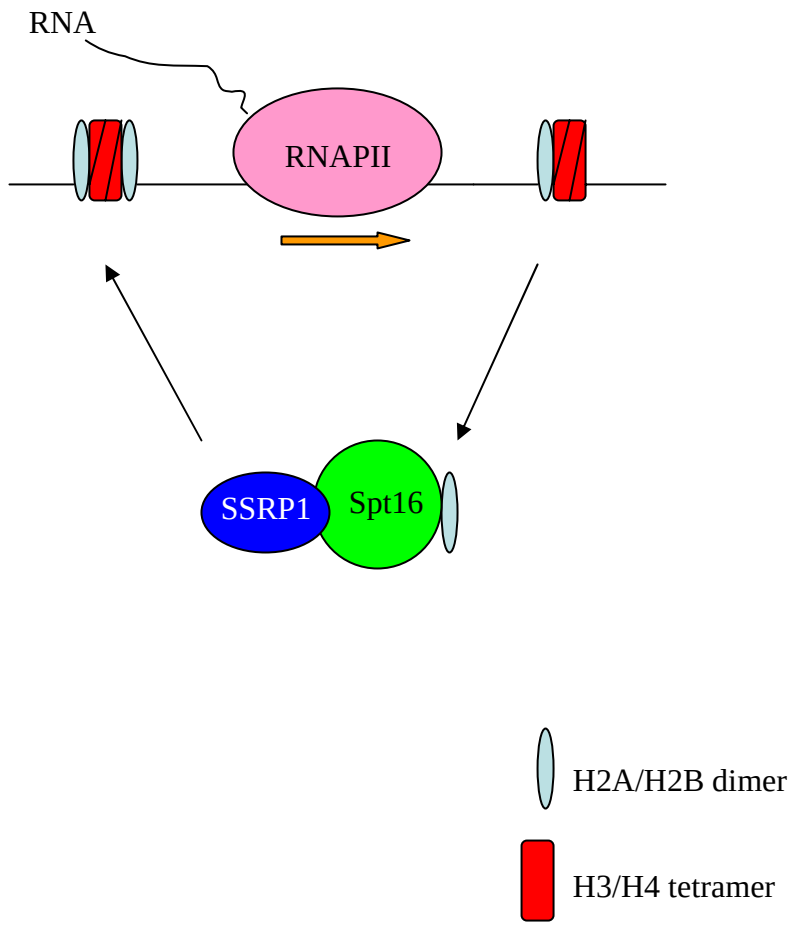
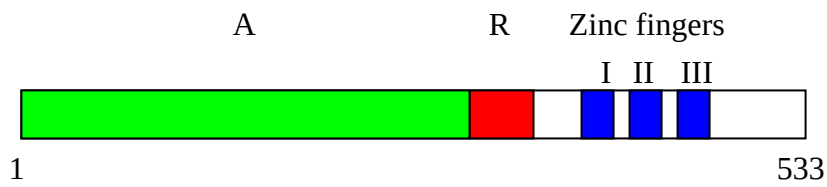


Fig. 1.3. Structural organization of the EGR protein and its cognate response element. (A) EGR1 has an activation domain (green), a repression domain (red) and three zinc-finger DNA-binding domains (blue). **(B)** EGR1 binds to the canonical EGR1 response element upstream of a hypothetical target gene. Each zinc finger (blue circle) binds to a three-nucleotide site. This figure is adapted from (O'Donovan, Tourtellotte et al. 1999).

Fig.1.3

A.


A
R
Zinc fingers
I II III
1
533

B.

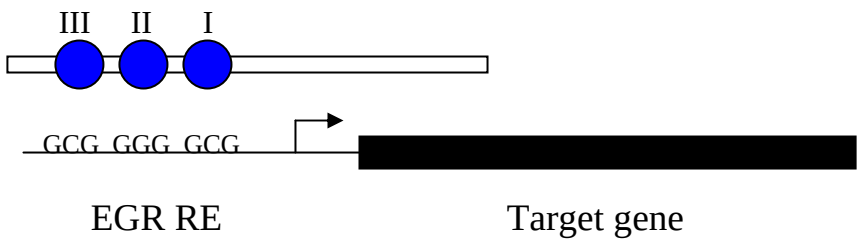
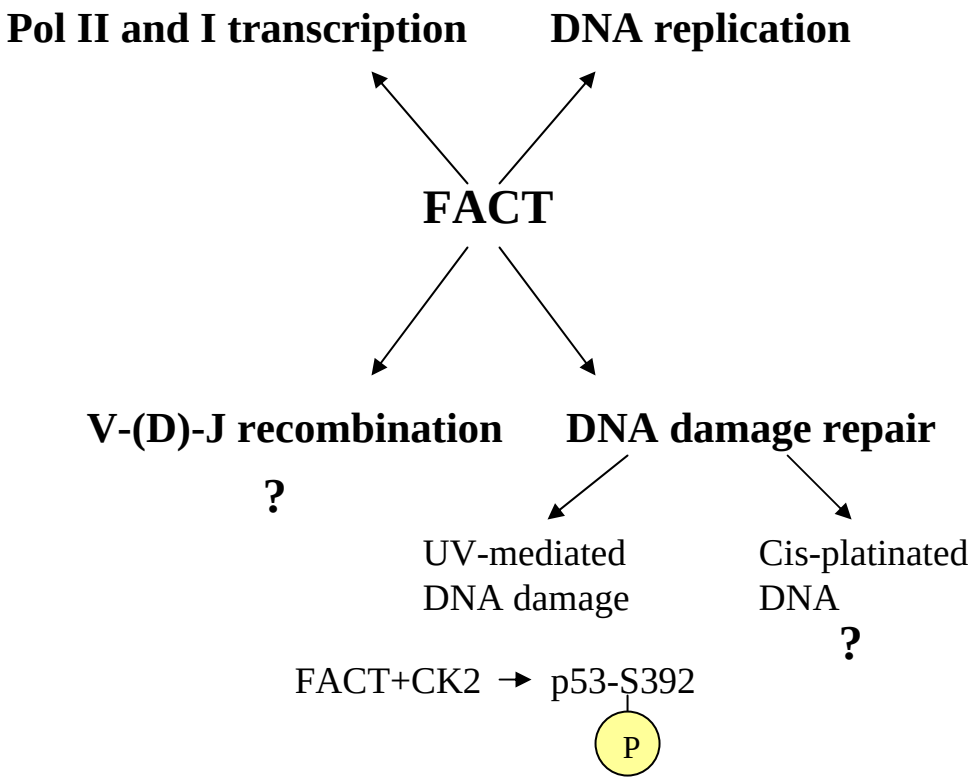

III II I
GCG GGG GCG
EGR RE
Target gene

Fig. 1.4. FACT is involved in several cellular processes. FACT facilitates RNA and DNA polymerase passage through nucleosomes. In response to UV-mediated DNA damage, CK2 forms a complex with FACT and specifically phosphorylates serine 392 of p53. Via its HMG domain, SSRP1 binds to the V-(D)-J recombination signal sequence and to cisplatin modified DNA. Thus FACT may be involved in V-(D)-J recombination and the repair of cisplatin modified DNA. This figure is adapted from (Belotserkovskaya, Saunders et al. 2004).

Fig.1.4


Pol II and I transcription
DNA replication
FACT
V-(D)-J recombination
?
DNA damage repair
UV-mediated
DNA damage
Cis-platinated
DNA
?
FACT+CK2
p53-S392
P

CHAPTER TWO

Human SSRP1 has Spt16-dependent and independent roles in gene transcription

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SUMMARY

The facilitating chromatin transcription (FACT) complex, a heterodimer of SSRP1 and Spt16, has been shown to regulate transcription elongation through a chromatin template *in vitro* and on specific genes in cells. However, its global role in transcription regulation in human cells remains largely elusive. We conducted spotted microarray analysis using arrays harboring 8308 human genes to assess the gene expression profile after knocking down SSRP1 or Spt16 levels in human non-small cell lung carcinoma (H1299) cells. Although the changes of these transcripts were surprisingly subtle, there were ~170 genes whose transcript levels were either reduced or induced >1.5 - fold. Approximately 106 genes with >1.2-fold change at the level of transcripts were the common targets of both SSRP1 and Spt16 (~1.3%). A subset of genes was regulated by SSRP1 independent of Spt16. Further analysis of some of these genes not only verified this observation but also identified the serum-responsive gene, *egr1*, as a novel target for both SSRP1 and Spt16. We further showed that SSRP1 and Spt16 are important for the progression of elongation RNA pol II on the *egr1* gene. These results suggest that SSRP1 has Spt16-dependent and -independent roles in regulating gene transcription in human cells.

INTRODUCTION

In eukaryotic cells, DNA is packaged with core histones and other chromosomal proteins in the form of chromatin, which limits the accessibility of DNA and inhibits the progression of RNA polymerases as they copy genetic information from the DNA. Thus altering the repressive nature of chromatin is necessary for the cells to implement all of

the nuclear activities on chromatin (Formosa 2003). There are at least three types of protein complexes for this function (Formosa 2003; Singer and Johnston 2004). The first type acts by covalently modifying the histones and non-histone chromatin proteins through phosphorylation, acetylation, ubiquitylation, and/or methylation (Formosa 2003; Singer and Johnston 2004). The second type of complex uses ATP hydrolysis to mobilize and/or to alter the structure of nucleosomes, such as the SWI/SNF complex (Vignali, Hassan et al. 2000; Svejstrup 2002; Akey and Luger 2003; Singer and Johnston 2004). The third type of chromatin-modulating complex disrupts and deposits the nucleosomes without utilizing ATP during transcriptional elongation (Svejstrup 2002; Belotserkovskaya and Reinberg 2004; Belotserkovskaya, Saunders et al. 2004). One of the latter members is facilitating chromatin transcription (FACT) (Orphanides, LeRoy et al. 1998; Svejstrup 2002; Belotserkovskaya and Reinberg 2004).

FACT is a heterodimeric complex consisting of Spt16 and SSRP1 (structure-specific recognition protein-1) (Bruhn, Pil et al. 1992; Orphanides, Wu et al. 1999). Both Spt16 and SSRP1 are highly conserved in all eukaryotes. Human Spt16 is a 120-kDa protein and contains a highly acidic and serine-rich carboxyl terminus. It binds to H2A-H2B dimers and to mononucleosomes (Belotserkovskaya, Oh et al. 2003). It has 36% identity to its *Saccharomyces cerevisiae* ortholog Spt16/Cdc68 (Belotserkovskaya, Oh et al. 2003). The yeast Spt16/Cdc68 was identified in two independent screens for genes involved in transcription regulation (Malone, Clark et al. 1991; Rowley, Singer et al. 1991). Genetic studies in yeast suggest that Spt16/Cdc68 is required for the normal transcription of many loci (Malone, Clark et al. 1991) and has both positive and negative effects on gene expression (Rowley, Singer et al. 1991). Spt16/Cdc68 is essential for

yeast cell growth because *spt16* null mutants are nonviable (Malone, Clark et al. 1991). The mechanism of how Spt16/Cdc68 affects transcription is suggested by the placement of Spt16/Cdc68 into a histone group of *spt* (*suppressor of TY*) genes that encode histones and also functionally related proteins, including Spt4, Spt5, Spt6, Spt11 and Spt12 (Malone, Clark et al. 1991). This group of proteins functions by altering chromatin properties and increasing or decreasing their dosage affects on transcription regulation (Evans, Brewster et al. 1998). The partner of Spt16 in the FACT complex, SSRP1, is a high mobility group (HMG) domain containing protein. It binds to cruciform or linear duplex DNA as well as DNA modified by the anticancer drug cisplatin (Shirakata, Huppi et al. 1991; Bruhn, Pil et al. 1992; Hertel, Foresta et al. 1997; Yarnell, Oh et al. 2001). The conserved amino terminus of SSRP1 is homologous to the yeast Pob3 protein, whereas the function of the HMG domain is provided by the small HMG-box polypeptide Nhp6 in yeast (Wittmeyer, Joss et al. 1999; Brewster, Johnston et al. 2001; Formosa, Eriksson et al. 2001). The human SSRP1 counterparts of the yeast Pob3 and Nhp6 proteins were detected during apoptosis as the products of caspases 3- and 7-mediated cleavage of SSRP1 (Landais, Lee et al. 2006), indicating the importance of SSRP1 for cell survival. Indeed, both yeast Pob3 and mammalian SSRP1 are essential for cell (Wittmeyer, Joss et al. 1999; Brewster, Johnston et al. 2001; Formosa, Ruone et al. 2002) and animal (Cao, Bendall et al. 2003) viability. Similar to Spt16, SSRP1 also has an acidic and serine-rich carboxyl terminus that most likely facilitates its binding to histone proteins. Supporting this is the observation that SSRP1 can bind to H3-H4 tetramers (Belotserkovskaya, Oh et al. 2003). The current model proposes that FACT disrupts nucleosomes, which allow RNA polymerases to access DNA, and then it reassembles the

nucleosomes (Formosa, Ruone et al. 2002; Belotserkovskaya, Oh et al. 2003). This property gives the FACT complex the ability to regulate transcription initiation (Mason and Struhl 2003; Biswas, Yu et al. 2005), elongation (Orphanides, LeRoy et al. 1998; Mason and Struhl 2003; Saunders, Werner et al. 2003) and DNA replication (Okuhara, Ohta et al. 1999; Wittmeyer, Joss et al. 1999; Schlesinger and Formosa 2000) and also to be involved in DNA damage response (Keller, Zeng et al. 2001; Keller and Lu 2002). In addition to its general role, SSRP1 also functions as a co-regulator for several transcription activators, such as serum-response factor (SRF) (Spencer, Baron et al. 1999) and p63 (Zeng, Dai et al. 2002). Despite the current knowledge, it remains obscure if SSRP1 has an Spt16-independent role in gene regulation. Also, it is still unclear if FACT plays a global or gene-specific role in transcriptional regulation in human cells.

To address these questions, we generated tet-inducible siRNA cell lines for each of these two proteins using H1299 cells that are p53-deficient (Zeng, Dai et al. 2002). Using these cell lines, we performed spotted microarray analysis and found that the expression of many genes was altered after ablation of the endogenous SSRP1 or Spt16 levels by siRNA. Surprisingly, the effect was moderate. However, there was a subset of genes (~170) whose expression was either up-regulated or down-regulated after induction of siRNA against SSRP1 or Spt16. We further characterized some of the genes that displayed more apparent changes and found that SSRP1 and Spt16 shared common targets, as well as individually regulated genes. In particular, we identified the serum responsive gene, *egr1* (early growth response 1), as a novel target for both SSRP1 and Spt16. Either SSRP1 or Spt16 was indispensable for the expression of EGR1 in response to serum stimulation. We further elucidated that SSRP1 and Spt16 are important for the

progression of RNA pol II on the coding region of the *egr1* gene. Hence, our study suggests that SSRP1 and Spt16 indeed work together for the expression of a number of genes, whereas SSRP1 also appears to have an independent role in regulating the expression of a subset of genes in human cells.

MATERIALS AND METHODS

Plasmids and Antibodies. pHteto siRNA cloning vectors were the generous gifts of Mathew Thayer and Dan Stauffer (Oregon Health & Science University, Portland, OR) (Kuninger, Stauffer et al. 2004). Oligonucleotides ctagGCTCAGGACTGCTCTACCCttcaagagaGGGTAGAGCAGTCCTGAGCttttttggaaa and agcttttccaaaaaGCTCAGGACTGCTCTACCCtctcttgaaGGGTAGAGCAGTCCTGAGC (capital letters indicate the targeting sequences) containing human SSRP1 19-nucleotide targeting sequences or ctagGGAATTAAGACATGGTGTGttcaagagaCACACCATGTCTTAATTCCttttttggaaa and agcttttccaaaaaGGAATTAAGACATGGTGTGtctcttgaaCACACCATGTCTTAATTCC (capital letters indicate the targeting sequences) containing human Spt16 19-nucleotide targeting sequences were annealed and ligated into SpeI and HindIII sites. Oligomers of *ssrp1* siRNA, *spt16* siRNA, and scrambled siRNA (5'-AAGCGCGCTTTGTAGGATTC-3') were synthesized (Dharmacon). pcDNA3 FLAGSSRP1 was described previously (Zeng, Dai et al. 2002). Anti-SSRP1 and anti-Spt16 antibodies were used for Western blot assays, as described previously (Zeng, Dai

et al. 2002; Li, Keller et al. 2005). Mouse monoclonal SSRP1 antibody 5B10 was generated by Zymed Laboratories Inc. and purified as described previously (Landais, Lee et al. 2006). The anti-EGR1, anti-SRF, and anti-Spt16 (used in chromatin immunoprecipitation assay) antibodies were purchased from Santa Cruz Biotechnology. The mouse monoclonal RNA polymerase II H5 antibody, which recognizes theRNApol II Ser-2 phospho-isoform, was purchased from Babco-Covance. The anti-FLAG, anti-α-tubulin, rabbit polyclonal IgG, mouse monoclonal IgG, and mouse monoclonal IgM antibodies were purchased from Sigma.

Cell Culture. H1299 and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 0.1 mg/ml streptomycin, at 37 °C in 5% CO2.

Generation of Inducible Tet-On Cell Line. H1299pcDNA6- TR cells, which stably express the tet repressor, were transfected with pHtetoScramble, pHtetoSSRP1siRNA, or pHtetoSpt16siRNA plasmid and then selected in the medium containing 90 µg/ml hygromycin. Individual colonies were expanded into 12-well plates. To induce the siRNA expression, doxycycline was added to the media at a final concentration of 5 µg/ml. After doxycycline induction, cells were harvested for cell lysate preparation. SSRP1 or Spt16 expression level was checked by Western blot with anti-SSRP1 or anti-Spt16 antibodies. The colonies, which express significantly reduced levels of SSRP1 or Spt16, were maintained and used for further study.

Spotted Microarray. One clone for each cell line (clone 19 of H1299pHtetoSSRP1siRNA and clone 20 of H1299pHteto-Spt16siRNA) was used in the spotted microarray experiment. RNA for the Tet-On (doxycycline treatment) samples and

the Tet-Off (no doxycycline treatment) samples were prepared from three independent experiments using the Qiagen RNeasy mini kit. RNAs prepared from the SSRP1 and Spt16 siRNA Tet-Off samples were pooled as a control to compare between SSRP1siRNA and Spt16siRNA samples. The samples were sent to the Microarray Core at Oregon Health & Science University. The SMChumC8400A array was used in the experiment. All samples were amplified using linear T7 amplification (Message-Amp, Ambion) and examined for integrity using a BioAnalyzer (Agilent). Reverse transcription was used to synthesize a cDNA containing aminoallyl-modified dUTP (CyScribe Post-labeling; Amersham Biosciences). Using aminoallyl-modified dUTP in both strands eliminates the requirement for dye swap experiments. Aminoallyl-modified cDNA was incubated with Cy-dye esters for a nonenzymatic and covalent attachment of either Cy5 or Cy3 to the cDNA. The experimental sample was labeled with Cy5, and the control sample was labeled with Cy3. Following cleanup, selected Cy5 and Cy3 targets were combined and applied to each of two identical slides. Arrays were hybridized using M-series LifterSlips (Erie Scientific) and deep well hybridization chambers (TeleChem). Hybridized arrays were scanned on a ScanArray 4000 XL (PerkinElmer Life Sciences) using ScanArray Express software, and ImaGene (BioDiscovery) was used to extract data from the image. The resulting data file was loaded into GeneSight for normalization using intensity-based local regression (Lowess). The normalized data were used for further segregation and clustering as described in the figure legend.

RT-PCR. Total RNA was isolated from cells after different treatments, using the TRIzol (Invitrogen) protocol or Qiagen RNeasy mini kit. Reverse transcription of 5 μg of total RNA was performed in a 20 μl reaction using SuperscriptII reverse transcriptase

(Invitrogen) reagent, dNTP, and oligo (dT) 15 primer. After reverse transcription, 30 µl of diethyl pyrocarbonate H_2O was added to the reaction. 1 µl of reverse transcription reaction was used in the following PCRs with the following primers:

egr1 F, 5'-CTGACCGCAGAGTCTTTTCCTG-3', and R, 5'-TGGGTGCCGCTGAGTAAATG- 3';

dusp5 F, 5'-GTGTTGCGTGGATGTAAAACCC-3', and R, 5'-GCTCCTCCTCTGCTTGATGTAATC-3';

plau F, 5'-CACACACTGCTTCATTGATTACCC-3', and R, 5'-TTTTGGTGGTGACTTCAGAGCC-3';

id2 F, 5'-CGTGAGGTCCGTTAGGAAAAACAG-3', and R,5'-CTGACAATAGTGGGATGCGAGTC-3';

gapdh F, 5'-TCTAGACGGCAGGTCAGGTCCACC-3', and R, 5'-CCACCCATGGCAAATTCCATGGCA-3';

ssrp1 F, 5'-GAGCGATGACTCAGGAGAAG-3', and R, 5'-TTACTCATCGGATCCTG-3';

spt16 F, 5'-AGATATGTGACGTGTATAACG-3', and R, 5'-CTTCAGCTTCTCGAGTTTTAT-3';

and β-*actin* F, 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3', and R, 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'.

Real Time PCR. 1 µl of RT reaction was used in the real time PCR. A 15 µl reaction was performed using the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol, and amplified on the ABI7900HT. Threshold cycles (*Ct*)

for three replicate reactions were determined using SDS2. The relative transcript abundance was calculated following normalization with the GAPDH amplicon. The data for p21 and EGR1 were collected at 80 °C, and the data for other target genes were collected at 83 °C. The following primers were used:

egr1 F, 5'-CCTCCCTCTCTACTGGAGTGGAA- 3', and R, 5'-GAAGAACTTGGACATGGCTGTTTC- 3';

dusp5 F, 5' CTCAGGGTAGGTTCTCGGGACT-3', and R, 5'-GGCGAACTCTGAGGTGCAAG-3';

plau F, 5'-ATTCCTGCCAGGGAGACTCAG- 3', and R, 5'-TTGTCCTTCAGGGCACATCC- 3';

id2 F, 5'-CAGTCCCGTGAGGTCCGTTA-3',and R, 5'-CACCAGCTCCTTGAGCTTGG-3';

p21 F, 5' CTGGACTGTTTTCTCTCGGCTC- 3', and R, 5'-TGTATATTCAGCATTGTGGGAGGA- 3';

gapdh (83 °C) F, 5'-TGGAGTCCACTGGCGTCTTC- 3', and R, 5'-TTCACACCCATGACGAACATG-3';

and *gapdh* (80 °C) F, 5'-GATTCCACCCATGGCAAATTC- 3', and R, 5'-AGCATCGCCCCACTTGATT-3'.

Transient Transfection. H1299 cells (60% confluence in 60-mm plates) were transfected with 3μg of pcDNA3 or pcDNA3-FLAGSSRP1 using TransFectin (Bio-Rad). Total RNA was extracted after 48 h of transfection.

siRNA Transient Transfection and Serum Stimulation Assays. H1299 cells (60% confluence in 60-mm plates) were transfected with 30 nM of the scramble, SSRP1siRNA, or Spt16siRNA using SiLentFect (Bio-Rad). At the same time, DMEM containing 10% FBS was changed to DMEM containing 0.25% FBS for serum starvation. After 48 h, cells were cultured in DMEM with 20% FBS for serum stimulation. The cells were harvested at different time points for Western blotting or RNA extraction. For Western blotting, the cell lysates were prepared as described (Zeng, Dai et al. 2002), and 30 μg of cell lysates were used.

Cell Growth Assays. H1299pHScramble, H1299pHteto- SSRP1siRNA, and H1299pHtetoSpt16siRNA inducible cell lines were seeded at $4X10^5$ cells/60-mm plate and induced for siRNA expression by adding 5 μg/ml doxycycline to the media. After 4 days of induction, the cell number was counted, and viable cells were compared among the scrambled siRNA-, SSRP1siRNA-, and Spt16siRNA-expressing cells.

Chromatin Immunoprecipitation (ChIP)-Real Time PCR. H1299 cells were cultured in DMEM containing 0.25% FBS for serum starvation. After 48 h, cells were switched to media containing 20% FBS and harvested at 0, 5, and 30 min post-serum stimulation. ChIP assays were carried out as described previously (Zeng, Dai et al. 2002; Gomes, Bjerke et al. 2006) with the indicated antibodies. After reverse of cross-linking, DNA was purified by miniprep kit (Qiagen) and eluted in 50 μlof elution buffer. 1 μl of ChIP DNA or input DNA was used as templates in real time PCRs. A 20 μl reaction was performed using the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol, and amplified on the ABI7300. Threshold cycles (*Ct*) for three replicate reactions were determined using the 7300 system SDS software. The relative

fold change among the ChIP DNA samples was calculated following normalization with the input DNA. The following primers were used in the real time PCR: primers for negative control, *egr1* upstream 5990F, 5'-CACGGCCTGAACAGTGCAC-3', and *egr1* upstream 5839R, 5'-AGAAAGCCAGTGGAACCATCC- 3'; primers for promoter region, *egr1* upstream440F, 5'-CCCGGAAATGCCATATAAGGAGC-3', and *egr1* upstream 291R, 5'-AGTTCCCGCGTTGCCCCT-3'; *egr1* upstream192F, 5'-GGGTGCAGGATGGAGGTGC-3', and *egr1* upstream37R, 5'-TTGAAGGGTCTGGAACGGCA-3'; primers for coding region, *egr1* downstream 1292F, 5'-AACGAGAAGGTGCTGGTGGA- 3', and *egr1* downstream 1408R, 5'-CCACAAGGTGTTGCCACTGTT-3'; *egr1* downstream 2705F, 5'-TCAGAGCCAAGTCCTCCCTCT-3', and *egr1* downstream 2836R, 5'-GAAGAACTTGGACATGGCTGTTTC-3'; and c-*myc* downstream 4192F, CAGGCTCCTGGCAAAAGGT, and c-*myc* downstream 4266R, CAGTGGGCTGTGAGGAGGTT.

RESULTS

The Establishment of siRNA-inducible Cell Lines

To determine whether SSRP1 and Spt16 are required for global or genespecific transcription in human cells, we established inducible SSRP1 or Spt16 siRNA tet H1299 cell lines. In the presence of doxycycline, the expression of siRNA can be induced to downregulate its target mRNA. Indeed, as shown in Fig. 2.1, both the protein and the mRNA levels of either SSRP1 or Spt16 were markedly knocked down when the cells were cultured with doxycycline. As a control, the level of neither α-tubulin nor β-actin

was changed in either of the SSRP1- or Spt16-knockdown cells. Also, the mRNA level of Spt16 was not changed in the SSRP1 knockdown cells and vice versa for the Spt16-knockdown cells. These results indicate that we have established tet-inducible siRNA cell lines for SSRP1 and Spt16, respectively.

Reduction of SSRP1 or Spt16 Level by siRNA Alters the Transcription of a Common Set of Genes

To compare the gene expression profiles obtained from SSRP1- and Spt16-knockdown cells, we used a common reference (a pool of RNA samples from both of the siRNA cell lines without doxycycline treatment) in spotted microarray. The alterations of 8308 genes were compared between the SSRP1 and Spt16 siRNA samples (three samples per cell line). The genes with more than a 1.2- fold change in both SSRP1 siRNA and Spt16 siRNA samples were displayed by unsupervised hierarchical clustering. This analysis revealed that ~118 genes were either up-regulated or down-regulated with >1.2-fold change in both of the SSRP1 and Spt16 siRNA samples (Fig. 2.2A). Most of the genes (106 genes) appeared to be the common targets for both SSRP1 and Spt16, suggesting that the regulation of these genes may be executed by the FACT complex. There were more down-regulated genes (73 or 75 genes) than up-regulated genes (45 or 43 genes) in the SSRP1 or Spt16 siRNA samples. These results suggest that SSRP1 and Spt16 work together to enhance the expression of most of their target genes, although they may also act to repress the expression of a subset of genes in human cells. This result is consistent with previous results in yeast (Rowley, Singer et al. 1991). However, the 118 genes should not be the final number of SSRP1 and Spt16 targets in human cells

because the cDNA array used for our study only contained 8308 genes. For example, p21, a previously identified target for SSRP1 (Zeng, Dai et al. 2002), was not in this array. Even with this limited number of genes in the array, our gene expression profile data suggest that FACT may not play a global role in gene transcription, as only ~1.3% of the tested genes (106 of 8308 genes) displayed similar changes at their transcript levels in both SSRP1 and Spt16 knockdown samples (Fig. 2.2A).

To further analyze the 118 affected genes, we classified them into 10 different groups based on their functions in biological processes. As shown in Fig. 2.2B, they are involved in a broad spectrum of functions, including cell growth/maintenance (23 or 27 of 118), nucleic acid metabolism (19 or 21 of 118), signal transduction (8 or 9 of 118), protein metabolism (11 of 118), biosynthesis (3 of 118), cell adhesion (3 of 118), etc. Most of the SSRP1 and Spt16 target genes (73 or 81 of 118) encode either novel proteins with unknown functions or proteins with unclassified functions or hypothetical proteins, and thus are put into the unclassified group (Fig. 2.2B). These results indicate that SSRP1 and Spt16 have a relatively broad role in various cellular activities. However, a majority of them are involved in cell growth and/or maintenance and metabolism, which are essential for cell growth. These data support the notion that SSRP1 and Spt16 are essential for cell viability (Wittmeyer, Joss et al. 1999) (Brewster, Johnston et al. 2001) (Formosa, Ruone et al. 2002). Indeed, ablation of either SSRP1 or Spt16 by inducible siRNA in H1299 cells severely reduced the number of viable cells (Fig. 2.2D). This result was also repeated in 293 cells (data not shown). It was surprising that the changes were no more than 4-fold after knockdown for either SSRP1 or Spt16 (Fig. 2.2, A and C; Tables 2.1–3). These moderate changes could be due to two possibilities. 1) SSRP1 or

Spt16 might act as a cofactor in cells, so reduction of their levels would not drastically affect the gene expression profile; or 2) knockdown of SSRP1 or Spt16 was not 100%, so the residual protein might be still sufficient for transcription regulation, although to a lesser extent. Nevertheless, our results suggest that the FACT complex is important for up-regulating or down-regulating gene-specific transcription, rather than for global gene regulation. This notion was also confirmed in SSRP1 siRNA expressing 293 cells using cDNA microarray analysis, although with some variations in terms of specific target genes (data not shown).

SSRP1 and Spt16 May Have Independent Roles in Regulating the Expression of a Subset of Genes

In addition to sharing a common set of target genes (Fig. 2.2A), SSRP1 and Spt16 differentially regulated a subset of genes. As shown in the middle part of Fig. 2.2A, and also in Table 2.1, 12 of the 118 target genes were differentially regulated by SSRP1 and Spt16. These data suggest that SSRP1 and Spt16 may also have independent roles in gene regulation. Because the analysis for Fig. 2.2A discarded the data that showed changes in one (such as the SSRP1-knockdown), but not in the other (such as Spt16-knockdown), this approach would overlook what might be significant in either one of the SSRP1- and Spt16 knockdown samples. To avoid this bias and to identify more potential target genes for either SSRP1 or Spt16 in all the samples, we also analyzed all of the genes whose expression either increased or decreased >1.5-fold with a 95% confidence interval in at least one set of siRNA samples. As shown in Fig. 2.2C, 171 genes were identified among the six samples. Among these genes, some were down-regulated in the

SSRP1 siRNA samples, compared with the Spt16 siRNA samples, and another set of genes was up-regulated in SSRP1 siRNA samples but down-regulated in Spt16 siRNA samples. Although there were some variations among the triplicates per each siRNA line, most of the transcript levels were reproducible overall within one siRNA cell line (Fig. 2.2C).

To analyze the data further, we picked up the top five genes whose expression was more apparently up- or down-regulated in either SSRP1 siRNA samples or Spt16 siRNA samples, as listed in Table 2.2 and Table 2.3. As expected and discussed above, some of them appeared to be common targets for both SSRP1 and Spt16, such as *egr1*, *rpc32*, *cpne6*, *loc163782*, and *pro1073*. This result suggests that the expression of these genes may be regulated by the FACT complex. Surprisingly, there was also a subset of genes whose expression was specifically regulated by SSRP1 but not by Spt16. For example, in the presence of the siRNA against SSRP1, but not Spt16, the expression of some genes, such as *dusp5* (dual specificity phosphatase 5) (Kovanen, Rosenwald et al. 2003) or *plau* (plasminogen activator and urokinase) (Andreasen, Kjoller et al. 1997), decreased ~2-fold. By contrast, the expression of other genes, such as *id2* (inhibitor of differentiation) (Sun, Copeland et al. 1991), increased ~2-fold. These observations suggest that SSRP1, but not Spt16, may be involved in the activation or repression of these genes in cells. On the other hand, when knocking down Spt16, but not SSRP1, the expression of *arg99* decreased ~1.5-fold, suggesting that Spt16, but not SSRP1, may be involved in specifically activating the expression of *arg99*. In addition, there were some genes that were oppositely regulated by SSRP1 and Spt16, such as *hist1h1c* and *krt8*. These results suggest that SSRP1 and Spt16, besides their common roles in regulating the

expression of certain targets, may also have independent roles in gene regulation in human cells.

SSRP1 Has an Spt16-independent Role in Gene Transcription

As revealed in Table 2.2, knocking down SSRP1 led to the decrease of *egr1*, *plau*, and *dusp5* transcripts, but it also resulted in the increase of *id2* transcripts. Among them, only *egr1* appeared to require Spt16, as Spt16 knockdown did not cause apparent changes of *plau*, *dusp5*, and *id2* transcripts. These results suggest that SSRP1 may have Spt16-independent roles in regulating the expression of a subset of genes. To verify this possibility, we performed a series of RT-PCR and real time PCR analyses for these four genes using the same cell lines, as shown in Fig. 2.1. Indeed, we reproduced the microarray results. As shown in Fig. 2.3, the expression of *dusp5*, *plau*, and *id2* was altered in the presence of SSRP1siRNA but not of Spt16 siRNA, whereas the transcription of *egr1* was inhibited when either SSRP1 siRNA or Spt16 siRNA was induced. In addition, the expression of p21, a previously identified target for SSRP1 (Zeng, Dai et al. 2002), was markedly reduced when SSRP1, but not Spt16, was knocked down by siRNA (Fig. 2.3), verifying our previous study (Zeng, Dai et al. 2002). These effects were p53-independent, as H1299 cells are p53-deficient.

To exclude the possibility that the above results were tet cell line-specific, we transiently introduced oligomers of siRNA into H1299 cells and tested the expression of *egr1*, *plau*, and *id2* using a real time PCR assay. As shown in Fig. 2.4A, transient transfection of either SSRP1 or Spt16 siRNA significantly reduced the level of SSRP1 or

Spt16. Also, the alterations of the expression of the three target genes were consistent with the result in Fig. 2.3. This result was also repeated in 293 cells (data not shown).

To determine whether overexpression of SSRP1 may have the opposite effect on the expression of the four potential target genes, we transiently introduced exogenous FLAG-SSRP1 into H1299 cells. The mRNA levels of *egr1*, *dusp5*, *plau*, and *id2* were assessed using RT-PCR. Consistent with the results in Figs. 2.3 and 2.4A, overexpression of FLAG-SSRP1 induced the transcription of *egr1*, *dusp5*, and *plau* but reduced the transcription of *id2* (Fig. 2.4B). These results suggest that SSRP1 can up and down-regulate the expression of a subset of genes, some of which may be regulated independently of Spt16, although it remains to be determined whether this regulation is at the initiation or elongation step during transcription.

The Expression of EGR1 in Response to Serum Stimulation Requires Both SSRP1 and Spt16

The fold change of these target genes was moderate, suggesting that SSRP1 and Spt16 may work as a cofactor of transcriptional activators or repressors in human cells. One question was whether SSRP1 requires Spt16 for its regulatory function in transcription. Interestingly, *egr1*, one of our identified target genes, as described above, appeared to require both of these proteins for expression. Also, interestingly, the *egr1* gene contains six serum-response elements (CArG-box) in its promoter region (Tullai, Schaffer et al. 2004). The CArG-box is the binding site of SRF (Chai and Tarnawski 2002), for which SSRP1 has been previously identified as a co-activator (Spencer, Baron

et al. 1999). Thus the *egr1* gene serves as a proper target to address the above specific question.

EGR1 is a zinc finger containing transcription factor (Sukhatme, Cao et al. 1988). It belongs to the cellular immediate early genes that rapidly respond to serum stimulation (Sukhatme, Cao et al. 1988). To test whether SSRP1 is required in serum-induced EGR1 expression, we conducted a set of serum stimulation experiments. Under normal or serum starvation conditions, the EGR1 protein level was detected at extremely low levels (Fig. 2.5A). After 20% serum stimulation for 1.5 h, the EGR1 level was significantly increased. This induction was markedly inhibited when the cell was transiently transfected with SSRP1 siRNA before serum stimulation (Fig. 2.5A). This result demonstrates that SSRP1 is required for the EGR1 induction in response to serum stimulation.

The *egr1* gene was also identified as a potential target for Spt16 (Table 2.2). To investigate whether Spt16 is also required in the EGR1 induction in response to serum stimulation, we analyzed the effect of Spt16 siRNA on the EGR1 expression after serum stimulation. The induction of EGR1 was inhibited at both the RNA (Fig. 2.5C) and the protein level (Fig. 2.5B) when Spt16 was knocked down. To determine whether overexpression of SSRP1 can rescue this inhibition, we introduced FLAGSSRP1 into the Spt16 siRNA expressing cells and found that even overexpression of exogenous FLAG-SSRP1 could not rescue the inhibitory effect of Spt16 siRNA on the induction of EGR1 in response to serum stimulation (Fig. 2.5, B and C). Taken together, these results demonstrate that both Spt16 and SSRP1 are indispensable for the regulation of serum-responsive EGR1 expression.

To distinguish if the inhibition of SSRP1 and Spt16 siRNA on EGR1 expression is because of the inhibition of the EGR1 expression level or the delay of EGR1 expression during the serum stimulation course, we performed a double SSRP1 and Spt16 siRNA treatment to investigate the effect on the EGR1 expression after serum stimulation at different time points. As shown in Fig. 2.5D, EGR1 expression after serum stimulation displayed similar kinetics between scrambled siRNA-treated cells and double SSRP1 and Spt16 siRNA-treated cells, but its level was markedly reduced in the SSRP1 and Spt16 siRNA treated cells. These data suggest that SSRP1 and Spt16 regulate the expression level of EGR1 and not the onset of EGR1 expression during serum stimulation.

SSRP1 and Spt16 Play an Important Role in the Elongation of egr1 Transcription in Response to Serum Stimulation

Next we wanted to determine how SSRP1 and Spt16 regulate the expression of *egr1* by performing a set of ChIP analyses. Fig. 2.6A shows the location of five amplicons used in real time PCR quantification of the ChIP-enriched DNA. Region 1 is a control region, which is far upstream of the *egr1* gene. Regions 2 and 3 are in the *egr1* promoter region and contain 4 and 2 CArG-boxes, respectively. Regions 4 and 5 are in the *egr1* coding region. To catch the temporal distribution of the protein on the *egr1* gene, we performed cross-linking and ChIP experiments at various times after serum stimulation. The corresponding transcription level of *egr1* is shown in Fig. 2.6B. After 30 min of serum stimulation, the *egr1* transcript was readily detected.

Both endogenous SSRP1 and Spt16 levels at the downstream coding regions of the *egr1* gene (region 4 and particularly region 5) remarkably increased after serum stimulation compared with the nonstimulated control (0 min) (Fig. 2.6, C and D), suggesting that SSRP1 and Spt16 are recruited to the elongation complex after serum stimulation in a time-dependent fashion. The recruitment of SSRP1 and Spt16 to the *egr1* coding region (region 5) is specific as they did not increase at a similar position of the c-*myc* gene, which is a late serum-responsive gene (Paulsson, Bywater et al. 1987) (Fig. 2.7). Given the knowledge that SSRP1 is a co-activator for SRF (Spencer, Baron et al. 1999) and that the *egr1* promoter contains six SRF-binding sites (CArG-box) (Tullai, Schaffer et al. 2004), it was surprising that there was no apparent recruitment of SSRP1 to the promoter region after serum stimulation (Fig. 2.6C). To determine the reason for this lack of recruitment, we also checked the occupation status of SRF on the *egr1* gene before and after serum stimulation. Surprisingly, SRF was already bound to the *egr1* promoter (regions 2 and 3) under nonserum stimulation conditions, and serum simulation only slightly strengthened SRF binding to the *egr1* promoter (Fig. 2.6E). Thus it is possible that this process does not require SSRP1 or that SSRP1 plays a transient function.

The carboxyl-terminal domain (CTD) of the largest subunit of RNA pol II is comprised of multiple heptad repeats (YSPTSPS motifs), and its phosphorylation has been shown to be mediated by TFIIH at transcriptional initiation (Lu, Flores et al. 1991; Lu, Zawel et al. 1992) and by CDK9 during transcriptional elongation (Fu, Peng et al. 1999). Two major phosphorylations of the CTD happen *in vivo*. Phosphorylation of the CTD on Ser-5 occurs at the promoter region and mediates recruitment and regulation of the mRNA capping enzyme guanylytransferase; and phosphorylation of CTD on Ser-2

occurs on the elongating polymerase and couples transcription and 3' end processing (Ahn, Kim et al. 2004). To investigate further how SSRP1 and Spt16 are involved in the transcription elongation complex, we analyzed the effect of simultaneous depletion of SSRP1 and Spt16 on the recruitment of RNA pol II Ser-2 phospho-isoform to the elongating region of the *egr1* gene after serum stimulation. First, we checked the distribution of RNA pol II Ser-2 phospho-isoform on the *egr1* gene before and after serum stimulation. As shown in Fig. 2.6F, phosphorylation of RNA pol II on Ser-2 occurred on the promoter (region 3) and coding regions (region 4 and 5) after serum stimulation. In agreement with previous studies (Wang, Balamotis et al. 2005; Gomes, Bjerke et al. 2006), this RNA pol II Ser-2 phospho-isoform was more abundant at the 3' region of the gene (regions 4 and 5) than in the promoter region (region 3) after serum stimulation (Fig. 2.6F). When SSRP1 and Spt16 were depleted in the cells, much less RNA pol II Ser-2 phosphoisoforms associated with the downstream coding region (region 5) (Fig. 2.8). These results suggest that the progression of RNA pol II to the downstream coding region was facilitated by SSRP1 and Spt16.

DISCUSSION

It has been shown that the FACT complex functions in transcriptional elongation on a chromatin template *in vitro* and on specific genes in cells (Orphanides, LeRoy et al. 1998; Saunders, Werner et al. 2003). However, it is unclear whether this function is at the global level or for specific genes. Our study, as described here, indicates that SSRP1 and Spt16 share a number of common target genes (Figs. 2.3 and 2.4; Tables 2.2 and 2.3), although their global roles in gene transcription are not apparent (only~1.3% of the 8308

tested genes were affected by both SSRP1 and Spt16 siRNAs). These target genes encode proteins that are important for cell growth and maintenance, signal transduction, nucleotide and protein metabolism, biosynthesis, cell adhesion, and so on (Fig. 2.2B), reflecting the importance of SSRP1 and Spt16 for a broad spectrum of cellular activities, as well as for cell viability (Fig. 2.2D).

Surprisingly, the changes of the specific gene expression profiles in the SSRP1- or Spt16-knockdown cell lines were quite moderate (less than 4-fold). This moderate change could be due to three possibilities. First, the SSRP1 and Spt16 protein levels were not completely depleted in the cells after the siRNAs were induced. Residual SSRP1 and Spt16 may be sufficient for maintaining the basal level of transcriptional regulation. Otherwise, the FACT complex might not be the only factor involved in transcription elongation on the chromatin template. It has been shown that chromatin remodeling complexes can also play a role in transcription elongation, such as CHD1 or SWI/SNF (Belotserkovskaya and Reinberg 2004). Other histone groups of SPT proteins have also been implicated in the control of transcription elongation, such as the Spt4-Spt5 complex or Spt6 (Belotserkovskaya and Reinberg 2004). Another possibility is that SSRP1 and Spt16 may regulate transcription in a gene-specific fashion. Our data appear to favor this hypothesis, as ~106 genes were identified as the potential targets for both SSRP1 and Spt16. These target genes may vary in different cell types and/or under different physiological conditions. Indeed, siRNA against SSRP1 in 293 cells appeared to affect the expression of genes, which did not match 100% of those identified in H1299 cells (data not shown). However, both up- and down-regulation of a subset of genes were also observed (data not shown). Consistent with this observation, a recent study on yeast

FACT also showed a gene-specific requirement for FACT during transcription (Jimeno-Gonzalez, Gomez-Herreros et al. 2006). The best example, as further examined here, is *egr1*, whose expression in response to serum stimulation requires both SSRP1 and Spt16, as knocking down either of these two proteins drastically inhibited the serum responsive expression of *egr1* (Fig. 2.5).

An intriguing question is how *egr1* might be rapidly transcribed under conditions of serum stimulation. The high level of SRF on the *egr1* promoter region before serum stimulation suggests the presence of a preassembled initiation complex (PIC). It has been shown that the transcription of human p21, c-*fos*, c-*myc*, and *Drosophila hsp70* is regulated at post-initiation steps (Espinosa, Verdun et al. 2003; Aida, Chen et al. 2006; Yamada, Yamaguchi et al. 2006). Before stimulation, these promoters are preloaded with significant amounts of several components of the PIC, including RNA pol II itself. After stimulation, conversion of RNA pol II into a fully elongating form is achieved, which leads to rapid activation. Our data suggest that *egr1* is probably regulated in the same fashion. Before serum stimulation, SRF exists on the *egr1* promoter. The presence of SRF at the *egr1* promoter before serum stimulation indicates that SRF may play an important role for the PIC assembly. After serum stimulation, rapid transcriptional activation is likely fulfilled by the conversion of RNA pol II to the elongation form. Although this model needs to be tested, our data suggest that the progression of this elongating RNA pol II on the *egr1* coding region requires FACT (SSRP1 and Spt16) complex. FACT has been shown to facilitate transcriptional elongation by disassembling nucleosomes *in vitro* (Malone, Clark et al. 1991). Moreover, FACT has been shown to be involved in the regulation of other rapid inducible genes. In *Drosophila*, FACT is

involved in the rapid induction of HSP70 under thermal stress and displays kinetics of recruitment to the coding region of the *hsp70* gene (Saunders, Werner et al. 2003). Similarly in human cells, FACT is recruited to the coding region of *egr1* after serum stimulation in a time-dependent fashion and contributes to the progression of RNA polymerase II along the *egr1* gene. Hence, by participating in the regulation of the elongation of the *egr1* transcription, FACT is essential for the expression of this immediate early serum-responsive gene, although it remains to be elucidated how FACT becomes committed to the regulation of a specific gene. It was suggested that histone H2B monoubiquitination facilitates FACT function (Pavri, Zhu et al. 2006; Reinberg and Sims 2006). So it is possible that after serum stimulation, the *egr1* gene locus is one of the first under extensive histone tail modification and then recruits FACT to dissemble the nucleosome.

Another finding from our study was that a subset of the common target genes for SSRP1 and Spt16 was up-regulated when either SSRP1 or Spt16 was knocked down (Fig. 2.1, Fig. 2.2, Tables 2.1–3). These data are consistent with the previous reports showing that the yeast orthologs of human SSRP1 and Spt16 were involved in down-regulation of specific genes (Rowley, Singer et al. 1991) and that yeast Spt16 depletion causes YAT1 mRNA levels to increase (Jimeno-Gonzalez, Gomez-Herreros et al. 2006). These results suggest that SSRP1 and Spt16 may act as co-repressors as well. It has yet to be determined if this repression is at the initiation or elongation step of transcription.

Interestingly, our study also suggests that SSRP1 and Spt16 may have independent functions in cells. Validating some of the SSRP1-specific target genes, such as *plau*, *id2*, or *dusp5*, further strengthens this notion (Figs. 2.3 and 2.4). Correlated with

this notion, we previously isolated an SSRP1-associated protein complex free of Spt16 from HeLa nuclear extracts (Zeng, Dai et al. 2002). Therefore, SSRP1 also has an Spt16-independent role in regulating transcription.

ACKNOWLEDGMENTS

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Fig. 2.1. Establishment of the tet-inducible SSRP1 or Spt16 siRNA cell lines.

SSRP1 or Spt16 siRNA was induced by doxycycline treatment of H1299 pHteto-SSRP1 or Spt16 siRNA cells. The protein and RNA levels of SSRP1 or Spt16 were detected by Western blotting (WB) and RT-PCR analyses, respectively.

Fig. 2.1

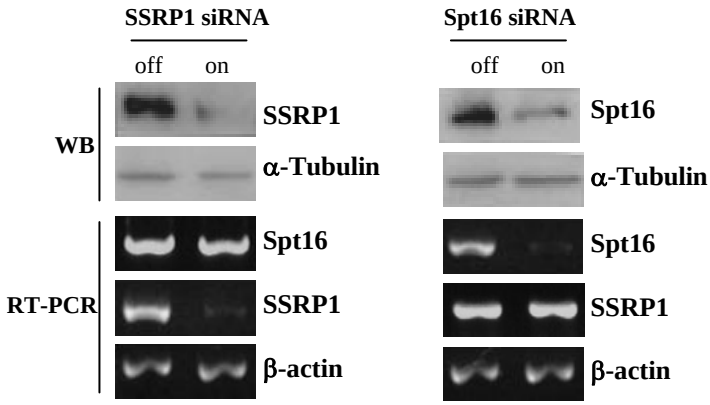
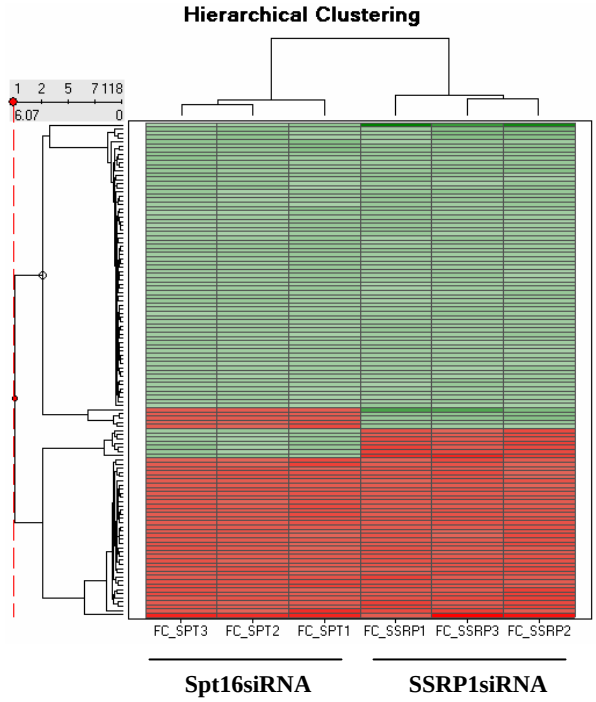

SSRP1 siRNA
Spt16 siRNA
off
on
off
on
WB
SSRP1
α-Tubulin
Spt16
α-Tubulin
RT-PCR
Spt16
SSRP1
β-actin
Spt16
SSRP1
β-actin

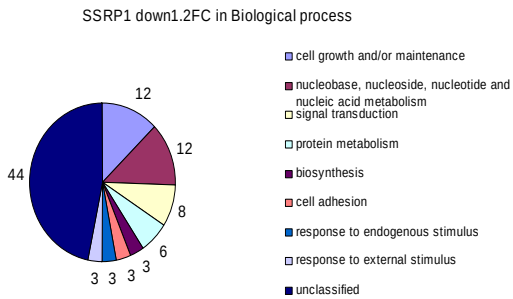
Fig. 2.2. (A) The hierarchical clustering analysis of the microarray gene expression profiles identified the common targets for SSRP1 and Spt16. The fold change of each gene (ratio of siRNA to control) and average fold change of three replicates were calculated. 118 genes had >1.2 average fold change (either up or down) in both of the SSRP1 and Spt16 siRNA samples. The data from these 118 genes were imported into "Statistica" software and visualized by the unsupervised hierarchical clustering. Green or red color indicates the genes that were down-regulated or up-regulated, respectively, after siRNA was induced in the cells. **(B)** The up-or down-regulated genes in SSRP1 or Spt16 siRNA samples were classified by biological process using an online annotation tool (http://apps1.niaid.nih.gov/david/). **(C)** The clustering analysis of the microarray gene expression profiles reveals a subset of genes that are specifically regulated by SSRP1 or Spt16. We segregated the genes that were significantly different in any of the six samples, using a 1.5 fold cut-off and a 95% confidence interval. The resulting gene set (171 genes) was clustered using a Pearson correlation metric. Log2 ratio of siRNA to control was used in this clustering. Green or red color indicates the genes that were down regulated or up regulated, respectively, after siRNA was induced in the cells. **(D)** SSRP1 and Spt16 are essential for cell viability. H1299pHScramble, H1299pHtetoSSRP1siRNA and H1299pHtetoSpt16siRNA inducible cell lines were split at equal number and induced for siRNA expression. Four days after induction, cell numbers were counted and the relative cell density was compared among the scrambled siRNA-, SSRP1siRNA- and Spt16siRNA-expressing cells. The experiment was repeated five times and error bars are indicated.

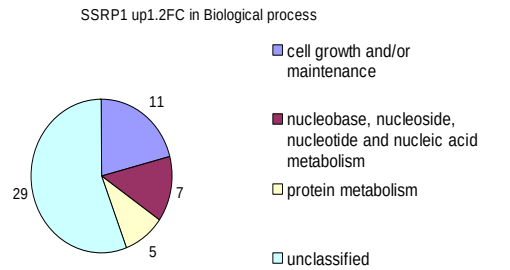
Fig. 2.2

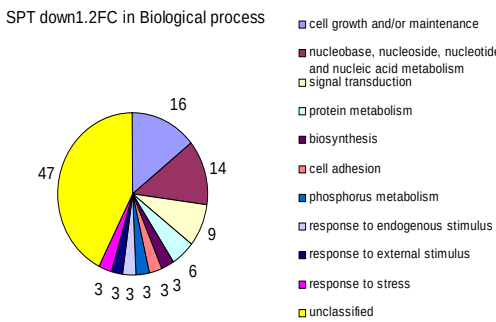
A.


Hierarchical Clustering
1 2 5 7 118
6.07
0
FC_SPT3
FC_SPT2
FC_SPT1
FC_SSRP1
FC_SSRP3
FC_SSRP2
Spt16siRNA
SSRP1siRNA

B.


SSRP1 down1.2FC in Biological process
12
12
44
8
6
3
3
3
3
cell growth and/or maintenance
nucleobase, nucleoside, nucleotide and nucleic acid metabolism
signal transduction
protein metabolism
biosynthesis
cell adhesion
response to endogenous stimulus
response to external stimulus
unclassified


SSRP1 up1.2FC in Biological process
11
7
29
5
cell growth and/or maintenance
nucleobase, nucleoside, nucleotide and nucleic acid metabolism
protein metabolism
unclassified


SPT down1.2FC in Biological process
16
14
47
9
6
3
3
3
3
3
cell growth and/or maintenance
nucleobase, nucleoside, nucleotide and nucleic acid metabolism
signal transduction
protein metabolism
biosynthesis
cell adhesion
phosphorus metabolism
response to endogenous stimulus
response to external stimulus
response to stress
unclassified

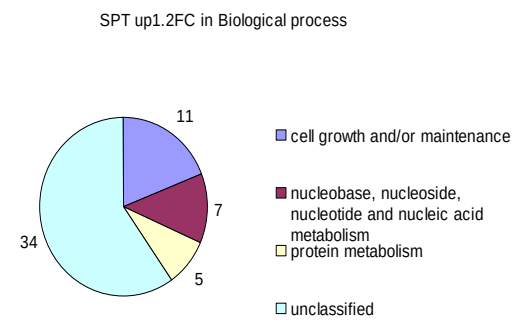
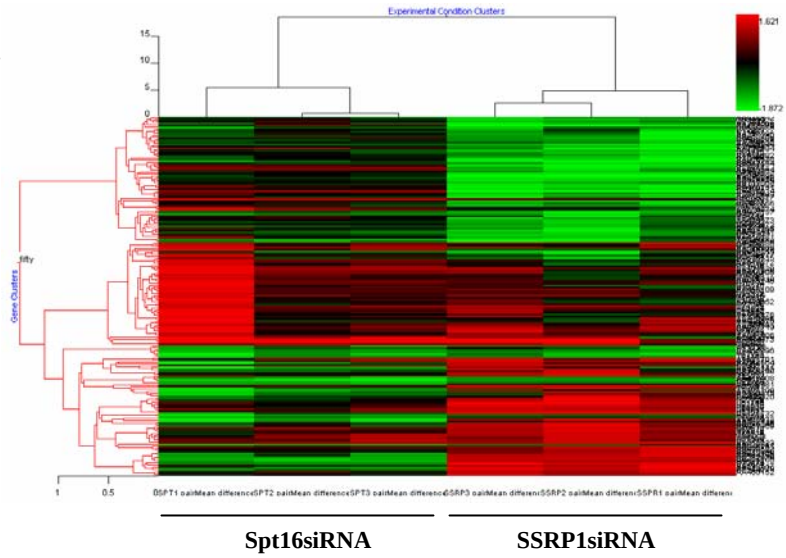

SPT up1.2FC in Biological process
11
7
34
5
cell growth and/or maintenance
nucleobase, nucleoside, nucleotide and nucleic acid metabolism
protein metabolism
unclassified

Fig. 2.2 (continued)

C.



D.

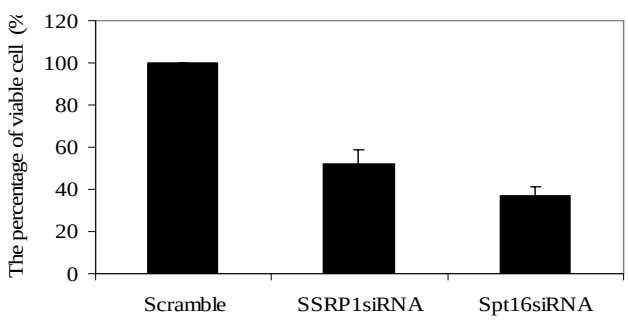


TABLE 2.1. The list of the 12 specific genes whose transcript levels were differentially regulated in the SSRP1 or Spt16 siRNA samples, as shown in Fig. 2.2A.

Ave_Spt16_FC means the average gene fold change in the three Spt16siRNA samples.

Ave_SSRP1_FC means the average gene fold change in the three SSRP1siRNA samples.

Table 2.1

GENBANK	Ave_Spt16_FC	Ave_SSRP1_FC	SYMBOL	GENENAME
R70601	1.25949	-2.4014		unknown protein
N78083	1.21344	-1.5576		unknown protein
R22977	1.24888	-1.5241	MSN	moesin
AA232856	1.21156	-1.3845	TOP1	topoisomerase (DNA) I
H00817	1.24823	-1.2726	LYPLA1	lysophospholipase I
AA620528	-1.2894	1.268		unknown protein
AA701502	-1.2371	1.25759	PDGFA	platelet-derived growth factor alpha polypeptide
AA676458	-1.2318	1.30321	LOXL2	lysyl oxidase-like 2
R08261	-1.3456	1.45108		unknown protein
AA610040	-1.3499	1.45112	HIST1H2BA	histone 1, H2ba
AA453105	-1.3104	1.51582	HIST1H2AC	histone 1, H2ac
AA598517	-1.4432	1.82049	KRT8	keratin 8

TABLE 2.2. Top 5 genes whose transcript levels were up- or down-regulated in the SSRP1 siRNA samples, as identified in Fig. 2.2C.

Table 2.2

GENBANK	Ave_Spt16_FC	Ave_SSRP1_FC	SYMBOL	GENENAME
AA486628	-1.303377	-3.43545	EGR1	early growth response 1
W65461	-1.09478	-2.43096	DUSP5	dual specificity phosphatase 5
R70601	1.2662815	-2.35666		unknown protein
AA284668	1.16133	-1.81744	PLAU	plasminogen activator, urokinase
H17960	1.02384	-1.7801	AP1G1	adaptor-related protein complex 1, gamma 1 subunit
H06273	-1.360353	1.74244	LOC90133	hypothetical protein LOC90133
H82706	1.0934903	1.778135	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
AA598517	-1.434228	1.82648	KRT8	keratin 8
N55540	1.9094455	2.078311	LOC163782	hypothetical protein LOC163782
AA064973	2.0401064	2.281155	PRO01073	PRO1073 protein

TABLE 2.3. Top 5 genes whose transcript levels were up- or down-regulated in the Spt16 siRNA samples, as identified in Fig. 2.2C.

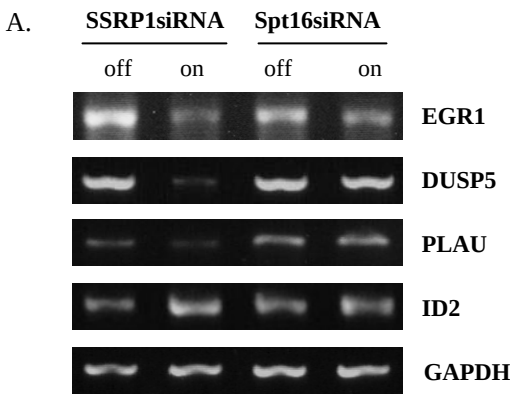
Table 2.3

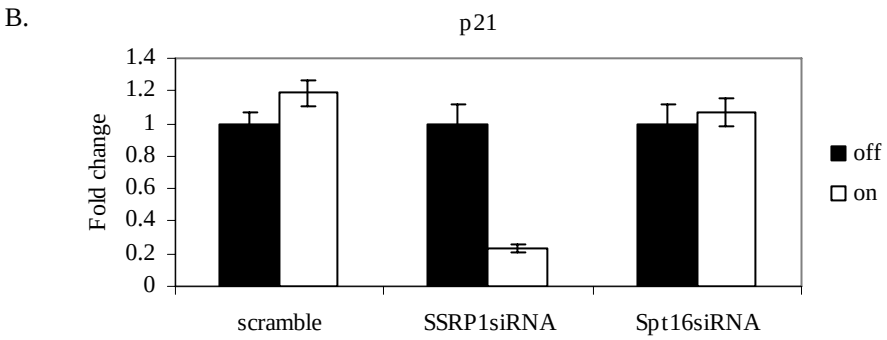
GENBANK	Ave_Spt16_FC	Ave_SSRP1_FC	SYMBOL	GENENAME
T66816	-1.916707	1.509468	HIST1H1C	histone 1, H1c
AA418408	-1.704988	-1.46134	RPC32	polymerase (RNA) III (DNA directed) (32kD)
H11051	-1.627403	-1.48982	CPNE6	copine VI (neuronal)
AA058323	-1.552067	-1.23807	IFITM1	interferon induced transmembrane protein 1 (9-27)
T60061	-1.514326	-1.14426	ARG99	ARG99 protein
W85697	1.4734588	-1.11418	HNRPK	heterogeneous nuclear ribonucleoprotein K
R53406	1.5113547	1.308622		unknown protein
AA041406	1.7223561	1.37524	COP1	constitutive photomorphogenic protein
N55540	1.9094455	2.078311	LOC163782	hypothetical protein LOC163782
AA064973	2.0401064	2.281155	PRO1073	PRO1073 protein

Fig. 2.3. Validation of some SSRP1-specific target genes as identified from the microarray analysis.

(A) SSRP1 or Spt16 siRNA was induced in the Tet-On cell lines. Total RNAs from the Tet-Off and on samples were prepared 65 h after induction for RT-PCR with different primers. **(B)**–**(F)** scrambled, SSRP1, or Spt16 siRNA was induced in the Tet-On cell lines. Total RNAs from the Tet-Off and -On samples were prepared for real time PCR with different primers.

Fig. 2.3


A.
SSRP1siRNA
Spt16siRNA
off
on
off
on
EGR1
DUSP5
PLAU
ID2
GAPDH


B.
p21
Fold change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
scramble
SSRP1siRNA
Spt16siRNA
off
on

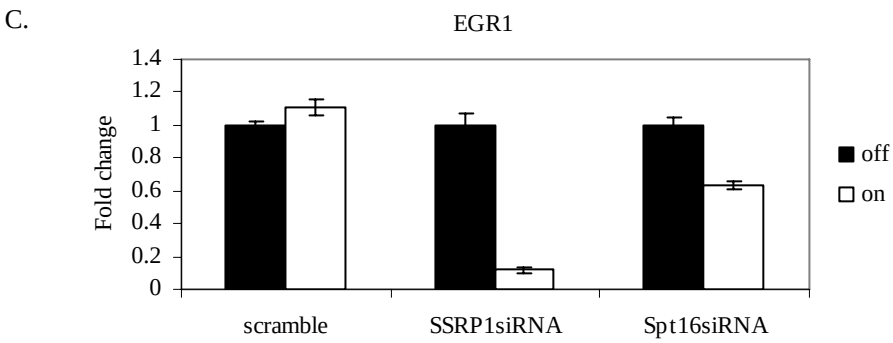
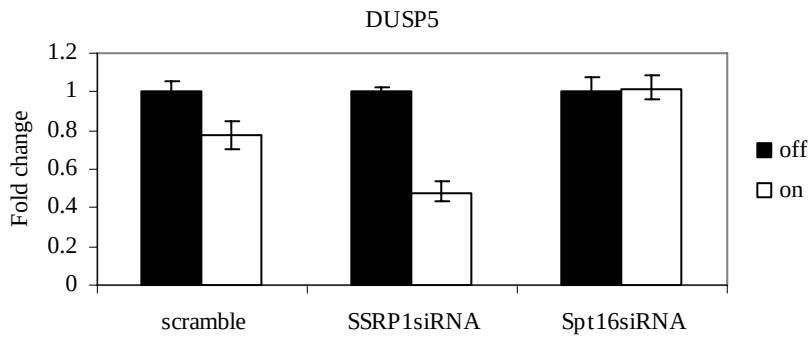
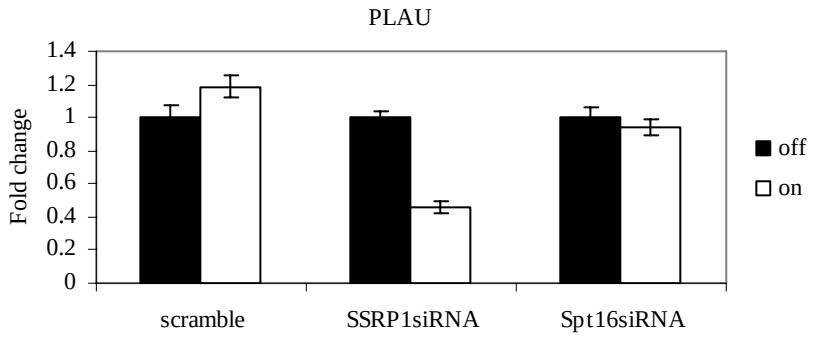

C.
EGR1
Fold change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
scramble
SSRP1siRNA
Spt16siRNA
off
on

Fig. 2.3 (continued)

D.



E.



F.

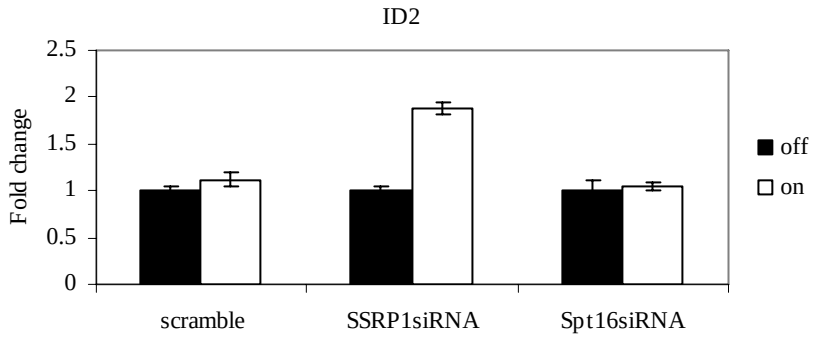


Fig. 2.4.SSRP1 has an Spt16-independent role in regulating the expression of *dusp5*, *id2*, and *plau*.

(A) H1299 cells were transiently transfected with scrambled, SSRP1, or Spt16 siRNA. Cells were harvested, 48 h after transfection, for real time PCR, as described in Fig. 2.3B.

(B) H1299 cells were transiently transfected with an empty vector (-) or FLAG-SSRP1 (+). Cells were harvested for RT-PCR as described in Fig. 2.3A, 48 h after transfection.

Fig. 2.4

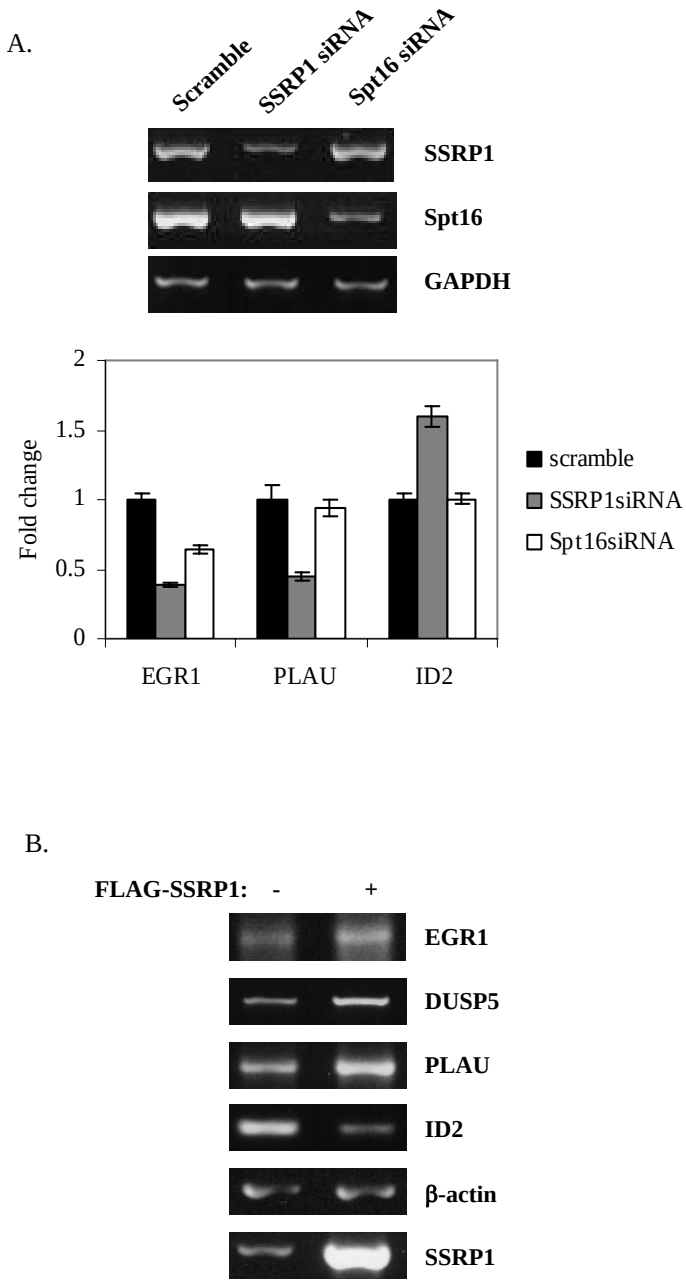

A.
Scramble
SSRP1 siRNA
Spt16 siRNA
SSRP1
Spt16
GAPDH
Fold change
2
1.5
1
0.5
0
EGR1
PLAU
ID2
scramble
SSRP1siRNA
Spt16siRNA
B.
FLAG-SSRP1: - +
EGR1
DUSP5
PLAU
ID2
β-actin
SSRP1

Fig. 2.5. SSRP1 and Spt16 are required for the expression of EGR1 in response to serum stimulation.

(A) Ablation of SSRP1 by siRNA inhibits the induction of EGR1 in response to serum stimulation. H1299 cells were transfected with 30 nM scrambled or SSRP1siRNA. At the same time, cells were incubated in DMEM containing 0.25% FBS for serum starvation. After 48 h, cells were cultured in DMEM containing 20% FBS for 1.5 h and harvested for Western blot using antibodies, as indicated. **(B)** and **(C)** Spt16 is required for the induction of EGR1 by serum. H1299 cells were transfected with scrambled, Spt16siRNA, or Spt16siRNA together with the plasmid FLAGSSRP1. At the same time, cells were incubated in DMEM containing 0.25% FBS for serum starvation. After 48 h, cells were cultured in DMEM containing 20% FBS for 1 h and harvested for real time PCR **(C)** and Western blot **(B)** analyses, as indicated. **(D)** SSRP1 and Spt16 regulate the expression level of EGR1 and not the onset of EGR1 expression during serum stimulation. H1299 cells were transfected with scrambled siRNA or siRNA for SSRP1 and Spt16 together. At the same time, cells were incubated in DMEM containing 0.25% FBS for serum starvation. After 48 h, cells were cultured in DMEM containing 20% FBS for various hours and harvested for Western blot using antibodies, as indicated.

Fig. 2.5

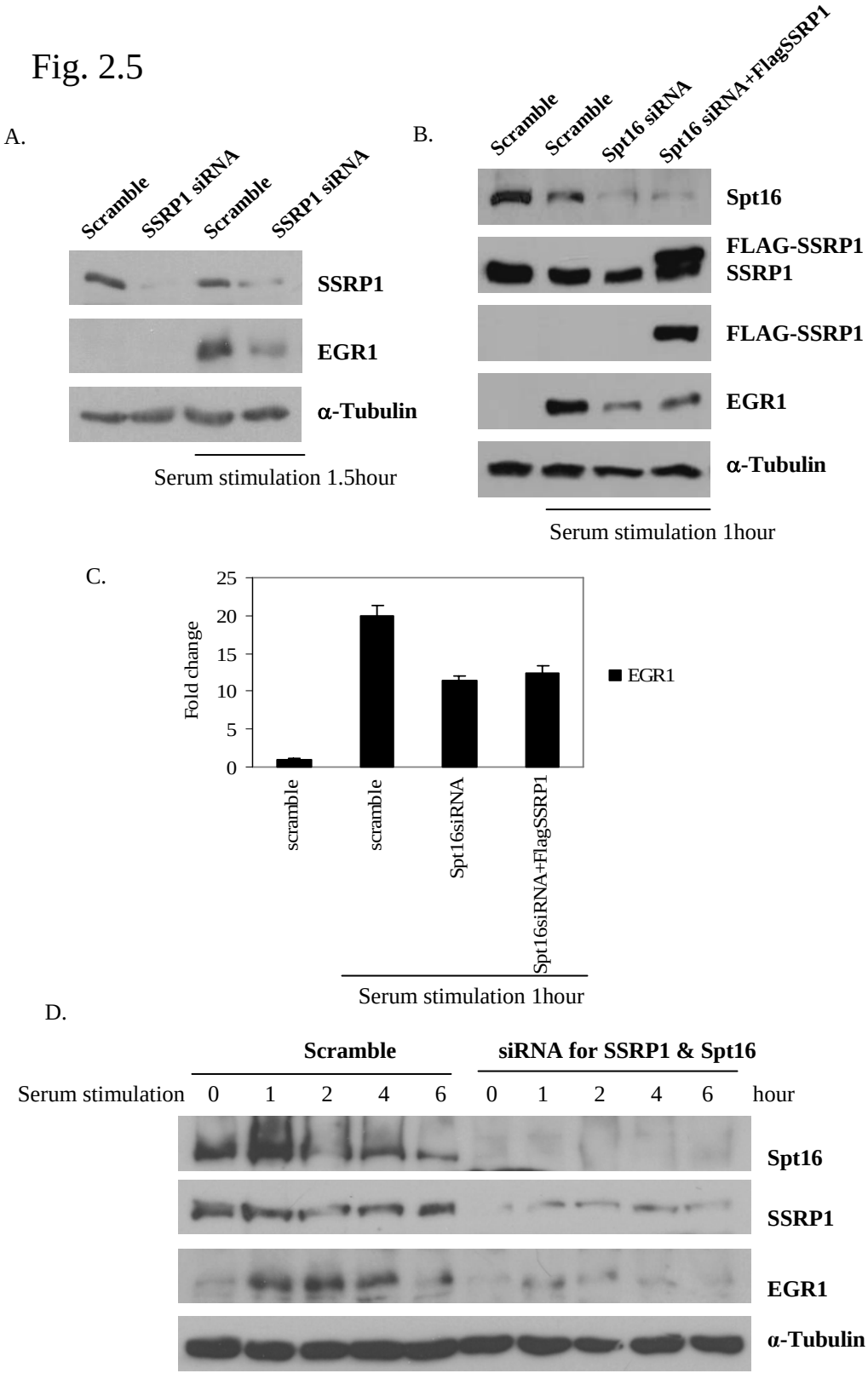
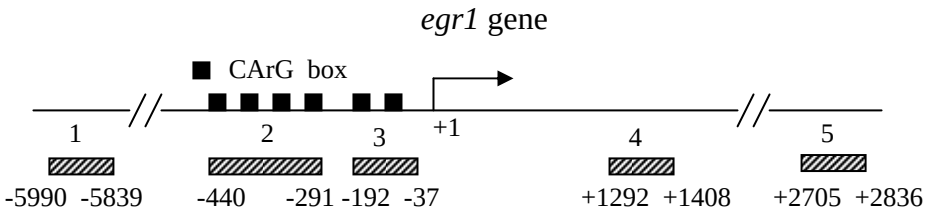

A.
Scramble
SSRP1 siRNA
Scramble
SSRP1 siRNA
SSRP1
EGR1
α-Tubulin
Serum stimulation 1.5hour
B.
Scramble
Scramble
Spt16 siRNA
Spt16 siRNA+FlagSSRP1
Spt16
FLAG-SSRP1
SSRP1
FLAG-SSRP1
EGR1
α-Tubulin
Serum stimulation 1hour
C.
Fold change
25
20
15
10
5
0
EGR1
scramble
scramble
Spt16siRNA
Spt16siRNA+FlagSSRP1
Serum stimulation 1hour
D.
Scramble
siRNA for SSRP1 & Spt16
Serum stimulation 0 1 2 4 6 0 1 2 4 6 hour
Spt16
SSRP1
EGR1
α-Tubulin

Fig. 2.6. SSRP1 and Spt16 play a role in the elongation of *egr1* transcription in response to serum stimulation.

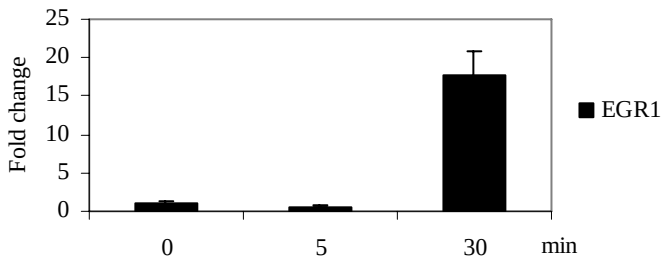
(A) Schematic showing location of the amplicons used in real time PCR quantification of chromatin immunoprecipitation-enriched DNA. **(B)** *T*he transcription level of *egr1* before (0 min) or after serum stimulation (5 and 30 min). Total RNA was extracted from H1299 cells before or after serum stimulation. After reverse transcription, real time PCR was performed using *egr1* primer. **(C)–(F)** Distribution of SSRP1, Spt16, SRF, and RNA pol II Ser-2 phospho-isoform on the *egr1* gene before and after serum stimulation. ChIP assays were performed with H1299 cells harvested before (0 min) or after serum stimulation (5 and 30 min) using the indicated antibodies. ChIP-enriched DNA was quantified by real time PCR using the indicated amplicons. Values are expressed as relative fold change over the IgG or IgM control.

Fig. 2.6

A.


egr1 gene
CArG box
+1
1
2
3
4
5
-5990 -5839
-440 -291 -192 -37
+1292 +1408
+2705 +2836

B.


Fold change
25
20
15
10
5
0
EGR1
0
5
30
min

C.

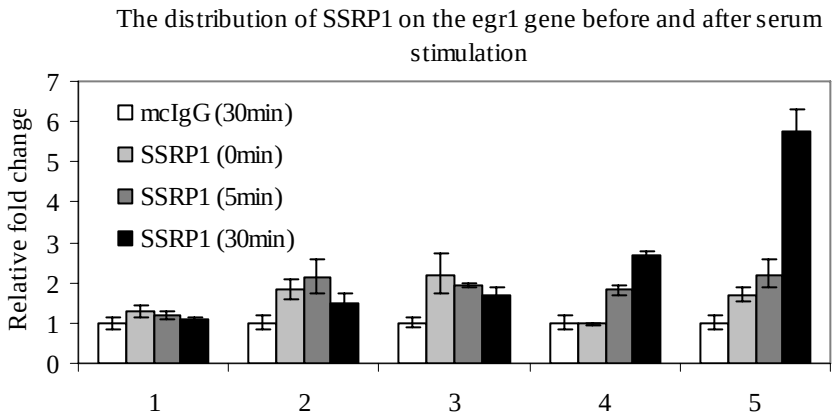
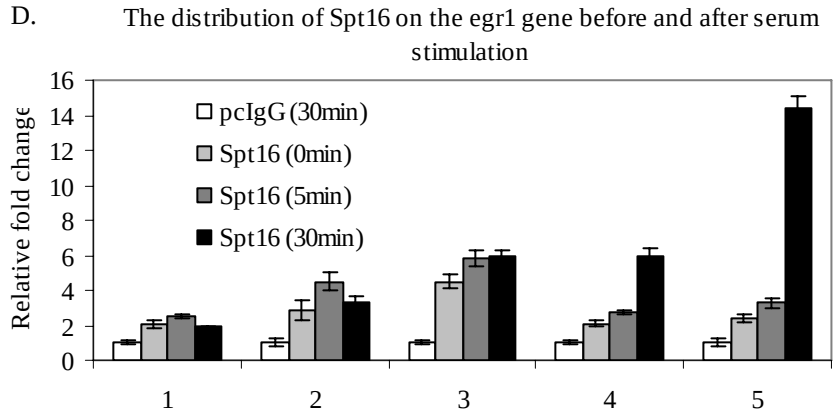
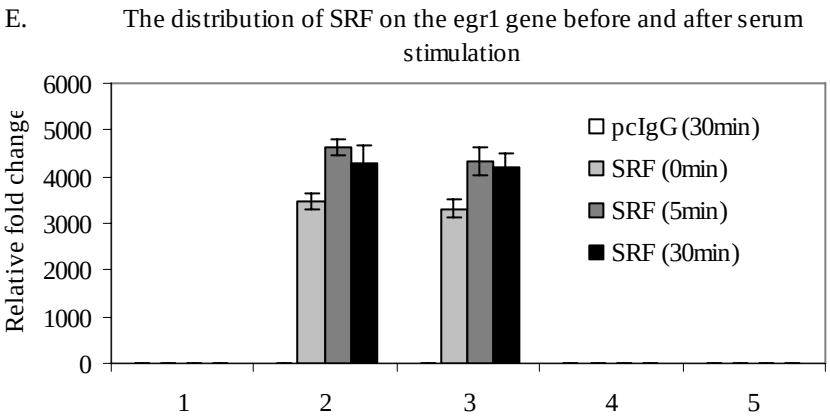

The distribution of SSRP1 on the egr1 gene before and after serum stimulation
Relative fold change
7
6
5
4
3
2
1
0
mcIgG (30min)
SSRP1 (0min)
SSRP1 (5min)
SSRP1 (30min)
1
2
3
4
5

Fig. 2.6 (continued)

D.



E.



F.

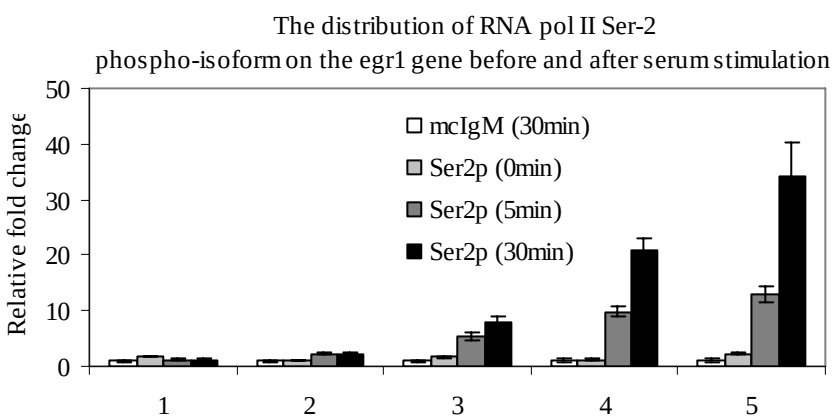
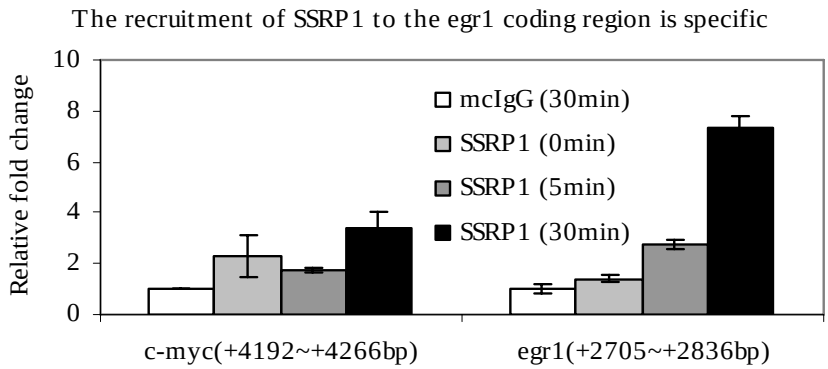


Fig. 2.7. The recruitment of SSRP1 and Spt16 to the *egr1* coding region (region 5) is specific. ChIP assays were performed with H1299 cells harvested before (0 min) or after serum stimulation (5 and 30 min) using the indicated antibodies. ChIP-enriched DNA was quantified by real-time PCR using the indicated amplicons. Values are expressed as relative fold changes over the IgG control.

Fig. 2.7


The recruitment of SSRP1 to the egr1 coding region is specific
Relative fold change
10
8
6
4
2
0
mcIgG (30min)
SSRP1 (0min)
SSRP1 (5min)
SSRP1 (30min)
c-myc(+4192~+4266bp)
egr1(+2705~+2836bp)

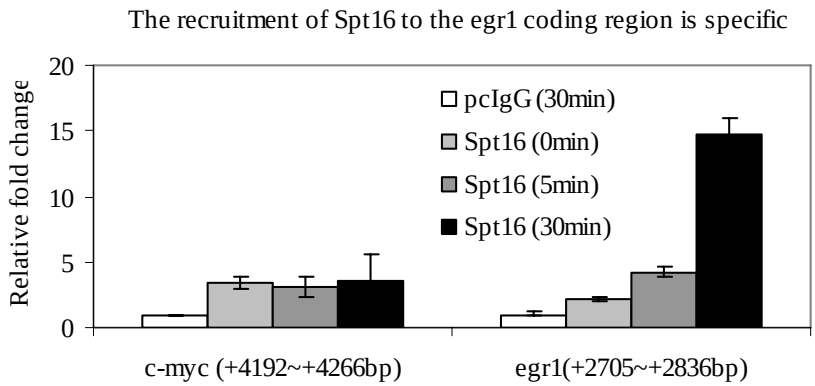
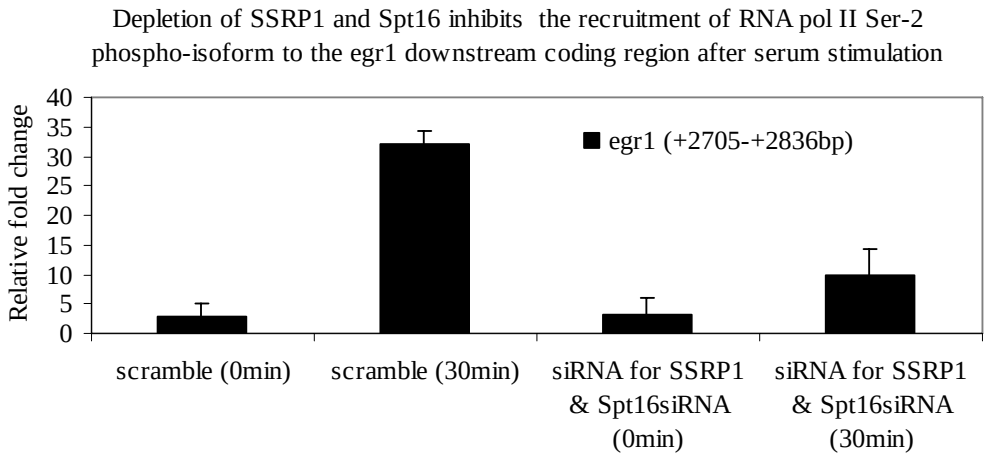

The recruitment of Spt16 to the egr1 coding region is specific
Relative fold change
20
15
10
5
0
pcIgG (30min)
Spt16 (0min)
Spt16 (5min)
Spt16 (30min)
c-myc (+4192~+4266bp)
egr1(+2705~+2836bp)

Fig. 2.8. SSRP1 and Spt16 are important for the progression of elongation RNA pol II on the *egr1* coding region.

H1299 cells were transfected with scrambled siRNA or siRNA for SSRP1 and Spt16 together. At the same time, cells were incubated in DMEM containing 0.25% FBS for serum starvation. After 48 h, nonstimulated or serum-stimulated cells were harvested for ChIP assay using antibodies that recognize the RNA pol II Ser-2 phosphoisoform. ChIP-enriched DNA was quantified by real time PCR using the indicated amplicons. Values are expressed as relative fold change over the IgM control.

Fig. 2.8


Depletion of SSRP1 and Spt16 inhibits the recruitment of RNA pol II Ser-2 phospho-isoform to the egr1 downstream coding region after serum stimulation
Relative fold change
40
35
30
25
20
15
10
5
0
egr1 (+2705-+2836bp)
scramble (0min)
scramble (30min)
siRNA for SSRP1 & Spt16siRNA (0min)
siRNA for SSRP1 & Spt16siRNA (30min)

CHAPTER THREE

CK2 Phosphorylates SSRP1 and Inhibits Its DNA-binding Activity

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Running title: Regulation of SSRP1 by CK2.

Key words: CK2, SSRP1, SPT16, phosphorylation, and DNA binding

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SUMMARY

We have previously shown that CK2 associates with the human high-mobility group protein SSRP1 and that this association increases in response to UV irradiation. CK2 also phosphorylates SSRP1 *in vitro*. Here we extend this work by investigating CK2 regulation of SSRP1 function through phosphorylation. Phosphorylation of SSRP1 by CK2 inhibited the nonspecific DNA-binding activity of SSRP1 and FACT (facilitating chromatin-mediated transcription) complex *in vitro*. Using a serine/ threonine-scanning Auto-spot peptide array coupled with a filter-based kinase assay with synthetic peptides as substrates, we identified serines 510, 657, and 688 as phosphorylation targets of CK2 *in vitro*. Mutagenesis of the three serines revealed that serine 510 was more important for the regulation of SSRP1 DNA-binding activity. Furthermore, we found that SSRP1 was phosphorylated in cells in response to UV (but not γ) irradiation. These results suggest that CK2 regulates the DNA-binding ability of SSRP1 and that this regulation may be responsive to specific cell stresses.

INTRODUCTION

CK2 is a ubiquitous and evolutionarily conserved kinase. This enzyme is a heterotetrameric protein complex consisting of two regulatory β subunits (28 kDa) and two catalytic α (42 kDa) or α' (38 kDa) subunits with the stoichiometry of either α2β2, α'2β2, or αα'β2 as the holoenzyme (Gietz, Graham et al. 1995). Genetic studies in yeast (Padmanabha, Chen-Wu et al. 1990) and in mice (Buchou, Vernet et al. 2003) demonstrate that this enzyme is essential for viability and animal embryogenesis. Biochemical and functional analyses of this enzyme reveal that it can phosphorylate a

broad spectrum of protein substrates *in vitro* and regulate a variety of cellular functions including transcription in the nucleus. Consistent with its regulatory role in the nucleus, CK2 associates with a number of chromatin and nuclear matrix proteins in the cell (Tawfic, Davis et al. 1997; Guo, Davis et al. 1998; Guo, Yu et al. 1999).

One of the mammalian CK2-interacting nuclear proteins is the previously identified DNA-binding protein called SSRP1 (structure-specific recognition protein 1) (Keller and Lu 2002). SSRP1 (named T160 in mice) (Shirakata, Huppi et al. 1991) is a member of the high-mobility group (HMG) 1 family of proteins (Bruhn, Pil et al. 1992), which represent the most abundant chromatin-associated nonhistone proteins. SSRP1 is essential for cell (Schlesinger and Formosa 2000) and animal (Cao, Bendall et al. 2003) viability. Sequence analysis reveals that this 80-kDa phosphoprotein possesses several interesting and evolutionarily conserved domains: a large N terminal region (aa 1–440) (~80% identity between *Drosophila* and human; 95% identity between *Xenopus* and human), an acidic domain (aa 440–496) with a limited homology to nucleolin, an HMG box domain (aa 539–614, 60% identity between *Drosophila* and human) with two flanking basic domains (aa 512–534 and aa 623–640), and a mixed charge domain at the extreme C-terminal region (aa 661–709). In yeast, Pob3 and Nhp6 form a bipartite SSRP1 analog (Brewster, Johnston et al. 2001). Pob3 is highly homologous with the N terminus of SSRP1, whereas Nhp6 is an HMG protein resembling the C terminus of SSRP1 (Brewster, Johnston et al. 2001). These domains are crucial for the functions of SSRP1. For example, the highly conserved N-terminal region of SSRP1 has been shown to directly interact with its partner, Spt16 (Keller and Lu 2002), forming a tight heterodimer complex (Orphanides, Wu et al. 1999). This complex was initially identified

from human cells as a facilitating chromatin-mediated transcription (FACT) complex to regulate transcriptional elongation with chromatin as a template (Orphanides, LeRoy et al. 1998). This complex was also shown to probably regulate DNA replication in yeast and *Xenopus* (Okuhara, Ohta et al. 1999; Schlesinger and Formosa 2000). Furthermore, the C-terminal HMG box is able to bind to DNA nonspecifically as well as to specific structures of DNA, such as DNA modified by the anti-tumor drug cisplatin (Bruhn, Pil et al. 1992) or cruciform DNA (Gariglio, Ying et al. 1997). Finally, SSRP1 functions as a co-regulator for transcription, and this regulation is executed by interacting with other transcriptional activators such as SRF (Spencer, Baron et al. 1999), *Drosophila* GATA factor (Shimojima, Okada et al. 2003), and p63 through its middle domain (Zeng, Dai et al. 2002). Thus, SSRP1 appears to possess multiple functions in the nucleus. Whether these functions of SSRP1 are regulated by CK2 remains unclear despite the fact that CK2 associates with SSRP1 in a protein kinase complex (Keller, Zeng et al. 2001).

As an initial step to address this question, we started to explore potential regulation of SSRP1 function by CK2 through phosphorylation. This idea originated from our previous findings that a UV irradiation-responsive p53 serine 392 kinase complex contains SSRP1, Spt16, and CK2 (Keller, Zeng et al. 2001), and CK2 efficiently phosphorylates SSRP1 (but not Spt16) *in vitro* (Keller, Zeng et al. 2001). Also, we found that the assembly of this ternary complex appears to be induced after UV irradiation of cells (Keller and Lu 2002). Consistent with our findings is that CK2 was also shown to phosphorylate maize SSRP1 at the HMG region *in vitro*, and this phosphorylation seemed to induce the recognition of UV irradiation-damaged DNA by SSRP1 *in vitro* (Krohn, Stemmer et al. 2003). Although SSRP1 possesses a number of CK2 consensus

sites, it is still unclear which amino acids CK2 specifically phosphorylates and whether phosphorylation of these potential residues regulates the ability of SSRP1 to bind to DNA. Also, it is unknown whether SSRP1 phosphorylation is responsive to UV irradiation in cells. Hence, we performed a series of biochemical analyses to address these specific questions. As detailed below, we show that SSRP1 is phosphorylated at serine and threonine residues in cells, and that one or more target sites are indeed induced by UV (but not γ) irradiation. Also, CK2 phosphorylation of SSRP1 that is either alone or in the FACT complex inhibits the ability of SSRP1 to bind to nonmodified linear DNA *in vitro*. Employing a serine/threonine-scanning Auto-spot peptide array technique coupled with a CK2 kinase assay, we identified serines 510, 657, and 688 of SSRP1 as CK2 targets *in vitro*. Mutagenesis of the three serines revealed that serine 510 might be critical for regulation of SSRP1•DNA binding *in vitro*. These results indicate that CK2 can regulate the ability of SSRP1 to bind to nonmodified DNA by specifically phosphorylating serine 510 and suggest that these amino acids, although not residing in the DNA-binding HMG domain, may play a critical role in regulating SSRP1 function.

EXPERIMENTAL PROCEDURES

Reagents and Buffers. Buffer C 100 (BC100) contains 20 mM Tris/ HCl (pH 7.9), 0.1 mM EDTA, 15% glycerol, 100 mM KCl, 1 mM dithiothreitol, and protease inhibitors including 0.2 mM phenylmethylsulfonyl fluoride, 4 mM pepstatin A, 1 mg/ml leupeptin, and 1 mg/ml aprotinin. Kinase buffer (1X) is 20 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, and 1 mM dithiothreitol. The buffer for circular dichroism (CD) analysis is 10 mM Tris/H_3PO_4 (pH 7.9) and 10% glycerol.

Plasmids and Antibodies. pET24a-CK2α', pET24a-CK2β, pET24a- SSRP1, and pGEX-KG-C-SSRP1 (aa 471–709) plasmids were as described previously (Keller and Lu 2002). The pGEX-KG-C-SSRP1 (aa 471–709) serine→alanine mutants were generated by site-directed mutagenesis using the QuikChangeTM kit (Stratagene). Polyclonal anti-SSRP1 and anti Spt16 antibodies were generated as described previously (Keller and Lu 2002).

Purification of Recombinant Proteins. Histidine-tagged proteins were expressed and purified from bacteria using nickel-nitrilotriacetic acid-agarose (Qiagen), as described previously (Keller and Lu 2002). GST fusion proteins were bound to glutathione-agarose beads (Sigma) and eluted with glutathione, followed by dialysis against BC100 buffer.

Purification of the FLAG-FACT Complex. FLAG-SSRP1 HEK293 cells (1 X 10^8 cells) were cultured in suspension and harvested for nuclear extracts as described previously (Flores, Lu et al. 1992). The extracts were loaded onto a phosphocellulose (P11) column and step-eluted at 0.1, 0.35, 0.5, and 1.0 M KCl. The 0.5 M KCl fraction was dialyzed against BC100 buffer and incubated with anti-FLAG M2-agarose affinity gel (Sigma). After washing rigorously, the immunoprecipitate was eluted from the beads with FLAG peptide, subjected to SDS-PAGE, and stained with colloidal blue. The FACT complex purified from the 0.5 M fraction was used for electrophoresis gel mobility shift assays.

Purification of the CK2 Kinase Complex. An equal molecular ratio of highly purified His-CK2α' and His-CK2β (Keller and Lu 2002) was incubated on ice for 1 h and run on a Superdex 200 (3.2/30) column (Smart HPLC, Pharmacia Corporation). The kinase-containing fractions were pooled and used for *in vitro* kinase assays.

***In Vivo* ^{32}P-Labeled Inorganic Phosphate and Phosphoamino Acid Analysis.** HeLa cells were either untreated, γ-irradiated (14 gray), or UVB-irradiated (1200 J/m2). Three hours after irradiation, ^{32}P inorganic phosphate was added to 375 μCi/ml, along with 500 nM phosphatase inhibitor okadaic acid, and the cells were allowed to grow for an additional 3 h. The cells were harvested for immunoprecipitation. After extensive washing, the immunoprecipitates were subjected to SDS-PAGE, and proteins were detected by autoradiography. The radioactive SSRP1 bands were sliced from the gel and subjected to phosphoamino acid analysis as described previously (van der Geer and Hunter 1994). After extraction from the gel, the samples, normalized by counts/min, were loaded onto a cellulose thin-layer chromatography plate (Fisher Scientific), which was subjected to two-dimensional electrophoresis. Signals were detected by autoradiography.

Electrophoresis Gel Mobility Shift Assay (EMSA). EMSA was performed as described previously (Keller, Zeng et al. 2001). Proteins, as indicated in each figure legend, were incubated with a 3' end-labeled 87-bp DNA fragment (5000 cycles/min, 0.1–1.0 ng of DNA/assay) derived from the *p21waf1/cip1* promoter (Zeng, Levine et al. 1998). After incubation at room temperature for 30 min, the reaction mixtures were loaded onto a 4.5% nondenatured gel. Protein•DNA complexes were detected by autoradiography. EMSA for SSRP1 was also carried out with different sizes and sequences of DNA. The results were similar to what are shown in this study (data not shown).

In Vitro Kinase Assay. Radioactive *in vitro* kinase assays were performed with [γ-^{32}P]ATP as described previously (Keller and Lu 2002). Substrates were either 50 ng of His-SSRP1 or 100 ng of GST-C-SSRP1. For the kinase reaction that was coupled with

EMSA, the reaction volume was 10 µl, and 1 mM unlabeled ATP, instead of [γ-^{32}P]ATP, was used.

In Vitro Kinase Assay on Peptide Arrays. Peptide arrays were synthesized on cellulose membranes with an Auto-Spot Robot ASP 222 (AbiMed, Langenfeld, Germany) as described previously (Tegge and Frank 1998). *In vitro* kinase assays on the peptide arrays with our purified recombinant CK2 complexes were also performed as described previously (Carnegie, Smith et al. 2004).

Circular Dichroism. CD analyses were performed on an automated AVIV 215 spectrometer at 25 °C as described previously (Yabuta, Subbian et al. 2003). All the protein concentrations were maintained at 0.1 mg/ml. Spectra were taken between 190–260 nm using a 0.5-mm path-length cuvette.

RESULTS

SSRP1 Phosphorylation Is Induced by UV (but Not by γ) Irradiation

Our previous findings that the CK2-SSRP1 interaction increased after UV irradiation and that CK2 efficiently phosphorylated SSRP1 *in vitro* prompted us to examine whether SSRP1 phosphorylation is induced by UV irradiation in cells. To this end, human cervical carcinoma HeLa cells were irradiated with either UVB or γ rays for 6 h, including 3 h of labeling with [^{32}P]orthophosphate. The cells were harvested for co-immunoprecipitation and ^{32}P-labeled proteins were detected by autoradiography. As shown in Fig. 3.1A, irradiation of the cells with either UV or γ rays did not change the steady-state level of SSRP1 (upper panel). Interestingly, and consistent with our previous work (Keller and Lu 2002), ^{32}P labeled SSRP1 increased 2.4-fold specifically in response

to UV (but not γ) irradiation (Fig. 3.1A, lower panel), suggesting that the UV irradiation-inducible CK2•SSRP1 complex might also lead to inducible SSRP1 phosphorylation by CK2. To determine which amino acids are phosphorylated after UV irradiation, the ^{32}P-labeled SSRP1 bands in Fig. 3.1A were gel-purified for phosphoamino acid analysis. Greater than 95% of phosphorylated amino acids in SSRP1 were serines, ~5% were threonines, and none was tyrosine (Fig. 3.1B). These results indicate that SSRP1 is indeed phosphorylated mainly at serines in cells and that this phosphorylation is UV irradiation-responsive.

CK2 Phosphorylation of SSRP1 Inhibits the Ability of SSRP1 and the FACT Complex to Bind to Nonmodified DNA in Vitro

To determine whether CK2-mediated phosphorylation of SSRP1 affects its DNA-binding activity, we compared a purified FACT complex containing both FLAG-SSRP1 and Spt16 from the FLAG-SSRP1 expressing HEK293 cells versus recombinant histidine-tagged SSRP1. The purity of FACT and His-SSRP1 is shown in Fig. 3.2A (left panel) and Fig. 3.2B (right panel), respectively. Recombinant CK2 α' and β subunits were either purified to homogeneity and reconstituted into a heterotetrameric complex as described previously (Keller and Lu 2002) or purchased from Promega. With these purified proteins, we carried out a set of electrophoresis gel mobility shift assays using ^{32}P-labeled 87-bp oligomers digested from the pWaf-1-Δ50 plasmid (Zeng, Levine et al. 1998) as probes. As shown in Fig. 3.2A (right panel), FACT bound to DNA efficiently *in vitro* as expected (Yarnell, Oh et al. 2001). This FACT•DNA complex was indeed formed with SSRP1 and Spt16, as both anti-SSRP1 and anti-Spt16 antibodies super-

shifted the complex (Fig. 3.2A, lanes 6 and 7). Interestingly, CK2 reduced the FACT•DNA complex in the presence (but not in the absence) of 1 mM ATP, suggesting that this inhibition may be phosphorylation dependent. Supporting this assumption is the fact that CK2 phosphorylated SSRP1 (but not Spt16) in FACT *in vitro* (Keller, Zeng et al. 2001).

To verify whether the reduction of the FACT•DNA complex formation is through CK2 phosphorylation of SSRP1, we conducted EMSA using purified His-SSRP1. As expected (Bruhn, Pil et al. 1992), recombinant SSRP1 efficiently bound to DNA (Fig. 3.2B) under the same conditions as those used for FACT (Fig. 3.2A), and the anti-SSRP1 antibody also supershifted this complex (Fig. 3.2B). Again, CK2 completely inhibited the formation of SSRP1•DNA complex in the presence (but not in the absence) of ATP (Fig. 3.2C). This inhibition was not because of the change of SSRP1 level, as its level stayed the same after incubation (data not shown). The elimination of SSRP1•DNA complexes by CK2 was closely related to SSRP1 phosphorylation by this kinase, as ^{32}P labeling of SSRP1 was detected in a CK2 dose-dependent manner (Fig. 3.2D). These results suggest that CK2 can phosphorylate SSRP1 and thus prevent SSRP1, either alone or in the FACT complex, from binding to nonmodified DNA in vitro.

CK2 Phosphorylates the C-terminal Domain of SSRP1 and Inhibits Its Ability to Bind to DNA in Vitro.

It was shown previously (Yarnell, Oh et al. 2001) that SSRP1 binds to DNA through its C-terminal HMG-containing domain. Next we wanted to determine whether CK2 inhibits the DNA-binding activity of SSRP1 by phosphorylating the C terminus of

SSRP1, although the middle domain of SSRP1 was also phosphorylated by CK2 (Keller and Lu 2002). GST-C-terminal SSRP1 (aa 471–709), encompassing the HMG box, was purified as described previously (Keller and Lu 2002) to homogeneity (Fig. 3.3A) for EMSA. As anticipated (Yarnell, Oh et al. 2001), this GST-C-SSRP1 fragment bound to DNA in a dose-dependent fashion (Fig. 3.3B). Again, this DNA-binding ability was inhibited by CK2 when only ATP was present (Fig. 3.3C). This inhibition was dose-dependent (Fig. 3.3C, lanes 5 and 6) and associated with phosphorylation of the C terminus of SSRP1 by CK2, as detected when [γ-^{32}P] ATP, instead of unlabeled ATP, was used (see Fig. 3.6D and data not shown). Taken together, these results demonstrated that CK2 inhibits the DNA-binding activity of SSRP1 by phosphorylating its C-terminal domain.

CK2 Phosphorylates Serines 510, 657, and 688 of SSRP1 in Vitro

Although the C terminus of SSRP1 was phosphorylated by CK2 *in vitro* (Fig. 3.6 and data not shown), it was challenging to determine which amino acids are CK2 target sites. There are 46 serines and four threonines in this region of SSRP1, seven of which exist in the HMG box (Fig. 3.4). Also, 20 of these potential phosphorylation sites display a similarity to the CK2 consensus sequence ((S/T)*XX*(D/E) or (S/T)(D/E)). Moreover, mass spectrometry of phosphorylated SSRP1 failed to map CK2 sites (data not shown) mainly because of the relatively low efficiency of SSRP1 phosphorylation (<30%) *in vitro* and dense distribution of phosphorylation target residues in the middle and C terminal regions of SSRP1 (Fig. 3.4 and data not shown). To surmount these obstacles, we employed an Auto-spot peptide array technique coupled with a filter-based kinase

assay (Tegge and Frank 1998; Carnegie, Smith et al. 2004). Using this approach with pairs of synthetic wild type and corresponding serine→alanine mutant 10mer peptides spotted on a nitrocellulose filter as substrates, we identified three serines as CK2 sites. As shown in Fig. 3.4, peptide pair 10 (note, each pair of peptides has a wild type and a serine→alanine mutant peptide), encompassing serine 510, peptide pair 15 with serines 657 and 659, and peptide pair 19 with serines 685 and 688, was phosphorylated by CK2. By contrast, phosphorylation of the peptides by CK2 was markedly reduced when their corresponding serine→alanine mutants were used as substrates. Although peptides 15 and 19 harbored two serines in each case, only the S657A mutation of peptide 15 (in comparison with peptide 16 that contains S657A and S659A) or the S688A of peptide 19 had reduced phosphorylation (Fig. 3.4, right column), suggesting that serines 657 and 688 are the CK2 sites (of note, the residual signals of the mutant S510A-, S657A-, or S657A/S659A-containing peptides 10, 15, and 16 might be due to less stringent washing, as these peptides should not be phosphorylated). Consistently, the three sites also display a similarity to the CK2 consensus sequence (Fig. 3.4). Surprisingly, all of these three serines reside outside of the HMG domain of SSRP1 (Fig. 3.4, lower panel). Although some of the synthetic peptides might not serve as ideal substrates for CK2 in this assay, perhaps because of improper folding (thereby giving false negative results), this study at least identifies serines 510, 657, and 688 as CK2 phosphorylation sites for further analysis.

Serine 510 (but Not Serines 657 and 688) Plays a Role in CK2 Regulation of SSRP1 DNA-binding Activity

To investigate whether phosphorylation of SSRP1 at serines 510, 657, and 688 by CK2 is important for regulating the DNA-binding activity of SSRP1, we generated an SSRP1 C-terminal triple mutant S3A (denoting S510A/S657A/S688A) and a double mutant S2A (denoting S657A/S688A) in the GST-C-SSRP1 fusion protein using a mutagenesis kit from Stratagene. These mutants were confirmed by DNA sequencing (data not shown). These GST fusion proteins and its wild type counterpart were purified to homogeneity as shown in Fig. 3.5A. To ensure that these mutant proteins are still able to bind to DNA, we carried out EMSA with the purified proteins, as described in Fig. 3.2. As shown in Fig. 3.5B, at the same concentrations, the wild type and two mutant GST-C-SSRP1 proteins were able to bind to DNA, although the DNA-binding ability of the mutant proteins was a bit weaker. CD analysis of the wild type and mutant proteins showed that these proteins displayed a similar secondary structure (Fig. 3.5C).

Next, we performed a set of EMSA experiments to determine whether these three serines are crucial for CK2 phosphorylation of the SSRP1 C terminus and thus its regulation of SSRP1 DNA-binding activity. Again phosphorylation of the GSTSSRP1-C terminus by CK2 markedly inhibited the formation of SSRP1•DNA complexes (Fig. 3.6A). By striking contrast, the triple mutant GST-C-SSRP1-S3A was still able to bind to DNA in the presence of CK2 and ATP (Fig. 3.6B), suggesting that substitution of the three serines by alanines may prevent CK2 phosphorylation of the C terminus of SSRP1 at these residues and thus rescue CK2-mediated inhibition of SSRP1 DNA binding. Consistent with this assumption was that phosphorylation of the triple mutant GST-C-SSRP1-S3A by CK2 was reduced to ~30% of that of the wild type protein (Fig. 3.6, D and E). Although the triple mutant was still phosphorylated by CK2 (Fig. 3.6D), the

remaining phosphorylation did not appear to efficiently block the DNA-binding activity of the C terminus of SSRP1 (Fig. 3.6B). To further determine which serine is more critical for the inhibitory effect of CK2 on SSRP1 DNA-binding activity, we conducted the same EMSA assay with the SSRP1 C-terminal double mutant S2A (S657A/S688A). We found that this double mutant was unable to prevent the inhibition of CK2-mediated phosphorylation on SSRP1 DNA-binding activity (Fig. 3.6C). Taken together, these results suggest that serine 510 may play a crucial role in mediating CK2 regulation of SSRP1 DNA-binding function.

DISCUSSION

It has been shown that CK2 forms a complex with SSRP1 and Spt16 in cells and phosphorylates SSRP1 (but not Spt16) *in vitro* (Keller, Zeng et al. 2001). Little is known about the regulation of SSRP1 function by CK2. As an initial step to understand the role of CK2 in human SSRP1 regulation, we have performed a series of biochemical experiments using purified proteins. We found that CK2-mediated phosphorylation of SSRP1 at its HMGcontaining C-terminal region led to a reduction of SSRP1•DNA binding (Fig. 3.3). This inhibition occurred when either FACT or recombinant SSRP1 was used as the substrate of CK2 (Fig. 3.2). Moreover, by means of the Auto-spot peptide array/filter kinase approach, we identified serines 510, 657, and 688 as CK2 target sites *in vitro* (Fig. 3.4). Replacing all three serines (but not merely the serine 657 and 688 residues together) with alanines alleviated the inhibition of SSRP1 DNA-binding activity by CK2 (Figs. 3.5 and 3.6), indicating that serine 510 is more critical for regulating SSRP1 DNA-binding activity. Consistent with these results is that CK2 was also

previously reported to inhibit the DNA-binding activity of maize HMGB proteins through phosphorylation (Stemmer, Schwander et al. 2002). Hence, our results demonstrate that CK2 can phosphorylate the serines outside of the HMG box domain and thereby inhibit its ability to interact with nonmodified DNA.

Intriguingly, phosphorylation of maize SSRP1 by CK2 was recently shown to enhance the ability of the SSRP1 protein to bind to UV irradiation-damaged DNA *in vitro* (Krohn, Stemmer et al. 2003), although specific phosphorylation amino acids were not identified and mutated. This finding, along with our current study, suggests that inhibiting the nonspecific DNA-binding activity of SSRP1 by CK2 would, in effect, increase the specificity of SSRP1 for UV irradiation-damaged DNA. In this scenario, DNA-damaging agents, such as UV irradiation, would signal through these molecules. Because SSRP1, together with Spt16, often associates with chromatin for its regulatory role in transcription and replication (Orphanides, LeRoy et al. 1998; Okuhara, Ohta et al. 1999; Schlesinger and Formosa 2000; Belotserkovskaya, Oh et al. 2003; Mason and Struhl 2003; Saunders, Werner et al. 2003), it is also likely that by dissociating SSRP1 from nonspecific DNA sequences, CK2 may enhance its ability to bind to specific structures of chromatin, where transcription, replication, or DNA repair may take place. Alternatively and conversely, by phosphorylating certain residues of SSRP1, CK2 may negatively regulate transcription or replication in response to UV irradiation-caused DNA damage. It is known that UV irradiation often causes global inhibition of transcription or replication (Takeda, Naruse et al. 1967). Yet, detailed mechanisms underlying this effect remain elusive, although one general thought is that intrastrand cross-linking of neighboring bases in DNA caused by UV irradiation may block elongation of

transcription or replication. Given that SSRP1/Spt16 has been proposed to be functional in transcriptional elongation and replication (Orphanides, LeRoy et al. 1998; Okuhara, Ohta et al. 1999; Schlesinger and Formosa 2000; Belotserkovskaya, Oh et al. 2003; Mason and Struhl 2003; Saunders, Werner et al. 2003), one mechanism would be that CK2 may inhibit this function by phosphorylating SSRP1 and thus preventing it from associating with chromatin, consequently leading to transcriptional stall or replication pause in response to UV irradiation. Consistent with this speculation is that the assembly of the CK2•SSRP1•Spt16 complex, as well as phosphorylation of SSRP1, is induced in response to UV irradiation (Fig. 3.1) (Keller, Zeng et al. 2001). Another possibility is that by dissociating SSRP1 or FACT from chromatin, CK2 would form a kinase complex with these proteins, which in turn assist CK2 in selectively phosphorylating and activating p53 in response to UV irradiation (Keller, Zeng et al. 2001; Keller and Lu 2002). In sum, our finding, as described here, suggests a potential mechanism underlying the regulation of SSRP1 function by CK2 in response to UV irradiation.

ACKNOWLEDGMENTS

We thank Mary MacPartlin for proofreading this manuscript, Shelya Zeng and Hunjoo Lee for technique assistance, and Ezhilkani Subbian and Ujwal Shinde for their assistance in CD analysis.

Fig. 3.1. UV irradiation induction of SSRP1 phosphorylation at Ser and Thr *in vivo*. **(A)** Phosphorylation of SSRP1 in cells was induced by UV irradiation. Hela cells were labeled *in vivo* by ^{32}P-labeled inorganic phosphate 3 h after irradiation by UVB or γ rays, followed by an additional 3 h of incubation before being harvested for immunoprecipitation (IP). 200 μg of protein was used for the immunoprecipitation. Immunoprecipitates were subjected to SDS-PAGE and labeled SSRP1 was detected by autoradiography. **(B)** Phosphoamino acid analysis was carried out as described under "Experimental Procedures", revealing that both serines and threonines are phosphorylated on SSRP1. Samples (1720 cycles/min) were loaded onto a cellulose thin-layer chromatography plate, which was subjected to two-dimensional electrophoresis and autoradiography. Only the UVB irradiation-treated sample is shown in the figure, although nontreated or γ-treated samples show the same pattern. WB, Western blot.

Fig. 3.1

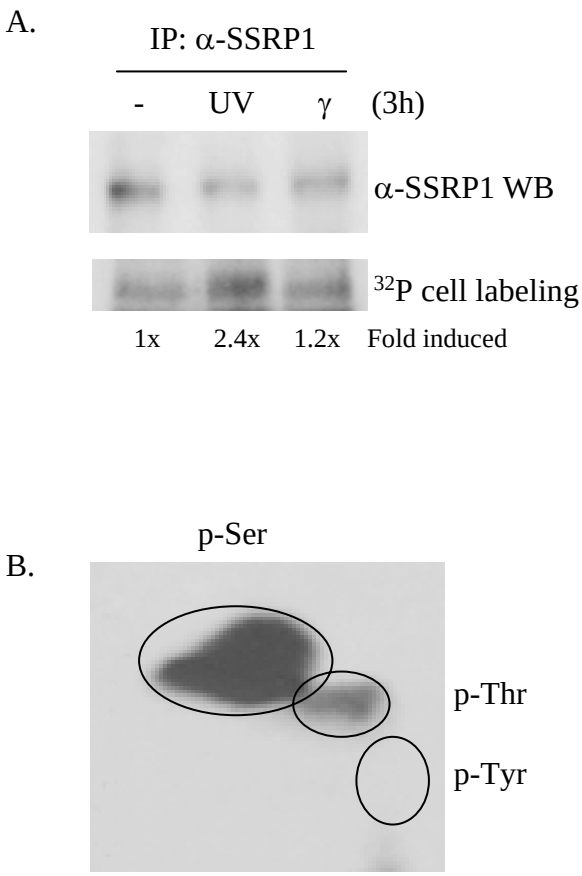

A.
IP: α-SSRP1
-
UV
γ
(3h)
α-SSRP1 WB
32P cell labeling
1x
2.4x
1.2x
Fold induced
B.
p-Ser
p-Thr
p-Tyr

Fig. 3.2. Phosphorylation of SSRP1 by CK2 inhibits its DNA-binding activity.

(A) Phosphorylation of FACT by CK2 inhibited its DNA-binding ability. The FACT complex was purified from FLAG-SSRP1-expressing HEK293 cells, as described under "Experimental Procedures". The 0.5 M fraction, which contains the FACT complex, is shown in the left panel. 4 µl of this fraction was used in an in vitro kinase assay followed by EMSA. 10µl CK2 kinase reactions were carried out with the FACT complex for 1 h at 30 °C and then incubated with 3'end-labeled DNA (87 bp) probes for 20–25 min. Also, antibodies specific against SSRP1 (lane 6) and Spt16 (lane 7) were used. **(B)** The purified recombinant His-SSRP1 protein bound to DNA. The purity of the recombinant His-SSRP1 is shown in the right panel. The EMSA was done using 250 and 500 ng of purified His-SSRP1. The His-SSRP1•DNA complex was supershifted by an anti-SSRP1 antibody (lanes 4 and 5). **(C)** Phosphorylation of SSRP1 by CK2 inhibited its DNA-binding activity. The same kinase/EMSA assay was conducted as in A, except that 50 ng of His-SSRP1 was used. **(D)** The purified recombinant His-SSRP1 was phosphorylated by CK2. In vitro kinase reactions were conducted for 30 min using [γ-^{32}P] ATP and 50 ng of His-SSRP1 as substrates. Coom, Coomassie.

Fig. 3.2

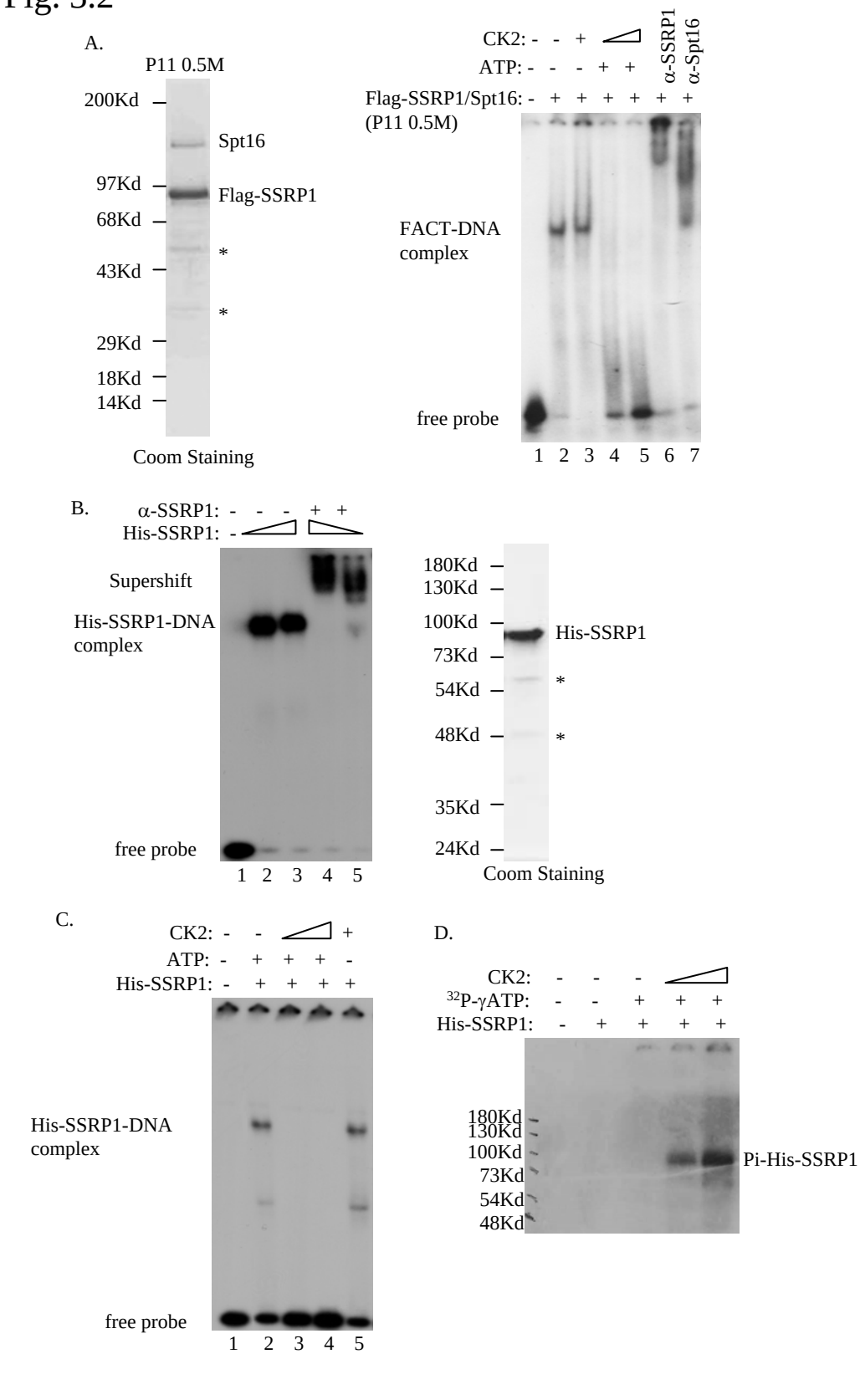
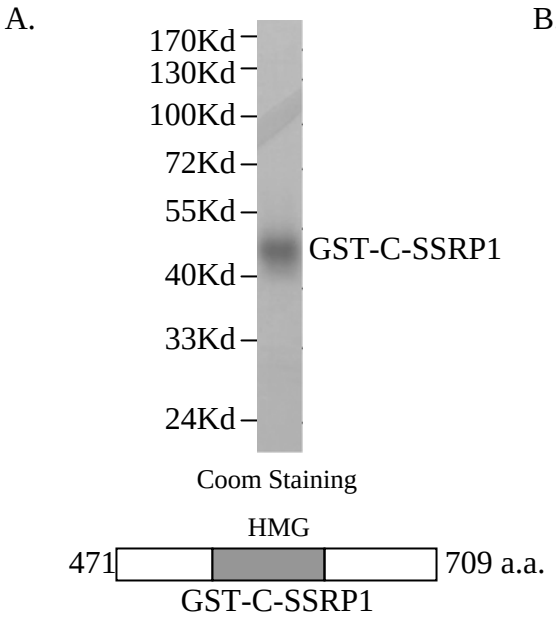
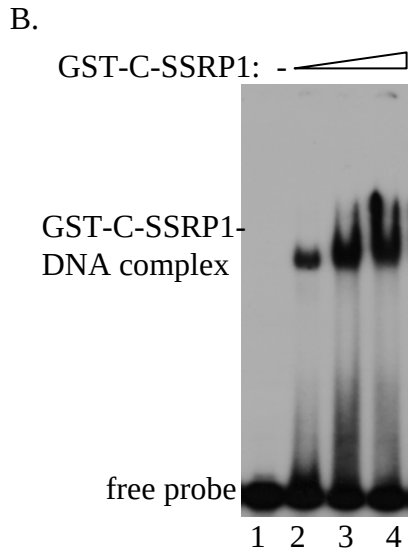

A.
P11 0.5M
200Kd
97Kd
68Kd
43Kd
29Kd
18Kd
14Kd
Spt16
Flag-SSRP1
*
*
Coom Staining
CK2: - - +
ATP: - - - + +
α-SSRP1
α-Spt16
Flag-SSRP1/Spt16: - + + + + + +
(P11 0.5M)
FACT-DNA
complex
free probe
1 2 3 4 5 6 7
B.
α-SSRP1: - - - + +
His-SSRP1: -
Supershift
His-SSRP1-DNA
complex
free probe
1 2 3 4 5
180Kd
130Kd
100Kd
73Kd
54Kd
48Kd
35Kd
24Kd
His-SSRP1
*
*
Coom Staining
C.
CK2: - - +
ATP: - + + + -
His-SSRP1: - + + + +
His-SSRP1-DNA
complex
free probe
1 2 3 4 5
D.
CK2: - - -
32P-γATP: - - + + +
His-SSRP1: - + + + +
180Kd
130Kd
100Kd
73Kd
54Kd
48Kd
Pi-His-SSRP1

Fig. 3.3. Phosphorylation of the C terminus of SSRP1 by CK2 inhibits its DNA-binding ability.

(A) A schematic shows the GST fusion C terminus of SSRP1 (bottom of the panel). GST-C-SSRP1 was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (Coom). **(B)** The purified GST-C-SSRP1 fragment bound to DNA. The EMSA was conducted using increasing amounts of GST-C-SSRP1 (50, 100, and 200 ng). **(C)** Phosphorylation of GST-C-SSRP1 by CK2 inhibited its DNA-binding activity. The same kinase/EMSA assay was conducted as described in the legend to Fig. 3.2 using 100 ng of GST-C-SSRP1.

Fig. 3.3


A.
170Kd
130Kd
100Kd
72Kd
55Kd
40Kd
33Kd
24Kd
GST-C-SSRP1
Coom Staining
HMG
471
709 a.a.
GST-C-SSRP1


B.
GST-C-SSRP1: -
GST-C-SSRP1-
DNA complex
free probe
1 2 3 4

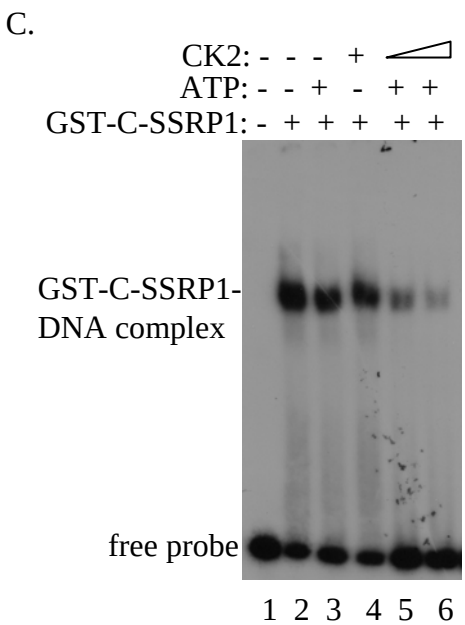

C.
CK2: - - - +
ATP: - - + - + +
GST-C-SSRP1: - + + + + +
GST-C-SSRP1-
DNA complex
free probe
1 2 3 4 5 6

Fig. 3.4. Identification of serine 510, 657, and 688 of SSRP1 as CK2 phosphorylation sites *in vitro*.

A panel of designed SSRP1 10mer peptides was spotted on a nitrocellulose membrane. *In vitro* CK2 kinase reactions were conducted on the membrane using [γ-^{32}P] ATP. After extensive washing, radioactive signals were detected by autoradiography. The *asterisks* denote potential phosphorylation sites, and the underlined asterisks indicate the identified CK2 sites. Only serines or threonines and their corresponding mutant alanines are shown here. The corresponding positions of these amino acids in the C terminus of SSRP1 are approximatelymarked on the schematic at the bottom. The sequences for peptide pairs 10, 15, and 19 are shown in corresponding positions, and CK2 target sites are underlined.

Fig. 3.4

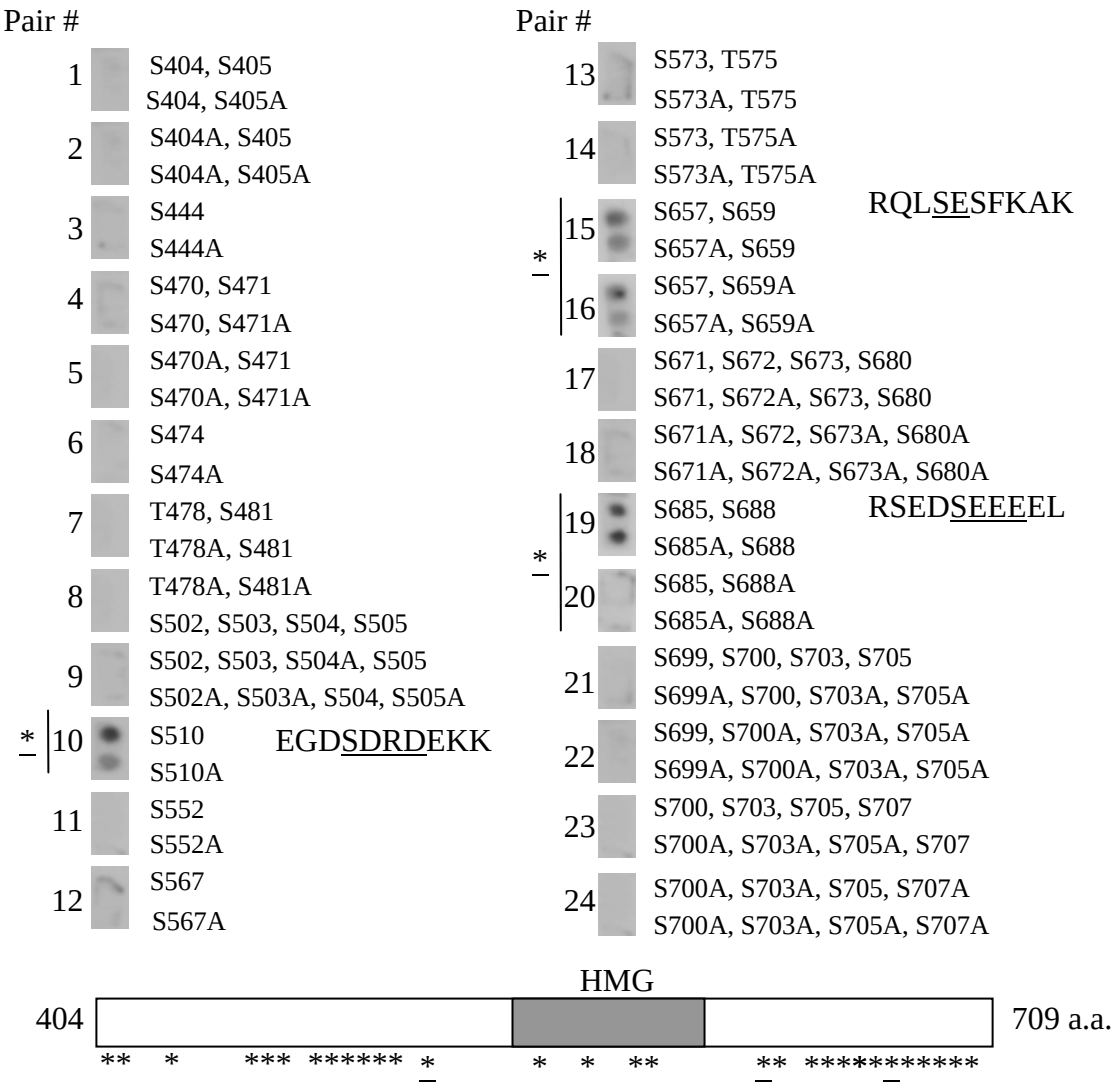

Pair #
1
S404, S405
S404, S405A
2
S404A, S405
S404A, S405A
3
S444
S444A
4
S470, S471
S470, S471A
5
S470A, S471
S470A, S471A
6
S474
S474A
7
T478, S481
T478A, S481
8
T478A, S481A
S502, S503, S504, S505
9
S502, S503, S504A, S505
S502A, S503A, S504, S505A
*
10
S510
S510A
EGDSDRDEKK
11
S552
S552A
12
S567
S567A
Pair #
13
S573, T575
S573A, T575
14
S573, T575A
S573A, T575A
*
15
S657, S659
S657A, S659
RQLSESFKAK
16
S657, S659A
S657A, S659A
17
S671, S672, S673, S680
S671, S672A, S673, S680
18
S671A, S672, S673A, S680A
S671A, S672A, S673A, S680A
*
19
S685, S688
S685A, S688
RSEDSEEEEL
20
S685, S688A
S685A, S688A
21
S699, S700, S703, S705
S699A, S700, S703A, S705A
22
S699, S700A, S703A, S705A
S699A, S700A, S703A, S705A
23
S700, S703, S705, S707
S700A, S703A, S705A, S707
24
S700A, S703A, S705, S707A
S700A, S703A, S705A, S707A
HMG
404
709 a.a.
** * *** ****** * * * ** ** **********

Fig. 3.5. GST-C-SSRP1-S3A triple and S2A double mutants can bind to DNA but with relatively lower affinity.

(A) A schematic shows the positions of S510A, S657A, and S688A on GST-C-SSRP1 (upper panel). GST-C-SSRP1, GST-C-SSRP1-S3A, and GST-C-SSRP1-S2A (S657A/ S688A) were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (Coom, lower panel). The amounts of proteins used are indicated on top. **(B)** Comparison between GST-C-SSRP1, GST-C-SSRP1-S3A, and GST-C-SSRP1-S2A in EMSA. 25, 50, and 100 ng of GST-C-SSRP1, GST-C-SSRP1-S3A, or GST-C-SSRP1-S2A were used in the EMSA. **(C)** CD analysis of GST-C-SSRP1, GST-C-SSRP1-S3A, and GST-C-SSRP1-S2A. CD spectra of the wild type, triple, and double mutant GST-C-SSRP1 proteins were recorded in the wavelength range of 190–260 nm.

Fig. 3.5

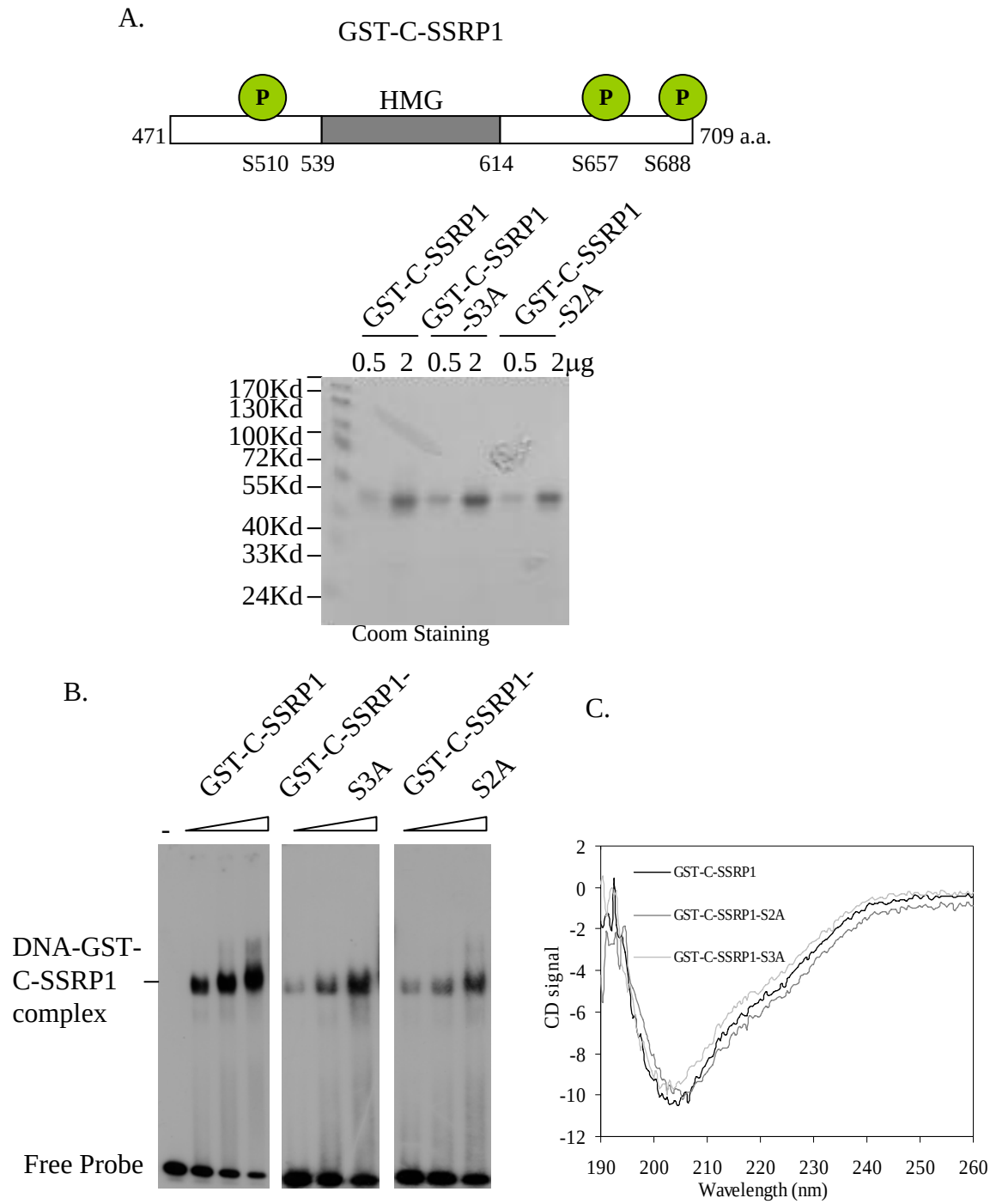
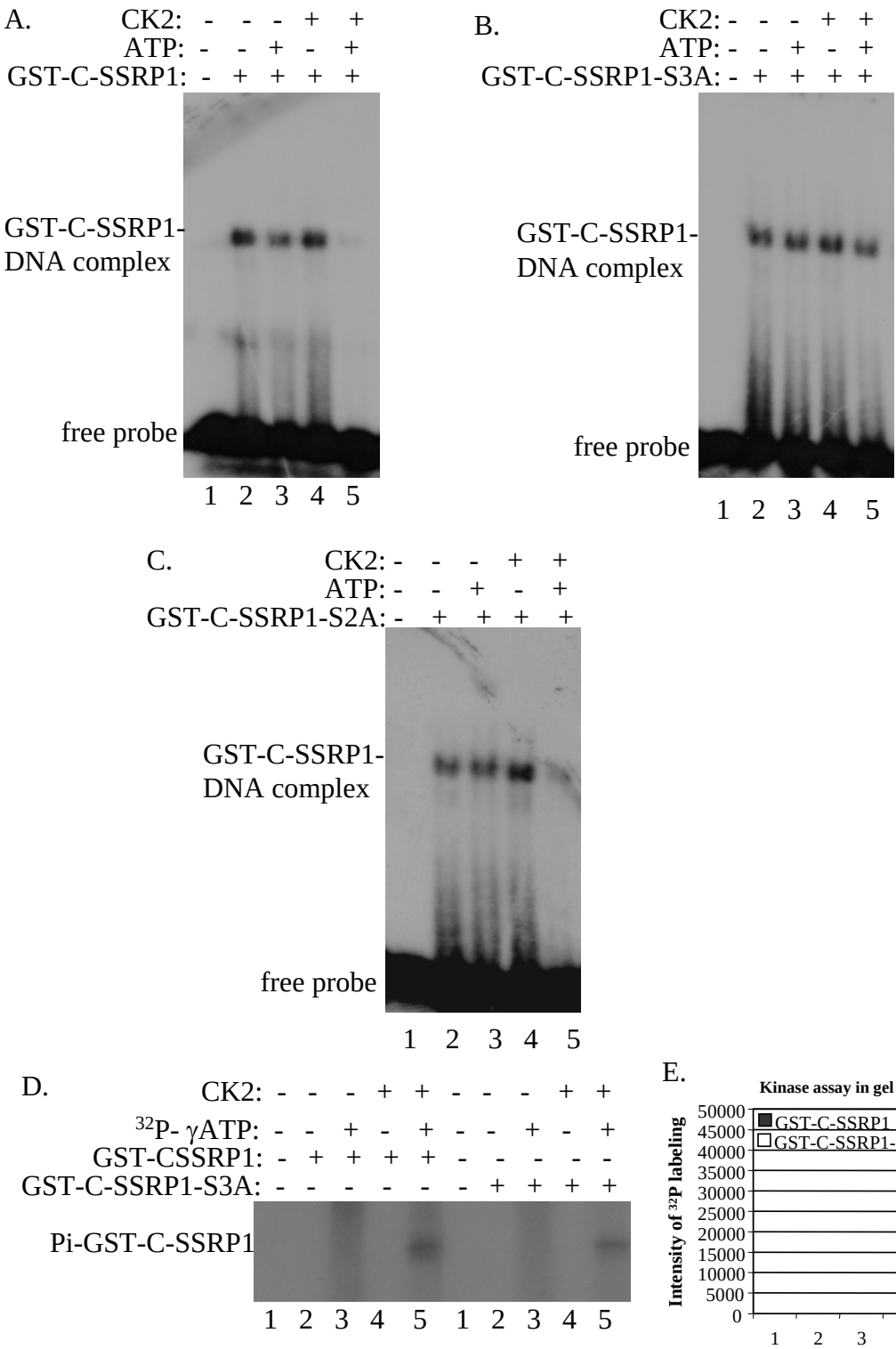

A.
GST-C-SSRP1
P
HMG
P
P
471
709 a.a.
S510
539
614
S657
S688
GST-C-SSRP1
GST-C-SSRP1 -S3A
GST-C-SSRP1 -S2A
0.5 2 0.5 2 0.5 2μg
170Kd
130Kd
100Kd
72Kd
55Kd
40Kd
33Kd
24Kd
Coom Staining
B.
GST-C-SSRP1
GST-C-SSRP1-S3A
GST-C-SSRP1-S2A
-
DNA-GST-C-SSRP1 complex
Free Probe
C.
GST-C-SSRP1
GST-C-SSRP1-S2A
GST-C-SSRP1-S3A
CD signal
2
0
-2
-4
-6
-8
-10
-12
190
200
210
220
230
240
250
260
Wavelength (nm)

Fig. 3.6. Phosphorylation of GST-C-SSRP1-S3A (but not GST-C-SSRP1-S2A) by CK2 fails to inhibit the DNA-binding activity of this protein.

(A) Phosphorylation of GST-C-SSRP1 by CK2 inhibits its DNA-binding activity. The same kinase/EMSA assay was conducted as described in the legend to Fig. 3.3, and 50 ng of GST-C-SSRP1 was used. **(B)** Phosphorylation of GST-C-SSRP1-S3A by CK2 failed to inhibit its DNA-binding ability. The kinase/EMSA assay on the triple mutant of GST-C-SSRP1 was conducted under the same conditions as those for wild type GST-C-SSRP1 in A. **(C)** Phosphorylation of GST-C-SSRP1-S2A by CK2 still inhibited its DNA-binding ability. The kinase/EMSA assay on the double mutant of GST-C-SSRP1 was conducted under the same conditions as those for wild type GST-C-SSRP1 in A. **(D)** GST-C-SSRP1-S3A is partially phosphorylated by CK2. *In vitro* radioactive kinase assays were carried out under the same conditions as used for the EMSA reactions in A and B. The ^{32}P-labeled SSRP1 fragment signals were quantified and plotted in a graph shown in **(E)** using imaging software.

Fig. 3.6


A.
CK2: - - - + +
ATP: - - + - +
GST-C-SSRP1: - + + + +
GST-C-SSRP1-DNA complex
free probe
1 2 3 4 5
B.
CK2: - - - + +
ATP: - - + - +
GST-C-SSRP1-S3A: - + + + +
GST-C-SSRP1-DNA complex
free probe
1 2 3 4 5
C.
CK2: - - - + +
ATP: - - + - +
GST-C-SSRP1-S2A: - + + + +
GST-C-SSRP1-DNA complex
free probe
1 2 3 4 5
D.
CK2: - - - + + - - - + +
32P- γATP: - - + - + - - + - +
GST-CSSRP1: - + + + + - - - - -
GST-C-SSRP1-S3A: - - - - - - + + + +
Pi-GST-C-SSRP1
1 2 3 4 5 1 2 3 4 5
E.
Kinase assay in gel shift
Intensity of 32P labeling
GST-C-SSRP1
GST-C-SSRP1-S3A
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
1 2 3 4 5

CHAPTER FOUR

SSRP1 facilitates microtubule growth and bundling required for mitosis

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Running title: Role of SSRP1 in mitosis.
Key words: SSRP1, Spt16, mitosis, microtubules, and cytokinesis.

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SUMMARY

Tight regulation of microtubule (MT) dynamics is essential for proper chromosome movement during mitosis. Here we report the identification of structure-specific recognition protein 1 (SSRP1) as a novel regulator of MT dynamics. SSRP1 co-localizes with the mitotic spindle and midbody in cells, and associates with MTs *in vitro*. Purified SSRP1 facilitates tubulin polymerization and MT bundling *in vitro*. Depletion of SSRP1 leads to disorganized centrosomes, spindles, and midbodies. Hence, SSRP1 plays a crucial role in MT growth and regulation during mitosis.

INTRODUCTION

Mitosis is the final and critical stage of cell division essential for cell proliferation, embryogenesis, and tumorigenesis. During mitosis, duplicated chromosomes are condensed, aligned, segregated, and equally packed into two daughter cells through cytokinesis (Scholey, Brust-Mascher et al. 2003). This chromosome movement is driven by bipolar spindles working in concert with stabilizing and destabilizing proteins (Kline-Smith and Walczak 2004). At the core of this mitotic machinery are the microtubules (MTs) (Kline-Smith and Walczak 2004), which consists of polymerized α-tubulin/β-tubulin heterodimers (Desai and Mitchison 1997; Wang and Nogales 2005). Bipolar MTs grow from two centrosomes containing γ-tubulin-associated complexes (Wiese and Zheng 2006) at prophase, and are organized into bundles that become the prime entities of mitotic spindles at metaphase, and midbody matrices at telophase (Alsop and Zhang 2003; Kline-Smith and Walczak 2004) in dividing cells. MTs are highly dynamic structure with two intrinsic properties: treadmilling and dynamic

instability. Both treadmilling and dynamic instability are likely to coexist in cells and may account for the execution of various processes such as kinetochore capture and chromosome movement during mitosis. However, the polymerization or depolymerization of microtubule must be properly regulated to happen in right time and place (Kline-Smith and Walczak 2004). *In vivo*, a large amount of microtubule-associated proteins regulate microtubule dynamics (Maiato, Sampaio et al. 2004).

Despite the identification of many cytoplasmic and nuclear proteins that have been shown to be crucial for spindle assembly and midbody formation (Wilde, Lizarraga et al. 2001; Prasanth, Prasanth et al. 2002; Kline-Smith and Walczak 2004; Skop, Liu et al. 2004; Vagnarelli and Earnshaw 2004; Helenius, Brouhard et al. 2006; Tsai, Wang et al. 2006), it is not resolved how bipolar MTs grow, and become organized into the bundles of spindle and midbody matrices in dividing cells. Several mitosis-specific proteins responsible for directly facilitating both MT polymerization and bundling have been identified (MacRae 1992). One such protein is MT-associated protein 4 (MAP4) (Aizawa, Murofushi et al. 1987; Kotani, Murofushi et al. 1988; West, Tenbarge et al. 1991; Ookata, Hisanaga et al. 1995). MAP4 directly binds to polymerized tubulin: it promotes both MT growth and bundling in mitosis (Aizawa, Murofushi et al. 1987; Kotani, Murofushi et al. 1988; West, Tenbarge et al. 1991)**.** Although MAP4 may act as a cross-linker to tether MT into bundles *in vitro* (Murofushi, Kotani et al. 1986; Aizawa, Murofushi et al. 1987), this activity may not be absolutely essential for MT assembly *in vivo* (Wang, Peloquin et al. 1996), raising the question of whether there are other proteins displaying similar activities. Another group of proteins important for spindle assembly is the family of plus end motor kinesin-5 proteins (Compton 2000; Scholey, Brust-Mascher et al. 2003)**.** *In*

vitro studies suggest that kinesin-5 proteins may utilize a "sliding" mechanism to recruit MTs into bundles (Kapitein, Peterman et al. 2005). Furthermore, two nuclear proteins, NuMA and TPX2, have been shown to stabilize MT and to organize polar MTs during mitosis (Lydersen and Pettijohn 1980; Wittmann, Boleti et al. 1998; Harborth, Wang et al. 1999; Merdes, Heald et al. 2000; Wittmann, Wilm et al. 2000; Gordon, Howard et al. 2001). Recently, the nuclear lamin B and RanGTP proteins also have been reported to facilitate spindle assembly (Tsai, Wang et al. 2006).

We now report the identification of nuclear protein SSRP1 as a novel MT-binding protein that facilitates MT growth and bundling, and is essential for mitosis. SSRP1 is a member of the abundant, non-histone, high-mobility group (HMG) family proteins that are associated with chromatin in interphase cells (Grosschedl, Giese et al. 1994). SSRP1 initially was identified as a protein that bound to DNA modified by the anti-cancer drug cisplatin (Bruhn, Pil et al. 1992), and later found in a heterodimic complex with Spt16, which regulates transcription elongation (Orphanides, Wu et al. 1999; Saunders, Werner et al. 2003; Belotserkovskaya and Reinberg 2004), and possibly replication (Wittmeyer and Formosa 1997; Okuhara, Ohta et al. 1999). Also, this heterodimer could bind to the protein kinase CK2, forming a specific kinase complex for the tumor suppressor protein p53 (Keller, Zeng et al. 2001; Keller and Lu 2002). In addition, SSRP1 appears to act as a transcriptional co-activator, physically modifies chromatin, and is cleaved during apoptosis (Landais, Lee et al. 2006). However, the biological role of SSRP1 remains largely obscure, since mice homozygous for the *ssrp1* deletion mutant were lethal at E3.5 (Cao, Bendall et al. 2003). The observations that SSRP1 is expressed at high levels in proliferating tissues in the mouse (Hertel, De Andrea et al. 1999) and cancerous tissues

(Xiang, Wang et al. 1996), but at low levels in less-renewable and differentiated tissues (Hertel, De Andrea et al. 1999) or cells (our unpublished data), suggest that SSRP1 may be important for the cell cycle. Our current results support this hypothesis.

MATERIALS AND METHODS

Buffers. Lysis buffer, Radioimmune precipitation assay (RIPA) and buffer C 100 (BC-100) were as previously described (Zeng, Dai et al. 2002). All of the above buffers contained 1 mM DTT, and protease inhibitors 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 4μM pepstatin A, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. Tubulin assembly buffer was 80 mM Pipes, pH 6.9, 0.5 mM EGTA, 2 mM $MgSO_4$, and 5% vol/vol glycerol.

Plasmids and antibodies. The pHTO2 siRNA cloning vectors were previously described (Li, Zeng et al. 2007). siRNA derived from the SSRP1 gene sequence 5' GCTCAGGACTGCTCTACCC 3' (nt 1043-1062) was cloned into the pHTO2 vector. pcDNA3-Flag-SSRP1 plasmid was previously described (Zeng, Dai et al. 2002). Polyclonal and monoclonal anti-SSRP1 (5B10) antibodies were previously described (Li, Keller et al. 2005; Li, Zeng et al. 2007). Monoclonal anti-α-tubulin was purchased from Sigma. For immuno-staining procedures, fluorescent secondary goat-anti-rabbit Alexa-Fluor (AF) 488, goat-anti-rabbit AF 546, and goat-anti-mouse AF 488 (Molecular Probes, Eugene, OR) were used.

Cell culture. Human embryonic kidney (HEK) 293, human lung small cell carcinoma H1299, and human cervical carcinoma HeLa cells were cultured in Dulbeccos's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS),

50 U/ml penicillin and 0.1 mg/ml streptomycin at 37^{o}C in a 5% CO_2 humidified atmosphere. All transfections were performed with LipofectAmine 2000 (Invitrogen).

Generation of H1299 pHTO2-SSRP1siRNA inducible Tet on cell line. H1299 pcDNA6-TR or HEK 293 pcDNA6-TR cells, which express Tet repressor, were transfected with 3 µg of pHTO2-SSRP1siRNA plasmid. 24 h post-transfection, cells were trypsinized and transferred to 10-cm plates at low density. 90 µg/ml Hygromycin was added to cells for a 2-3 week selection until colonies became visible. SSRP1 siRNA was induced by addition of doxycycline (5 µg/ml) and cells were harvested for immunoflourescent staining and Western blot (WB) with the anti-SSRP1 antibody. The colonies with the marked reduction of SSRP1 were maintained in future use.

GST fusion protein association assay Equal amount of GST fusion proteins immobilized on Glutathione agarose beads were incubate with S100 extract from HEK 293 cells at 30^{o}C for 1 hour. Beads were washed three times with lysis buffer. Proteins bound to beads were resolved onto a SDS-PAGE gel and analyzed by immunoblot.

***In vitro* tubulin-protein cosedimentation assay.** His-SSRP1 was purified using NTA beads followed by running though HS column. The purified His-SSRP1 protein was dialyzed in BC100 to get rid of high salt. Pure tubulin dimer was purchased from Cytoskeleton Inc. The purity of these proteins was shown in Fig. 4.2B. Tubulins were polymerized at 37^{o}C for 40 min. Then His-SSRP1 or BSA was incubated with polymerized microtubulins at room temperature for 30 min (see the legend of Fig. 4.2C for the amount of proteins used). The mixtures were subjected to centrifugation over 40% sucrose cushions at 13K rpm for 20 min at room temperature. Supernatants and pellets were analyzed by SDS-PAGE, and proteins were detected by WB using anti-SSRP1, and

anti-tubulin antibodies or by Commassie brilliant blue staining for the BSA-tubulin reaction.

Immunofluorescent microscopy. For mitotic analysis, HeLa cells were plated on glass cover slip 4-well chamber slides, synchronized by double-thymidine block, and fixed with 2% paraformaldehyde when 70-80% of the cells were in G2/M phase as previously determined by flow cytometry. The cells were permeablized in 0.1% Triton X-100 (in PBS), and blocked with 2% goat serum. The antibodies used for staining are described in the figure legend, and cells were visualized by deconvolultion microscropy using a DeltaVision Applied Precision Nikon TE200 inverted fluorescent microscope at 60x magnification. For all other staining, cells were visualized with a Zeiss Axiovert 200M microscope at magnification of 40x. Representative images for HeLa cells were shown in Fig. 4.1. The same procedure was used for examining SSRP1 and tubulin after doxycycline-induced SSRP1 siRNA in H1299 pHTO2-SSRP1siRNA tet-inducible cells.

***In vitro* tubulin polymerization assay.** The *in vitro* kinetics of tubulin polymerization was measured using the pure tubulin dimer purchased from Cytoskeleton, Inc. according to the manufacturer's instructions. In brief, 20μM (final concentraion) of pure tubulin dimer in tubulin assembly buffer and 1 mM of GTP (final concentration) were mixed on ice with recombinant proteins (as indicated in Fig. 4.3), which were dialyzed against tubulin assembly buffer, in 50 μl (final volume) of reaction. The mixtures were transferred to a 96-well plate. Polymerization was started by incubation at 37°C followed by optical density reading at the wavelength of 340 nm every minute for up to 30 min in a temperature-controlled 96-well microtitre plate spectrophotometer. Duplicate reaction

mixtures also were processed for IF staining with antibodies against α-tubulin and SSRP1 as well as EM analysis, as described below.

***In vitro* MT bundling assay**. 20µl of tubulin polymerization reaction containing 100µM (final concentration) of tubulin in tubulin assembly buffer and 1mM GTP was incubated at 37°C for 30 min. 180µl of pre-warmed tubulin assembly buffer containing 20µM of taxol (final concentration) was added to the reaction and incubated for additional 10 min. This step diluted the final concentration of the tubulin to 10µM in the reaction. 10 µl of the taxol-stablized MTs (the final concentration of tubulin is 5µM) were mixed with 10 µl of tubulin assembly buffer containing 0, 2, or 10µM of His-SSRP1 (0, 1 or 5µM in the final concentration) at 37^0C for 15 more min. The mixture was dropped onto poly-L-lysine treated glass slides for 5 min and fixed in 4% paraformaldehyde for 30 min. The slides were pre-blocked with 8% BSA for 30 min and incubated with anti-SSRP1 and anti-α-tubulin antibodies overnight and secondary antibodies for 40 min. The slides were washed with PBS three times after incubating with each antibody. Polymerized MTs were analyzed under a fluorescence microscope.

Analysis of *in vitro* polymerized tubulins by electron microscopy (EM). Tubulin polymerization mixtures were incubated at 37^0C for 30 min and immediately used for EM analysis. Briefly, 300 mesh copper grids were coated with a thin carbon film, and irradiated for 15 min with ultraviolet light prior to sample application. Grids were floated on top of 9 µl sample drops for 3 min, after which excess sample was removed gently by wicking with Whatman 1M filter paper. The grids were stained by incubation on 2% uranyl acetate for 1 min, and then wicked and dried. Samples were imaged on a Philips CM120 transmission EM equipped with a Gatan multiscan 625 CCD camera.

***In vitro* monitoring the kinetics of MT bundling mediated by SSRP1.** Rhodamine-conjugated tubulins [20μM (final concentration) of tubulin dimmer mixtures containing 1:5 ratio of Rhodamine-tubulins to tubulins] were incubated with 1mM of GTP and 20μM of taxol in the absence or presence of 8μM of His-SSRP1 at 37^0C and the reactions were stopped by addition of 2 reaction volumes of antifade at different time points for fluorescence microscopic analysis.

RESULTS

SSRP1 co-localizes with mitotic MT.

To examine the cellular localization of SSRP1 protein during cell cycle, we did immunofluorescence (IF) staining. The specificity of polyclonal (Fig. 4.1A) or monoclonal (Fig. 4.1B) SSRP1 antibodies was demonstrated by Western blot (WB) using HeLa whole cell lysates (WCL) or nuclear extracts (NE). A light band around 50 Kd detected by the polyclonal antibody (Fig. 4.1A) has been shown to be a cleaved SSRP1 fragment (Landais, Lee et al. 2006). In interphase cells, SSRP1 predominantly localized to the nucleus as would be expected for a transcription factor (Fig. 4.1C). Immediately after prophase, most of SSRP1 surprisingly co-localized with the centrosomal and spindle MTs (Fig. 4.1C). Conversely, SSRP1 was excluded from the condensed chromosomes as evident by the absence of SSRP1 staining in the DAPI-stained region (Fig. 4.1C). This persisted until the nucleus was re-established during late telophase, at which time SSRP1 was observed in two locations: the midbody, which is the separation point between the two daughter cells and important for cytokinesis; and the nucleus (Fig. 4.1C). The co-localization of SSRP1 with centrosomal and midbody MTs was also verified with a

monoclonal antibody (5B10) specifically against SSRP1 (Fig. 4.1B; data not shown for IF staining). This localization phenomenon was specific to SSRP1, as its partner Spt16 did not co-localize with centrosomal and midbody MTs; instead it was evenly distributed in mitotic cells (data not shown). These results are consistent with two proteomic studies, listing SSRP1, but not Spt16, as one of the components identified in the midbody (Skop, Liu et al. 2004) and spindle complex (Sauer, Korner et al. 2005), and suggest that SSRP1 may associate with the spindle and midbody MTs.

SSRP1 binds to MT *in vitro*.

To investigate the potential association of SSRP1 with MTs, a cytoplasmic S100 extract from HEK 293 cells was incubated with equal amount of wild type (WT) or deletion mutants of SSRP1 fused with glutathione-S-transferase (GST) (Keller and Lu 2002) at 30^{o}C *in vitro*. GST-WT-SSRP1, GST-N-SSRP1 (a.a.1-242), and GST-Mid-SSRP1 (a.a.235-475), but not the GST-0 control and GST-C-SSRP1 (a.a.471-709), bound to MTs, as shown by WB with antibodies against α-, β-, and γ-tubulins (Fig. 4.2A). This data demonstrates that SSRP1 binds to MTs through the highly conserved N-terminal two-thirds of SSRP1. Moreover, SSRP1, but not BSA, directly bound to MTs *in vitro* (Fig. 4.2C), when purified proteins (Fig. 4.2B) were used in sucrose co-sedimentation centrifugation assays. SSRP1-MT binding was confirmed by IF staining of MTs that were formed *in vitro* in the presence of SSRP1 (Figs. 4.3B and D).

SSRP1 facilitates MT polymerization and bundling *in vitro*.

To determine if the association of SSRP1 with MTs affects MT formation *in vitro*, we performed *in vitro* tubulin polymerization assays using purified proteins (Fig. 4.2B). After incubation of protein cocktails in the presence of GTP at 37^0C, as indicated in the figure legend and described in the "material and method", MTs were detected via UV-spectrophotometry by light absorbance 340nm, by fluorescence microscopy after IF staining and by electron microscopy (EM). Interestingly, SSRP1 stimulated MT formation in a dose-dependent fashion, as 2μM (1:10 molar ration of SSRP1 to tubulins in the reaction) of SSRP1 was more effective than 1μM of SSRP1 in promoting MT formation (Fig. 4.3A). Validation that assemblies induced by SSRP1 were not simply random protein aggregates was shown by the appearance of MTs by fluorescence (Fig. 4.3B) and electron (Fig. 4.3C) microscopy. Control experiments employing nocodazole, a chemical that destabilizes MTs, demonstrated that SSRP1 stimulation of MT polymerization was sensitive to this drug (Fig. 4.3B).

The above results demonstrate that SSRP1 is able to promote MT formation *in vitro* by directly associating with this structure. Additionally, when examining electron micrographs of MTs formed *in vitro*, we surprisingly found that the MTs assembled in the presence of SSRP1 appeared to be much longer and more likely to occur in parallel bundles than those assembled in the presence of taxol (Fig. 4.3C), a chemical that stabilizes MTs (Schiff and Horwitz 1980). This observation suggests that SSRP1 may possess an activity to promote MT elongation and bundling. To test this idea, we conducted a set of MT bundling assays. MTs were examined by fluorescence microscopy. Remarkably, SSRP1 not only extended the short and needle-like MTs formed in the presence of taxol, but also organized net-like MTs into root-like architectures in a dose-

dependent manner (Fig. 4.3D). At an equal molar ratio of SSRP1 to tubulins, MTs were regrouped into gigantic bundles (bottom panels). Kinetic analysis of MT bundle formation *in vitro* clearly showed that SSRP1 effectively facilitated MT bundling in a time-dependent fashion (Fig. 4.3E). Hence these results demonstrate that SSRP1, like MAP4, also possesses a dual activity to promote MT elongation and bundling.

SSRP1 is required for maintaining spindle and midbody architectures.

To determine whether the depletion of SSRP1 would influence the formation of spindle and midbody structures in cells, we analyzed mitotic cells using the human p53-deficient lung non-small cell carcinoma H1299 cells that harbored Tet-inducible SSRP1 siRNA. As shown in Fig. 4.4A, both of the SSRP1 mRNA and protein levels were reduced dramatically after siRNA induction. This reduction was specific to SSRP1, as the levels of MDM2, aurora B, survivin, and L23 were not changed (Fig. 4.4A). In the absence of doxycycline, mitotic cells with SSRP1 co-localizing to mitotic spindle and the midbody showed a normal mitotic phenotype (Fig. 4.4B). By contrast, the two centrosomes stained with anti-α-tubulin antibodies were reduced in size, and appeared considerably closer in representative SSRP1-depleted cells. Additionally, the architecture of the spindles, and the midbodies were altered significantly in dividing cells (Fig. 4.4B). These abnormal MT structures were observed in ~60% of mitotic cells with the reduction of SSRP1, whereas only ~5% of mitotic cells with normal levels of SSRP1 showed abnormal mitotic structures (Fig. 4.4C), indicating that SSRP1 is crucial for the formation of the spindles and midbodies. Correlated with these aberrant changes of mitotic MTs, chromosomes were not aligned properly on metaphase plates, and lagging chromosomes

frequently were seen in telophase cells (Fig. 4.4B). These results demonstrate that SSRP1 is required for the formation of normal mitotic spindle and midbody structures.

DISCUSSION

Our studies demonstrate that SSRP1, in addition to its role in regulating transcription (Orphanides, Wu et al. 1999; Saunders, Werner et al. 2003; Belotserkovskaya and Reinberg 2004), and replication (Wittmeyer and Formosa 1997; Okuhara, Ohta et al. 1999) in interphase cells, also plays a direct role in mitosis. SSRP1 not only directly associates with polymerized tubulin *in vitro* and in cells, but also promotes MT growth *in vitro* and in cells (data not shown). To our surprise, SSRP1 exhibits an activity that tethers MTs together and to organizes them into bundle-like architectures. These novel activities of SSRP1 are important for mitosis, as knock down of SSRP1 by siRNA impairs the formation of mitotic machinery and chromosome movement during mitosis (data not shown). Interestingly, the mitotic role of SSRP1 is evolutionarily conserved in *Xenopus*. *Xenopus* SSRP1 co-localizes with mitotic spindle and depletion of SSRP1 from egg extract using SSRP1 antibody inhibits the formation of the mitotic spindle *in vitro*, as shown by our collaborators Stacie Stone and Maureen Hoatlin (data not shown). Therefore, this mitotic role of SSRP1 is crucial for the cell division (Fig. 4.4) and possibly for embryogenesis (Cao, Bendall et al. 2003).

The mitotic and nuclear functions of SSRP1 are not in direct contradiction to each other, because SSRP1 separates from the condensed chromosomes during mitosis (Fig. 4.1). There are other nuclear proteins, such as the DNA replication factor Orc6 (Prasanth, Prasanth et al. 2002), tankyrase1 (a telomeric poly(ADP-ribose) polymerase) (Dynek and

Smith 2004), NuMA (Lydersen and Pettijohn 1980; Harborth, Wang et al. 1999; Merdes, Heald et al. 2000; Gordon, Howard et al. 2001), TPX2 (Wittmann, Wilm et al. 2000), and nuclear lamin B (Tsai, Wang et al. 2006), which have also been shown to play a role in mitosis. Thus mammalian cells appear to effectively utilize their limited resource of proteins for different cellular functions in order to maintain normal cell growth.

For some of its functions, such as transcription elongation and DNA replication, SSRP1 works with Spt16 as a heterodimer (Wittmeyer and Formosa 1997; Okuhara, Ohta et al. 1999; Orphanides, Wu et al. 1999; Belotserkovskaya, Oh et al. 2003; Saunders, Werner et al. 2003). SSRP1 may have Spt16-independent functions as well (Dyer, Hayes et al. 1998; Zeng, Dai et al. 2002; Li, Zeng et al. 2007). In mitosis, SSRP1 appears to function independently of Spt16. Spt16 did not co-localize with SSRP1 to the spindle and midbody (data not shown). Consistent with this result, SSRP1, but not Spt16, was recently found to reside in the mitotic spindle (Sauer, Korner et al. 2005) and midbody complex (Skop, Liu et al. 2004). Thus, the direct role of SSRP1 in mitosis may be Spt16-independent.

The mitotic role of SSRP1 is linked to its interaction with MTs during mitosis, as SSRP1 co-localized with the spindle and midbody MTs (Fig. 4.1) and associated with polymerized MTs *in vitro* (Fig. 4.2). Through a direct association with MTs, SSRP1 enhanced MT polymerization (Fig. 4.3). Thus, SSRP1 appears to play a role in MT dynamics. Because SSRP1 associates with MTs only in mitotic cells, its role in maintaining MT dynamics is exclusive for mitotic MTs. Consequently, depletion of SSRP1 by siRNA resulted in disorganized mitotic machinery (Fig. 4.4). The ability of SSRP1 to organize MTs into bundles remarkably resembles that of MAP4. It has been

shown that MAP4 performs its MT bundling activity by cross-linking MTs through its intrinsic MT-binding domain (Aizawa, Emori et al. 1990; Aizawa, Emori et al. 1991). Although there is no significant similarity between SSRP1 and MAP4, SSRP1 may still utilize the mechanism similar to that for MAP4 to promote MT growth and bundling, which is supported by the fact that SSRP1 associates with MTs throughout the entire MT architecture (Figs. 4.3B and D). At substoichiometric levels, SSRP1 appeared to associate with a portion of MTs and to promote partial MT bundling (Fig. 4.3D), whereas at a stoichiometry of 1 to 1, the effect of SSRP1 on MT bundling was more compelling (Fig. 4.3D). Although detailed biochemical mechanisms underlying SSRP1-mediated MT growth and bundling remain to be studied, it is conceivable that this dual activity of SSRP1 may be pivotal for chromosome movement, as the deficiency of this protein severely impaired this cellular process (data not shown) and inhibited cell division (Fig. 4.4). Therefore, our study uncovers a novel and direct function of SSRP1 for mitosis, though it might also regulate mitosis in part by controlling the expression of as yet unidentified target genes critical for mitosis. Future studies are also necessary to elucidate how SSRP1 regulates the dynamics of mitotic MTs by working in concert with other regulatory proteins, such as NuMA, TPX2, lamin B, Survivin, or MAP4, and how this mitotic function of SSRP1 is regulated during mitosis.

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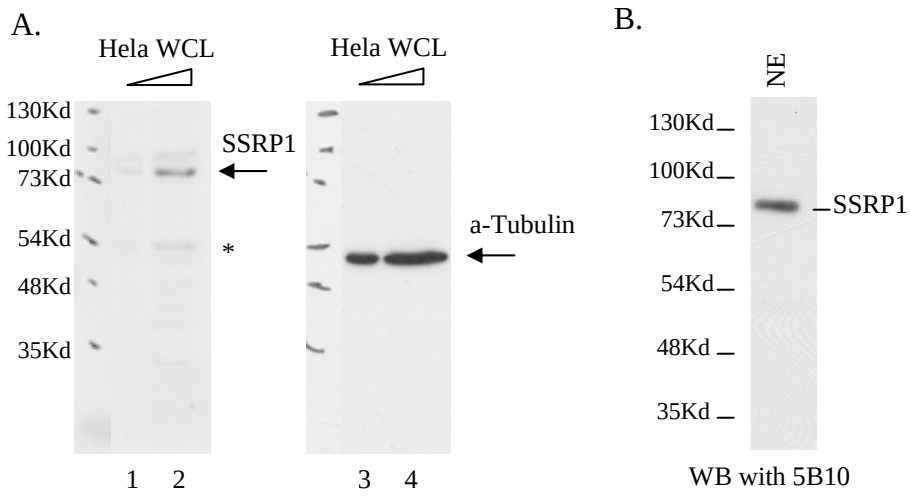
Fig. 4.1. SSRP1 co-localizes with centrosomal and midbody MTs during mitosis. **(A)** The specificity of the polyclonal anti-SSRP1 antibody. 10 and 30 μg of HeLa whole cell lysate (WCL) was subjected to SDS-PAGE for WB with either anti-SSRP1 (left panel) or anti-α-tubulin (right panel) antibodies. The asterisk (*) indicates a truncated SSRP1 fragment. **(B)** The monoclonal anti-SSRP1 antibody 5B10 specifically detects a single band of SSRP1. 50 μg of HeLa nuclear extract (NE) were used for WB analysis with affinity purified 5B10 antibodies. **(C)** SSRP1 co-localizes with MTs during mitosis in HeLa cells. HeLa cells were synchronized by double-thymidine block and stained by IF at interphase and the different phases of mitosis, using anti-SSRP1 (red) or anti-tubulin (green) antibodies and DAPI (blue) for DNA. Co-localization of SSRP1 with tubulins was also observed in H1299 and 293 cells (Fig. 4.4 and data not shown).

Fig. 4.1


A.
Hela WCL
Hela WCL
130Kd
100Kd
73Kd
54Kd
48Kd
35Kd
SSRP1
*
a-Tubulin
1 2
3 4
B.
NE
130Kd
100Kd
73Kd
54Kd
48Kd
35Kd
SSRP1
WB with 5B10

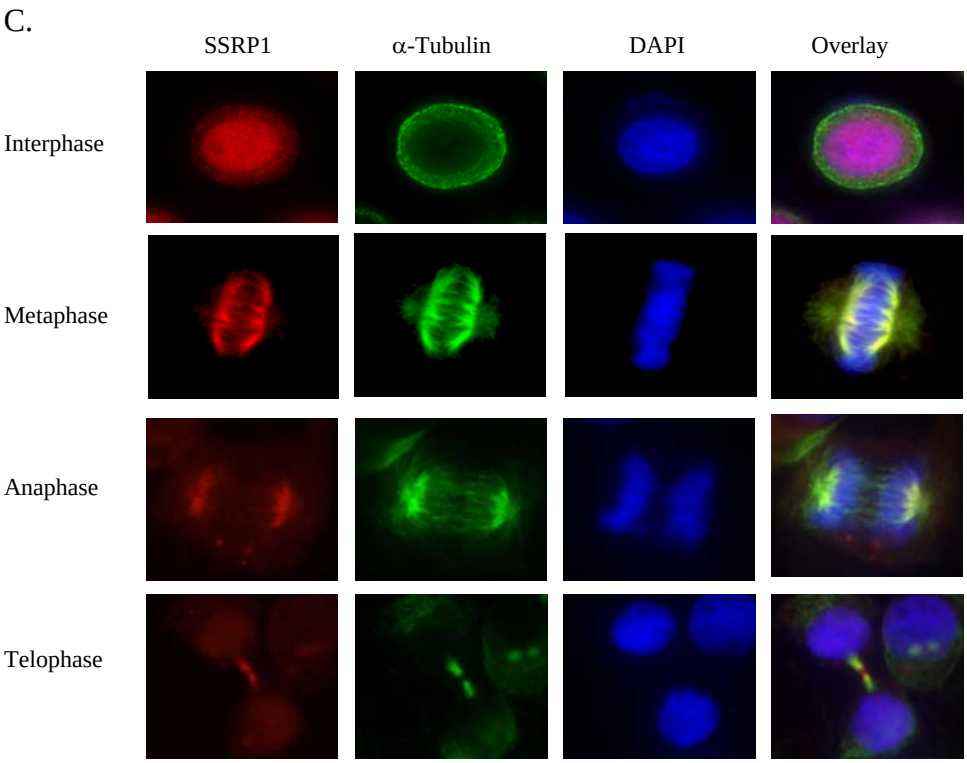

C.
SSRP1
α-Tubulin
DAPI
Overlay
Interphase
Metaphase
Anaphase
Telophase

Fig. 4.2. SSRP1 binds to microtubules *in vitro*.

(A) SSRP1 associates with MTs *in vitro*. GST-SSRP1 fusion proteins (2 μg) were incubated with S100 from HEK 293 cells (500 μg of total proteins) at 30^{o}C. Bound proteins were detected by WB using anti-α-, β-, and γ-tubulin antibodies. **(B)** Coommassie brilliant blue staining of purified proteins used for the assays in Figs. 4.2C and 4.3. 300 ng of His-SSRP1, 1.0 μg of tubulins, or 1.0 μg of bovine serum albumin (BSA) were loaded onto an SDS gel and stained. **(C)** SSRP1, but not BSA, interacts with polymerized tubulins *in vitro.* 2μM of purified His-SSRP1 or BSA was incubated alone or together with 4 μM of polymerized tubulin at 37^{o}C for 30 min. Mixtures were subjected to sucrose co-sedimentation assay. Proteins in supernatants/low molecular weight fractions (S) or pellets/high molecular weight fractions (P) were subjected to SDS-PAGE for WB with antibodies indicated on right. For BSA, the gel was stained with commassie brilliant blue. [Of note, the full-length SSRP1 was unstable at 37^{0}C when incubated alone, because although the amount of the protein input for lanes 1-2 and 3-4 of panel A was the exact same, much more SSRP1 in the presence of tubulins was detected than that in the absence of tubulins. This result was consistently reproduced and thus very convincing].

Fig. 4.2

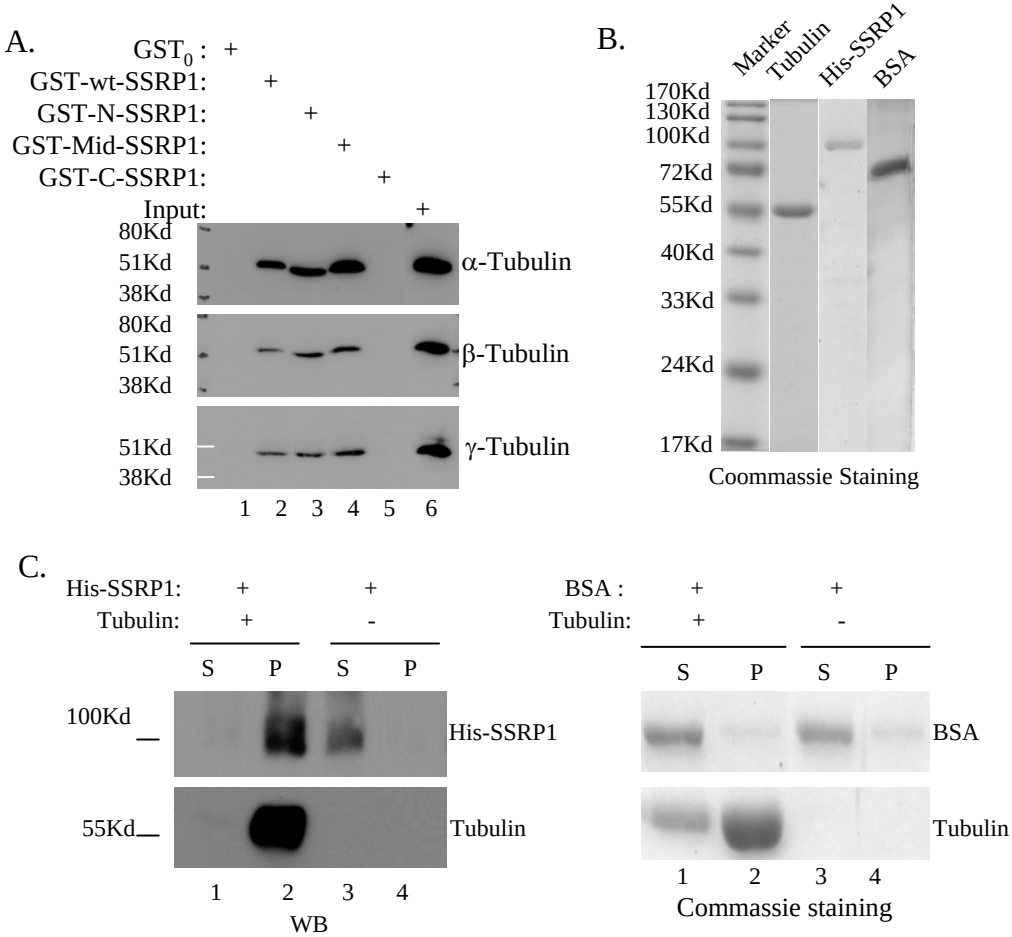
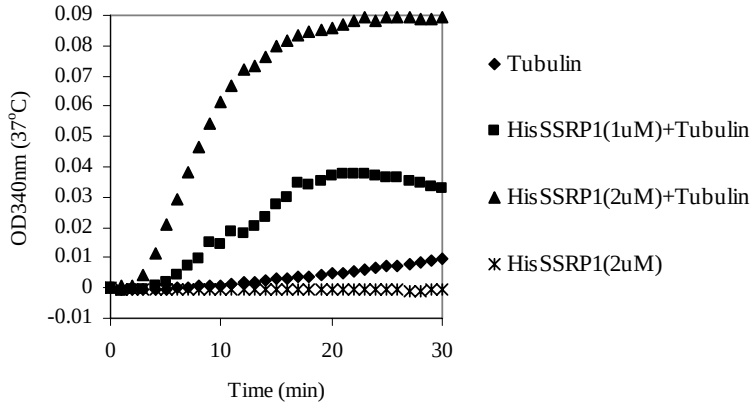

A.
GST0 :
GST-wt-SSRP1:
GST-N-SSRP1:
GST-Mid-SSRP1:
GST-C-SSRP1:
Input:
80Kd
51Kd
38Kd
α-Tubulin
β-Tubulin
γ-Tubulin
1 2 3 4 5 6
B.
Marker
Tubulin
His-SSRP1
BSA
170Kd
130Kd
100Kd
72Kd
55Kd
40Kd
33Kd
24Kd
17Kd
Coommassie Staining
C.
His-SSRP1:
Tubulin:
BSA :
S P S P
100Kd
55Kd
His-SSRP1
Tubulin
BSA
1 2 3 4
WB
Commassie staining

Fig. 4.3. SSRP1 facilitates MT growth and bundling *in vitro*.

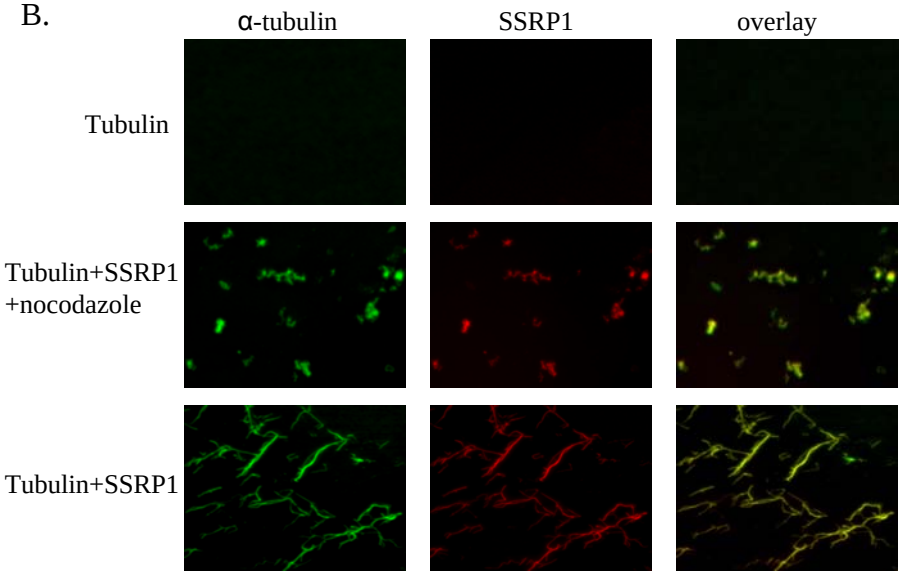
(A) SSRP1 promotes tubulin polymerization *in vitro* in a dose-dependent fashion. *In vitro* tubulin polymerization assays were conducted as described in "material and method". Reaction mixtures containing the following reagents, GTP + tubulin + 2 μM SSRP1 (triangles), GTP + tubulin + 1 μM SSRP1 (squares), GTP + tubulin (diamonds), 2 μM SSRP1 (crosses) were incubated at 37^{o}C for different time points for measuring absorbance at 340 nm as indicated. **(B)** IF analyses of polymerized tubulins *in vitro*. Aliquots from the above reactions were deposited on glass slides for IF analyses with monoclonal anti-tubulin (green) and polyclonal anti-SSRP1 (red) antibodies. Representative IF images are shown. These IF results were also confirmed with polyclonal anti-tubulin and monoclonal anti-SSRP1 antibodies (data not shown). **(C)** EM analysis of *in vitro* polymerized MTs. The same reactions as those described in panels A and B were used for EM analysis. Representative images are shown here as indicated. Images in the top row are 26.5 x 26.5 microns; images in the bottom row are 0.54 x 0.54 microns. **(D)** SSRP1 facilitates MT bundling *in vitro*. 20μM of tubulins were incubated with 20μM of taxol at 37^{0}C for 1 hrs. Tubulins from the reaction cocktail were incubated with purified His-SSRP1 at the concentration as indicated for additional 30 min. Proteins in the reactions were stained with antibodies as described for panel B. **(E)** Kinetics of MT bundling mediated by SSRP1. 20μM Rhodamine-conjugated tubulins (1:5 ratios of Rhodamine-tubulins to tubulins) were incubated with 1mM of GTP and 20μM of taxol in the absence or presence of 8μM of His-SSRP1 at 37^{0}C and the reactions were stopped by addition of 2 reaction volumes of antifade at different time points as indicated for fluorescence microscopic analysis.

Fig. 4.3

A.


0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-0.01
OD340nm (37°C)
0
10
20
30
Time (min)
Tubulin
HisSSRP1(1uM)+Tubulin
HisSSRP1(2uM)+Tubulin
HisSSRP1(2uM)

B.


α-tubulin
SSRP1
overlay
Tubulin
Tubulin+SSRP1
+nocodazole
Tubulin+SSRP1

C.

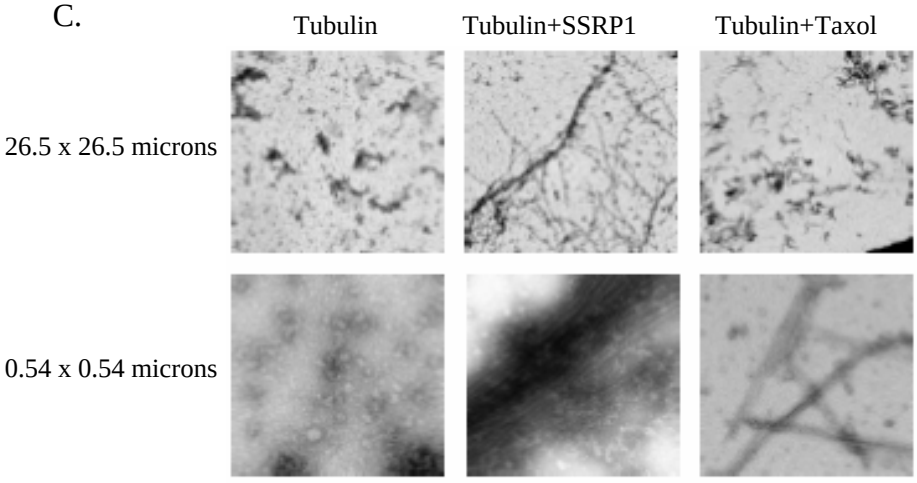
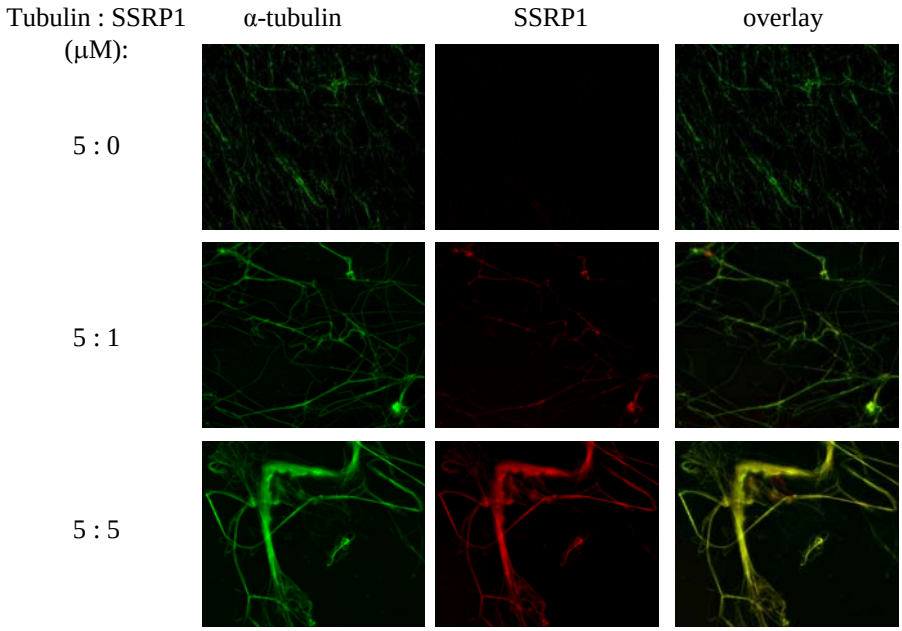

Tubulin
Tubulin+SSRP1
Tubulin+Taxol
26.5 x 26.5 microns
0.54 x 0.54 microns

Fig. 4.3 (continued)

D.



E.

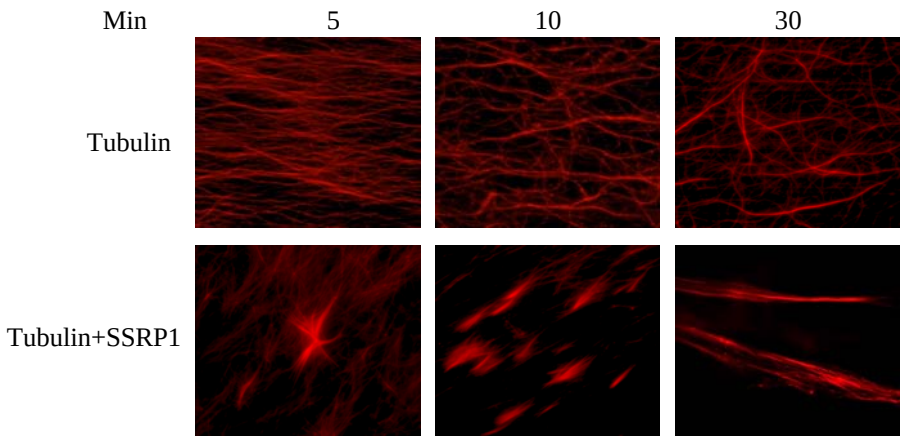
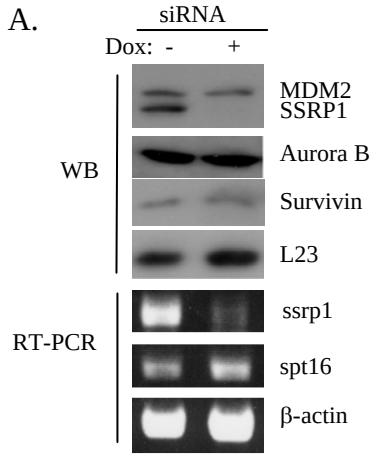
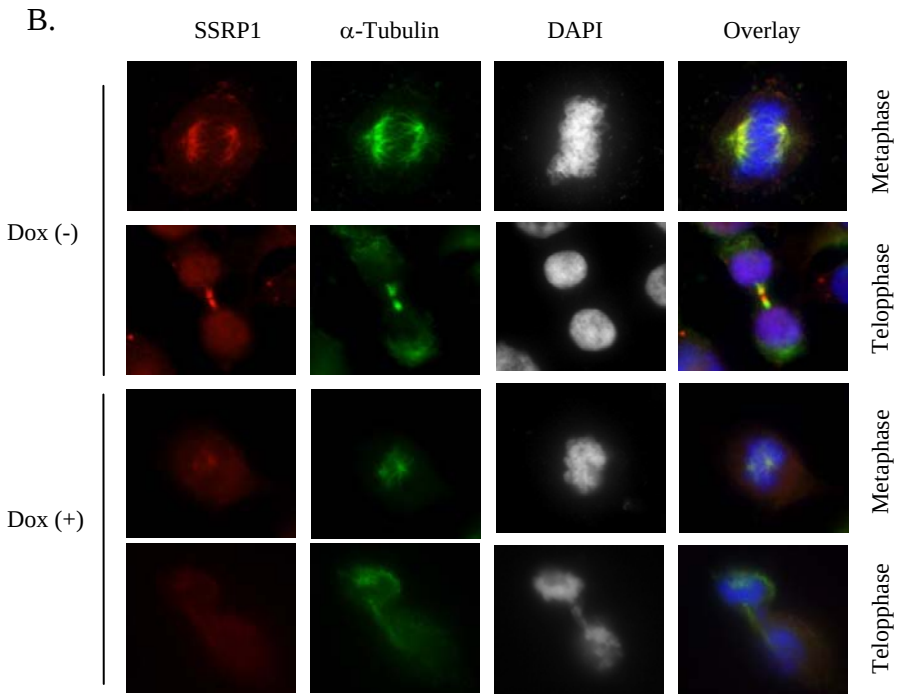


Fig. 4.4. SSRP1 is required for maintaining spindle and midbody architectures.

(A) Establishment of an inducible SSRP1 siRNA expression cell line. H1299 cells that harbor a Tet-inducible SSRP1-siRNA vector were established and were treated with or without doxycycline to induce siRNA expression for 2 days. Cell lysates (50 μg) were harvested for WB (four upper panels) and RT-PCR (three lower panels) analyses with antibodies and DNA primers as indicated on the right. RNAs were detected by 23 cycles of RT-PCR and stained with ethidium bromide. **(B)** Ablation of SSRP1 by siRNA results in disorganized centrosomes, spindles, and midbodies. H1299 cells harboring the inducible SSRP1-siRNA vector were treated as described in panel A. Cells were fixed for IF staining with antibodies against SSRP1 (red) and α-tubulin (green) as indicated on top. DNA was stained with DAPI. **(C)** Percentages of abnormal mitotic cells after ablation of SSRP1 by siRNA. Mitotic cells from mock or siRNA-induced samples were counted to determine the percentages of cells that displayed defects in mitotic spindles or midbodies, as shown in panel B.

Fig. 4.4





C.

	Abnormal/normal mitotic cells	Percentage
(-) siRNA	5/92	5.4 %
(+) siRNA	56/94	59.6 %

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

The FACT complex, a heterodimer of SSRP1 and Spt16, is evolutionarily conserved in all eukaryotic species. Its function in transcription has been established using biochemical assays. FACT facilitates RNAPII-driven transcription through chromatin by destabilizing the nucleosomal structure so that one of the H2A/H2B dimers is removed upon RNAPII passage and maintains nucleosome integrity after RNAPII passage by promoting core histone deposition onto DNA *in vitro* (Fig.1.2). *In vivo*, FACT has been shown to be recruited to several actively transcribed genes in a variety of organisms, such as *hsp70* in *Drosophila* and *p21* in human, by IF staining and ChIP. To assess the global role of FACT in transcription regulation in human cells, I conducted spotted microarray analysis using arrays harboring 8308 human genes. This analysis revealed that FACT up-regulated and down-regulated a subset of gene expression. SSRP1 and Spt16 shared common and individual targets. Further analysis of some of these genes not only verified these observations but also identified the serum-responsive gene, *egr1*, as a novel target for both SSRP1 and Spt16. SSRP1 and Spt16 were recruited to the coding region of *egr1* after serum stimulation. Depletion of SSRP1 and Spt16 inhibited the progression of elongation RNAPII on the *egr1* gene. This study is the first time the FACT complex was shown to be required for specific gene transcription in human cells. The identification of *egr1* as a novel target for the FACT complex establishes a model system to further study how the FACT complex is recruited to a specific gene. In addition, discovering new target genes using microarray opens up new research areas into their functions and regulations by or beyond the FACT complex. In the case of *egr1*, I

provided the novel observation that FACT is required for the transcription elongation of *egr1* and SRF pre-exists on the *egr1* promoter before serum stimulation. The presence of SRF on the *egr1* promoter before serum stimulation suggests the formation of a preassembled initiation complex (PIC) complex. According to my observations, it is most likely that the transcription of human *egr1* is regulated at post-initiation steps (Fig.5.1). This direction would be interesting to explore in the future.

In addition to the role in transcription, SSRP1 is regulated by phosphorylation. In this dissertation, I showed that phosphorylation of SSRP1 by CK2 inhibited the nonspecific DNA-binding activity of SSRP1 and the FACT complex. I further identified serines 510, 657, and 688 of SSRP1 as phosphorylation targets of CK2 *in vitro* and found that serine 510 was more important for the regulation of SSRP1 DNA-binding activity. To further investigate the role of phosphorylation on SSRP1 in cells, I generated an antibody which specifically recognizes the phosphorylation signal on serine 510 of SSRP1 (Fig.A.1 and A.2). Further, I found that serine 510 of SSRP1 is an *in vivo* phosphorylation site. There are two SSRP1 serine 510 phosphoisoforms. The hyperphosphorylated SSRP1 is free from the chromatin and the hypophosphorylated SSRP1 still associates with chromatin (Fig.A.2). In addition, I observed that much more Spt16 associates with the hypophosphorylated SSRP1 than the hyperphosphorylated SSRP1 (data not shown). It was reported that FACT binds to mononucleosomes through Spt16 (Belotserkovskaya, Oh et al. 2003). So it is likely that hyperphosphorylation on SSRP1 inhibits its dimerization with Spt16 and accociation with chromatin. When using this antibody to do ChIP assays, I found that serine 510 phosphorylated SSRP1 is recruited to the *egr1* coding region after serum stimulation (Fig.A.3). It is possible that

the chromatin-associated hypophosphorylated SSRP1 serine 510 phosphoisoform is in the FACT complex and is involved in transcription elongation of *egr1* (Fig.5.1). The additional phosphorylation on SSRP1 frees it from Spt16 and the chromatin. This hyperphosphorylated SSRP1 may play role in Spt16-independent process. One such process might be mitosis.

As shown by this thesis, SSRP1 has a novel function in mitosis. SSRP1 is a nuclear protein during interphase, while it co-localizes with the mitotic spindle and midbody during mitosis. SSRP1 binds to MTs and facilitates tubulin polymerization and MT bundling *in vitro*. Depletion of SSRP1 leads to disorganized spindles and midbodies and causes defects in chromosome alignment and segregation in cells. These results suggest that SSRP1 is a novel MT-stabilizing protein and essential for mitosis. Both tubulin polymerization and depolymerization happen during mitosis and depletion of SSRP1 causes similar defects as depletion of passenger proteins. Thus the function of SSRP1 during mitosis might be regulated by crosstalk with passenger proteins. Our preliminary data showed that SSRP1 interact with Aurora B and Survivn. How SSRP1 is regulated during mitosis and SSRP1's relationship with passenger proteins would be interesting questions for future study.

In conclusion, our studies not only enrich the knowledge of the known transcriptional function for SSRP1 and Spt16, but also uncover the regulation of SSRP1 by CK2 and its previously un-recognized role in regulating MT dynamics. These functions are able to co-exist and are most likely coordinated through SSRP1's subcellular localization. SSRP1 is a nuclear protein in interphase and separated from the condensed chromosomes during mitosis. In addition, SSRP1 is regulated by

phosphorylation and multiple phosphoisoforms exist in cells provide a second level of control. Based on these data, I propose a model for SSRP1's diverse functions and its regulation (Fig.5.2). The unphosphorylated SSRP1 dimerizes with Spt16 to form the FACT complex and non-specifically associates with the chromatin (Fig. 5.2A). In response to active transcription signals, FACT-associated SSRP1 is phosphorylated on serine 510 and recruited to the actively-transcribed region (Fig. 5.2B). Since the phosphorylation of SSRP1 on serine 510 was suggested to weaken the interaction between SSRP1 and DNA based on my *in vitro* assay (Chapter Three), this phosphorylation may provide a favorable mechanism for the FACT complex to facilitate the progression of RNAPII through the chromatin template. After the termination of the transcription, the SSRP1 serine 510 phosphoisoform might be either dephosphorylated to return to the basal status (Fig. 5.2A) or further phosphorylated on other sites (Fig. 5.2C). This hyperphosphorylation might change the conformation of SSRP1 and dissociate it from Spt16 and the chromatin. Thus, there might be two nuclear pools of SSRP1- one that is FACT bound and one that is FACT-independent (Fig. 5.2A-C). During mitosis, the nuclear membrane is broken and the free hyperphosphorylated SSRP1 is released to the cytosol where it binds to MTs (Fig. 5.2D) and plays novel role in cell division. Since SSRP1 does not co-localize with chromosomes during mitosis (Fig. 4.1), most, if not all, of the SSRP1 has likely been hyperphosphorylated before entering mitosis. After mitosis, the hyperphosphorylated SSRP1 might be dephosphorylated to associate with Spt16 and the chromatin again. To accomplish this model, several issues still need to be addressed. First, is phosphorylation on serine 510 of SSRP1 important for its recruitment to the actively transcribed gene? Second, what are the additional phophorylation sites on SSRP1,

and their specific kinases? Third, is the hyperphosphorylated SSRP1 involved in mitosis? In conclusion, my thesis advances what is known about SSRP1, and also uncovers new roles of SSRP1 in gene regulation and mitosis.

Fig. 5.1. The model for how the EGR1 transcription is regulated immediately in response to serum stimulation. The high level of SRF on the *egr1* promoter region before serum stimulation suggests the presence of a PIC including RNAPII itself. After serum stimulation, rapid transcriptional activation is likely fulfilled by the conversion of RNAPII to the elongation form (Ser2 phosphoisoform). The progression of this elongating RNAPII on the *egr1* coding region requires the FACT (SSRP1 and Spt16) complex. The SSRP1 S510 phosphoform is involved in the transcriptional regulation of *egr1* expression.

Fig.5.1

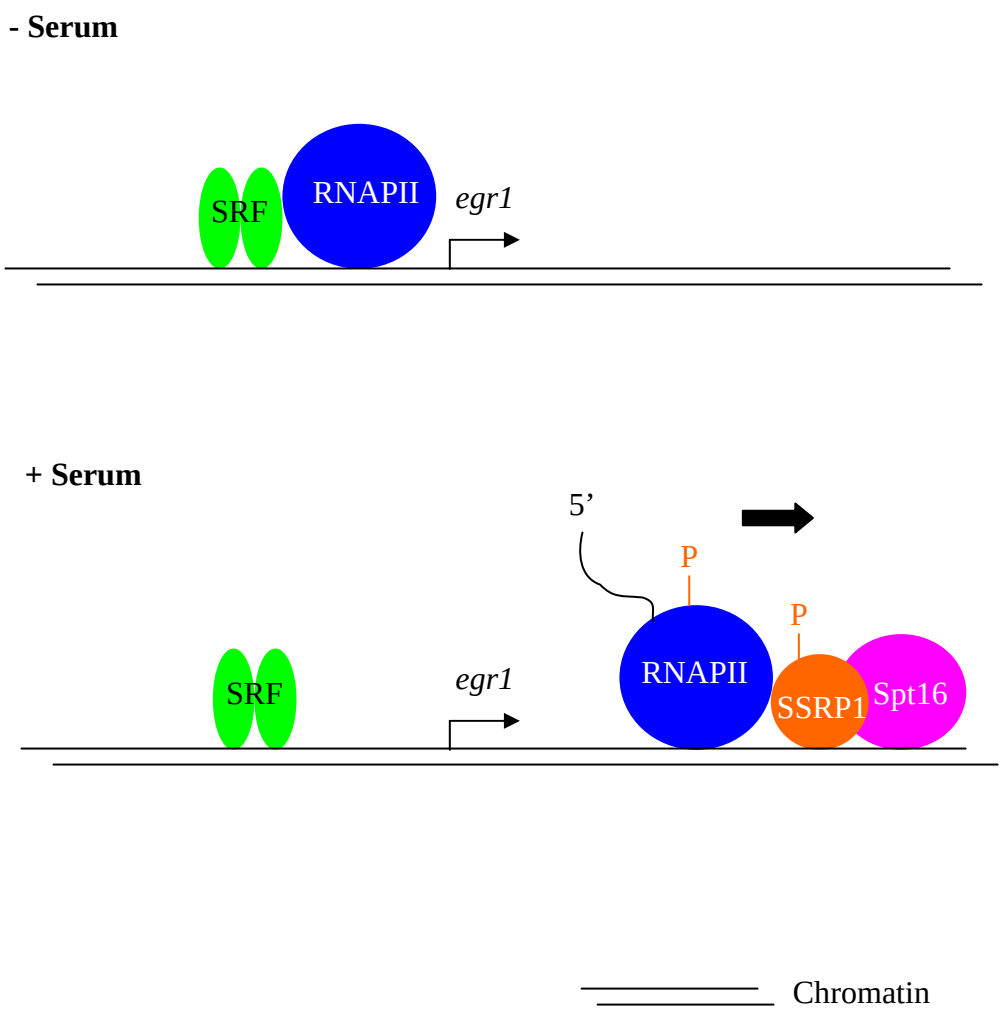
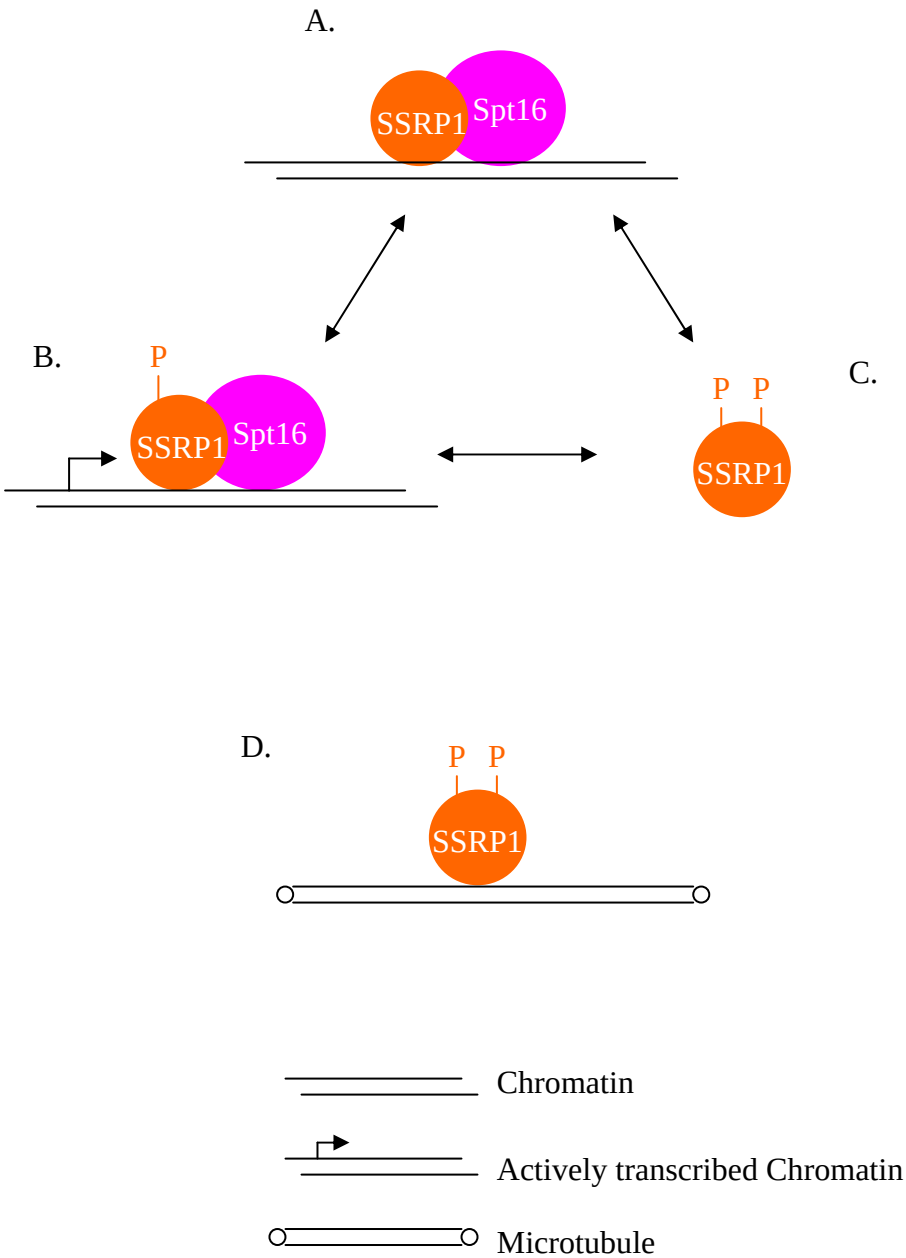

- Serum
SRF
RNAPII
egr1
+ Serum
5’
P
P
SRF
egr1
RNAPII
SSRP1
Spt16
Chromatin

Fig. 5.2. The model for how the diverse functions of SSRP1 are regulated through phosphorylation.

(A) The FACT complex, formed by unphosphorylated SSRP1 and Spt16, non-specifically associates with the chromatin. **(B)** SSRP1 is phosphorylated on serine 510 and, together with Spt16, is recruited to the actively transcribed region in response to active transcription signals. After the termination of the transcription, SSRP1 serine 510 phosphoisoform might be either dephosphorylated to return to the basal status **(A)** or further phosphorylated on other sites **(C)**. **(C)** The hyperphosphorylated SSRP1 serine 510 phosphoisoform become a free form in the nucleus. It might return to **(A)** or **(B)** through dephosphorylation. **(D)** During mitosis, the nuclear membrane is broken and the free hyperphosphorylated SSRP1 is released to the cytosol where it binds to MTs and plays novel role in mitosis.

Fig.5.2


A.
SSRP1
Spt16
B.
P
SSRP1
Spt16
C.
P P
SSRP1
D.
P P
SSRP1
Chromatin
Actively transcribed Chromatin
Microtubule

REFERENCE

Abdulkadir, S. A., J. M. Carbone, et al. (2001). "Frequent and early loss of the EGR1 corepressor NAB2 in human prostate carcinoma." Hum Pathol **32**(9): 935-9.

Adams, R. R., S. P. Wheatley, et al. (2000). "INCENP binds the Aurora-related kinase AIRK2 and is required to target it to chromosomes, the central spindle and cleavage furrow." Curr Biol **10**(17): 1075-8.

Ahn, S. H., M. Kim, et al. (2004). "Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing." Mol Cell **13**(1): 67-76.

Aida, M., Y. Chen, et al. (2006). "Transcriptional pausing caused by NELF plays a dual role in regulating immediate-early expression of the junB gene." Mol Cell Biol **26**(16): 6094-104.

Ainsztein, A. M., S. E. Kandels-Lewis, et al. (1998). "INCENP centromere and spindle targeting: identification of essential conserved motifs and involvement of heterochromatin protein HP1." J Cell Biol **143**(7): 1763-74.

Aizawa, H., Y. Emori, et al. (1991). "Functional analyses of the domain structure of microtubule-associated protein-4 (MAP-U)." J Biol Chem **266**(15): 9841-6.

Aizawa, H., Y. Emori, et al. (1990). "Molecular cloning of a ubiquitously distributed microtubule-associated protein with Mr 190,000." J Biol Chem **265**(23): 13849-55.

Aizawa, H., H. Murofushi, et al. (1987). "Limited chymotryptic digestion of bovine adrenal 190,000-Mr microtubule-associated protein and preparation of a 27,000-

Mr fragment which stimulates microtubule assembly." J Biol Chem **262**(8): 3782-7.

Akey, C. W. and K. Luger (2003). "Histone chaperones and nucleosome assembly." Curr Opin Struct Biol **13**(1): 6-14.

Allende, J. E. and C. C. Allende (1995). "Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation." Faseb J **9**(5): 313-23.

Alsop, G. B. and D. Zhang (2003). "Microtubules are the only structural constituent of the spindle apparatus required for induction of cell cleavage." J Cell Biol **162**(3): 383-90.

Andreasen, P. A., L. Kjoller, et al. (1997). "The urokinase-type plasminogen activator system in cancer metastasis: a review." Int J Cancer **72**(1): 1-22.

Baron, V., E. D. Adamson, et al. (2006). "The transcription factor Egr1 is a direct regulator of multiple tumor suppressors including TGFbeta1, PTEN, p53, and fibronectin." Cancer Gene Ther **13**(2): 115-24.

Baron, V., S. Duss, et al. (2003). "Antisense to the early growth response-1 gene (Egr-1) inhibits prostate tumor development in TRAMP mice." Ann N Y Acad Sci **1002**: 197-216.

Belotserkovskaya, R., S. Oh, et al. (2003). "FACT facilitates transcription-dependent nucleosome alteration." Science **301**(5636): 1090-3.

Belotserkovskaya, R. and D. Reinberg (2004). "Facts about FACT and transcript elongation through chromatin." Curr Opin Genet Dev **14**(2): 139-46.

Belotserkovskaya, R., A. Saunders, et al. (2004). "Transcription through chromatin: understanding a complex FACT." Biochim Biophys Acta **1677**: 87-99.

Biswas, D., R. Dutta-Biswas, et al. (2006). "Opposing roles for Set2 and yFACT in regulating TBP binding at promoters." Embo J **25**(19): 4479-89.

Biswas, D., Y. Yu, et al. (2005). "The yeast FACT complex has a role in transcriptional initiation." Mol Cell Biol **25**(14): 5812-22.

Boldyreff, B. and O. G. Issinger (1995). "Structure of the gene encoding the murine protein kinase CK2 beta subunit." Genomics **29**(1): 253-6.

Bortvin, A. and F. Winston (1996). "Evidence that Spt6p controls chromatin structure by a direct interaction with histones." Science **272**(5267): 1473-6.

Brewster, N. K., G. C. Johnston, et al. (2001). "A bipartite yeast SSRP1 analog comprised of Pob3 and Nhp6 proteins modulates transcription." Mol Cell Biol **21**(10): 3491-502.

Bruhn, S. L., P. M. Pil, et al. (1992). "Isolation and characterization of human cDNA clones encoding a high mobility group box protein that recognizes structural distortions to DNA caused by binding of the anticancer agent cisplatin." Proc Natl Acad Sci U S A **89**(6): 2307-11.

Buchou, T., M. Vernet, et al. (2003). "Disruption of the regulatory beta subunit of protein kinase CK2 in mice leads to a cell-autonomous defect and early embryonic lethality." Mol Cell Biol **23**(3): 908-15.

Buchwalter, G., C. Gross, et al. (2004). "Ets ternary complex transcription factors." Gene **324**: 1-14.

Cao, S., H. Bendall, et al. (2003). "The high-mobility-group box protein SSRP1/T160 is essential for cell viability in day 3.5 mouse embryos." Mol Cell Biol **23**(15): 5301-7.

Cao, X., R. Mahendran, et al. (1992). "Protein phosphatase inhibitors induce the sustained expression of the Egr-1 gene and the hyperphosphorylation of its gene product." J Biol Chem **267**(18): 12991-7.

Cao, X. M., R. A. Koski, et al. (1990). "Identification and characterization of the Egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals." Mol Cell Biol **10**(5): 1931-9.

Carnegie, G. K., F. D. Smith, et al. (2004). "AKAP-Lbc nucleates a protein kinase D activation scaffold." Mol Cell **15**(6): 889-99.

Chai, J. and A. S. Tarnawski (2002). "Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing." J Physiol Pharmacol **53**(2): 147-57.

Christy, B. and D. Nathans (1989). "DNA binding site of the growth factor-inducible protein Zif268." Proc Natl Acad Sci U S A **86**(22): 8737-41.

Christy, B. and D. Nathans (1989). "Functional serum response elements upstream of the growth factor-inducible gene zif268." Mol Cell Biol **9**(11): 4889-95.

Clark, C. D., D. Norris, et al. (1988). "Changes in histone gene dosage alter transcription in yeast." Genes Dev **2**: 150-159.

Compton, D. A. (2000). "Spindle assembly in animal cells." Annu Rev Biochem **69**: 95-114.

de Belle, I., R. P. Huang, et al. (1999). "p53 and Egr-1 additively suppress transformed growth in HT1080 cells but Egr-1 counteracts p53-dependent apoptosis." Oncogene **18**(24): 3633-42.

Desai, A. and T. J. Mitchison (1997). "Microtubule polymerization dynamics." Annual Review of Cell and Developmental Biology **13**: 83-117.

Dominguez, I., J. Mizuno, et al. (2005). "A role for CK2alpha/beta in Xenopus early embryonic development." Mol Cell Biochem **274**(1-2): 125-31.

Dyer, M. A., P. J. Hayes, et al. (1998). "The HMG domain protein SSRP1/PREIIBF is involved in activation of the human embryonic beta-like globin gene." Mol Cell Biol **18**(5): 2617-28.

Dynek, J. N. and S. Smith (2004). "Resolution of sister telomere association is required for progression through mitosis." Science **304**(5667): 97-100.

Earnshaw, W. C. and R. L. Bernat (1991). "Chromosomal passengers: toward an integrated view of mitosis." Chromosoma **100**(3): 139-46.

Eid, M. A., M. V. Kumar, et al. (1998). "Expression of early growth response genes in human prostate cancer." Cancer Res **58**(11): 2461-8.

Eisenmann, D. M., C. Dollard, et al. (1989). "SPT15, the gene encoding the yeast TATA binding factor TFIID, is required for normal transcription initiation in vivo." Cell **58**(6): 1183-91.

Espinosa, J. M., R. E. Verdun, et al. (2003). "p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage." Mol Cell **12**(4): 1015-27.

Evans, D. R., N. K. Brewster, et al. (1998). "The yeast protein complex containing cdc68 and pob3 mediates core-promoter repression through the cdc68 N-terminal domain." Genetics **150**(4): 1393-405.

Flores, O., H. Lu, et al. (1992). "Factors involved in specific transcription by mammalian RNA polymerase II. Identification and characterization of factor IIH." J Biol Chem **267**(4): 2786-93.

Formosa, T. (2003). "Changing the DNA landscape: putting a SPN on chromatin." Curr Top Microbiol Immunol **274**: 171-201.

Formosa, T., P. Eriksson, et al. (2001). "Spt16-Pob3 and the HMG protein Nhp6 combine to form the nucleosome-binding factor SPN." Embo J **20**(13): 3506-17.

Formosa, T., S. Ruone, et al. (2002). "Defects in SPT16 or POB3 (yFACT) in Saccharomyces cerevisiae cause dependence on the Hir/Hpc pathway: polymerase passage may degrade chromatin structure." Genetics **162**(4): 1557-71.

Fu, T. J., J. Peng, et al. (1999). "Cyclin K functions as a CDK9 regulatory subunit and participates in RNA polymerase II transcription." J Biol Chem **274**(49): 34527-30.

Gadde, S. and R. Heald (2004). "Mechanisms and molecules of the mitotic spindle." Curr Biol **14**(18): R797-805.

Gariglio, M., P. Foresta, et al. (1997). "Suppression of high mobility group protein T160 expression impairs mouse cytomegalovirus replication." J Gen Virol **78 (Pt 3)**: 665-70.

Gariglio, M., G. G. Ying, et al. (1997). "The high-mobility group protein T160 binds to both linear and cruciform DNA and mediates DNA bending as determined by ring closure." Exp Cell Res **236**(2): 472-81.

Gashler, A. L., S. Swaminathan, et al. (1993). "A novel repression module, an extensive activation domain, and a bipartite nuclear localization signal defined in the immediate-early transcription factor Egr-1." Mol Cell Biol **13**(8): 4556-71.

Gassmann, R., A. Carvalho, et al. (2004). "Borealin: a novel chromosomal passenger required for stability of the bipolar mitotic spindle." J Cell Biol **166**(2): 179-91.

Gatica, M., M. V. Hinrichs, et al. (1993). "Effect of metal ions on the activity of casein kinase II from Xenopus laevis." FEBS Lett **315**(2): 173-7.

Gietz, R. D., K. C. Graham, et al. (1995). "Interactions between the subunits of casein kinase II." J Biol Chem **270**(22): 13017-21.

Gomes, N. P., G. Bjerke, et al. (2006). "Gene-specific requirement for P-TEFb activity and RNA polymerase II phosphorylation within the p53 transcriptional program." Genes Dev **20**(5): 601-12.

Gordon, M. B., L. Howard, et al. (2001). "Chromosome movement in mitosis requires microtubule anchorage at spindle poles." J Cell Biol **152**(3): 425-34.

Graham, K. C. and D. W. Litchfield (2000). "The regulatory beta subunit of protein kinase CK2 mediates formation of tetrameric CK2 complexes." J Biol Chem **275**(7): 5003-10.

Grosschedl, R., K. Giese, et al. (1994). "HMG domain proteins: architectural elements in the assembly of nucleoprotein structures." Trends Genet **10**(3): 94-100.

Guerra, B., B. Boldyreff, et al. (1999). "CK2: a protein kinase in need of control." Pharmacol Ther **82**(2-3): 303-13.

Guerra, B. and O. G. Issinger (1999). "Protein kinase CK2 and its role in cellular proliferation, development and pathology." Electrophoresis **20**(2): 391-408.

Guo, C., A. T. Davis, et al. (1998). "Dynamics of protein kinase CK2 association with nucleosomes in relation to transcriptional activity." J Biol Chem **273**(22): 13675-80.

Guo, C., S. Yu, et al. (1999). "Nuclear matrix targeting of the protein kinase CK2 signal as a common downstream response to androgen or growth factor stimulation of prostate cancer cells." Cancer Res **59**(5): 1146-51.

Hahn, S., S. Buratowski, et al. (1989). "Isolation of the gene encoding the yeast TATA binding protein TFIID: a gene identical to the SPT15 suppressor of Ty element insertions." Cell **58**(6): 1173-81.

Harborth, J., J. Wang, et al. (1999). "Self assembly of NuMA: multiarm oligomers as structural units of a nuclear lattice." Embo J **18**(6): 1689-700.

Hartzog, G. A., T. Wada, et al. (1998). "Evidence that Spt4, Spt5, and Spt6 control transcription elongation by RNA polymerase II in Saccharomyces cerevisiae." Genes Dev **12**(3): 357-69.

Helenius, J., G. Brouhard, et al. (2006). "The depolymerizing kinesin MCAK uses lattice diffusion to rapidly target microtubule ends." Nature **441**(7089): 115-9.

Hertel, L., M. De Andrea, et al. (1999). "The HMG protein T160 colocalizes with DNA replication foci and is down-regulated during cell differentiation." Exp Cell Res **250**(2): 313-28.

Hertel, L., P. Foresta, et al. (1997). "Decreased expression of the high-mobility group protein T160 by antisense RNA impairs the growth of mouse fibroblasts." Biochimie **79**(12): 717-23.

Hirschhorn, J. N., S. A. Brown, et al. (1992). "Evidence that SNF2/SWI2 and SNF5 activate transcription in yeast by altering chromatin structure." Genes Dev **6**(12A): 2288-98.

Honda, R., R. Korner, et al. (2003). "Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis." Mol Biol Cell **14**(8): 3325-41.

Huang, R. P. and E. D. Adamson (1995). "A biological role for Egr-1 in cell survival following ultra-violet irradiation." Oncogene **10**(3): 467-75.

Huang, R. P., Y. Fan, et al. (1998). "Egr-1 inhibits apoptosis during the UV response: correlation of cell survival with Egr-1 phosphorylation." Cell Death Differ **5**(1): 96-106.

Jimeno-Gonzalez, S., F. Gomez-Herreros, et al. (2006). "A gene-specific requirement for FACT during transcription is related to the chromatin organization of the transcribed region." Mol Cell Biol **26**(23): 8710-21.

Kaitna, S., M. Mendoza, et al. (2000). "Incenp and an aurora-like kinase form a complex essential for chromosome segregation and efficient completion of cytokinesis." Curr Biol **10**(19): 1172-81.

Kapitein, L. C., E. J. Peterman, et al. (2005). "The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks." Nature **435**(7038): 114-8.

Kaplan, C. D., L. Laprade, et al. (2003). "Transcription elongation factors repress transcription initiation from cryptic sites." Science **301**(5636): 1096-9.

Keller, D. M. and H. Lu (2002). "p53 serine 392 phosphorylation increases after UV through induction of the assembly of the CK2.hSPT16.SSRP1 complex." J Biol Chem **277**(51): 50206-13.

Keller, D. M., X. Zeng, et al. (2001). "A DNA damage-induced p53 serine 392 kinase complex contains CK2, hSpt16, and SSRP1." Mol Cell **7**(2): 283-92.

Kelley, D. E., D. G. Stokes, et al. (1999). "CHD1 interacts with SSRP1 and depends on both its chromodomain and its ATPase/helicase-like domain for proper association with chromatin." Chromosoma **108**(1): 10-25.

Klein, U. R., E. A. Nigg, et al. (2006). "Centromere targeting of the chromosomal passenger complex requires a ternary subcomplex of Borealin, Survivin, and the N-terminal domain of INCENP." Mol Biol Cell **17**(6): 2547-58.

Kline-Smith, S. L. and C. E. Walczak (2004). "Mitotic spindle assembly and chromosome segregation: refocusing on microtubule dynamics." Mol Cell **15**(3): 317-27.

Knapska, E. and L. Kaczmarek (2004). "A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK?" Progress in Neurobiology **74**: 183-211.

Kotani, S., H. Murofushi, et al. (1988). "Isolation of rat liver microtubule-associated proteins. Evidence for a family of microtubule-associated proteins with molecular mass of around 200,000 which distribute widely among mammalian cells." J Biol Chem **263**(11): 5385-9.

Kouskouti, A. and I. Talianidis (2005). "Histone modifications defining active genes persist after transcriptional and mitotic inactivation." Embo J **24**(2): 347-57.

Kovanen, P. E., A. Rosenwald, et al. (2003). "Analysis of gamma c-family cytokine target genes. Identification of dual-specificity phosphatase 5 (DUSP5) as a regulator of mitogen-activated protein kinase activity in interleukin-2 signaling." J Biol Chem **278**(7): 5205-13.

Krogan, N. J., M. Kim, et al. (2002). "RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach." Mol Cell Biol **22**(20): 6979-92.

Krohn, N. M., C. Stemmer, et al. (2003). "Protein kinase CK2 phosphorylates the high mobility group domain protein SSRP1, inducing the recognition of UV-damaged DNA." J Biol Chem **278**(15): 12710-5.

Kuninger, D., D. Stauffer, et al. (2004). "Gene disruption by regulated short interfering RNA expression, using a two-adenovirus system." Hum Gene Ther **15**(12): 1287-92.

Landais, I., H. Lee, et al. (2006). "Coupling caspase cleavage and ubiquitin-proteasome-dependent degradation of SSRP1 during apoptosis." Cell Death Differ **13**(11): 1866-78.

Lau, L. F. and D. Nathans (1987). "Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc." Proc Natl Acad Sci U S A **84**(5): 1182-6.

Lee, S. L., Y. Sadovsky, et al. (1996). "Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1)." Science **273**(5279): 1219-21.

Lee, S. L., L. C. Tourtellotte, et al. (1995). "Growth and differentiation proceeds normally in cells deficient in the immediate early gene NGFI-A." J Biol Chem **270**(17): 9971-7.

Lee, S. L., Y. Wang, et al. (1996). "Unimpaired macrophage differentiation and activation in mice lacking the zinc finger transplantation factor NGFI-A (EGR1)." Mol Cell Biol **16**(8): 4566-72.

Lemaire, P., C. Vesque, et al. (1990). "The serum-inducible mouse gene Krox-24 encodes a sequence-specific transcriptional activator." Mol Cell Biol **10**(7): 3456-67.

Lens, S. M., G. Vader, et al. (2006). "The case for Survivin as mitotic regulator." Curr Opin Cell Biol **18**(6): 616-22.

Li, F., G. Ambrosini, et al. (1998). "Control of apoptosis and mitotic spindle checkpoint by survivin." Nature **396**(6711): 580-4.

Li, Y., D. M. Keller, et al. (2005). "CK2 phosphorylates SSRP1 and inhibits its DNA-binding activity." J Biol Chem **280**(12): 11869-75.

Li, Y., S. X. Zeng, et al. (2007). "Human SSRP1 has Spt16-dependent and -independent roles in gene transcription." J Biol Chem **282**(10): 6936-45.

Lindstrom, D. L., S. L. Squazzo, et al. (2003). "Dual roles for Spt5 in pre-mRNA processing and transcription elongation revealed by identification of Spt5-associated proteins." Mol Cell Biol **23**(4): 1368-78.

Litchfield, D. W. (2003). "Protein kinase CK2: structure, regulation and role in cellular decisions of life and death." Biochem J **369**(Pt 1): 1-15.

Litchfield, D. W. and B. Luscher (1993). "Casein kinase II in signal transduction and cell cycle regulation." Mol Cell Biochem **127-128**: 187-99.

Lu, H., O. Flores, et al. (1991). "The non-phosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex." Proc. Natl. Acad. Sci. **USA 88**: 10004-10008.

Lu, H., L. Zawel, et al. (1992). "Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II." Nature **358**: 641-645.

Lycan, D., G. Mikesell, et al. (1994). "Differential effects of Cdc68 on cell cycle-regulated promoters in Saccharomyces cerevisiae." Mol Cell Biol **14**(11): 7455-65.

Lydersen, B. K. and D. E. Pettijohn (1980). "Human-specific nuclear protein that associates with the polar region of the mitotic apparatus: distribution in a human/hamster hybrid cell." Cell **22**(2 Pt 2): 489-99.

Mackay, A. M., D. M. Eckley, et al. (1993). "Molecular analysis of the INCENPs (inner centromere proteins): separate domains are required for association with microtubules during interphase and with the central spindle during anaphase." J Cell Biol **123**(2): 373-85.

MacRae, T. H. (1992). "Microtubule organization by cross-linking and bundling proteins." Biochim Biophys Acta **1160**(2): 145-55.

Maiato, H., P. Sampaio, et al. (2004). "Microtubule-associated proteins and their essential roles during mitosis." Int Rev Cytol **241**: 53-153.

Malone, E. A., C. D. Clark, et al. (1991). "Mutations in SPT16/CDC68 suppress cis- and trans-acting mutations that affect promoter function in Saccharomyces cerevisiae." Mol Cell Biol **11**(11): 5710-7.

Marin, O., F. Meggio, et al. (1999). "Tyrosine versus serine/threonine phosphorylation by protein kinase casein kinase-2. A study with peptide substrates derived from immunophilin Fpr3." J Biol Chem **274**(41): 29260-5.

Mason, P. B. and K. Struhl (2003). "The FACT complex travels with elongating RNA polymerase II and is important for the fidelity of transcriptional initiation in vivo." Mol Cell Biol **23**(22): 8323-33.

Meggio, F., B. Boldyreff, et al. (1992). "The effect of polylysine on casein-kinase-2 activity is influenced by both the structure of the protein/peptide substrates and the subunit composition of the enzyme." Eur J Biochem **205**(3): 939-45.

Meggio, F. and L. A. Pinna (2003). "One-thousand-and-one substrates of protein kinase CK2?" Faseb J **17**(3): 349-68.

Merdes, A., R. Heald, et al. (2000). "Formation of spindle poles by dynein/dynactin-dependent transport of NuMA." J Cell Biol **149**(4): 851-62.

Milbrandt, J. (1987). "A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor." Science **238**(4828): 797-9.

Munstermann, U., G. Fritz, et al. (1990). "Casein kinase II is elevated in solid human tumours and rapidly proliferating non-neoplastic tissue." Eur J Biochem **189**(2): 251-7.

Murofushi, H., S. Kotani, et al. (1986). "Purification and characterization of a 190-kD microtubule-associated protein from bovine adrenal cortex." J Cell Biol **103**(5): 1911-9.

Nagulapalli, S., J. M. Pongubala, et al. (1995). "Multiple proteins physically interact with PU.1. Transcriptional synergy with NF-IL6 beta (C/EBP delta, CRP3)." J Immunol **155**(9): 4330-8.

Nair, P., S. Muthukkumar, et al. (1997). "Early growth response-1-dependent apoptosis is mediated by p53." J Biol Chem **272**(32): 20131-8.

Niefind, K., M. Putter, et al. (1999). "GTP plus water mimic ATP in the active site of protein kinase CK2." Nat Struct Biol **6**(12): 1100-3.

O'Donovan, K. J., W. G. Tourtellotte, et al. (1999). "The EGR family of transcription-regulatory factors:progress at the interface of molecular and systems neuroscience." Trends Neurosci. **22**(4): 167-173.

Okuhara, K., K. Ohta, et al. (1999). "A DNA unwinding factor involved in DNA replication in cell-free extracts of Xenopus eggs." Curr Biol **9**(7): 341-50.

Ookata, K., S. Hisanaga, et al. (1995). "Cyclin B interaction with microtubule-associated protein 4 (MAP4) targets p34cdc2 kinase to microtubules and is a potential regulator of M-phase microtubule dynamics." J Cell Biol **128**(5): 849-62.

Orphanides, G., G. LeRoy, et al. (1998). "FACT, a factor that facilitates transcript elongation through nucleosomes." Cell **92**(1): 105-16.

Orphanides, G., W. H. Wu, et al. (1999). "The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins." Nature **400**(6741): 284-8.

Padmanabha, R., J. L. Chen-Wu, et al. (1990). "Isolation, sequencing, and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in Saccharomyces cerevisiae." Mol Cell Biol **10**(8): 4089-99.

Paulsson, Y., M. Bywater, et al. (1987). "Effects of epidermal growth factor and platelet-derived growth factor on c-fos and c-myc mRNA levels in normal human fibroblasts." Exp Cell Res **171**(1): 186-94.

Pavletich, N. P. and C. O. Pabo (1991). "Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A." Science **252**(5007): 809-17.

Pavri, R., B. Zhu, et al. (2006). "Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II." Cell **125**(4): 703-17.

Pinna, L. A. (1990). "Casein kinase 2: an 'eminence grise' in cellular regulation?" Biochim Biophys Acta **1054**(3): 267-84.

Pinna, L. A. and F. Meggio (1997). "Protein kinase CK2 ("casein kinase-2") and its implication in cell division and proliferation." Prog Cell Cycle Res **3**: 77-97.

Prasanth, S. G., K. V. Prasanth, et al. (2002). "Orc6 involved in DNA replication, chromosome segregation, and cytokinesis." Science **297**(5583): 1026-31.

Prowald, K., H. Fischer, et al. (1984). "Enhanced casein kinase II activity in human tumour cell cultures." FEBS Lett **176**(2): 479-83.

Reinberg, D. and R. J. Sims, 3rd (2006). "de FACTo nucleosome dynamics." J Biol Chem **281**(33): 23297-301.

Rowley, A., R. A. Singer, et al. (1991). "CDC68, a yeast gene that affects regulation of cell proliferation and transcription, encodes a protein with a highly acidic carboxyl terminus." Mol Cell Biol **11**(11): 5718-26.

Russo, M. W., B. R. Sevetson, et al. (1995). "Identification of NAB1, a repressor of NGFI-A- and Krox20-mediated transcription." Proc Natl Acad Sci U S A **92**(15): 6873-7.

Sauer, G., R. Korner, et al. (2005). "Proteome analysis of the human mitotic spindle." Mol Cell Proteomics **4**(1): 35-43.

Saunders, A., J. Werner, et al. (2003). "Tracking FACT and the RNA polymerase II elongation complex through chromatin in vivo." Science **301**(5636): 1094-6.

Schiff, P. B. and S. B. Horwitz (1980). "Taxol stabilizes microtubules in mouse fibroblast cells." Proc Natl Acad Sci U S A **77**(3): 1561-5.

Schlesinger, M. B. and T. Formosa (2000). "POB3 is required for both transcription and replication in the yeast Saccharomyces cerevisiae." Genetics **155**(4): 1593-606.

Scholey, J. M., I. Brust-Mascher, et al. (2003). "Cell division." Nature **422**(6933): 746-52.

Schratt, G., B. Weinhold, et al. (2001). "Serum response factor is required for immediate-early gene activation yet is dispensable for proliferation of embryonic stem cells." Mol Cell Biol **21**(8): 2933-43.

Shaw, P. E. and J. Saxton (2003). "Ternary complex factors: prime nuclear targets for mitogen-activated protein kinases." Int J Biochem Cell Biol **35**(8): 1210-26.

Shimojima, T., M. Okada, et al. (2003). "Drosophila FACT contributes to Hox gene expression through physical and functional interactions with GAGA factor." Genes Dev **17**(13): 1605-16.

Shirakata, M., K. Huppi, et al. (1991). "HMG1-related DNA-binding protein isolated with V-(D)-J recombination signal probes." Mol Cell Biol **11**(9): 4528-36.

Singer, R. A. and G. C. Johnston (2004). "The FACT chromatin modulator: genetic and structure/function relationships." Biochem Cell Biol **82**(4): 419-27.

Skop, A. R., H. Liu, et al. (2004). "Dissection of the mammalian midbody proteome reveals conserved cytokinesis mechanisms." Science **305**(5680): 61-6.

Spencer, J. A., M. H. Baron, et al. (1999). "Cooperative transcriptional activation by serum response factor and the high mobility group protein SSRP1." J Biol Chem **274**(22): 15686-93.

Stemmer, C., A. Schwander, et al. (2002). "Protein kinase CK2 differentially phosphorylates maize chromosomal high mobility group B (HMGB) proteins modulating their stability and DNA interactions." J Biol Chem **277**(2): 1092-8.

Sukhatme, V. P., X. M. Cao, et al. (1988). "A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization." Cell **53**(1): 37-43.

Sun, X. H., N. G. Copeland, et al. (1991). "Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins." Mol Cell Biol **11**(11): 5603-11.

Svaren, J., T. Ehrig, et al. (2000). "EGR1 target genes in prostate carcinoma cells identified by microarray analysis." J Biol Chem **275**(49): 38524-31.

Svaren, J., B. R. Sevetson, et al. (1996). "NAB2, a corepressor of NGFI-A (Egr-1) and Krox20, is induced by proliferative and differentiative stimuli." Mol Cell Biol **16**(7): 3545-53.

Svejstrup, J. Q. (2002). "Chromatin elongation factors." Curr Opin Genet Dev **12**(2): 156-61.

Takeda, S., S. Naruse, et al. (1967). "Effects of ultra-violet microbeam irradiation of various sites of HeLa cells on the synthesis of RNA, DNA and protein." Nature **213**(5077): 696-697.

Tan, B. C., C. T. Chien, et al. (2006). "Functional cooperation between FACT and MCM helicase facilitates initiation of chromatin DNA replication." Embo J **25**(17): 3975-85.

Tawfic, S., A. T. Davis, et al. (1997). "Association of protein kinase CK2 with nuclear matrix: influence of method of preparation of nuclear matrix." J Cell Biochem **64**(3): 499-504.

Tegge, W. J. and R. Frank (1998). "Analysis of protein kinase substrate specificity by the use of peptide libraries on cellulose paper (SPOT-method)." Methods Mol Biol **87**: 99-106.

Thigpen, A. E., K. M. Cala, et al. (1996). "Increased expression of early growth response-1 messenger ribonucleic acid in prostatic adenocarcinoma." J Urol **155**(3): 975-81.

Thyss, R., V. Virolle, et al. (2005). "NF-kappaB/Egr-1/Gadd45 are sequentially activated upon UVB irradiation to mediate epidermal cell death." Embo J **24**(1): 128-37.

Tsai, M. Y., S. Wang, et al. (2006). "A mitotic lamin B matrix induced by RanGTP required for spindle assembly." Science **311**(5769): 1887-93.

Tullai, J. W., M. E. Schaffer, et al. (2004). "Identification of transcription factor binding sites upstream of human genes regulated by the phosphatidylinositol 3-kinase and MEK/ERK signaling pathways." J Biol Chem **279**(19): 20167-77.

Vader, G., J. J. Kauw, et al. (2006). "Survivin mediates targeting of the chromosomal passenger complex to the centromere and midbody." EMBO Rep **7**(1): 85-92.

Vader, G., R. H. Medema, et al. (2006). "The chromosomal passenger complex: guiding Aurora-B through mitosis." J Cell Biol **173**(6): 833-7.

Vagnarelli, P. and W. C. Earnshaw (2004). "Chromosomal passengers: the four-dimensional regulation of mitotic events." Chromosoma **113**(5): 211-22.

van der Geer, P. and T. Hunter (1994). "Phosphopeptide mapping and phosphoamino acid analysis by electrophoresis and chromatography on thin-layer cellulose plates." Electrophoresis **15**(3-4): 544-54.

Vignali, M., A. H. Hassan, et al. (2000). "ATP-dependent chromatin-remodeling complexes." Mol Cell Biol **20**(6): 1899-910.

Virolle, T., E. D. Adamson, et al. (2001). "The Egr-1 transcription factor directly activates PTEN during irradiation-induced signalling." Nat Cell Biol **3**(12): 1124-8.

Virolle, T., A. Krones-Herzig, et al. (2003). "Egr1 promotes growth and survival of prostate cancer cells. Identification of novel Egr1 target genes." J Biol Chem **278**(14): 11802-10.

Wada, T., T. Takagi, et al. (1998). "DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs." Genes Dev **12**(3): 343-56.

Wang, G., M. A. Balamotis, et al. (2005). "Mediator requirement for both recruitment and postrecruitment steps in transcription initiation." Mol Cell **17**(5): 683-94.

Wang, H. W. and E. Nogales (2005). "Nucleotide-dependent bending flexibility of tubulin regulates microtubule assembly." Nature **435**(7044): 911-5.

Wang, X. M., J. G. Peloquin, et al. (1996). "Removal of MAP4 from microtubules in vivo produces no observable phenotype at the cellular level." J Cell Biol **132**(3): 345-57.

West, R. R., K. M. Tenbarge, et al. (1991). "A model for microtubule-associated protein 4 structure. Domains defined by comparisons of human, mouse, and bovine sequences." J Biol Chem **266**(32): 21886-96.

Wiese, C. and Y. Zheng (2006). "Microtubule nucleation: {gamma}-tubulin and beyond." J Cell Sci **119**(Pt 20): 4143-53.

Wilde, A., S. B. Lizarraga, et al. (2001). "Ran stimulates spindle assembly by altering microtubule dynamics and the balance of motor activities." Nat Cell Biol **3**(3): 221-7.

Wilson, L. K., N. Dhillon, et al. (1997). "Casein kinase II catalyzes tyrosine phosphorylation of the yeast nucleolar immunophilin Fpr3." J Biol Chem **272**(20): 12961-7.

Winston, F., C. DT, et al. (1984). "Mutations affecting Ty-mediated expression of the HIS4 gene of Saccharomyces cerevisiae." Genetics **107**: 179-197.

Wirkner, U., H. Voss, et al. (1994). "The human gene (CSNK2A1) coding for the casein kinase II subunit alpha is located on chromosome 20 and contains tandemly arranged Alu repeats." Genomics **19**(2): 257-65.

Wittmann, T., H. Boleti, et al. (1998). "Localization of the kinesin-like protein Xklp2 to spindle poles requires a leucine zipper, a microtubule-associated protein, and dynein." J Cell Biol **143**(3): 673-85.

Wittmann, T., M. Wilm, et al. (2000). "TPX2, A novel xenopus MAP involved in spindle pole organization." J Cell Biol **149**(7): 1405-18.

Wittmeyer, J. and T. Formosa (1997). "The Saccharomyces cerevisiae DNA polymerase alpha catalytic subunit interacts with Cdc68/Spt16 and with Pob3, a protein similar to an HMG1-like protein." Mol Cell Biol **17**(7): 4178-90.

Wittmeyer, J., L. Joss, et al. (1999). "Spt16 and Pob3 of Saccharomyces cerevisiae form an essential, abundant heterodimer that is nuclear, chromatin-associated, and copurifies with DNA polymerase alpha." Biochemistry **38**(28): 8961-71.

Xiang, Y. Y., D. Y. Wang, et al. (1996). "Expression of structure-specific recognition protein mRNA in fetal kidney and Fe-nitrilotriacetate-induced renal carcinoma in the rat." Cancer Lett **106**(2): 271-8.

Xu, Q., G. C. Johnston, et al. (1993). "The Saccharomyces cerevisiae Cdc68 transcription activator is antagonized by San1, a protein implicated in transcriptional silencing." Mol Cell Biol **13**(12): 7553-65.

Xu, X., P. A. Toselli, et al. (1999). "Globozoospermia in mice lacking the casein kinase II alpha' catalytic subunit." Nat Genet **23**(1): 118-21.

Yabuta, Y., E. Subbian, et al. (2003). "Folding pathway mediated by an intramolecular chaperone. A functional peptide chaperone designed using sequence databases." J Biol Chem **278**(17): 15246-51.

Yamada, T., Y. Yamaguchi, et al. (2006). "P-TEFb-mediated phosphorylation of hSpt5 C-terminal repeats is critical for processive transcription elongation." Mol Cell **21**(2): 227-37.

Yamaguchi, Y., T. Wada, et al. (1999). "Structure and function of the human transcription elongation factor DSIF." J Biol Chem **274**(12): 8085-92.

Yang-Feng, T. L., T. Naiman, et al. (1994). "Assignment of the human casein kinase II alpha' subunit gene (CSNK2A1) to chromosome 16p13.2-p13.3." Genomics **19**(1): 173.

Yarnell, A. T., S. Oh, et al. (2001). "Interaction of FACT, SSRP1, and the high mobility group (HMG) domain of SSRP1 with DNA damaged by the anticancer drug cisplatin." J Biol Chem **276**(28): 25736-41.

Yoh, S. M., H. Cho, et al. (2007). "The Spt6 SH2 domain binds Ser2-P RNAPII to direct Iws1-dependent mRNA splicing and export." Genes Dev **21**(2): 160-74.

Yu, J., I. de Belle, et al. (2004). "Coactivating factors p300 and CBP are transcriptionally crossregulated by Egr1 in prostate cells, leading to divergent responses." Mol Cell **15**(1): 83-94.

Zangenehpour, S. and A. Chaudhuri (2002). "Differential induction and decay curves of c-fos and zif268 revealed through dual activity maps." Brain Res Mol Brain Res **109**(1-2): 221-5.

Zeng, S. X., M. S. Dai, et al. (2002). "SSRP1 functions as a co-activator of the transcriptional activator p63." Embo J **21**(20): 5487-97.

Zeng, X., A. J. Levine, et al. (1998). "Non-p53 p53RE binding protein, a human transcription factor functionally analogous to P53." Proc Natl Acad Sci U S A **95**(12): 6681-6.

APPENDIX—Serine 510 is phosphorylated *in vivo* and this phosphorylated SSRP1 is still associated with the actively transcribing *egr1* gene in response to serum stimulation.

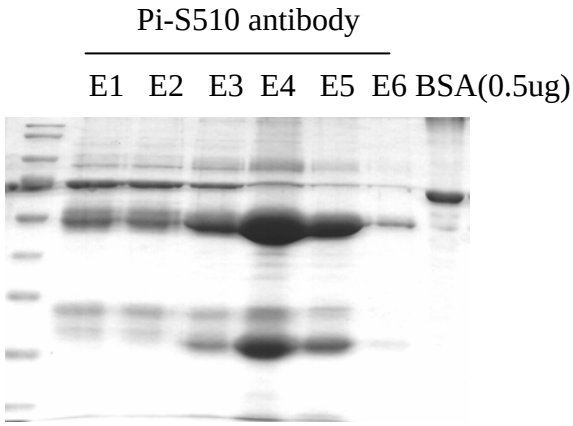
As a logical continuation of one part of my thesis work as detailed in Chapter 3, I have also investigated whether serine 510 of SSRP1 is an *in vivo* phosphorylation site. To do so, we generated an antibody using a SSRP1 S510 phosphopeptide (15aa) and purified the antibody (Fig. A.1A). As shown in Fig. A.2B, the purified antibody specifically recognized the phosphopeptide, not the non-phosphopeptide. When the antibody was used to detect the endogenous SSRP1 protein, it detected two populations of SSRP1 serine 510 phosphoisoforms. The slower-migrating form was free from chromatin (Fig. A.2A, supernatant) and the faster-migrating form still associated with chromatin (Fig. A.2A, pellet). Both populations of SSRP1 serine 510-phosphorylated isoforms were recognized by the monoclonal SSRP1 antibody 5B10, though to different degrees. To further confirm that these two populations are phosphorylated forms of SSRP1, we used CIP treatment. Indeed, after CIP treatment, the serine 510 phospho-antibody did not recognize either of these putative phosphorylated isoforms, although the same level of SSRP1 protein was present in the CIP treated versus the CIP non-treated samples (Fig. A.2B). I also generated a serine 510 to alanine mutant (S510A). As shown in Fig. A.2C, this mutation completely abolished the signal detected by the serine 510 phospho-antibody. These results demonstrate that serine 510 is indeed an *in vivo* phosphorylation site and two populations of SSRP1 serine 510-phosphorylated isoforms co-exist in cells. However, serine 510 phosphorylation does not appear to dissociate SSRP1 from chromatin. Indeed, ChIP analyses using this serine 510 antibody showed that serine 510

phosphorylated SSRP1 was involved in the transcriptional regulation of *egr1* expression (Fig. A.3), as the serine 510 phosphorylated SSRP1 was recruited to the coding region of *egr1* gene after serum stimulation. Therefore, phosphorylation at additional sites in SSRP1 may be necessary to affect the association of this protein with chromatin. Future studies will focus on identification of possible phosphorylation sites that are important for regulating SSRP1 activity during transcription.

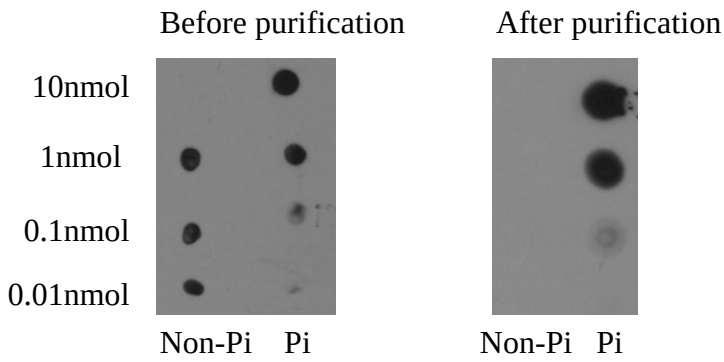
Fig. A.1. The purification of the SSRP1 serine 510 phospho-antibody. (A), The rabbit polyclonal SSRP1 serine 510 phospho-antibody (Pi-S510) was generated by GenScript Corporation using S510 phosphopeptide (SSNEGDSpiDRDEKKRC). The non-phospho antibody was cleared by incubating the serum with CNBr-activated sepharose 4B beads coupled with the non-phosphopeptide. Then the phospho-antibody was affinity purified using CNBr-activated sepharose 4B beads coupled with the phosphopeptide. 30 μl of each elution was subjected to SDS-PAGE and antibody was visualized by coomassie blue staining. **(B)**, The purified SSRP1 Pi-S510 antibody only recoginizes the phosphopeptide, not the non-phosphopeptide. Different concentration of phosphopeptide or non-phosphopeptide was spotted on the nitrocellulose membrane (OSMONICS INC.) and immunoblot with Pi-S510 antibody before or after purification.

Fig. A.1

A.



B.



Non-Pi: SSNEGDSDRDEKKR

Pi: SSNEGDSpiDRDEKKRC

Fig. A.2. The SSRP1 serine 510 phospho-antibody specifically recognizes the phosphorylation signal on SSRP1 S510 residue. (A), Both hyperphosphorylated and hypophosphorylated SSRP1 were found in cells. H1299 cells were lysed in lysis buffer. 50 μg of WCL was centrifuged for 5 mins (13,000 rpm, 4^{o}C). The pellet was washed with lysis buffer, resuspended in 20 μl of 1x SDS loading buffer and boiled at 95^{o}C for 30 min. The supernatant and pellet were analyzed by WB with different antibodies. **(B)**, CIP treatment abolished the phosphorylation signal on serine 510 of SSRP1. 30 μg of H1299 cell lysis was subjected to CIP treatment at 37^{o}C for 1 h and analyzed by WB with different antibodies. **(C)**, S510A mutant ablated the phosphorylation of SSRP1 serine 510 residues. GFP-SSRP1-S510A mutant was generated by site-directed mutagenesis. Equal amount of GFP-SSRP1 wild type and mutant was transiently transfected into H1299 cells. 30 μg of cell lysis was analyzed by WB with different antibodies.

Fig. A.2

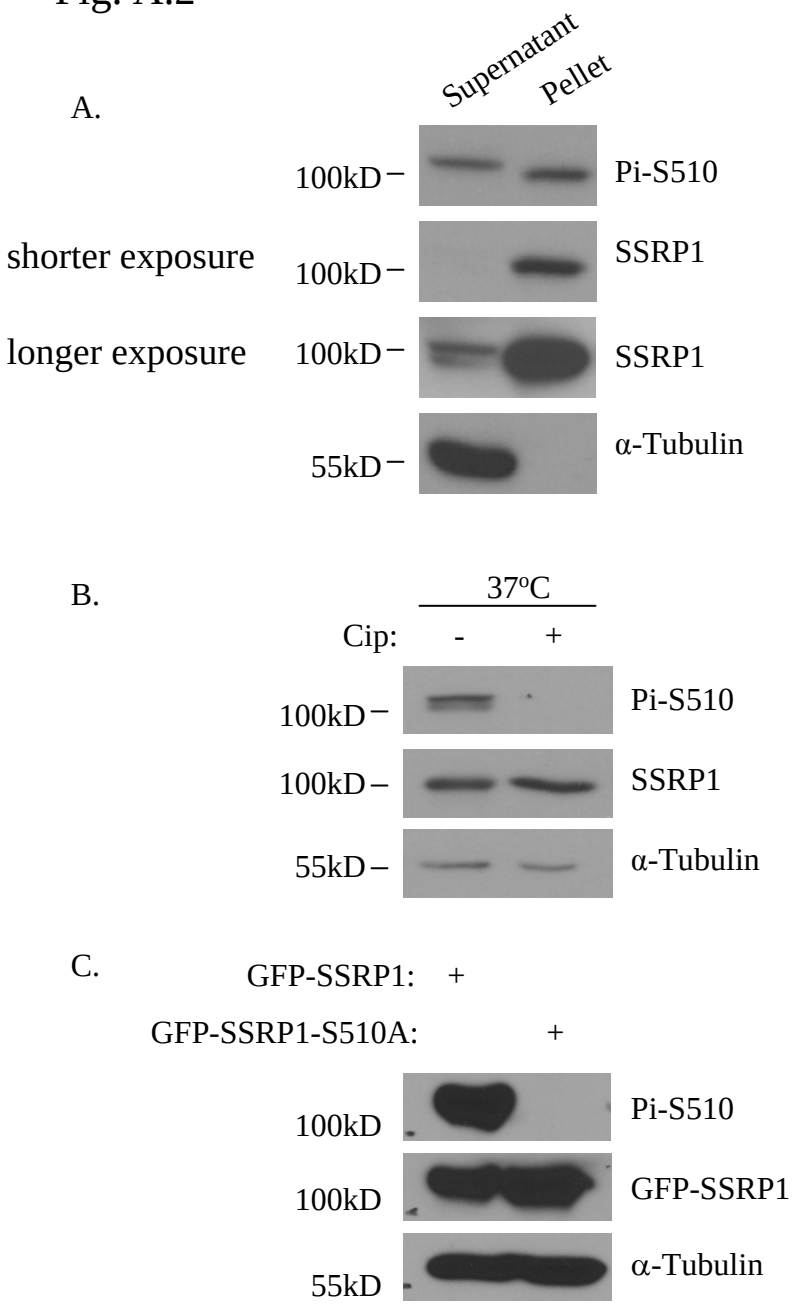
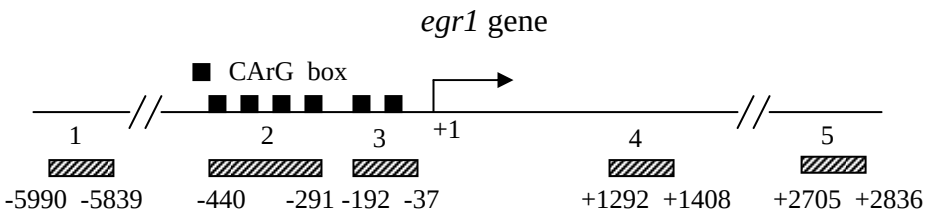
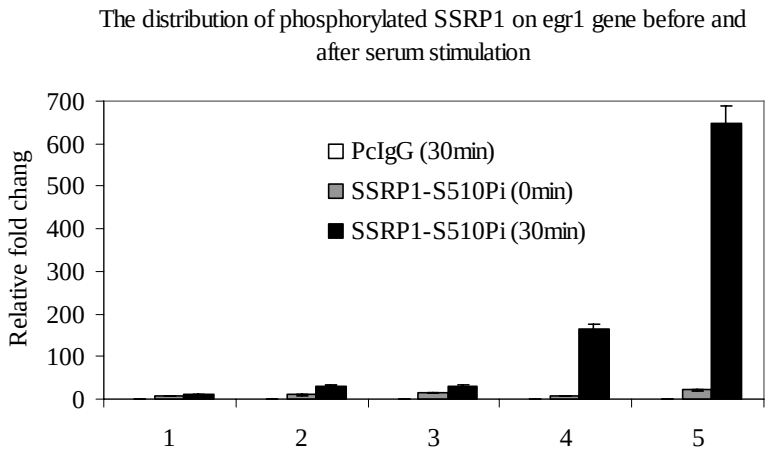

A.
Supernatant
Pellet
100kD
Pi-S510
shorter exposure
100kD
SSRP1
longer exposure
100kD
SSRP1
55kD
α-Tubulin
B.
37°C
Cip:
-
+
100kD
Pi-S510
100kD
SSRP1
55kD
α-Tubulin
C.
GFP-SSRP1:
+
GFP-SSRP1-S510A:
+
100kD
Pi-S510
100kD
GFP-SSRP1
55kD
α-Tubulin

Fig. A.3. serine 510 phosphorylated SSRP1 is recruited to the coding region of *egr1* gene after serum stimulation. H1299 cells were cultured in DMEM containing 0.25% FBS for serum starvation. After 48 h, cells were switched to media containing 20% FBS and harvested at 0, 5, and 30 min post-serum stimulation. ChIP assays were carried out as described previously (Zeng, Dai et al. 2002; Gomes, Bjerke et al. 2006) with the purified SSRP1 Pi-S510 antibody antibody. After reverse of cross-linking, DNA was purified by miniprep kit (Qiagen) and eluted in 50 µl of elution buffer. 1 µl of ChIP DNA or input DNA was used as templates in real time PCRs. A 20 µl reaction was performed using the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol, and amplified on the ABI7300. Threshold cycles (*Ct*) for three replicate reactions were determined using the 7300 system SDS software. The relative fold change among the ChIP DNA samples was calculated following normalization with the input DNA. The same primers which were used in Fig. 2.6 were used here.

Fig. A.3


egr1 gene
CArG box
+1
1
2
3
4
5
-5990 -5839
-440 -291 -192 -37
+1292 +1408
+2705 +2836


The distribution of phosphorylated SSRP1 on egr1 gene before and after serum stimulation
Relative fold chang
700
600
500
400
300
200
100
0
PcIgG (30min)
SSRP1-S510Pi (0min)
SSRP1-S510Pi (30min)
1
2
3
4
5