

# UC San Diego

## UC San Diego Electronic Theses and Dissertations

### Title

Stress-induced non-coding RNAs allosterically regulate TLS, a HAT-inhibitory RNA binding protein, to mediate transcriptional repression

### Permalink

<https://escholarship.org/uc/item/2249443g>

### Author

Wang, Xiangting

### Publication Date

2007

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Stress-Induced Non-coding RNAs Allosterically  
Regulate TLS, A HAT-inhibitory RNA Binding Protein,  
to Mediate Transcriptional Repression**

A dissertation submitted in partial satisfaction of the  
requirements for the degree Doctor of Philosophy

in

Molecular Pathology

by

Xiangting Wang

Committee in charge:

Professor Michael G. Rosenfeld, Chair  
Professor Steven F. Dowdy  
Professor Xiangdong Fu  
Professor Christopher K. Glass  
Professor Yang Xu

2007

Copyright  
Xiangting Wang, 2007  
All rights reserved.

The dissertation of Xiangting Wang is approved, and it is acceptable in quality and form for publication on microfilm:

---

---

---

---

---

Chair

University of California, San Diego

2007

# TABLE OF CONTENTS

SIGNATURE PAGE .....	iii
TABLE OF CONTENTS.....	iv
LIST OF ABBREVIATIONS.....	vii
LIST OF FIGURES.....	x
LIST OF TABLES.....	xi
ACKNOWLEDGEMENTS.....	xii
VITA.....	xv
ABSTRACT.....	xvi
CHAPTER 1. INTRODUCTION .....	1
1.1 TLS IS A MULTIFUNCTIONAL RNA/DNA BINDING PROTEIN.....	1
1.1.1 TLS fusions are frequently found in human cancers.....	1
1.1.2 TLS functions in transcription.....	3
1.1.3 TLS functions in RNA process.....	4
1.1.4 TLS promotes DNA damage/repair.....	6
1.2 CBP AND P300.....	7
1.2.1 CBP and p300 are essential coactivators for sequence-specific transcription factors.....	7
1.2.2 CBP/p300 and human disease.....	10
1.2.3 CBP and p300 are E4 ubiquitin ligases.....	10
1.3 NON-CODING RNA AND GENE REGULATION.....	11
1.3.1 NcRNAs in monoallelic gene expression.....	12
1.3.2 <i>SRA</i> is a transcriptional coactivator for nuclear receptor.....	12
1.3.3 <i>Eyf-1</i> and <i>Eyf-2</i> ncRNAs and <i>Dlx5/6</i> .....	13
1.3.4 <i>Bxd</i> ncRNAs and <i>Ubx</i> .....	14
1.3.5 <i>DHFR</i> ncRNA and DHFR.....	15

<b>CHAPTER 2. MATERIALS AND METHODS .....</b>	<b>19</b>
2.1 GST FUSION PROTEIN EXPRESSION.....	19
2.2 GST PULL-DOWN ASSAY .....	20
2.3 CELL CULTURE .....	20
2.4 siRNAs AND RNA OLIGONUCLEOTIDES TRANSFECTION .....	20
2.5 <i>IN VIVO</i> UBIQUITINATION ASSAY .....	22
2.6 VERY LOW-COPY PLASMID PURIFICATION USING QIAGEN-TIP 100 .....	23
2.7 HAT ASSAYS.....	24
2.8 BIOCHEMISTRY PURIFICATION .....	25
2.9 PROTEIN IDENTIFICATION.....	25
2.10 NUCLEOSOME PREPARATION .....	26
2.11 RNASE TREATMENT .....	27
2.12 DEPHOSPHORYLATING PROTEINS WITH CIP .....	27
2.13 RNA BINDING ASSAY .....	27
2.14 RAPID AMPLIFICATION OF CDNA ENDS (RACE) .....	27
2.15 LABELING THE LIVING CELLS WITH BIOTIN.....	28
2.16 SINGLE CELL NUCLEAR MICROINJECTION ASSAY .....	28
2.17 REPORTER ASSAY.....	28
2.18 CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY .....	28
2.19 RNA IMMUNOPRECIPITATION (RIP) ASSAY .....	31
2.20 SMALL RNA NORTHERN BLOT.....	32
2.21 COMPLEX PURIFICATION.....	32
<b>CHAPTER 3. RESULTS .....</b>	<b>35</b>
3.1 TLS SPECIFICALLY INHIBITS CBP/p300 HAT-DEPENDENT TRANSCRIPTION.....	35
3.1.1 TLS was identified as a component of flag-CBP complex in HeLa nuclear extracts.....	35
3.1.2 TLS specifically inhibited CBP/p300 HAT activities in a substrate specific manner.....	36

3.1.3	Mapping of CBP/p300 interacting domain on TLS and TLS interacting domain on CBP. ....	37
3.1.4	TLS is a co-repressor of CREB-mediated transcription. ....	38
3.1.4.1	TLS represses the forskolin induced CREB activation of a CRE-dependent promoter.....	38
3.1.4.2	TLS represses endogenous CREB target gene, <i>CCND1</i> .....	39
3.1.5	Two TLS homologues-EWS and TAF15 selectively binds with CBP/p300 and inhibits the HAT activity of CBP/p300 as well. ....	40
3.2	NCRNAS MEDIATE THE INHIBITORY EFFECT OF TLS. ....	41
3.2.1	TLS specifically bound to and functionally required GGUG containing RNA. ....	41
3.2.2	New identified ncRNA generated from <i>CCND1</i> 5' regulatory region recruited TLS to <i>CCND1</i> promoter. ....	43
3.2.2.1	Identification of ncRNA from <i>CCND1</i> 5' regulatory region.....	43
3.2.2.2	TLS functionally required <i>CCND1</i> ncRNA. ....	44
<b>CHAPTER 4. DISCUSSION</b> .....		<b>59</b>
<b>REFERENCE</b> .....		<b>69</b>

# LIST OF ABBREVIATIONS

**AFH:** Angiomatoid Fibrous Histiocytoma

**AML:** Acute Myeloid leukemia

**ATM:** Ataxia Telangiectasia Mutated

**ATF1:** Activating Transcription Factor 1

**CBP:** CREB Binding Protein

**CHOP:** C/EBP Homologous Protein

**Cisplatin:** *cis*-diamminedichloroplatinum

**DSBs:** (DNA) Double Strand Breaks

**Dlx:** Distal-less homeobox

**E1A:** Enhancer 1 Adenovirus

**ER:** Estrogen Receptor

**ERG:** ETS-Related Gene

**Evf-1/2:** Embryonic ventral forebrain-1/2

**EWS:** Ewing Sarcomas

**GR:** Glucocorticoid Receptor



**H19:** H19, imprinted maternally expressed untranslated mRNA

**hnRNP:** heterogeneous nuclear Ribonucleoprotein

**HR repair:** homologous recombination repair

**IPW:** Imprinted in Prader-Willi syndrome

**LGFS:** Low-Grade FibroMyxoid Sarcoma

**miRNA:** microRNA

**MLL:** Mixed Lineage Leukemia

**NHEJ:** Non-Homologous End Joining

**p300:** E1A binding protein p300

**PML:** ProMyelocytic Leukemia

**RA:** Retinoic Acid

**RAR:** Retinoic Acid Receptor

**RARE:** Retinoic Acid Response Element

**RGG:** Arginine-Glycine-Glycine

**roX:** RNA on the X

**RRM:** RNA Recognition Motif

**RSK:** Ribosomal S6 kinase

**RXR:** Retinoid X Receptor

**SARFH:** Stands for sarcoma-Associated RNA-binding Fly Homolog

**Shh:** Sonic hedgehog

**siRNA:** small interfering RNA

**snRNP:** small nuclear RiboNucleoProtein

**TAF:** TBP Associated Factor

**TASR:** TLS associated serine-arginine protein

**TBP:** TATA-binding protein

**TLS:** Translocated in LipoSarcomas

**TR:** Thyroid Receptor

**ZnF:** Zinc Finger

**Xist:** X inactive specific transcript

## LIST OF FIGURES

Figure 1. Diagrams of TLS and CBP.....	17
Figure 2. Identification of TLS as a specific CBP/p300 HAT inhibitor. ....	46
Figure 3. TLS binds to the p160-interacting domain of CBP. ....	47
Figure 4. N-terminus of TLS interacts with and inhibits CBP HAT. ....	48
Figure 5. TLS negatively regulates CREB target <i>CCND1</i> through modulating CBP HAT activity on histone acetylation.....	49
Figure 6. TLS is recruited to a subset of CREB target genes.....	50
Figure 7. TLS is recruited to the <i>CCND1</i> promoter when <i>CCND1</i> is reduced upon IR...	51
Figure 8. EWS and TAFII68 interact with and inhibit CBP/p300 HAT activity.....	52
Figure 9. Both TLS and EWS negatively regulate <i>CCND1</i> . ....	53
Figure 10. Specific GGUG-containing RNA promotes the inhibitory effect of TLS on CBP/p300 HAT activity and <i>CCND1</i> transcription.....	54
Figure 11. GGUG-containing RNA oligonucleotides specifically interact with TLS and inhibit <i>CCND1</i> promoter activity. ....	55
Figure 12. NcRNAs generated from the 5' regulatory region of human <i>CCND1</i> gene (ncRNA <sub><i>CCND1</i></sub> ) are induced upon IR.....	56
Figure 13. NcRNA <sub><i>CCND1</i></sub> negatively regulates <i>CCND1</i> transcription through TLS.....	57
Figure 14. TLS is required for genotoxic stress activated NFκB-mediated genes.....	67
Figure 15. The model.....	68

## LIST OF TABLES

Table 1. Function of ncRNA.....	18
Table 2. Amount of Lipofectamin2000 and tested RNA for transfection.....	21
Table 3. Preparation of 1M Sodium Phosphate Buffer at room temperature.....	23
Table 4. Binding Characteristics of Immunoglobulins to ProteinA/G-sepharose beads...	30

## ACKNOWLEDGEMENTS

Many people have contributed to the work with this dissertation. Without their help, support and encouragement, I would never have been able to finish this work.

First of all, I would like to gratefully and sincerely thank my advisor Prof. Michael Geoff Rosenfeld, for guiding me towards a deeper understanding of knowledge work, and for his invaluable comments and always being supportive to my research work. For everything you have done for me, Geoff, I thank you.

I would like to thank my committee members, Prof. Steven F. Dowdy, Prof. Xiangdong Fu, Prof. Christopher K. Glass, and Prof. Yang Xu, for their valuable input and accessibility. In particular, I would like to give a special thanks to Prof. Christopher K. Glass for his numerous critical helps to this work.

I am very grateful to my collaborators, Prof. Riki Kurokawa and his group, Prof. David W. Rose, Prof. Paul Tempst, Ms. Donna Reichart, Dr. Gabriel Pascual, and Dr. Xiaoyuan Song. Thank you for the wonderful work and valuable feedbacks.

I would also like to express my thankfulness to all of the members of the Rosenfeld lab, especially Bong-Gun, Kasey, Chi-Jen, Feng, Ivan, Michelle, Ping, Victoria and Wenlai for their valuable discussions; Chuck, Kenny and Jane for their technique support.

Additionally, I am very grateful to Prof. Amy Pasquinelli for her insightful comments and constructive criticisms on the manuscript and technique suggestions. Thanks to Janet for figure preparation. Thanks to Chris Benner for the useful discussion on bioinformatics analysis. Thanks to Joe Aguiler for ionizing irradiation.

Finally, I would like to thank my husband, Xiaoliang Bai, for supporting me with infinite love, through success and setback, joy and sorrow. I thank my parents, Jitang Wang and Baozhu Wang, and my sister Meiting Wang, for their faith in me and allowing me to be as ambitious as I wanted. With love, we have the world.

The Chapter 2, 3, and 4 with this dissertation, in full or in part, are based on material that has been submitted for publication to Nature, as “Stress-induced Non-coding RNAs Allosterically Regulate a HAT-inhibitory RNA Binding Protein to Mediate Transcriptional Repression. X.T. Wang, S. Arai, D. Reichart, T. Oyoshi, X.Y. Song, G. Pascual, D.W. Rose, P. Tempst, M.G. Rosenfeld, C.K. Glass and R. Kurokawa.” I was the primary researcher and author of the work, and the co-authors listed in the work collaborated on, or supervised the research which forms the basis for the work with this dissertation.

# VITA

## EDUCATION

- 1997 M.D., Hebei Medical University, Hebei, P. R. China
- 2000 Master, Peking University, Beijing, P. R. China
- 2007 Ph.D., Department of Molecular Pathology, University of California at San Diego, La Jolla, CA, USA



ABSTRACT OF THE DISSERTATION

**Stress-Induced Non-coding RNAs Allosterically  
Regulate TLS, A HAT-inhibitory RNA Binding Protein,  
to Mediate Transcriptional Repression**

by

Xiangting Wang

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2007

Professor Michael G. Rosenfeld, Chair

A large number of coactivators and corepressors are required for regulating programs of gene expression in a transcription factor- and gene-specific manner, suggesting that they serve as both sensors and integrators of signaling information to coordinate complex homeostatic responses[1, 2]. A central emerging goal in gene regulation is to fully understand the roles of previously unrecognized non-coding RNAs in regulated gene transcription programs. Here, we report that the RNA-binding protein, TLS, serves as a key transcriptional regulatory sensor of DNA damage signals by specifically binding to and allosterically inhibiting CBP/p300 HAT activities on repressed gene targets.

Recruitment of TLS to the *CCND1* promoter and gene-specific repression are directed by non-coding RNA transcripts from the 5' regulatory regions of *CCND1*, acting as molecular beacons that are themselves induced in response to DNA damage signals. These data suggest that non-coding RNAs induced in regulatory regions of transcription units may act as selective ligands, recruiting and modulating the activities of distinct classes of RNA binding co-regulators in response to specific signaling programs, providing an unexpected RNA-based strategy to integrate transcriptional programs.

# **Chapter 1. INTRODUCTION**

## **1.1 TLS is a multifunctional RNA/DNA binding protein.**

TLS (translocated in liposarcoma), also called FUS (fusion), has been suggested as a multifunctional RNA/DNA-binding protein involved in transcription[3-6], RNA processing[7-13], and DNA damage/repair[14-17].

The human TLS gene consists of 15 exons located within 11kb of genomic DNA[18]. The TLS protein has an N-terminal SYQQQS-rich region[19] and C-terminal RNA binding domain. The RNA binding domain of TLS consists of one RNA recognition motif (RRM), three RGG rich regions, and a RanBP2-type Cys2/Cys2 ZnF motif (Fig. 1).

### **1.1.1 TLS fusions are frequently found in human cancers.**

Chromosomal translocation is the most conspicuous reason to raise cancer cells. TLS was first identified and is frequently found as part of the fusion protein in human cancer cells. For example, TLS fuses to CHOP in human liposarcoma[20-22], to ETS-like protein ERG as a result of the translocation t(16;21)(p11;q22) in AML and Ewing's sarcoma[23, 24], to ATF-1 in AFH, and to CREB3L2/BBF2H7 in LGFMS. The common feature of TLS fusions is that the promoter and 5' coding region of TLS link to the transcription factors.

TLS-CHOP fusion protein was the first to be identified as a result of the translocation t(12;16)(q13;p11), the most common translocation in liposarcoma. CHOP is a transcription repressor[25-29]. Its expression is strictly regulated and usually repressed under normal conditions[30-33]. In contrast, TLS promoter is constitutively activated. In TLS-CHOP fusion protein, multiple RNA binding motifs in TLS C-terminus are replaced by the DNA-binding and basic leucine zipper domain of CHOP[20]. Therefore, CHOP gene is under the control of TLS promoter and constitutively expressed in TLS-CHOP fusion. CHOP regulated genes are misexpressed as a result, which can be pro-oncogenic. TLS N-terminus binds to RNA polymerase II, and possesses transformation activity[19, 34]. Injection of CHOP-carrying cells into nude mice produces only small tumors with longer latency than that of TLS-CHOP-carrying cells, suggesting that the presence of TLS N-terminal sequence in the fusion protein is necessary for full oncogenic potential[35-37].

EWS and TAFII68/TAF15[38, 39] are two homologues to TLS. The three genes are called TET (TLS, ESW, TAFII68) family[6, 34]. In addition to RNA binding motifs at their C-termini, mammalian TLS, EWS and TAFII68 share glutamine rich N-termini consisting of a series of degenerate repeats[19]. EWS-transcriptional factor fusion proteins are found in human cancers[34, 40-42] and share the common feature as that of TLS fusions, such as EWS-FLI1 and EWS-ERG in Ewing's sarcoma, EWS-CHOP in myxoid liposarcoma, EWS-ATF-1 in malignant melanoma of soft parts/soft tissue clear cell sarcoma. EWS-FLI1 fusion protein shows no apparent *in vitro* DNA binding activity and retained some transformation potency[41], suggesting that EWS or TLS fusions promotes oncogenesis more than simply changing the affinity to their specific target genes.

### 1.1.2 TLS functions in transcription.

SARFH/Cabezza, a *Drosophila* homologue of TLS, is colocalized with the active chromatin revealed by phosphorylated RNA polymerase II[5]. TLS has been shown to associate with a subpopulation of the TFIID complex in cells. Inhibition of RNA polymerase II results in subcellular translocation of TLS from the nucleus to the cytoplasm[37]. EWS and TAFII68 have been shown to be complexed with both the TFIID and RNA polymerase II fractions in nuclear cell extracts[6, 39]. The association with RNA polymerase II appears to involve the N-terminus of EWS. It is hypothesized that TET family proteins act as adapters between transcription and mRNA processing by interacting with components of the transcription apparatus and splicing factors[6]. However, it makes TET family proteins as non-classical transcription factors, as they contain RNA binding motifs, and bind to both RNA and DNA[6].

TLS directly interacts with p65 of NF $\kappa$ B in a TNF $\alpha$  dependent manner[4, 43]. Such interaction is thought to be mediated through the RNA binding motifs located at TLS C-terminus, though weak interaction by TLS N-terminus was observed as well. NF $\kappa$ B-mediated transactivation is enhanced on model reporters by TLS under treatments of TNF $\alpha$ , IL1 $\beta$  and overexpression of NF $\kappa$ B inducing kinase NIK. These data suggest that TLS is a coactivator of NF $\kappa$ B and plays a pivotal role in the NF $\kappa$ B-mediated transactivation.

TLS also interacts with YB-1[7], a transcriptional regulator in response to DNA damage. Such interaction augments a further 2-fold activation of YB-1-mediated transcription activation on MMP-1.

TLS was coimmunoprecipitated with  $\beta$ -catenin in human colorectal cancer cell line DLD1[44]. In DLD1,  $\beta$ -catenin is accumulated in the nucleus and cell membrane. TLS was found exclusively in the nucleus and colocalized with nuclear  $\beta$ -catenin. Modulating TLS level regulates  $\beta$ -catenin-mediated gene transactivation on TCF/LEF luciferase in human colorectal cancer cells in a way of repression. Moreover, overexpression of TLS markedly suppressed the colony formation in human colorectal cancer cell HCT116 cells.

TLS also interacts with steroid, thyroid hormone and retinoid receptors[45]. The physiologic significance of these wide ranging associations is unexplored.

### **1.1.3 TLS functions in RNA process.**

TLS binds to RNA both *in vitro* and *in vivo*[9]. Multiple domains, including RRM, ZnF and RGG domains of TLS, are required for RNA binding. By using a synthesised RNA oligonucleotide pool, TLS is found mainly binding to GGUG-containing oligoribonucleotides *in vitro*[46]. The GGUG-containing RNAs have higher affinity than other RNAs for the ZnF of TLS[47]. In contrast, screening of TLS-binding RNAs in adult mouse brain reveals that TLS associates with mRNA without GGUG-motif as well, among them an actin-stabilizing protein Nd1-L[48]. Though some other TLS-binding RNAs have been reported, the overall picture of TLS-binding RNAs is not dissolved.

The role of TLS in splicing is suggested by identification of TLS as hnRNP P2[49], and by its interacting proteins. TLS interacts with hnRNP A1, hnRNP C1/C2 in RNA maturation, and many RNA splicing (co)factors such as SF1, SC35, TASR1, TASR2, PTB (hnRNP I), and SRm160[10-12]. *In vitro* splicing experiments show that

immunodepletion of TLS results in an inhibition of the splicing reaction of  $\beta$ -globin pre-mRNA, which can be partially restored by giving recombinant TLS[8]. In the case of a plant rubisco activase pre-mRNA, depletion of TLS results in not only an inhibition of regular splicing, but also an appearance of alternative splicing. These data suggest a role of TLS in regular and alternative splicing events in opposite directions.

Since the C-terminal domain of TLS is the domain missing in TLS fusion proteins and required for interaction with splicing factors such as SC35, TASR1 and TASR2[10], a dysfunction of TLS in splicing profile could be oncogenic.

Evidences suggest that TLS participates in nucleo-cytoplasmic shuttling and RNA translocation as well. TLS is specifically localized in the mature neuronal dendrites and recruited in the spines upon mGluR5 activation[13]. In adult mouse brain, TLS binds to Nd1-L, an actin-stabilizer[48]. Nd1-L mRNA level is reduced and Nd1-L mRNA fails to translocate to the corresponding spines in TLS-null neurons. Considering the critical role of actin cytoskeleton in spine architecture maintenance, such observations may explain the abnormal spine morphology in TLS-null neurons.

ZFM1 is a transcriptional repressor and identical to presplicing factor SF1[50, 51]. ZFM1 is activated in the process of p53-induced apoptosis[52]. ZFM1 has been shown to bind to TET family proteins and repress the transcriptional activity of EWS[53]. It has been shown that EWS-FLI1 fusion protein binds U1C, a member of the small nuclear ribonucleoprotein family and that such interaction inhibits EWS-FLI1 transactivation on model reporter[54].

The N-terminal regions of TLS and EWS are distinct from those of other RRM-carrying proteins, suggesting that TET family may be a new family of RNA-binding proteins.

#### **1.1.4 TLS promotes DNA damage/repair.**

DNA damage, due to environment or normal metabolic processes inside the cells occurs every day and needs to be repaired[55]. Double-strand breaks (DSBs) are particularly hazardous because they can lead to genome rearrangements[56]. Two mechanisms exist to repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR) repair. NHEJ is especially important before the cell has replicated its DNA and also required for V(D)J recombination, the process that generates diversity in B-cell and T-cell receptors in the vertebrate immune system. The faithful HR repair use a sister chromatid or a homologous chromosome as template. Unrepaired DNA lesions can be oncogenic or induces apoptosis.

Surprising phenotypes of TLS knocked out were reported by two independent groups. Inbred TLS  $-/-$  mice have high neonatal death rate, and are defected on lymphocyte development, B-cell activation and spermatogenesis[15]. Outbred TLS null mice live into adulthood but exhibit enhanced radiation sensitivity and male sterility due to chromosome pairing defects during meiosis in addition to the genomic instability[14]. These suggest pathways related to DNA repair are defect in TLS knock outs. Indeed, TLS was found identical to POMp75[16, 17], a protein promotes  $Mg^{2+}$  dependent/ATP-independent DNA pairing in HR repair. TLS binds to both ssDNA and dsDNA, with a 2-fold higher affinity for ssDNA. TLS-CHOP fusion protein does not promote DNA



pairing, suggesting a function of TLS C-terminal RNA binding motifs in DNA damage/repair. Moreover, some Phenotypes of TLS knock outs are reminiscent of studies involving *Atm*<sup>-/-</sup>[57], *Abl*<sup>-/-</sup>[58, 59], or *H2ax*<sup>-/-</sup>[60] mice, suggesting that TLS may act in the *Atm/Abl/γH2ax* signal transduction pathway during DNA damage/repair. So far, the knowledge is limited to that binding activity of TLS to DNA requires a functional BCR/ABL tyrosine kinase necessary to induce PKCβII[61, 62].

It is still far beyond clear how TLS functions in transcription, splicing and damage repair, though the TLS has been extensively investigated in the clinical research. It will be very helpful to understand the development of human liposarcomas and leukemias if the mechanisms of TLS involved can be clarified.

## **1.2 CBP and p300.**

CBP and p300 are transcriptional coactivators with intrinsic histone acetyltransferase (HAT) activities[1, 2, 63-67]. CBP and p300 HAT activities are essential for normal development and homeostasis[68-70]. Recently, CBP and p300 are found as new ubiquitin ligases mediating polyubiquitination for protein degradation[71-73]. A diagram of CBP protein structure is illustrated in Fig. 1[74].

### **1.2.1 CBP and p300 are essential coactivators for sequence-specific transcription factors.**

CBP and p300 play essential roles as coactivators of multiple classes of signal-dependent transcription factors by mediating protein-protein interactions and by acetylating histones and other protein substrates[75-77]. Previous studies demonstrated

that the histone acetyltransferase (HAT) activity of CBP/p300 is required for transcriptional activity of CREB on model promoters, but not for transcriptional activity of retinoic acid receptor (RAR), suggesting that modulation of CBP/p300 HAT function might provide a strategy for differential control of CBP/p300 HAT-dependent and CBP/p300 HAT-independent transcription units[66].

CBP and p300 interact with the kinase-inducible domain (KID) of CREB through signal-induced phosphorylation of CREB at Ser-133[78]. Interaction between CBP/p300 and CREB enhances transcription elongation and reinitiation of CREB-mediated transcription. Biochemical studies suggest that CBP serves as a coactivator of CREB in part by recruitment of RNA polymerase II (pol II) complexes through the C/H3 region of CBP. Disassociation of CBP:CREB complex by phosphorylation of CREB is also reported. In this case, the phosphorylation of CREB at Ser-121 instead is mediated by ATM upon DNA damage[79]. Forskolin is a strong inducer of CREB-mediated transcription by provoking phosphorylation of CREB and CBP:CREB interaction. T cell receptor-mediated PKC activation provokes phosphorylation of CREB without inducing the CBP:CREB interaction in nucleus, suggesting mechanisms other than CREB phosphorylation regulate CBP:CREB interaction. Indeed, the transcription factor C/EBP- $\beta$  may attract CBP:CREB interaction in a serine-133-independent manner, to create a stronger CREB:C/EBP- $\beta$ :CBP transcription complex.

The protein-protein interaction is mediated by distinct structural domains of CBP/p300, such as KIX, C/H1, C/H3, and bromodomain domains. Such interactions display partial specificity, having both distinct and overlapping binding partner profiles. It

is suggested that the recruitment of CBP/p300 to the steroid receptors is achieved *via* the p160 proteins. The p160-interacting domain of CBP is located within amino acids 2058-2163[77].

CBP/p300 HAT activities can be modulated by their associating factors, most of which are viral proteins. E1A enhances histone acetylation *in vivo*, whereas it inhibits HAT activity of CBP and P/CAF in biochemical assays at high levels[74, 80]. During the preparation of this work, HOX proteins have been shown to bind to CBP or p300. Surprisingly, interaction between CBP/p300 and HOX blocks the DNA binding activity of HOX proteins and inhibits the HAT activity of CBP/p300[81]. In quiescent cells CBP forms an enzymatic inactive complex with unphosphorylated Rsk-2[82].

CBP and p300 acetylate non-histone proteins as well, such as p53. Activation of p53-mediated transcription is a critical cellular response to DNA damage. Activation of p53 is modulated by post-translational modifications including acetylation. CBP/p300 acetylates p53 at multiple lysine residues in the carboxy-terminal region. In contrast, another HAT protein, the p300/CBP associated factor (P/CAF) acetylates p53 in the nuclear localization signal. Acetylation prevents p53 from degradation and enhances sequence-specific DNA binding of p53. In addition, acetylation of p53 is important for efficient recruitment of CBP and P/CAF complexes to promoter regions and for activation of p53-targeted gene expression.

### **1.2.2 CBP/p300 and human disease.**

Mutations in CBP and p300, together with a microdeletion at chromosome 16p13.3, occur in ~55% of Rubenstein-Taybi syndrome (RTS) patients. Abolishing the CBP HAT activity by a point mutation is sufficient to cause RTS in mouse model[68]. This data suggests that haploinsufficiency of CBP is the basis for RTS. Expansion of a polyglutamine repeat in the huntingtin protein enables it to inhibit the HAT activities of CBP, p300 and p/CAF[83, 84]. Histone deacetylase inhibitors inhibit neurodegeneration induced by *Drosophila* huntingtin protein, suggesting that disruption of the balance between HAT and HDAC activity contributes to the pathogenesis of Huntington's disease[84]. Translocations of CBP-MOZ and MLL-CBP have been linked to AML[69]. Fusion of both the bromodomain and HAT domain of CBP to MLL is required for full transformation *in vitro* and is sufficient to induce the leukemic phenotype *in vivo*[69].

### **1.2.3 CBP and p300 are E4 ubiquitin ligases.**

Recently, CBP and p300 were found to exhibit intrinsic ubiquitin ligase activity as well[71]. CBP and p300 have no clear homology to known E3 ubiquitin ligase motifs, such as RING, PHD, HECT, and U-box domains. p300 provokes polyubiquitination of p53 dependent on the monoubiquitination of p53 mediated by the ring finger E3 ligase MDM2/HDM2. Therefore, CBP and p300 are classified as new E4 ubiquitin ligases. TIP60 (Tat-interacting protein, 60 kDa) possesses intrinsic HAT activity and is required for DNA repair[85]. Tat provokes CBP/p300-mediated polyubiquitination on TIP60, thus resulting in a dramatic impairment of the TIP60-dependent apoptosis upon DNA

damage[72]. More recently, P/CAF was identified as an ubiquitin ligase for HDM2[73]. In each case, the ubiquitin activity is HAT-independent.

### **1.3 Non-coding RNA and gene regulation.**

Non-coding RNA (ncRNA) is a class of transcripts whose end products are RNA rather than proteins[86-96]. The significance of ncRNAs is suggested from the results of analyses of the protein-encoding capacity of sequenced genomes, which indicates that ncRNAs make up a larger population of transcription output in higher eukaryotes[97-103]. In fact, recent studies of the transcriptome suggest that at least half of the human genome is transcribed. A majority of these previously unrecognized transcripts are putative ncRNAs.

NcRNAs are key regulators in many cellular processes (Table 1). Transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) are well-known ncRNAs for their function in making proteins[104]. They are abundant and have no variety in size. In contrast, the non-rRNA/non-tRNA ncRNAs are non-abundant and complex. Small nuclear RNAs (snRNAs) have roles in mRNA splicing through interaction with RNP complexes[105, 106]. Small nucleolar RNAs (snoRNAs) are ~ 70-250 nt and use base complementarity to guide catalytic modification on rRNA[107-109].

Other ncRNAs have been involved in monoallelic gene expression[110-114], nuclear receptor regulation, and recently gene transcription[115-120].

### **1.3.1 NcRNAs in monoallelic gene expression.**

NcRNAs are crucial in monoallelic gene expression. The large human Xist (16.5 kb) is the best characterized ncRNA in X-inactivation. Xist itself is under the control of an antisense ncRNA, Tsix. Though the mechanism by which Xist and Tsix accomplish silencing remains unclear, they are clearly capable of establishing epigenetic marks on the X chromosome, such as histone acetylation/methylation and DNA methylation. There are ~70 imprinted genes in mammals. Most imprinted clusters have ncRNAs, which seem to be especially rich in cis-antisense ncRNAs and usually large, such as *Igf2r/Air*. Inappropriate expressions of ncRNAs have been linked to human diseases[121]. Mutations in *SCA8*, a cis-antisense RNA in human, are found in spinocerebellar ataxia patients[122]. Other human disease associated antisense ncRNAs include *DGCR5* in DiGeorge syndrome[123], *Kvlqtas/Lit1* in Beckwith-Wiedeman syndrome[124], *CMPD* associated RNA in Campomelic dysplasia.

### **1.3.2 SRA is a transcriptional coactivator for nuclear receptor.**

SRA, steroid receptor RNA activator, is the only known transcriptional coactivator for nuclear receptor as a RNA[125-128]. Though evidence shows that an alternative splice variant of SRA encodes protein[129, 130], it is conclusively clear that SRA can function as a RNA. SRA plays an important role in mediating estrogen receptor action, and its expression level is aberrant in human breast tumors[131]. Recently, protein partners of SRA have been found, including p72, SHARP (SMRT/HDAC1 associated repressor protein)[132], and SLIRP (SRA stem-loop interacting RNA binding protein)[133]. SHARP has three RNA binding motifs – RRM, and SLIRP is composed almost entirely

of a RRM region. Intact RRM domains are required for their association with SRA, and for their function as SRA corepressors. Recruitment of SLIRP to the target promoter is SRA dependent.

### **1.3.3 *Evf-1* and *Evf-2* ncRNAs and *Dlx5/6*.**

The vertebrate homeodomain protein family *Dlx* genes are important for neuronal differentiation and migration[134-139]. They are expressed in bi-gene clusters and conserved intergenic enhancers were found for the *Dlx-5/6* and *Dlx-1/2* loci[140, 141]. *Evf-1* was isolated from a genetic screen as one out of eight novel *Shh* targets in rat telencephalon at embryonic day 12[142]. *Evf-1* is a 2.7 kb, polyadenylated, spliced ncRNA. Its 3.8 kb alternative splicing form, *Evf-2*, was identified recently[115]. Both are located at the ultraconserved sequences region of *Dlx-5/6* loci. It has been reported that transcriptional activation of *Dlx5/6* occurs by the direct action of *Dlx2* proteins on this ultraconserved region. Therefore, it is possible that *Dlx2* action is achieved through *Evf-1* and *Evf-2* ncRNAs. Indeed, *Evf-1* and *Evf-2* are developmentally regulated, and their expression overlaps to those of *Dlx2/5/6* genes. During ventral telencephalic differentiation, *Evf-1* is expressed by immature neurons upon migration out of the ventricular zone into the subventricular zone. The ectopic expression of both *Evf-1/2* and *Dlx-2/5/6* were observed in the same region of the brain after injection of a virus based *Shh* expressing vector. *Evf-2* associated with *Dlx-2* protein in rat embryonic brain nuclear extracts by RNA-immunoprecipitation, which was further proved by combination of in situ hybridization and immunostaining. *Evf-2* activates *Dlx-5/6* enhancer activity in a *Dlx-2* dependent mechanism. However, *Evf-2* does not cooperate with *Dlx-2* on a known

Dlx-2 target, Wnt-1[143] enhancer, suggesting that *Evf-2*/Dlx-2 cooperation is target specific. Despite a potentially encoded 19 aa peptide from *Evf-2*, it is strongly supported that *Evf-2* RNA is functional. In addition, the active form of *Evf-2* is the sense strand. These data provide a new mechanism that Shh activates Dlx genes through ncRNAs during mouse development.

#### **1.3.4 *Bxd* ncRNAs and *Ubx*.**

Many spliced long ncRNAs have been found from the intergenic regions of the *Drosophila* Hox genes as well[144-146]. Several ncRNAs are transcribed from the maintenance elements (MEs) in the *bithoraxoid* (*bxd*) regulatory region[147-149]. PcG and trxG are two genes maintaining the state of Hox genes as repression or activation, respectively[150-152]. The acts of PcG and trxG are partially overlapped on MEs[153]. The observation that *bxd* ncRNAs precede activation of Hox gene *Ubx* suggests a positive role of *bxd* ncRNAs on *Ubx*. Genetic studies suggest that transcription of *bxd* ncRNAs interferes with PcG-mediated silencing[154]. Indeed, Sanchez-elsner *et.al.* show that *bxd* ncRNAs recruit trxG proteins to MEs[120]. More recently, Petruk *et.al.*[119] report that *Ubx* and several tested *bxd* ncRNAs are expressed in nonoverlapping patterns in both embryos and imaginal discs. Ectopic expression of the *GFP* reporter gene is observed in the *Ubx* transgene which lacks several promoters driving *bxd* ncRNAs. These data suggest that *bxd* ncRNAs repress the *Ubx* gene instead. Since the *bxd* ncRNAs extend into the region of upstream of *Ubx* initiation site, the *bxd* ncRNAs may mediate transcriptional repression by disrupting protein-DNA interactions required for *Ubx* initiation, or by promoter competition.



### 1.3.5 *DHFR* ncRNA and DHFR.

Human DHFR gene contains two promoters, with 99% of the DHFR transcript generated from the major promoter[155, 156]. The minor promoter is located upstream of the major promoter and terminated within intron 2 of DHFR. It does not encode protein, thus referred as *DHFR* ncRNA[117]. *DHFR* ncRNA is accumulated when the DHFR gene is repressed in quiescent cells. Reporter assays show that *DHFR* ncRNA negatively regulates the DHFR major transcript, which is further supported by knocking-down experiment using specific siRNA against *DHFR* ncRNA. Both *in cis* and *in trans* mechanisms might be involved. Though the repression does not required the entire *DHFR* ncRNA, evidence shows that the sequence corresponding to very 5' of the major promoter is essential. General transcription factors, TBP and TFIIB, are recruited to DHFR major promoter when the gene is activated. Introduction of *DHFR* ncRNA greatly reduces the binding of TBP and TFIIB on the DHFR major promoter. DHFR major promoter is GC-rich and contains several G-track sequences[157-159]. Such sequences have been shown to form stable purine-purine-pyrimidine triplex structure (H form) between DNA and RNA, thus inhibiting the transcription. A stable H form is observed between the DHFR promoter DNA and a synthetic oligonucleotide corresponding to the major promoter region of the *DHFR* ncRNA.

New ncRNAs continue to appear, and a full understanding of ncRNA in gene transcription is needed. In this work, I will provide evidence suggesting that new ncRNAs generated from the 5' regulatory region of *CCND1* gene can negatively regulate *CCND1*. Through base complementarity, *CCND1* ncRNAs recruit the RNA/DNA binding protein

– TLS to the *CCND1* promoter. *CCND1* is an endogenous CREB target gene and its activation requires CBP/p300 HAT activity. TLS is a specific CBP/p300 HAT inhibitor. Therefore, the recruitment of TLS on the *CCND1* promoter by *CCND1* ncRNAs results in transcriptional gene silencing of *CCND1*.

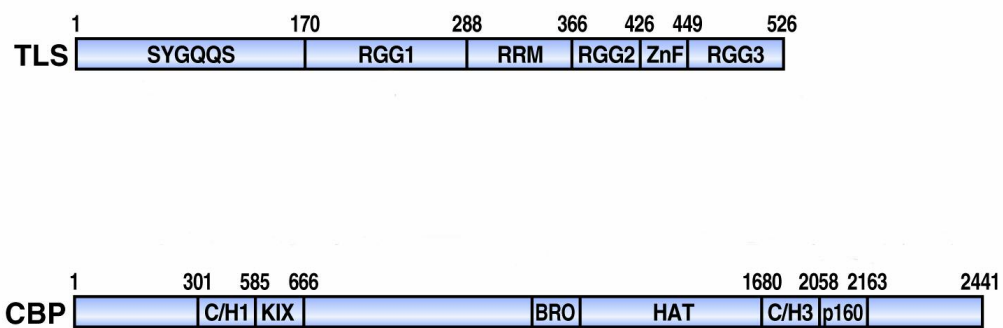


Figure 1. Diagrams of TLS and CBP.

**Figure 1. Diagrams of TLS and CBP.**

Table 1. Function of ncRNA.

Process	Example	Length	Function
Transcription	<i>SRA</i> <i>Evf-1, Evf-2</i> <i>Bxd</i> ncRNA <sup>Δ</sup> <i>DHFR</i> ncRNA	875 bp 2.7kb; 3.8kb	NR Activation <i>Dlx-5/6</i> activation <i>Ubx</i> repression/activation <i>DHFR</i> repression
Heterochromatic silencing	siRNA <sup>ψ</sup>	21-24 bp	
Dosage compensation	<i>Xist</i> <i>IPW</i> <i>Air</i> <i>H19</i> <i>roX</i> <sup>Δ</sup>	16.5kb	X-inactivation Imprinting Imprinting Imprinting Chromatin entry site
RNA modification	snoRNA	70-250 bp	2'-O-ribose methylation and Pseudouridylation of target rRNA
mRNA splicing	snRNA		mRNA splicing
Posttranscriptional gene silencing	miRNA siRNA	21-24 bp	mRNA degradation, translation repression
mRNA translation	rRNA tRNA	70-80 bp	Translation

<sup>Δ</sup> Drosophila ncRNA.  
<sup>ψ</sup> Plant and yeast.

## **Chapter 2. MATERIALS AND METHODS**

### **2.1 GST fusion protein expression**

The plasmid expressing GST or GST fusion protein was transformed into *E. coli* cells. A single colony was inoculated into 5 ml of LB/Ampicilin medium at 37°C for overnight. The next day, the culture was 1:100 diluted into fresh LB/Ampicilin medium to 500 ml and kept growing around 4 hr until the A600 reached to 0.6-0.8. Then a final concentration of 0.1 mM of IPTG was added to induce the protein expression for 2 hr. Cell cultures were centrifuged at 3,000 g, 4°C for 10 min. The pellets were re-suspended in 10 ml of PBS containing proteinase inhibitors (PBS/PI) and aliquoted into 1 ml each for sonication (10 sec, 3 times). The sonicated products were added Triton X-100 at a final concentration of 1%, mixed gently, and rotated at 4°C for 30 min. The cell debris was discarded after centrifugation at 12,000 g, 4°C for 10 min. The supernatant were pooled into a fresh tube and incubated with Glutathoin Sepharose 4B beads (1 ml of 50% slurry of beads for each 500 ml of culture) at 4°C on a rotator for overnight. Collected the beads by centrifugation at 300 g for 5 min, and washed the beads with PBS/PI for 3 times. The proteins were eluted from the beads by incubating with 100 ul of 10 mM reduced glutathione/50 mM Tris-HCl (pH 8.0) buffer on a rotator at room temperature for 30 min. The supernatant was collected and stored at -80°C for future use. An aliquote, together with different amount of BSA, was performed for SDS-PAGE electrophoresis to test the

expression and concentration of the interesting protein. Repeat elution steps 2 to 3 times if needed.

If the purified proteins were subjected to microinjection assay, the normal bacterial lysis buffer with 50 mg/ml of lysozyme instead of detergent was used. The same amount of buffer was added, and the buffer/lysozyme/cells were incubated on ice for 1 hr prior to sonication.

## **2.2 GST pull-down Assay**

GST fusion proteins were produced as described in “**GST fusion protein expression**”. TLS protein was translated *in vitro* using <sup>35</sup>S-labeled methionine and TnT-coupled reticulocyte lysate system (Promega). CBP, p300, TIP60, P/CAF, EWS, and TAF15 were tagged with flag sequence, and synthesized in a baculovirus expression system (Invitrogen).

## **2.3 Cell culture**

RAW 264.7 cells (ATCC) and HeLa (ATCC) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gemini), 100 U/ml penicillin and 100 mg/ml streptomycin.

## **2.4 siRNAs and RNA oligonucleotides Transfection**

All siRNA duplex and RNA oligonucleotides were transfected with Lipofectamine2000 (Invitrogen). Dependent on the cell lines, different amount of Lipofectamine2000 and siRNA duplex or RNA oligonucleotides were used as shown in

Table 2. Briefly, cells were plated the day before transfection. At the day of transfection, Lipofectamine2000 or tested RNA was diluted in Opti-MEM in two separate RNase-free tubes. After 5 min incubation at room temperature, the diluted Lipofectamine2000 was combined with the diluted duplex and further incubated for 20 min at room temperature. During the 20 min incubation, cells were changed into fresh no-antibiotics medium. 4-6 hr after adding the Lipofectamine2000 mix, cells were changed into fresh growing medium and incubated for further treatment or harvest 48 hr after the first transfection. For RAW cells, a second transfection was performed as described above the next day after first transfection.

Table 2. Amount of Lipofectamin2000 and tested RNA for transfection

	RAW (6-well)	RAW (100mm)	Hela (6-well)
siRNA/RNA oligonucleotide (20 uM)	20 ul	120 ul	2 ul
Opti-MEM	250 ul	1.5 ml	100 ul
Lipofectamine2000	10 ul	60 ul	2 ul
Medium on plate	500 ul	3 ml	2000 ul
Final concentration of siRNA	400 nM	400 nM	20 nM
Final dilution of Lipofectamine2000	1:100	1:100	1:1000

## 2.5 *In vivo* ubiquitination assay

The day before transfection,  $10^6$  of HeLa cells were plated in 15 ml of growth medium without antibiotics. Lipofectamin2000 (Invitrogen) was used to transfect cells with 4  $\mu$ g of pHis6-Ubi and desired plasmid at a 1:3 ratio, following the manufacturer's instruction. Transfected cells were cultured for a further 48 hr. To enhance the accumulation of the ubiquitinated proteins, MG132 (Calbiochem) was added into the medium 4 hr prior to harvest. The cells were then washed twice with ice-cold PBS. 1/10 volume of amount was saved as inputs and lysed in NP40 buffer (50 mM HEPES pH 8, 250 mM NaCl, 1% NP40) to determine the total amount of the protein of interest by Western Blotting. Remaining cells were re-suspended in 7 ml of Guanidine lysis buffer (100 mM  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  pH 8, 10 mM Tris-HCl pH 8, 6 M guanidine-HCl, 10 mM 2-Mercaptoethanol, and 5 mM imidazole fresh added), and 100  $\mu$ l of Ni-NTA Agarose beads (Qiagen) was added to allow binding of the His-ubiquitinated proteins. After incubation at room temperature on a rotator, the beads were precipitated by centrifugation at 5,000 g for 5 min. Then the beads were consecutively washed for 10 min each in 750  $\mu$ l of Guanidine lysis buffer without imidazole, Urea buffer (100 mM  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  pH 8, 10 mM Tris-HCl pH 8, 8 M urea, 10 mM 2-Mercaptoethanol fresh added), Buffer A (100 mM  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  pH 6.3, 10 mM Tris-HCl pH 6.3, 8 M urea, 10 mM 2-Mercaptoethanol fresh added), and Buffer A with 0.1% Triton X-100. Finally His-ubiquitinated proteins were eluted from the beads by incubating in 100  $\mu$ l elution buffer (150 mM Tris-HCl pH 6.8, 200 mM imidazole, 5% SDS, 30% glycerol, 0.72 M 2-Mercaptoethanol) for 40 min at room temperature. Supernatants containing ubiquitinated proteins, together with the inputs, were then subjected to Western blotting analysis.



Table 3. Preparation of 1M Sodium Phosphate Buffer at room temperature

pH	1M Na <sub>2</sub> HPO <sub>4</sub> (ml)	1M NaH <sub>2</sub> PO <sub>4</sub> (ml)
6.2	17.8	82.2
6.8	35.2	64.8
8.0	93.2	6.8

## 2.6 Very low-copy plasmid purification using QIAGEN-tip 100

This protocol is suitable for QIAGEN-tip 100, and use centrifugation to clear lysates, due to the large culture volumes. After alkaline lysis, there is an additional isopropanol precipitation step to decrease the amount of lysate before DNA is bound to the QIAGEN-tip 100. The expected yields are 20-100 µg. It may be beneficial to increase the lysis buffer volumes in order to increase the efficiency of alkaline lysis, and thereby the DNA yield.

Grow a culture of 500 ml at 37°C for overnight with vigorous shaking. Harvest the bacterial cells by centrifugation at 4,000 rpm for 15 min at 4°C. Resuspend the bacterial pellet in 20-60 ml Buffer P1/RNase A. Add 1 x volume of Buffer P2, mix well and incubate at room temperature for 5 min. Add 1 x volume of pre-chilled Buffer P3, mix well and incubate on ice for 30 min. Transfer the sample to polypropylene tube, and centrifuge at 12,000 rpm for 30 min at 4°C. Collect the supernatant to a fresh tube, and filter the supernatant over a prewetted, folded filter. Add 0.7 x volumes of room-temperature isopropanol to the lysate and precipitate the DNA by centrifugation at 10,000

rpm for 30 min at 4°C. Carefully decant the supernatant thoroughly, and redissolve the DNA pellet in 500 µl TE buffer, pH 8.0. Add Buffer QBT to obtain a final volume of 5 ml. At the same time, equilibrate a QIAGEN-tip 100 by applying 4 ml Buffer QBT. Then apply the 5 ml DNA/QBT solution to the QIAGEN-tip. Wash the QIAGEN-tip with 2 x 10 ml Buffer QC, followed by elution with 5 ml Buffer QF. For constructs larger than 45-60 kb, prewarming the elution buffer to 50°C may help to increase yield. Precipitate DNA by adding 0.7 x volumes of room-temperature isopropanol and centrifugation at 9,000 rpm for 30 min at 4°C. Wash DNA pellet with 2 ml room-temperature 70% ethanol, and centrifuge at 9,000 rpm for 10 min. Air-dry the pellet for 5-10 min, and redissolve the DNA in 20-50 µl of desired buffer.

## **2.7 HAT assays**

Solution HAT assays were performed by incubating HeLa extracts, histones (Sigma), and [<sup>14</sup>C] acetyl-CoA with baculovirus-expressed CBP as previously described. Pull down HAT assays were performed by capturing baculovirus-expressed, flag-tagged CBP on anti-flag agarose beads. Beads were incubated with the indicated HeLa extracts for 1 hr and washed three times with HAT assay buffer to remove unbound proteins. Anti-flag agarose beads with CBP-protein complexes were then incubated with histones and [<sup>14</sup>C] acetyl-CoA. CBP and histones were subsequently resolved by SDS-PAGE and acetylation was detected by autoradiography.

## 2.8 Biochemistry purification

The nuclear extracts (0.3 M NaCl, 20 mM HEPES, pH 7.9, 25% Glycerol, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, and 0.5 mM DTT; 10 L culture of HeLa cells) with the protease inhibitor mix (10 µg/ml Aprotinin, 10 µg/ml leupeptin, 0.4 mM bezamidine, and 0.2 mM PMSF) were dialyzed against 0.1 M NaCl containing dialysis buffer (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.5 mM DTT). After the dialysis, the sample was applied onto the 500 ml column (ID, 2.5 cm x Length, 100 cm; volume, 491 ml) of Sephacryl S-300 equilibrated with the dialysis buffer and fractionated into 40 fractions. The fractions were analyzed with HAT assay to detect inhibitory activity. Two peaks of the inhibitory activity were incubated with baculovirus-expressed flag-tagged CBP bound flag antibody agarose beads. After extensive washing beads with buffer H (20 mM HEPES pH 7.9, 50 mM KCl, 20% Glycerol, 0.5% NP40), the sample was extracted with the 0.3 M NaCl extraction buffer and separated on SDS-PAGE gel. The protein bands stained were analyzed by mass spectrometry.

## 2.9 Protein Identification

Gel-resolved proteins were digested with trypsin, batch purified on a reversed-phase micro-tip, and resulting peptide pools individually analyzed by matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (MS) (*UltraFlex TOF/TOF*; BRUKER; Bremen, Germany) for peptide mass fingerprinting (PMF), as described before [160, 161]. Selected peptide ions ( $m/z$ ) were taken to search a “non-redundant” human protein database (NR; 134,604 entries on 26 July 2005; National Center for Biotechnology Information; Bethesda, MD) utilizing the PeptideSearch

algorithm (Matthias Mann, Max-Planck Institute for Biochemistry, Martinsried, Germany; an updated version of this program is currently available as 'PepSea' from Applied Biosystems/MDS Sciex; Foster City, CA). A molecular mass range up to twice the apparent molecular weight (as estimated from electrophoretic relative mobility) was covered, with a mass accuracy restriction of less than 35 ppm, and maximum one missed cleavage site allowed per peptide. To confirm PMF results with scores  $\leq 40$ , mass spectrometric sequencing of selected peptides was done by MALDI-TOF/TOF (MS/MS) analysis on the same prepared samples, using the UltraFlex instrument in 'LIFT' mode. Fragment ion spectra were taken to search NR using the MASCOT MS/MS Ion Search program, version 2.0.04 for Windows (Matrix Science Ltd., London, UK). Any tentative confirmation (Mascot score  $\geq 30$ ) of a PMF result thus obtained was verified by comparing the computer-generated fragment ion series of the predicted tryptic peptide with the experimental MS/MS data.

## **2.10 Nucleosome preparation**

Poly-nucleosomes were prepared from HeLa cell nuclei[162, 163] as previously described. The quality of the nucleosome assembly was monitored by micrococcal nuclease digestion and mass of core histones, with 15% SDS-PAGE and 1.2% agarose gel. Mononucleosomes (5 mg core histones)  $\gamma$ [ $^{14}\text{C}$ ] acetyl-CoA (50 mCi/mmol), 1 ng of each CBP protein (flag-CBP incubated with GST or GST-TLS) in 50 mM Tris-HCl pH 8.0, 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butylate.

### **2.11 RNase treatment**

The cell nuclear extracts were treated with RNase A at a final concentration of 50 ng/ul at room temperature for 30 min, followed by immunoprecipitation or HAT assay.

### **2.12 Dephosphorylating proteins with CIP**

For every 50 ul of protein sample, 1 ul of CIP was added for incubation at 37°C, 30 min. If the source was proteins dissolved in H<sub>2</sub>O, NEBuffer 3 was used as reaction buffer.

### **2.13 RNA binding assay**

RNA oligo nucleotides were labeled with  $\gamma$ [<sup>32</sup>P]-ATP using 4 units of T4 polynucleotide kinase (Toyobo) at 25°C for 30 min. The RNA oligo was separated on Sephadex p10 column (1 ml) from free  $\gamma$  [<sup>32</sup>P]. The reactions containing baculovirus expressed CBP, 10 mM Tris-HCl pH 7.5 buffer with 5% glycerol, 10 mM EDTA, 1 mM DTT, and 50  $\mu$ g of yeast tRNA, plus 0.2  $\mu$ g of [<sup>32</sup>P] RNA oligo nucleotides were incubated at 25°C for 15 min. The samples were analyzed on 6% PAGE gel with 0.3 x TBE buffer at 150 volts for 1.5 hr. The gel was dried and analyzed with autoradiography overnight.

### **2.14 Rapid Amplification of cDNA Ends (RACE)**

RACE was performed using SMART RACE cDNA Amplification Kit (clontech) based on user manual with some modification. Briefly, 1  $\mu$ g of Trizol-extracted total RNA (less than 3  $\mu$ l) was used for first-strand synthesis. Gene specific primers (GSP) were used. The first-strand reaction product was diluted 2 times with TE buffer, and 3  $\mu$ l

of diluted product was used as template for PCR using the GSP and 5'-RACE TFR primer. Two rounds of PCR were performed followed by sequencing.

### **2.15 Labeling the living cells with biotin**

The assays were performed as previously described with some modification. Briefly, permeabilize cells with lysolecithin and biotinyl-11-deoxyuridine triphosphate (Bio-UTP) (200 uM final concentration) added to cell media for 1 h, maximum incorporation of Bio-UTP lysolecithin concentration (80-100 pg/ml).

### **2.16 Single cell nuclear microinjection assay**

The single cell nuclear microinjection assays were performed as previously described. Each experiment was performed on three independent coverslips with >300 injected cells per point, and rhodamine-conjugated dextran was used as a negative control in each experiment. Before injection, cells were rendered quiescent by incubation in serum-free medium for 24-36 hr.

### **2.17 Reporter assay**

RAW 264.7 or Hela cells were transfected with *CCND1* promoter-driven luciferase reporter gene, together with tested plasmids or RNA samples. A  $\beta$ -Gal reporter plasmid was also co-transfected as an internal control (Promega).

### **2.18 Chromatin immunoprecipitation (ChIP) assay**

Monolayer grown cells were washed twice with PBS and fixed with 1% formaldehyde at room temperature for 10 min, followed by twice of quick rinse with PBS. Then 0.25 M

Glycine/PBS solution was added on the plate to stop the cross-link for 10 min at room temperature, followed by a quick wash with ice-cold PBS. The cells were collected into a fresh tube and sequentially washed in ice-cold buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH 6.5) and buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH 6.5). Cell pellets were re-suspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, 1 x protease inhibitor cocktail) and sonicated three times for 10 sec each at the setting of 3.5 (Fisher Sonic Dismembrator, Model 300). The samples were kept on ice during the sonication. The efficiency of sonication was checked by running 10 ul of the sample on a 2% agarose gel after boiling at 100°C for 10 min. The supernatant (soluble chromatin) was transferred to a new tube after centrifugation at 16,000 g for 10 min at 4°C. The soluble chromatin was then 1:10 diluted in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1, 1 x protease inhibitor cocktail). 45 ul of protein A/G-sepharose beads (50% slurry in TE buffer) were added and incubated for 1 hr at 4°C on a rotator. The beads were discarded after quick spin and specific antibody (2-4 ug) was added to the supernatant and incubated at 4°C overnight on a rotator. At the next day, 45 ul of protein A/G-sepharose beads (50% slurry in TE buffer) were added for a further 2-3 hr incubation at 4°C. Beads were harvested by centrifugation and washed sequentially for 10 min each at 4°C in TSE I buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), TSE II buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1 mM EDTA, 10 mM Tris-HCl pH 8.1). Beads were then quickly washed with TE buffer twice and incubated with 300 ul of 1% SDS, 0.1 M NaHCO<sub>3</sub> at 65°C overnight to elute

DNA and reverse the formaldehyde cross-link. Supernatant containing the DNA fragments were collected after centrifugation at 12,000 g, room temperature for 10 min. DNA fragments were then purified with a QIAquick Spin Kit (Qiagen) and eluted in 50 ul of H<sub>2</sub>O. 2 ul of DNA was used for real-time PCR. The binding characteristics of immunoglobulins to proteinA/G-sepharose beads is shown in Table 4.

Table 4. Binding Characteristics of Immunoglobulins to ProteinA/G-sepharose beads

Immunoglobulin		Protein A	Protein G
Rabbit	IgG1	+++	++
Mouse	IgG1	+	++
	IgG2a	+++	+++
	IgG2b	++	++
	IgG3	+	+++
	IgM	-	-
	IgA	-	-
	IgE	-	-
guinea pig	IgG	+++	-
Rat	IgG1	+	+
	IgG2a	-	+++
	IgG2b	-	++
	IgG2c	+	++
Human	IgG1	+++	+++
	IgG2	+++	+++
	IgG3	-	+++
	IgG4	+++	+++



## 2.19 RNA immunoprecipitation (RIP) assay

RIP assay was performed according to the method described before[164] with some modifications. Cultured cells were harvested and treated with formaldehyde and Glycine/PBS as described in “Chromatin immunoprecipitation (ChIP) assay”. The cells were then re-suspended in 300 ul of RIPA buffer (50 mM Tris-HCl pH 7.5, 1% NP40, 0.05% SDS, 1 mM EDTA, 150 mM NaCl) containing protease inhibitors, and lysed by sonication as described in “Chromatin immunoprecipitation (ChIP) assay. After centrifugation, an aliquot of solubilized cell lysate (25 ul, 10%) was saved as input. Protein A/G-Sepharose beads (30 ul of 50% slurry) were coated with the specific antibody (2 ug) of interest for 2 h at 4°C followed by extensive washing with RIPA buffer containing protease inhibitors. Before immunoprecipitation, the coated beads were incubated for 10 min with 0.5 ul of RNasein (40 U/ul). The lysate (250 ul) was diluted with same volume of RIPA buffer (250 ul), mixed with antibody-coated beads, and incubated with rotation at 4°C overnight. The beads were washed six times in high stringency RIPA buffer (50 mM Tris-HCl pH 7.5, 1% NP40, 0.1% SDS, 1 mM EDTA, 0.5 M NaCl, 3 M urea) by 10 min rotation at room temperature. The beads containing the immunoprecipitated samples were collected and re-suspended in 100 ul of RIP elution buffer (50 mM Tris-HCl pH 7.0, 5 mM EDTA, 10 mM DTT, 1% SDS). The de-crosslink was performed by incubating samples (re-suspended beads) for 45 min at 70°C. RNA was extracted using Qiagen RNeasy Micro kit with some modifications. 250 ul of RLT buffer was added to 100 ul of elutant, and DNaseI treatment was performed during RNA extraction as manufactura’s instruction. Extracted RNA was collected in 16 ul and 8 ul were subjected to reverse transcription using random primers.

## 2.20 Small RNA Northern blot

Total cellular RNA was extracted using Trizol (Invitrogen) and treated with RNase-free DNaseI (DNA-free; Ambion). Small transcript northern blot was performed as follows. 15 % TBE-Urea PAGE gels (Invitrogen) were loaded with 30 µg of total RNA, and then run at 200 volts for 1 h until the bromophenol blue dye reached the gel bottom. RNA was then electro-transferred to Hybond N+ Nylon membranes (GE Healthcare) for 3 h at 25 volts in 0.25X TBE. Membranes were then UV cross-linked (Stratalinker, Stratagene), and prehybridized in expressHyb hybridization solution (Clontech) at 68°C for 1 h. 25 ng of DNA probes were labeled with [ $\alpha$ -<sup>32</sup>P]-dCTP using Rediprime II Random Prime Labelling system (Amersham). Probes were then purified using Nick column (Amersham). After hybridization at 42°C for overnight, membranes were extensively washed in high stringency wash buffer (0.1X SSC, 0.1% SDS) at 65°C and exposed to film.

## 2.21 Complex purification

**Cell culture and extract preparation** HEK293 cells were transfected with a flag-tagged plasmid or an empty vector. Stable transfectants were selected with 500 µg/ml of G418 and tested by Western Blotting using anti-flag antibody. Two transfectants with relatively low expression level of flag-tagged proteins were pooled and further cultured in medium with 300 µg/ml of G418. Cells were harvested and  $1 \times 10^{8-9}$  cells were used for each data point. Cells were washed with ice-cold PBS twice. Cell pellets were then lysed in Hypotonic Buffer (10 mM KCl, 1 mM MgCl<sub>2</sub>, 50 mM Tris-HCl pH 7.6, 0.3% NP40, proteinase inhibitors fresh added) on ice for 10 min, followed by centrifugation at 1,000 g

for 10 min at 4°C. Cell pellets, as nuclei, were stored at -80°C. At the day for complex purification, nuclei were thawed and lysed in 15 ml of 300 mM Low Salt Lysis Buffer (300 mM NaCl, 1 mM MgCl<sub>2</sub>, 20 mM Tris-HCl pH 7.6, 0.25 mM EDTA, 0.5% NP40, 20% glycerol) on a rotator at 4°C for 30 min. After centrifugation on a table centrifuge at max speed for 10 min at 4°C, the supernatants were transferred as nuclear extract to a fresh tube. Same volume of Hypotonic Buffer without NaCl was added to generate a final concentration of 150 mM NaCl.

**Resin preparation** Anti-flag M2 affinity gel/resin (Sigma) was used for flag-tagged protein complex purification. The anti-flag M2 affinity gel/resin was prepared as manufacturer's instruction. After the final step, resin was re-suspended in 1ml of TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl) and used in batch format.

**Complex purification** The protein extraction from “**Cell culture and extract preparation**” was added 330 ul of prepared anti-flag resin from “**Resin preparation**” and incubated for 2 hr at 4°C with gentle rotation to capture the flag fusion proteins. After binding, the resin was collected by filtration in columns, followed by consecutively washed with 10 volume of TBS Buffer, 30 volume of Wash Buffer (50 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.5% NP40), and 10 volume of TBS. The protein being eluted from the resin was monitored by measuring the absorbance of the eluant at 280 nm. The resin was continued washed until the absorbance difference of the wash solution coming off the column was less than 0.05 versus a wash solution blank. The flag-tagged proteins were then eluted from the resin by competition with 1 ml of 500 ug/ml 3Xflag peptide. 20 ul of eluant was saved and the complex was separated by SDS-PAGE electrophoresis for silver

staining. The remaining eluants were concentrated with MICROCON centrifugal filter devices YM10 (Millipore). Concentrated eluant was electrophoresed on SDS-PAGE gel for Coomassie Staining. Desired bands were cut for Mass Spectrometry. Resin was regenerated as manufactur's instruction.

This Chapter (Chapter 2), in part, is based on material that has been submitted for publication to Nature, as “Stress-induced Non-coding RNAs Allosterically Regulate a HAT-inhibitory RNA Binding Protein to Mediate Transcriptional Repression. X.T. Wang, S. Arai, D. Reichart, T. Oyoshi, X.Y. Song, G. Pascual, D.W. Rose, P. Tempst, M.G. Rosenfeld, C.K. Glass and R. Kurokawa.”

## **Chapter 3. RESULTS**

### **3.1 TLS specifically inhibits CBP/p300 HAT-dependent transcription.**

#### **3.1.1 TLS was identified as a component of flag-CBP complex in HeLa nuclear extracts.**

To search for cellular factors that might regulate CBP HAT activity, full-length, flag-tagged CBP immobilized on anti-flag IgG affinity beads was incubated with HeLa whole cell extracts or control whole cell extract buffer. The CBP affinity beads were subsequently washed to remove unbound proteins and tested for HAT activity. These experiments revealed marked inhibition of CBP HAT activity by HeLa whole cell extracts in “pull-down” HAT assays (Fig. 2a, lane 2). Similar results were obtained when “solution” HAT assays were performed in which CBP was not separated from soluble extract proteins prior to addition of radiolabeled Acetyl Co A (Fig. 2a, lane 5).

Subcellular fractionation studies indicated the presence of two classes of inhibitory activities: one that bound to CBP and was primarily present in nuclear extracts (Fig. 2a, lane 3), and a second that did not bind to CBP and was present in both nuclear and cytoplasmic extracts (Fig. 2a, lanes 6-7). The second activity is apparently corresponding to the INHAT complex that exerts inhibitory effects on HAT activity by direct binding to

histone substrates[165]. In contrast, the HAT activity of CBP was not inhibited if the HeLa cytoplasmic extracts were used in “pull-down” HAT assay (Fig. 2a, lane 4).

We further characterized the nuclear activity that bound to and inhibited CBP in pull-down HAT assays. The nuclear activity that bound to and inhibited CBP in “pull-down” HAT assays fractionated as two main peaks using gel filtration chromatography (Fig. 2b). While the majority of inhibitory activity eluted in high molecular weight fractions 13-17, the lower molecular weight fractions 30-32 were also observed to have inhibitory activity. Each pooled fraction was further purified using flag-tagged, full length CBP linked to anti-flag IgG beads as an affinity matrix. SDS-PAGE analysis revealed a large number of proteins in the high molecular weight fractions and a major band of approximately 60 kDa molecular weight in the low molecular weight fractions (Fig. 2c). Using MALDI-reTOF MS analysis, this 60 kDa protein was identified in three independent purifications to be TLS, which was shown to also be present in the high molecular weight fractions by Western blotting (Fig. 2d).

### **3.1.2 TLS specifically inhibited CBP/p300 HAT activities in a substrate specific manner.**

To confirm and extend our findings, we tested the ability of recombinant TLS to bind to and influence the enzymatic activities of CBP and several other HATs. As expected from the purification studies, recombinant TLS bound to CBP (Fig. 2e, lane 2) and inhibited its HAT activity on all four core histones (Fig. 2f, lanes 1-3). Intriguingly, GST-TLS inhibition of CBP HAT activity exhibited substrate-specificity as CBP-dependent acetylation of p53 was not affected (Fig. 2f, lanes 4-6). TLS also bound to p300 and

TIP60 with similar affinities (Fig. 2e, lanes 3-4), but not to p/CAF (Fig. 2e, lane 5). GST-TLS inhibited the HAT activity of p300, but not that of TIP60 (Fig. 2g), indicating that the inhibitory effect of TLS is specific to CBP/p300 HAT. TLS was able to inhibit the CBP acetylation on histones in nucleosomes prepared from HeLa cell nuclei as well (Fig. 2h).

### **3.1.3 Mapping of CBP/p300 interacting domain on TLS and TLS interacting domain on CBP.**

To determine whether the inhibitory activity on CBP HAT is through binding directly to the HAT domain, “pull down” and “solution” HAT assays were performed using a GST-CBP-HAT fusion protein containing a catalytically active HAT domain extending from amino acids 1099-1760. The CBP HAT activity was strongly inhibited in “solution” HAT assays, but not in “pull-down” assays (data not shown). This observation suggested that TLS inhibits CBP HAT activity through binding to a region outside of the HAT domain of CBP.

To determine the domains of CBP involved in interaction with TLS, GST-CBP fusion proteins containing distinct CBP domains were tested for their ability to interact with *in vitro* translated TLS in GST pull-down assays. TLS interacted strongly with a fragment of CBP corresponding to amino acids 1892-2241 (CBP 1892-2241), and weakly with several other fragments (Fig. 3a). The TLS-binding domain on CBP 1892-2241 was further precisely mapped to CBP amino acids 2058-2163, which overlaps with the p160-interaction domain of CBP (Fig. 3b). This result indicates that TLS is an allosteric

regulator of CBP HAT activity, consistent with its ability to inhibit histone, but not p53 acetylation.

Parallel assays performed with GST-TLS deletion mutants defined amino acids 1-211 in the N-terminal domain of TLS (TLS1-211) as the minimum region capable of full CBP binding activity (Fig. 4a). Consistent with this observation, TLS1-211 was found to possess the similar, but even stronger activity as full length TLS in inhibiting CBP HAT activity (Fig. 4b). Deletion of amino acids 1-211 (TLS211-526) resulted in a significant reduction in TLS binding to CBP (Fig. 4a) and loss of inhibition of CBP HAT activity (Fig. 4b). In concert, these findings suggest that TLS inhibits CBP HAT activity by binding *via* the TLS N-terminus to the p160-interaction domain of CBP.

### **3.1.4 TLS is a co-repressor of CREB-mediated transcription.**

#### **3.1.4.1 TLS represses the forskolin induced CREB activation of a CRE-dependent promoter.**

Previous studies demonstrated that the HAT activity of CBP/p300 is required for transcriptional activity of CREB on model promoters, but not for transcriptional activities of RAR, suggesting that modulation of CBP/p300 HAT function might provide a strategy for differential control of CBP/p300 HAT-dependent and CBP/p300 HAT-independent transcription units. To investigate whether the ability of TLS to inhibit CBP/p300 HAT activity could influence signal-dependent transcription in a promoter-specific manner, we performed single cell microinjection experiments. Overexpression of *TLS* abolished forskolin-induced CREB activation of a CRE-dependent promoter, but did not affect



retinoic acid activation of a RAR-responsive promoter (Fig. 5a). These results are consistent with previous studies indicating that the HAT activity of CBP is required for CREB-dependent transcription, but not for an RAR-dependent transcription unit and support possible roles of TLS as an integrator of complex programs of gene regulation.

### **3.1.4.2 TLS represses endogenous CREB target gene, *CCND1*.**

To investigate roles of TLS as a negative regulator of gene expression, we focused on *CCND1*, an endogenous CREB target gene[166, 167] that is induced in RAW264.7 cells by forskolin. SiRNA-mediated knockdown of *TLS* mRNA in RAW264.7 cells resulted in a marked increase in both basal and forskolin-stimulated expression of *CCND1* mRNA levels (Fig. 5b). To understand whether TLS regulates *CCND1* through inhibiting CBP/p300 HAT activity, we performed a series of reporter assays using a *CCND1* promoter-driven luciferase expression plasmid. As expected, overexpression of *TLS* repressed *CCND1* promoter activity (Fig. 5c). Introduction of wild-type *CBP*, but not a *CBP* HAT mutant, rescued the repression of *CCND1* promoter activity by *TLS* (Fig. 5c).

ChIP experiments were performed in quiescent RAW264.7 cells to test whether TLS might be directly bound to the *CCND1* promoter. When cells were cultured with carrier in serum-starved media, both CBP/p300, and TLS were bound to *CCND1* in the region of the CRE site (Fig. 5d). Forskolin treatment caused TLS to be dismissed from the *CCND1* promoter, while both CBP and p300 remained bound (Fig. 5d). ChIP analysis of histone acetylation marks on the *CCND1* promoter revealed hyper-acetylation of histone (Ace H3-K9K14) upon forskolin treatment (Fig. 5d). Transfection of *TLS* siRNA resulted in an increase of histone acetylation in the region of the *CCND1* CRE site (Fig. 5e). We did not

observe binding of TLS on all CREB target promoters, such as the *TGF $\beta$ 2* and *FZD4* promoters (Fig. 6), suggesting that the negative regulation of CREB target genes by TLS is promoter context-dependent.

Down regulation of *CCND1* expression is linked to G1 cell cycle arrest in response to DNA damage. Consistent with this, *CCND1* mRNA levels were significantly reduced following exposure of HeLa cells to IR (Fig. 7a). This response was accompanied by the recruitment of TLS to the *CCND1* promoter and a reduction in acetylation of histone H3 as detected by ChIP assay (Fig. 7b). In contrast, the expression level of TLS is not regulated by IR (Fig. 7a).

### **3.1.5 Two TLS homologues-EWS and TAF15 selectively binds with CBP/p300 and inhibits the HAT activity of CBP/p300 as well.**

The two homologues of TLS, EWS and TAFII68 also bound to CBP and TIP60, but not to p/CAF (Fig. 8a) and exerted inhibitory effects on the HAT activity of CBP/p300 (Fig. 8b), but not of TIP60 (data not shown). More interestingly, overexpression of either *TLS* from a heterogenous species or *EWS* could overcome the effect of *TLS* siRNA on *CCND1* promoter activity, suggesting an overlapped function of TLS and EWS (Fig. 9). These data suggest conserved roles of TLS, EWS, and TAFII68 as inhibitors of CPB/p300 HAT activity.

## **3.2 NcRNAs mediate the inhibitory effect of TLS.**

### **3.2.1 TLS specifically bound to and functionally required GGUG containing RNA.**

RNAs are now well-established to function in diverse regulatory strategies, from gene silencing to RNA stability. Because TLS is an RNA binding protein, we next tested the potential function of RNA in TLS actions, and first performed co-immunoprecipitation assays using the RNaseA-treated, quiescent HeLa cell lysates. The assay showed a greatly reduced interaction of TLS and p300 upon RNaseA treatment (Fig. 10a). Further, treatment of the flag-CBP complex with RNaseA caused a loss of its ability to inhibit histone acetylation (Fig. 10b). These data suggested that TLS inhibits CBP/p300 HAT activity by an RNA-dependent mechanism.

TLS has been reported to bind RNAs containing the consensus sequence GGUG, with mutations of the GGUG sequence to CCUC abolishing binding. We confirmed these findings (Fig. 11a) and further determined that this binding activity involved redundant functions of the RGG, RRM and ZnF motifs of TLS, as individual mutations of these motifs did not abolish binding (Fig. 11b). In contrast, the N-terminus of TLS (TLS1-211) alone was not sufficient to mediate interaction with GGUG-containing RNA (Fig. 11b). As predicted from our data, the RNA oligonucleotide containing GGUG, but not CCUC, enhanced the binding of TLS to p300 (Fig. 10c) and the inhibitory activity of TLS on p300 HAT (Fig. 10d). Similar results were also obtained with CBP (data not shown). A potential mechanism for this effect is provided by the observation that the N-terminus of TLS (TLS1-211) interacted with the C-terminus of TLS (TLS373-526) and that

addition of the consensus GGUG oligonucleotide prevented this interaction in a dose-dependent manner (Fig. 10e). These results suggest that RNA binding causes an allosteric modification of TLS, relieving an inhibitory function of the TLS C-terminus, and allowing the N-terminus of TLS to bind to CBP and allosterically regulate its HAT activity.

To test whether specific RNAs might regulate TLS function *in vivo*, we transfected the RNA oligonucleotides containing GGUG or CCUC into RAW264.7 cells and assessed their effect on *CCND1* promoter activity. GGUG-, but not CCUC-, containing RNA repressed *CCND1* promoter activity (Fig. 11c). Furthermore, GGUG, but not CCUC-containing RNA reduced endogenous *CCND1* and *CCNE1* mRNA (Fig. 10f). We next asked whether TLS-associated RNAs could be localized to the *CCND1* promoter by transfecting RAW264.7 cells with biotinylated RNA oligonucleotides containing the same GGUG or CCUC sequences and performing ChIP assays with anti-biotin antibody. Remarkably, transfection of the biotinylated GGUG-containing RNA permitted precipitation of the region of the *CCND1* promoter containing the CRE in a manner that was reduced by forskolin treatment (Fig. 10g). Consistent with this, GGUG-containing RNA enhanced TLS binding on the *CCND1* CRE site (Fig. 10h). In contrast, the recruitment on *CCND1* exon2 region or on control genes was not regulated (Fig. 10g and Fig. 10h).

### **3.2.2 New identified ncRNA generated from *CCND1* 5' regulatory region recruited TLS to *CCND1* promoter.**

#### **3.2.2.1 Identification of ncRNA from *CCND1* 5' regulatory region.**

Based on the enhanced sensitivity of TLS-deficient cells to sources of DNA damage that cause double strand breaks, such as IR, and the functionality of TLS as a RNA binding protein, we considered the possibility that previously unrecognized transcripts generated upstream of the *CCND1* promoter could serve as the source of endogenous TLS-regulatory RNAs. RNA was therefore isolated from HeLa cells treated with or without IR, followed by DNaseI treatment to remove DNA. As diagrammed in Fig. 12a, first strand synthesis was performed using random primers, followed by real-time PCR using a series of validated specific primer pairs that exhibited similar amplification efficiency on genomic DNA templates (data not shown), spanning from -1813 to -116 bp upstream of the established *CCND1* transcription start site. These experiments revealed the presence of previously unrecognized, IR-induced, RNAs transcribed from this region (Fig. 12b). Intriguingly, ncRNA transcripts were identified corresponding to distinct upstream regions of the *CCND1* gene (Fig. 12b). It is possible then that either multiple transcripts are generated from 5' regulatory regions of *CCND1*, or that a single transcript is highly labile and rapidly processed, exhibiting differential stability of the products. Independent evidence of transcripts from this region was further provided by Northern blot using non-overlapped probes (~200 bp each) targeting the 5' regulatory regions of *CCND1*, with these probes detecting the presence of IR-induced ~330 nt transcripts over this region (Fig. 12c, Fig. 12d, and data not shown). Further, our data revealed the presence of bidirectional transcripts, induced by IR (Fig. 12e), in contrast to the adjacent

5' UTR of the *CCND1* mRNA, which exhibited a decreased level in response to IR (Fig. 12e), further delimitating the differential regulation of ncRNA<sub>*CCND1*</sub> and *CCND1* mRNA. As is the case for other classes of regulatory non-coding transcripts, a precise determination of the identity of the limit digests, if any, remain to be identified.

### 3.2.2.2 TLS functionally required *CCND1* ncRNA.

To investigate the function of the ncRNAs on *CCND1* transcription, we synthesized siRNAs (sincRNA) to specifically target the ncRNA<sub>*CCND1*</sub>. Introduction of sincRNA in HeLa cells resulted in reduced ncRNA<sub>*CCND1*</sub> (Fig. 13a, left), while simultaneously significantly enhancing the level of *CCND1* mRNA (Fig. 13a, middle). As a control, the siRNA targeting the *CCND1* coding region (si*CCND1*) blocked *CCND1* expression (Fig. 13a, middle). In contrast, neither sincRNA<sub>*CCND1*</sub> nor si*CCND1* regulated the expression of *CCNE1* mRNA (Fig. 13a, right), arguing against a trans-acting model. Consistent with its putative local biological role, the sincRNA<sub>*CCND1*</sub>, but not control siRNA, caused a decrease in TLS recruitment to the *CCND1* promoter both in the absence of IR and under IR-induced conditions (Fig. 13b). These data argue that the ncRNA<sub>*CCND1*</sub> serves to directly recruit TLS to the promoter region of the *CCND1* transcription unit. Given that the synthetic 25 mer GGUG-containing RNA oligonucleotides bind to TLS and allosterically inhibit CBP/p300 HAT activities, we elected to investigate the effect on TLS by similar GGUG-containing oligonucleotides generated from *CCND1* 5' regulatory region corresponding to the ncRNA<sub>*CCND1*</sub>. Our data revealed that these RNA oligonucleotides were capable of binding to TLS (Fig. 13c and data not shown), with binding of the RNAs causing an inhibition of p300 HAT function (Fig. 13d).

Interestingly, a series of other evaluated GGUG-containing oligonucleotides were either ineffective at causing TLS to bind CBP/p300, or for a subset that did mediate binding, were unable to cause inhibition of CBP/p300 HAT functions (data not shown), consistent with the suggestion that RNA:TLS interactions exhibit RNA ligand specificity. This specificity of RNA binding effects on TLS is, in a sense, reminiscent of the specificity of ligand effects on nuclear receptors.

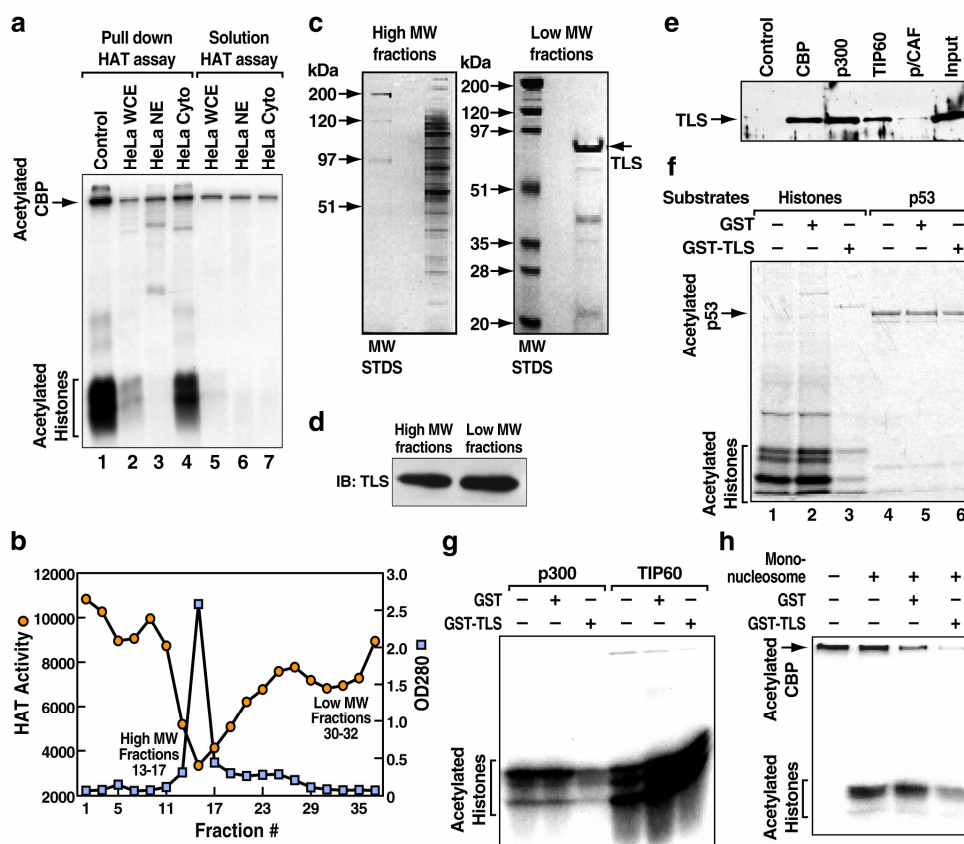


Figure 2. Identification of TLS as a specific CBP/p300 HAT inhibitor.

**Figure 2. Identification of TLS as a specific CBP/p300 HAT inhibitor.** **a**, Both ‘pull down’ and ‘solution’ HAT assays were performed using HeLa cell lysates. The experiments reveal marked inhibition of CBP HAT activity by HeLa whole cell extract (WCE) and nuclear extract (NE) in “pull down” HAT assay, and by HeLa WCE, NE and cytoplasmic extract (Cyto) in “solution” HAT assay. **b**, The elution profile of inhibitory activity upon gel filtration with Sephacryl S-300 shows two peaks of inhibitory activity at high molecular weight (MW) fractions 13-17, and low MW fractions 30-32. **c**, Representative silver-stained gels of pooled high and low MW weight fractions are illustrated. The major band in the low MW fractions is identified as TLS by mass spectrometry. **d**, TLS is present in both high MW fractions and low MW fractions as revealed by Western blotting using a TLS-specific antibody. **e**, TLS interacts with CBP, p300 and TIP60, but not p/CAF. Flag-tagged, baculovirus expressed CBP, p300, TIP60 and p/CAF were captured on anti-flag agarose beads and incubated with recombinant TLS. Beads were then extensively washed and specifically bound TLS was detected by Western blotting. **f**, GST-TLS inhibits the acetylation of core histones, but not of p53, by CBP. HAT assay of CBP was performed with core histones or purified GST-p53 as substrates. **g**, TLS selectively inhibits the HAT activity of p300, but not that of TIP60. **h**, Pull-down HAT assay of CBP with GST-TLS and mononucleosome fractions shows that TLS inhibits the acetylation of histones assembled as nucleosomes.



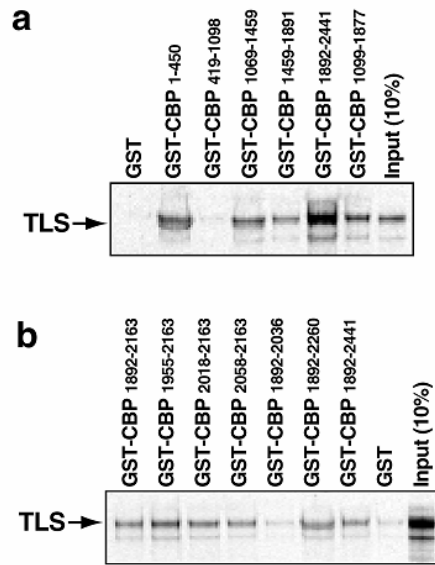


Figure 3. TLS binds to the p160-interacting domain of CBP.

**Figure 3. TLS binds to the p160-interacting domain of CBP. a,** Full-length CBP was divided into six GST fragments, of which GST-CBP 1892-2441 shows the strongest interaction with TLS. **b,** GST-CBP 1892-2441 was further divided into seven GST fragments for GST-pull down assay. The fragment from aa 2058-2163 is indicated to be the minimal domain able to mediate interaction of CBP with TLS.

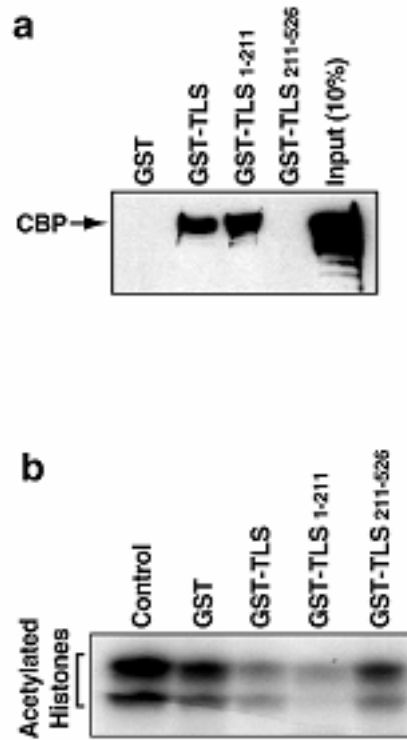


Figure 4. N-terminus of TLS interacts with and inhibits CBP HAT.

**Figure 4. N-terminus of TLS interacts with and inhibits CBP HAT.** **a**, Western blotting of flag-tagged CBP with anti-flag antibody shows that the N-terminus of TLS (GST-TLS1-211) interacts with CBP, while the C-terminus of TLS (GST-TLS211-526) does not. **b**, A pull-down HAT assay of CBP with GST-TLS fragments shows that GST-TLS 1-211, as well as full length TLS (GST-TLS), possess full inhibitory activity on CBP HAT while GST-TLS 211-526 does not inhibit CBP HAT activity.

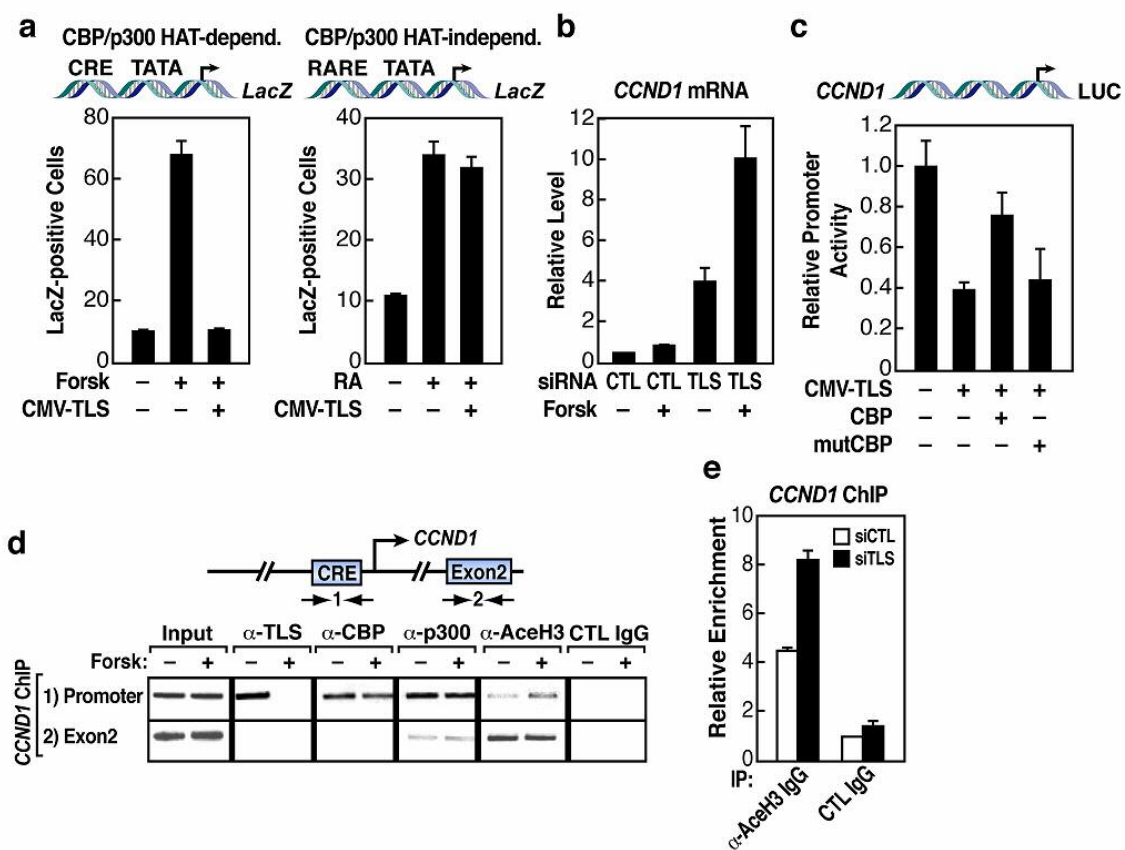


Figure 5. TLS negatively regulates CREB target *CCND1* through modulating CBP HAT activity on histone acetylation.

**Figure 5. TLS negatively regulates CREB target *CCND1* through modulating CBP HAT activity on histone acetylation.** **a**, Single cell microinjection assay shows that TLS blocks the forskolin (Forsk)-induced CREB activation of a CRE-dependent LacZ reporter, but does not block the RA-induced RAR activation of a retinoic acid response element (RARE)-containing-LacZ reporter. **b**, SiRNA-mediated knockdown of *TLS* mRNA in RAW264.7 cells results in a marked increase in both basal (no Forsk) and forskolin-stimulated expression of *CCND1* mRNA levels. **c**, Overexpression of *TLS* represses the activation of a *CCND1* promoter-driven luciferase (LUC) reporter, which can be rescued by wild-type *CBP*, but not a *CBP* HAT mutant. **d**, ChIP experiment demonstrates that CBP, p300 and TLS are bound to the *CCND1* promoter under basal condition. Forskolin treatment causes a dismissal of TLS from the *CCND1* promoter, while both CBP and p300 remain bound, and results in a hyperacetylation (Ace H3-K9K14) in the *CCND1* promoter. **e**, Transfection of *TLS* siRNA induces an enhanced histone acetylation in the *CCND1* promoter as detected by ChIP-real-time PCR. Error bars indicate  $\pm$  SEM.

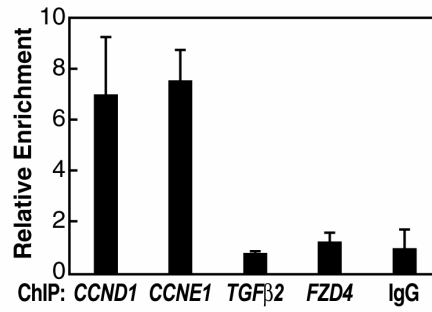


Figure 6. TLS is recruited to a subset of CREB target genes.

**Figure 6. TLS is recruited to a subset of CREB target genes.** TLS is recruited to the promoters of *CCND1* and *CCNE1*, but not to the promoter of *TGFβ2* or *FZD4* in quiescent RAW264.7 cells as shown by ChIP-real time PCR. Error bars indicate ± SEM.

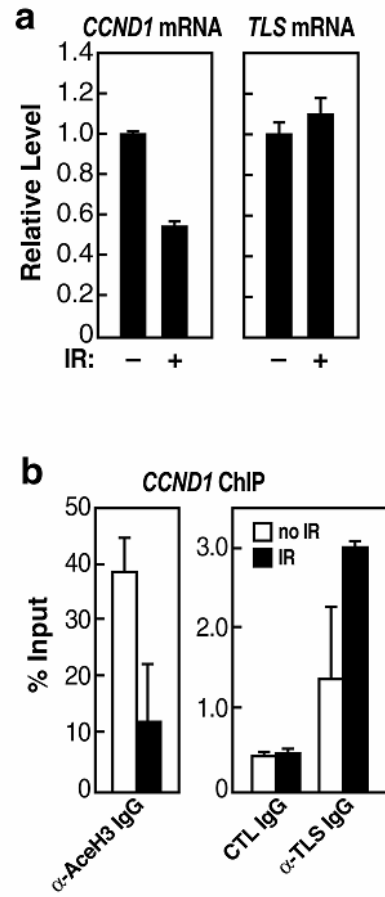


Figure 7. TLS is recruited to the *CCND1* promoter when *CCND1* is reduced upon IR.

**Figure 7. TLS is recruited to the *CCND1* promoter when *CCND1* is reduced upon IR.** **a**, *CCND1* mRNA is reduced upon IR, while *TLS* mRNA is not regulated. **b**, ChIP assay reveals an IR-induced hypoacetylation of histone H3 and increased occupancy of TLS to the *CCND1* promoter. Error bars indicate  $\pm$ SEM.

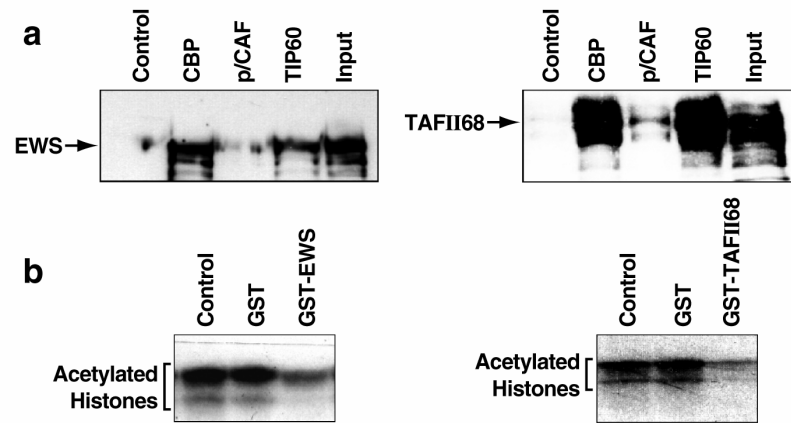


Figure 8. EWS and TAFII68 interact with and inhibit CBP/p300 HAT activity.

**Figure 8. EWS and TAFII68 interact with and inhibit CBP/p300 HAT activity.** a, EWS (left) or TAFII68 (right) interacts with CBP, p300 and TIP60, but not p/CAF. b, Pull-down HAT assays indicate that GST-EWS (left) and GST-TAFII68 (right) inhibit the acetylation of CBP on core histones.

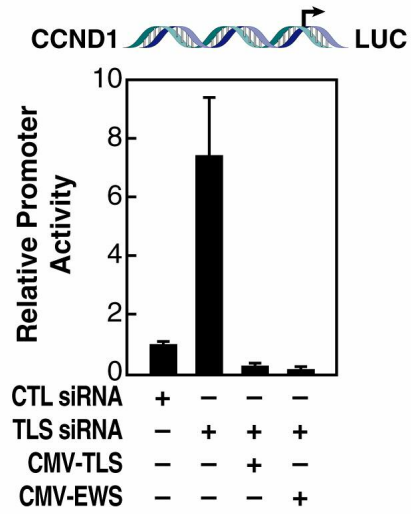


Figure 9. Both TLS and EWS negatively regulate *CCND1*.

**Figure 9. Both TLS and EWS negatively regulate *CCND1*.** Overexpression of *TLS* or *EWS* represses *CCND1* promoter-driven luciferase activity induced by an siRNA targeting *TLS*.

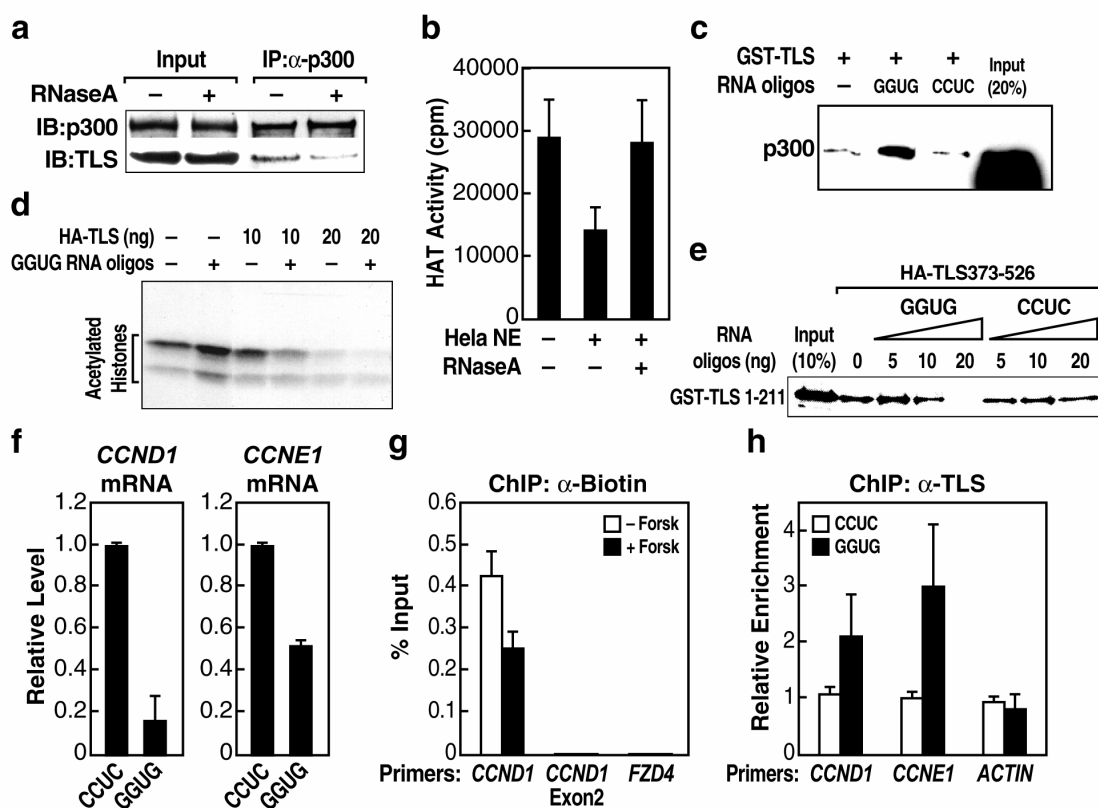


Figure 10. Specific GGUG-containing RNA promotes the inhibitory effect of TLS on CBP/p300 HAT activity and *CCND1* transcription.

**Figure 10. Specific GGUG-containing RNA promotes the inhibitory effect of TLS on CBP/p300 HAT activity and *CCND1* transcription.** **a**, The interaction of p300 and TLS is dramatically decreased in HeLa cells treated with RNaseA. **b**, Loss of HAT inhibition by flag-CBP/TLS complex when HeLa cells treated with RNaseA. **c**, GGUG-containing RNA, but not CCUC-containing RNA, enhances the binding of GST-TLS to p300. **d**, Pull-down HAT assay of CBP with baculovirus expressed TLS shows that GGUG-containing RNA enhances the inhibitory activity of TLS on CBP in a dose-dependent manner. **e**, The N-terminus of GST-TLS (GST-TLS 1-211) interacts with the C-terminus of TLS (HA-TLS 373-526) in a manner that is dissociated by GGUG-, but not CCUC-, containing RNA oligonucleotides. **f**, Transfection of GGUG-containing RNA oligonucleotides inhibits endogenous *CCND1* and *CCNE1* expression in proliferating RAW264.7, compared with CCUC-containing RNA oligonucleotides. **g**, Biotinylated GGUG-containing RNA oligonucleotides are associated with the *CCND1* promoter in quiescent RAW264.7 cells and are dismissed by forskolin treatment. As controls, no binding is observed on *CCND1* exon2 or *FZD4* promoter. **h**, Biotinylated GGUG-containing RNA oligonucleotides enhances the binding of TLS on the promoters of *CCND1* and *CCNE1* in proliferating RAW264.7 cells, compared with CCUC-containing RNA oligonucleotides. *ACTIN* promoter is used as a control. Error bars indicate  $\pm$  SEM.



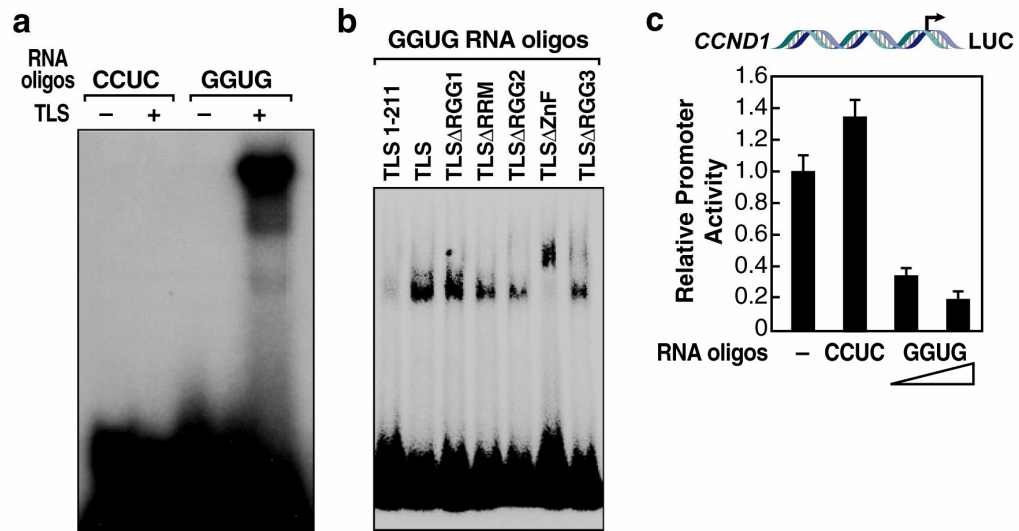


Figure 11. GGUG-containing RNA oligonucleotides specifically interact with TLS and inhibit *CCND1* promoter activity.

**Figure 11. GGUG-containing RNA oligonucleotides specifically interact with TLS and inhibit *CCND1* promoter activity.** **a**, Gel Shift assay of TLS with  $^{32}$ P-labeled RNA probes containing GGUG and CCUC motifs indicates that TLS binds to GGUG-containing RNA, but not CCUC-containing RNA. **b**, Interaction of TLS and TLS deletion mutants with GGUG-containing RNA oligonucleotides. **c**, Transfection of GGUG-, but not CCUC-, containing RNA oligonucleotides, inhibits *CCND1* promoter activity in proliferating RAW264.7 cells.

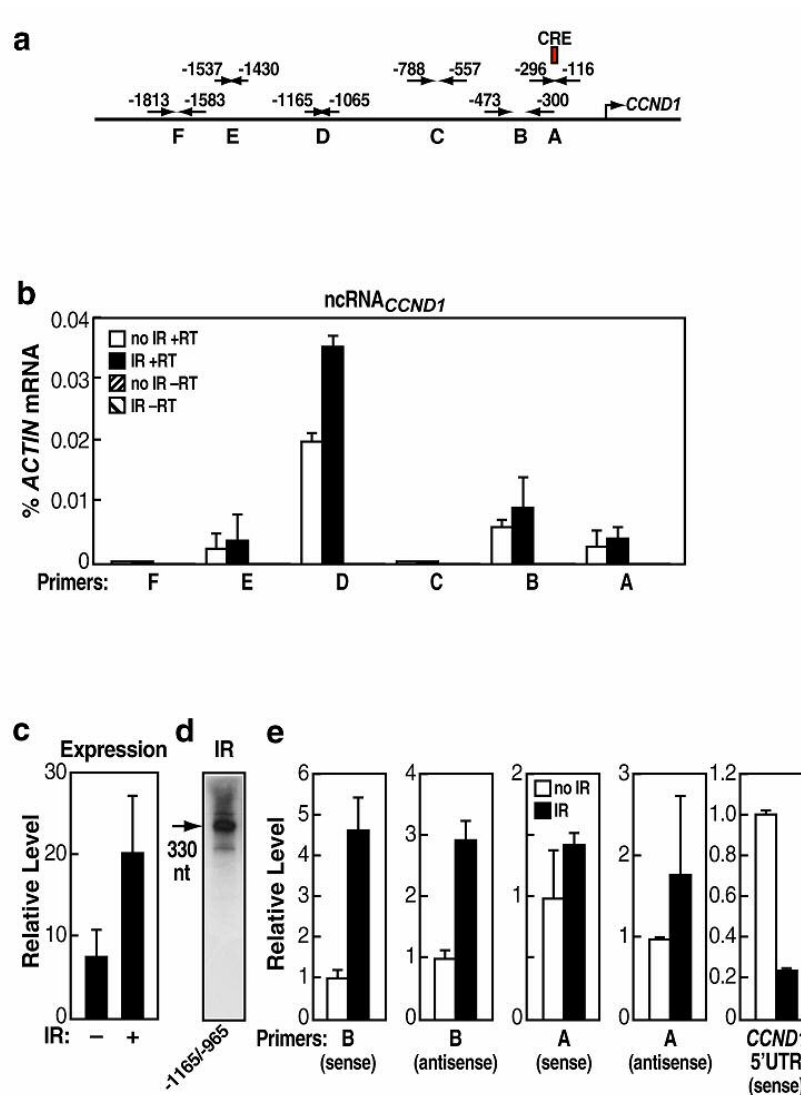


Figure 12. NcRNAs generated from the 5' regulatory region of human *CCND1* gene (ncRNA<sub>CCND1</sub>) are induced upon IR.

**Figure 12. NcRNAs generated from the 5' regulatory region of human *CCND1* gene (ncRNA<sub>CCND1</sub>) are induced upon IR.** **a**, Diagram of primers used to detect ncRNA<sub>CCND1</sub>. **b**, The expression levels of ncRNAs were assayed by real-time PCR, following reverse transcription (RT) using random primers. **c**, Ratio of the ~330 nt transcripts (no IR vs. IR) detected in Northern blot was quantified using NIH Image J program. **d**, Northern blot shows existence of ~330 nt transcripts by using a probe (-1165 to -965) targeted at the 5' regulatory region of *CCND1*. **e**, Strand specific RT real-time PCR shows both sense- and antisense-strand generated ncRNA<sub>CCND1</sub> are upregulated upon IR. In contrast, IR causes a decrease in 5'UTR of the *CCND1* transcript. The primers used and detected strand are indicated at the bottom. Error bars indicate  $\pm$  SEM.

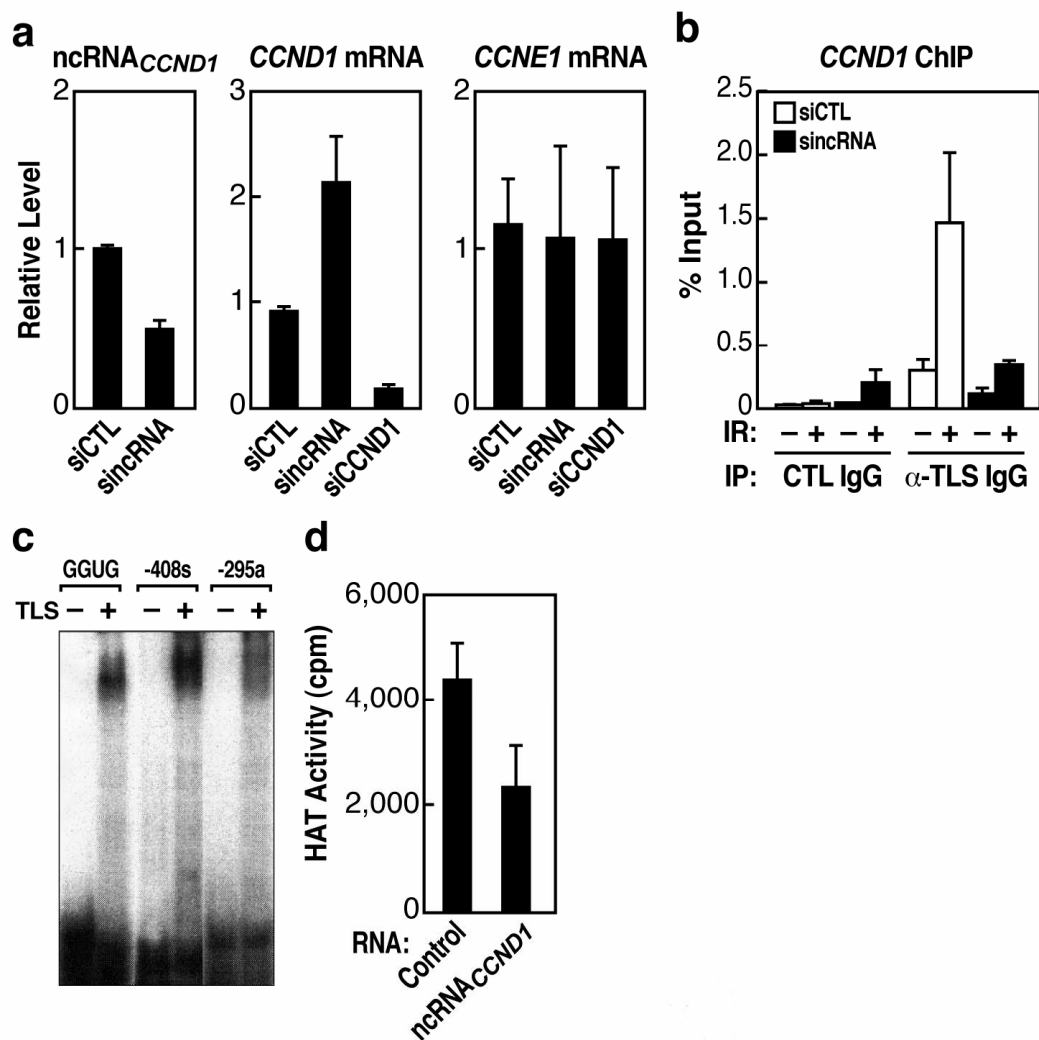


Figure 13. NcRNA<sub>CCND1</sub> negatively regulates *CCND1* transcription through TLS.

**Figure 13. NcRNA<sub>CCND1</sub> negatively regulates *CCND1* transcription through TLS.** **a, Left**, An siRNA targeting the ncRNA<sub>CCND1</sub> (sincRNA) knocks down the ncRNA<sub>CCND1</sub> in HeLa cells, compared with control siRNA (siCTL). **Middle**, Introduction of the sincRNA upregulates *CCND1* mRNA, while the siRNA targeting the *CCND1* coding region (siCCND1) knocks down *CCND1* mRNA. **Right**, Neither sincRNA or siCCND1 regulates the expression of *CCNE1* mRNA. **b**, Occupancy of TLS on the *CCND1* promoter is enhanced upon IR in the presence of siCTL. Introduction of the sincRNA results in a decreased binding of TLS on the *CCND1* promoter indicated by ChIP real-time PCR. **c**, Gel shift assay shows that TLS binds to synthesized GGUG-containing RNA oligonucleotides generated from *CCND1* 5' regulatory region corresponding to ncRNA<sub>CCND1</sub> (-408s and -295a RNA oligonucleotides). **d**, HAT assay shows that p300 HAT activity is inhibited by the synthesized GGUG-containing RNA oligonucleotides generated from *CCND1* 5' regulatory region corresponding to ncRNA<sub>CCND1</sub>. Error bars indicate  $\pm$ SEM.

This Chapter (Chapter 3), in full, is based on material that has been submitted for publication to Nature, as “Stress-induced Non-coding RNAs Allosterically Regulate a HAT-inhibitory RNA Binding Protein to Mediate Transcriptional Repression. X.T. Wang, S. Arai, D. Reichart, T. Oyoshi, X.Y. Song, G. Pascual, D.W. Rose, P. Tempst, M.G. Rosenfeld, C.K. Glass and R. Kurokawa.”

## Chapter 4. DISCUSSION

Forty years ago, it was proposed that sequence-specific RNA might interact with promoters to regulate gene transcription[168]. For a long time, this hypothesis has been underestimated, largely because that ncRNAs are usually less abundant and unstable, making them not easily found, though the interaction of transcription factors with RNA has been reported as well[169, 170]. A large-scale genomic comparison revealed a number of conserved DNA regions located close to transcription factors-encoded genes in the vertebrate genome[101, 103]. The average length of these regions is ~500bp, which is too long to explain their function solely as DNA-binding elements. One likely explanation is that unidentified ncRNAs are generated from such conserved region. The hypothesis is supported by the latest investigations of relative long ncRNAs in mammals *via* multiple mechanisms[115, 117, 119, 120]. On the other hand, the role of short ncRNAs[171, 172] in transcriptional gene silencing (TGS) has been intensively investigated in plant and yeast[173-175]. It is now widely accepted that siRNA can mediate TGS through epigenetic histone methylation and DNA methylation in plant and yeast. In human cells, siRNA-mediated TGS has been shown by transfecting the exogenous siRNA[176-178]. On the other hand, siRNA-mediated transcriptional activation has been reported recently as well[179]. To elucidate the roles of ncRNAs might lead us to solve some puzzles in well-studied regulatory events.

*CCND1* is a key G1 checkpoint regulator. Its expression is tightly regulated during cell cycle[55, 180]. I found, in this work, that previously unrecognized ncRNAs are generated from the 5' regulatory region of *CCND1*. Quantitative real-time PCR shows gaps between the detected ncRNAs in windows around 300-400 nt in length. Northern blot shows a ~330 nt length product induced upon IR. It is possible then that either multiple transcripts are generated from 5' regulatory regions of *CCND1*, or that a single transcript is highly labile and rapidly processed, exhibiting differential stability of the products. Interestingly, my preliminary data showed that the *CCND1* ncRNAs seem to be regulated by DROSHA, an RNase III-like enzyme involved in miRNA biogenesis[181]. TLS, EWS and TAFII15, have been reported as components of flag-DROSHA complex in HEK293 cells[95]. Though the significance of such interaction has not been explored, it is very likely that TET family members are involved in miRNA biogenesis as well. On the other hand, DROSHA could function more than simply as an enzyme in miRNA biogenesis. DROSHA is a nuclear protein and my preliminary ChIP experiments showed that DROSHA binds to multiple gene promoters, including *CCND1* promoter, suggesting a role of DROSHA in gene transcriptional regulation. It is under investigation now how the *CCND1* ncRNAs are regulated. Many questions need to be answered. How is DROSHA involved? Are the ncRNAs capped, polyadenylated or spliced? And are the ncRNAs pol II-regulated?

*CCND1* ncRNAs are detected from both sense and antisense strand of the genomic DNA. However, it is not clear whether a double-strand RNA is formed. It is possible that the antisense strand is the active form, though the significance of such bi-directional transcripts is unclear neither. NcRNAs can mediate both activation and repression in

transcription regulation event. In this work, the expression levels of *CCND1* ncRNAs are enhanced whereas *CCND1* is inhibited upon IR. Moreover, siRNA knocking down experiments proved that the *CCND1* ncRNAs inhibited *CCND1* through recruitment of TLS to the *CCND1* promoter.

Using RNA, instead of protein, makes transcriptional regulation in a more gene specific way through base complimentary between RNA and DNA. Both *CCND1* and *CCNE1* are CREB-mediated and TLS-regulated genes. Similar to the case that *Eyf-2* ncRNA did not regulate Wnt1 enhancer activity[115], *CCND1* ncRNAs do not regulate *CCNE1* gene. This result strongly suggests that *CCND1* ncRNAs regulate transcription in a sequence/target-specific manner. TLS does not bind to all of the tested CREB-mediated genes, such as *TGF $\beta$ 2* and *FZD4*, suggesting that the negative regulation of CREB target genes by TLS is context specific. The promoters of *TGF $\beta$ 2* and *FZD4* have TATA box, whereas *CCND1* and *CCNE1* are TATA-less genes. In fact, most TATA-less CREB targets are genes involved in cell cycle or DNA repair regulation. Quantitative real-time PCR was performed to test whether such difference is due to the existence of gene specific ncRNA. Surprisingly, my preliminary data showed that ncRNA was also detected from the *TGF $\beta$ 2* promoter. This result suggests that specificity of TLS regulation on CREB-targeted genes is more than simply due to the existence of promoter ncRNA.

Cancer is viewed in part as a cell cycle disease. As a key cell cycle regulator, mutations, amplification and overexpression of *CCND1*, are observed frequently in a variety of tumors and may contribute to tumorigenesis. NcRNAs are found to be tightly regulated by environmental stimuli. In fact, ncRNAs consist of the majority of the

transcripts expressed in a highly cell- and condition- specific manner. ncRNAs have been shown to play important roles in a number of cellular processes, especially in regulatory processes such as differentiation or control of apoptosis and cell cycle. It is, therefore, not surprising that a considerable amount of different disease- and particularly tumor-associated ncRNAs have been identified. Trans-acting effects of the *H19* ncRNA have been linked to tumor suppression. Mutations in *SCA8*, a cis-antisense RNA in human, are found in spinocerebellar ataxia patients. Other human disease associated antisense ncRNAs include *DGCR5* in DiGeorge syndrome, *Kvlqtas/Lit1* in Beckwith-Wiedeman syndrome, *CMPD* associated RNA in Campomelic dysplasia. Thus, ncRNAs constitute an important group of potential diagnostic markers, as well as drug targets. As the number of ncRNAs increases and their functions are more precisely understood, it is likely that dysregulation of ncRNA is the basis for many human diseases. Therefore, it would be extremely interesting to investigate the genome-wide expression pattern of ncRNAs in cancer tissues or cultured cells under different stimuli.

Besides their functions in RNA processing, RNA binding proteins have been reported to be involved in transcriptional regulation event, for example, hnRNP K1 is a transcriptional coactivator of p53 in response to DNA damage[182]. RNA binding proteins consist of a large group and are frequently co-immunoprecipitated with transcriptional (co)factors complex. Recent studies of the transcriptome revealed that at least 50% of the genome is transcribed. It would be interesting to investigate the involvement of other RNA binding proteins in ncRNA-mediated transcriptional regulation event, and to elucidate the protein binding specificity of ncRNA. For example, what is the RNA binding protein interacted with *TGFβ2* ncRNA?



The C-terminus of TLS might be an inhibitory domain of TLS function as a CBP/p300 HAT inhibitor is suggested by the observation that the N-terminus of TLS displays a stronger inhibitory effect on CBP HAT activity compared with the full-length TLS. This is supported by the observations that N-terminus, but not the C-terminus of TLS interacts with CBP, and that GGUG-containing oligonucleotides release the N-terminus from association with the C-terminus of TLS, and that adding GGUG-containing oligonucleotides results in an enhanced HAT inhibitory effect by TLS. This is also supported by the observations on EWS from other group during the preparation of this dissertation. They showed that multiple C-terminal RGG motifs of EWS played cis/trans-repression roles on the transcriptional activity of the N-terminus of EWS[183]. RNA binding to TLS may cause an allosteric modification of TLS. Indeed, my preliminary data showed that posttranslational modification of TLS is impeded in RNase A-treated cell extracts. TLS has been shown to be a target of the kinase PKCII $\beta$  or c-Abl[61, 62]. Phosphorylation of TLS by PKCII $\beta$  at serine 256 enhances the protein stability of TLS, which is associated with decreased proteasome-mediated degradation[62]. Interestingly, phosphorylation by c-Abl stimulates the DNA binding activity of TLS[61], whereas phosphorylation by PKCII $\beta$  impedes the RNA binding activity of TLS. EWS can be phosphorylated as well[184-186]. In addition, serum starvation results in EWS association with Pyk2, a cytoplasmic protein tyrosine kinase, and thus keeping EWS in the cytoplasm[185]. My preliminary data showed that phosphorylation of TLS is greatly enhanced in response to IR. It would be great interesting to test posttranslational modifications, such as phosphorylation, on the role of TLS as an RNA-mediated transcriptional coregulator.

TLS is required for NF- $\kappa$ B-mediated activation on model reporters[4]. Upon DNA damage, NF- $\kappa$ B is activated to mediate activation of cell survival genes[187]. Cisplatin is a DNA damaging anticancer drug used most successfully for the testicular cancers[188]. Many NF- $\kappa$ B target genes were greatly induced after treatments of cisplatin and IR, among them *IL6* and *IL8* (Fig. 14a). Knocking down TLS expression blocked the induction of *IL6* and *IL8* upon cisplatin and IR treatment (Fig. 14b). ChIP experiments showed TLS was strongly recruited to the promoters of *IL6* and *IL8* when the genes were activated upon IR and cisplatin treatments (Fig. 14c). In concert, these data suggest that TLS is required for positive regulation of genes that are activated by genotoxic stress. Detectable, low abundant, IR-induced transcripts are found from the 5' regulatory region of *IL6*. It is possible that TLS is recruited to the *IL6* promoter by such transcripts. If so, then it is intriguing to understand the mechanism that distinguishes TLS function as a coactivator on *IL6* and as a corepressor on *CCND1* as ncRNAs are found from both genes' regulatory regions.

To summarize, I have provided evidence that, in response to DNA damage signals, enhanced expression of previously unrecognized transcription units, overlapping with negatively regulated TLS gene targets, generate ncRNAs with ligand-like inhibitory properties that serve as molecular beacons to recruit specific RNA binding proteins, in this case TLS, and cause an allosteric modification that permits specific TLS interactions with CBP/p300, inhibiting CBP/p300 HAT function on histone substrates and causing repression of target genes (Fig. 15). This allows TLS to serve as an integrator of transcriptional programs that are regulated by homeostatic signals and genotoxic stress.

Because these regulatory RNA transcripts are derived from the promoter regions of TLS negatively-regulated genes, and themselves are activated by genotoxic stress, these RNAs, in a sense, actually function as ligands that instruct the inhibitory activities of TLS on CBP/p300 HAT. Only a subset of tested RNAs that can bind TLS are capable of causing the interaction with CBP/p300 or/and inhibiting HAT function. I am tempted to suggest that the regulated expression of these ncRNA transcripts, act *in situ* to recruit TLS to the *CCND1* promoter and cause the specific allosteric modifications resulting in inhibition of CBP/p300 HAT function; conversely, CREB/CBP-dependent transcription units that do not generate ncRNAs would not exhibit TLS-dependent repression. It is likely that different regulatory ncRNAs may have distinct consequences on the action of TLS under different cellular conditions. The impact of TLS interactions with CBP/p300 is proposed to depend primarily on the requirements of specific promoters for the HAT activities of CBP/p300. Further, TLS can now apparently function as a required coactivator, including a subset of genes induced by genotoxic stress. It is possible that a different class of activating ncRNAs exists and mediates TLS function as a transcriptional coactivator. In the case of promoters driven by CREB, the HAT activities of CBP/p300 are essential, and recruitment of TLS functions as a transcriptional corepressor. Intriguingly, TLS did not inhibit the ability of CBP/p300 to acetylate p53, and did not inhibit TIP60 HAT activity, which are crucial for DNA repair. These findings thus identify the roles of novel, genotoxic stress-induced ncRNA transcripts that, on processing, are capable of providing gene-specific recruitment/modulation of TLS, permitting it to exert dual functions in gene repression, or even activation, allowing integration of the transcriptional programs that control proliferation and DNA repair in

response to DNA damage signals. It is tempting to speculate that many other RNA binding factors exert functional roles on gene transcription by analogous regulation by other ncRNAs; conversely, specific RNA binding proteins are likely to be required to mediate known effects of other ncRNAs on transcriptional repression/silencing events.

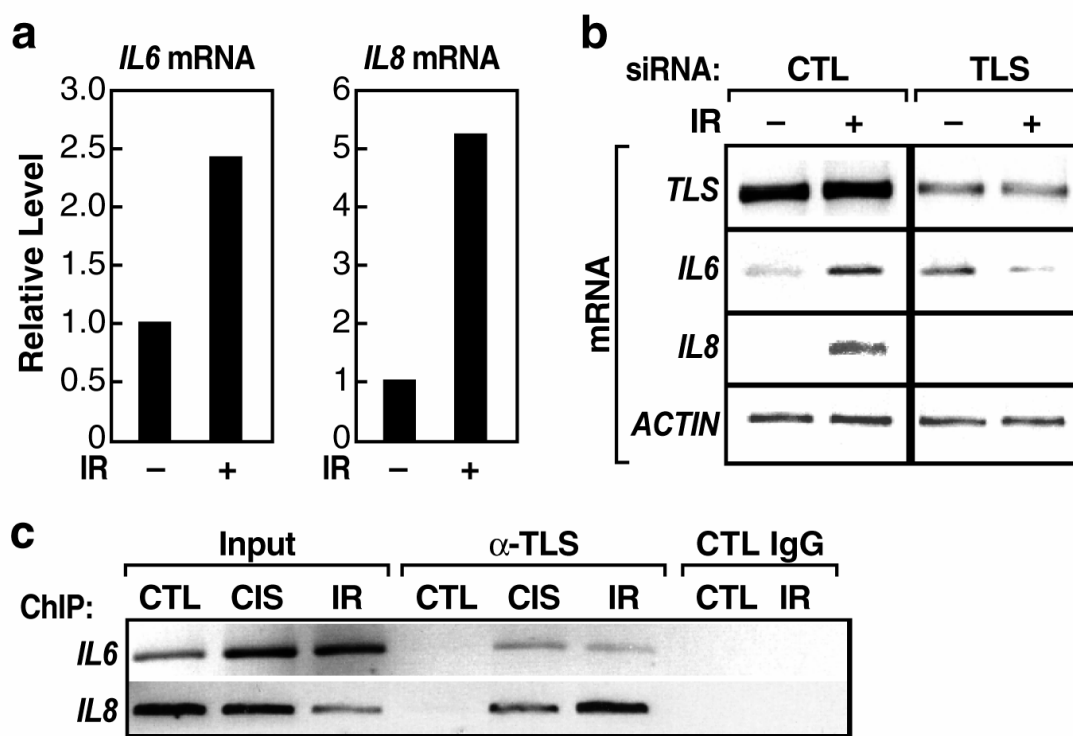


Figure 14. TLS is required for genotoxic stress activated NF $\kappa$ B-mediated genes.

**Figure 14. TLS is required for genotoxic stress activated NF $\kappa$ B-mediated genes..** **a**, *IL6* and *IL8* mRNAs are induced by IR. **b**, Knockdown of TLS expression blocks the induction of *IL6* and *IL8*. **c**, TLS is recruited to the *IL6* and *IL8* promoters upon cisplatin (CIS) and ioning irradiation (IR) treatment as shown by ChIP assay.

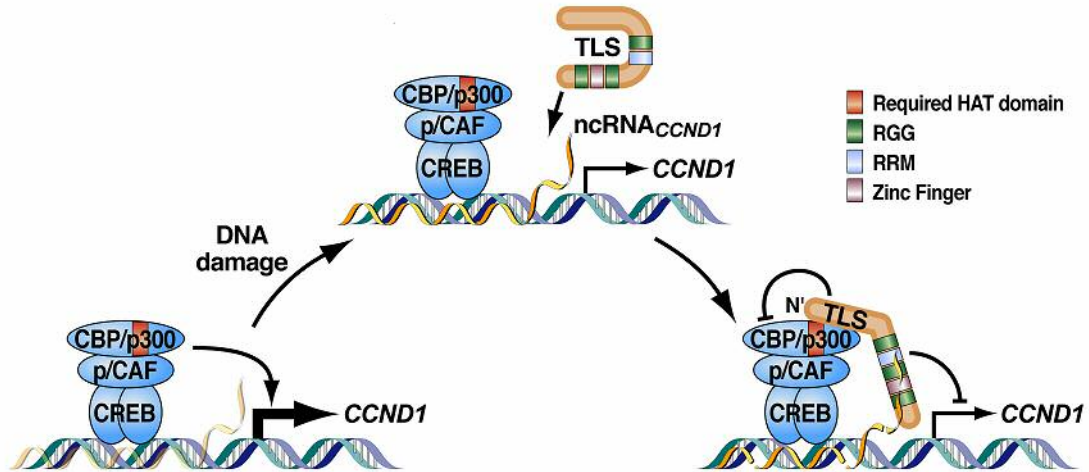


Figure 15. The model.

**Figure 15. The model.**

This Chapter (Chapter 4), in part, is based on material that has been submitted for publication to Nature, as “Stress-induced Non-coding RNAs Allosterically Regulate a HAT-inhibitory RNA Binding Protein to Mediate Transcriptional Repression. X.T. Wang, S. Arai, D. Reichart, T. Oyoshi, X.Y. Song, G. Pascual, D.W. Rose, P. Tempst, M.G. Rosenfeld, C.K. Glass and R. Kurokawa.”

## REFERENCE

- [1] Rosenfeld, M.G., V.V. Lunyak, and C.K. Glass, *Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response*. Genes Dev, 2006. **20**(11): p. 1405-28.
- [2] Glass, C.K. and M.G. Rosenfeld, *The coregulator exchange in transcriptional functions of nuclear receptors*. Genes Dev, 2000. **14**(2): p. 121-41.
- [3] Barone, M.V., et al., *CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G1/S arrest*. Genes Dev, 1994. **8**(4): p. 453-64.
- [4] Uranishi, H., et al., *Involvement of the pro-oncoprotein TLS (translocated in liposarcoma) in nuclear factor-kappa B p65-mediated transcription as a coactivator*. J Biol Chem, 2001. **276**(16): p. 13395-401.
- [5] Immanuel, D., H. Zinszner, and D. Ron, *Association of SARFH (sarcoma-associated RNA-binding fly homolog) with regions of chromatin transcribed by RNA polymerase II*. Mol Cell Biol, 1995. **15**(8): p. 4562-71.
- [6] Law, W.J., K.L. Cann, and G.G. Hicks, *TLS, EWS and TAF15: a model for transcriptional integration of gene expression*. Brief Funct Genomic Proteomic, 2006. **5**(1): p. 8-14.
- [7] Chansky, H.A., et al., *Oncogenic TLS/ERG and EWS/Fli-1 fusion proteins inhibit RNA splicing mediated by YB-1 protein*. Cancer Res, 2001. **61**(9): p. 3586-90.
- [8] Hallier, M., et al., *The transcription factor Spi-1/PU.1 interacts with the potential splicing factor TLS*. J Biol Chem, 1998. **273**(9): p. 4838-42.
- [9] Zinszner, H., et al., *TLS (FUS) binds RNA in vivo and engages in nucleocytoplasmic shuttling*. J Cell Sci, 1997. **110 ( Pt 15)**: p. 1741-50.
- [10] Meissner, M., et al., *Proto-oncoprotein TLS/FUS is associated to the nuclear matrix and complexed with splicing factors PTB, SRm160, and SR proteins*. Exp Cell Res, 2003. **283**(2): p. 184-95.

- [11] Yang, L., et al., *Oncoprotein TLS interacts with serine-arginine proteins involved in RNA splicing*. J Biol Chem, 1998. **273**(43): p. 27761-4.
- [12] Yang, L., L.J. Embree, and D.D. Hickstein, *TLS-ERG leukemia fusion protein inhibits RNA splicing mediated by serine-arginine proteins*. Mol Cell Biol, 2000. **20**(10): p. 3345-54.
- [13] Fujii, R., et al., *The RNA binding protein TLS is translocated to dendritic spines by mGluR5 activation and regulates spine morphology*. Curr Biol, 2005. **15**(6): p. 587-93.
- [14] Kuroda, M., et al., *Male sterility and enhanced radiation sensitivity in TLS(-/-) mice*. Embo J, 2000. **19**(3): p. 453-62.
- [15] Hicks, G.G., et al., *Fus deficiency in mice results in defective B-lymphocyte development and activation, high levels of chromosomal instability and perinatal death*. Nat Genet, 2000. **24**(2): p. 175-9.
- [16] Baechtold, H., et al., *Human 75-kDa DNA-pairing protein is identical to the pro-oncoprotein TLS/FUS and is able to promote D-loop formation*. J Biol Chem, 1999. **274**(48): p. 34337-42.
- [17] Bertrand, P., et al., *Human POMp75 is identified as the pro-oncoprotein TLS/FUS: both POMp75 and POMp100 DNA homologous pairing activities are associated to cell proliferation*. Oncogene, 1999. **18**(31): p. 4515-21.
- [18] Morohoshi, F., et al., *Genomic structure of the human RBP56/hTAFII68 and FUS/TLS genes*. Gene, 1998. **221**(2): p. 191-8.
- [19] Ichikawa, H., et al., *Dual transforming activities of the FUS (TLS)-ERG leukemia fusion protein conferred by two N-terminal domains of FUS (TLS)*. Mol Cell Biol, 1999. **19**(11): p. 7639-50.
- [20] Crozat, A., et al., *Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma*. Nature, 1993. **363**(6430): p. 640-4.
- [21] Kanoe, H., et al., *Characteristics of genomic breakpoints in TLS-CHOP translocations in liposarcomas suggest the involvement of Translin and topoisomerase II in the process of translocation*. Oncogene, 1999. **18**(3): p. 721-9.
- [22] Rabbitts, T.H., et al., *Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t(12;16) in malignant liposarcoma*. Nat Genet, 1993. **4**(2): p. 175-80.



- [23] Panagopoulos, I., et al., *Fusion of the FUS gene with ERG in acute myeloid leukemia with t(16;21)(p11;q22)*. Genes Chromosomes Cancer, 1994. **11**(4): p. 256-62.
- [24] Ichikawa, H., et al., *An RNA-binding protein gene, TLS/FUS, is fused to ERG in human myeloid leukemia with t(16;21) chromosomal translocation*. Cancer Res, 1994. **54**(11): p. 2865-8.
- [25] Ron, D. and J.F. Habener, *CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription*. Genes Dev, 1992. **6**(3): p. 439-53.
- [26] Batchvarova, N., X.Z. Wang, and D. Ron, *Inhibition of adipogenesis by the stress-induced protein CHOP (Gadd153)*. Embo J, 1995. **14**(19): p. 4654-61.
- [27] Wang, X.Z., et al., *Identification of novel stress-induced genes downstream of chop*. Embo J, 1998. **17**(13): p. 3619-30.
- [28] Wang, X.Z., et al., *Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153)*. Mol Cell Biol, 1996. **16**(8): p. 4273-80.
- [29] Wang, X.Z. and D. Ron, *Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase*. Science, 1996. **272**(5266): p. 1347-9.
- [30] Bruhat, A., et al., *Amino acid limitation induces expression of CHOP, a CCAAT/enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels*. J Biol Chem, 1997. **272**(28): p. 17588-93.
- [31] Silva, R.M., et al., *CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an in vivo neurotoxin model of parkinsonism*. J Neurochem, 2005. **95**(4): p. 974-86.
- [32] Sok, J., et al., *CHOP-Dependent stress-inducible expression of a novel form of carbonic anhydrase VI*. Mol Cell Biol, 1999. **19**(1): p. 495-504.
- [33] Ubeda, M., et al., *Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element*. Mol Cell Biol, 1996. **16**(4): p. 1479-89.
- [34] Zinszner, H., R. Albalat, and D. Ron, *A novel effector domain from the RNA-binding protein TLS or EWS is required for oncogenic transformation by CHOP*. Genes Dev, 1994. **8**(21): p. 2513-26.

- [35] Perez-Losada, J., et al., *The chimeric FUS/TLS-CHOP fusion protein specifically induces liposarcomas in transgenic mice*. *Oncogene*, 2000. **19**(20): p. 2413-22.
- [36] Perez-Losada, J., et al., *Liposarcoma initiated by FUS/TLS-CHOP: the FUS/TLS domain plays a critical role in the pathogenesis of liposarcoma*. *Oncogene*, 2000. **19**(52): p. 6015-22.
- [37] Zinszner, H., et al., *A topogenic role for the oncogenic N-terminus of TLS: nucleolar localization when transcription is inhibited*. *Oncogene*, 1997. **14**(4): p. 451-61.
- [38] Bertolotti, A., et al., *hTAF(II)68, a novel RNA/ssDNA-binding protein with homology to the pro-oncoproteins TLS/FUS and EWS is associated with both TFIID and RNA polymerase II*. *Embo J*, 1996. **15**(18): p. 5022-31.
- [39] Bertolotti, A., B. Bell, and L. Tora, *The N-terminal domain of human TAFII68 displays transactivation and oncogenic properties*. *Oncogene*, 1999. **18**(56): p. 8000-10.
- [40] Arvand, A. and C.T. Denny, *Biology of EWS/ETS fusions in Ewing's family tumors*. *Oncogene*, 2001. **20**(40): p. 5747-54.
- [41] Jaishankar, S., et al., *Transforming activity of EWS/FLI is not strictly dependent upon DNA-binding activity*. *Oncogene*, 1999. **18**(40): p. 5592-7.
- [42] Petermann, R., et al., *Oncogenic EWS-Fli1 interacts with hsRPB7, a subunit of human RNA polymerase II*. *Oncogene*, 1998. **17**(5): p. 603-10.
- [43] Sheppard, K.A., et al., *Transcriptional activation by NF-kappaB requires multiple coactivators*. *Mol Cell Biol*, 1999. **19**(9): p. 6367-78.
- [44] Sato, S., et al., *beta-catenin interacts with the FUS proto-oncogene product and regulates pre-mRNA splicing*. *Gastroenterology*, 2005. **129**(4): p. 1225-36.
- [45] Powers, C.A., et al., *TLS (translocated-in-liposarcoma) is a high-affinity interactor for steroid, thyroid hormone, and retinoid receptors*. *Mol Endocrinol*, 1998. **12**(1): p. 4-18.
- [46] Lerga, A., et al., *Identification of an RNA binding specificity for the potential splicing factor TLS*. *J Biol Chem*, 2001. **276**(9): p. 6807-16.
- [47] Iko, Y., et al., *Domain architectures and characterization of an RNA-binding protein, TLS*. *J Biol Chem*, 2004. **279**(43): p. 44834-40.

- [48] Fujii, R. and T. Takumi, *TLS facilitates transport of mRNA encoding an actin-stabilizing protein to dendritic spines*. J Cell Sci, 2005. **118**(Pt 24): p. 5755-65.
- [49] Calvio, C., et al., *Identification of hnRNP P2 as TLS/FUS using electrospray mass spectrometry*. Rna, 1995. **1**(7): p. 724-33.
- [50] Zhang, D. and G. Childs, *Human ZFMI protein is a transcriptional repressor that interacts with the transcription activation domain of stage-specific activator protein*. J Biol Chem, 1998. **273**(12): p. 6868-77.
- [51] Covini, N., et al., *ZFMI/SF1 mRNA in rat and gerbil brain after global ischaemia*. Eur J Neurosci, 1999. **11**(3): p. 781-7.
- [52] Amson, R.B., et al., *Isolation of 10 differentially expressed cDNAs in p53-induced apoptosis: activation of the vertebrate homologue of the drosophila seven in absentia gene*. Proc Natl Acad Sci U S A, 1996. **93**(9): p. 3953-7.
- [53] Zhang, D., A.J. Paley, and G. Childs, *The transcriptional repressor ZFMI interacts with and modulates the ability of EWS to activate transcription*. J Biol Chem, 1998. **273**(29): p. 18086-91.
- [54] Knoop, L.L. and S.J. Baker, *The splicing factor UIC represses EWS/FLI-mediated transactivation*. J Biol Chem, 2000. **275**(32): p. 24865-71.
- [55] Agami, R. and R. Bernards, *Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage*. Cell, 2000. **102**(1): p. 55-66.
- [56] Bassing, C.H. and F.W. Alt, *The cellular response to general and programmed DNA double strand breaks*. DNA Repair (Amst), 2004. **3**(8-9): p. 781-96.
- [57] Xu, Y. and D. Baltimore, *Dual roles of ATM in the cellular response to radiation and in cell growth control*. Genes Dev, 1996. **10**(19): p. 2401-10.
- [58] Kharbanda, S., et al., *Functional role for the c-Abl tyrosine kinase in meiosis I*. Oncogene, 1998. **16**(14): p. 1773-7.
- [59] Kharbanda, S., et al., *Determination of cell fate by c-Abl activation in the response to DNA damage*. Oncogene, 1998. **17**(25): p. 3309-18.
- [60] Celeste, A., et al., *Genomic instability in mice lacking histone H2AX*. Science, 2002. **296**(5569): p. 922-7.

- [61] Perrotti, D., et al., *TLS/FUS, a pro-oncogene involved in multiple chromosomal translocations, is a novel regulator of BCR/ABL-mediated leukemogenesis*. *Embo J*, 1998. **17**(15): p. 4442-55.
- [62] Perrotti, D., et al., *BCR-ABL prevents c-jun-mediated and proteasome-dependent FUS (TLS) proteolysis through a protein kinase CbetaII-dependent pathway*. *Mol Cell Biol*, 2000. **20**(16): p. 6159-69.
- [63] Ogryzko, V.V., et al., *The transcriptional coactivators p300 and CBP are histone acetyltransferases*. *Cell*, 1996. **87**(5): p. 953-9.
- [64] Spiegelman, B.M. and R. Heinrich, *Biological control through regulated transcriptional coactivators*. *Cell*, 2004. **119**(2): p. 157-67.
- [65] Bannister, A.J. and T. Kouzarides, *The CBP co-activator is a histone acetyltransferase*. *Nature*, 1996. **384**(6610): p. 641-3.
- [66] Korzus, E., et al., *Transcription factor-specific requirements for coactivators and their acetyltransferase functions*. *Science*, 1998. **279**(5351): p. 703-7.
- [67] McKenna, N.J. and B.W. O'Malley, *Combinatorial control of gene expression by nuclear receptors and coregulators*. *Cell*, 2002. **108**(4): p. 465-74.
- [68] Murata, T., et al., *Defect of histone acetyltransferase activity of the nuclear transcriptional coactivator CBP in Rubinstein-Taybi syndrome*. *Hum Mol Genet*, 2001. **10**(10): p. 1071-6.
- [69] Lavau, C., et al., *Chromatin-related properties of CBP fused to MLL generate a myelodysplastic-like syndrome that evolves into myeloid leukemia*. *Embo J*, 2000. **19**(17): p. 4655-64.
- [70] Snowden, A.W. and N.D. Perkins, *Cell cycle regulation of the transcriptional coactivators p300 and CREB binding protein*. *Biochem Pharmacol*, 1998. **55**(12): p. 1947-54.
- [71] Grossman, S.R., et al., *Polyubiquitination of p53 by a ubiquitin ligase activity of p300*. *Science*, 2003. **300**(5617): p. 342-4.
- [72] Col, E., et al., *HIV-1 Tat targets Tip60 to impair the apoptotic cell response to genotoxic stresses*. *Embo J*, 2005. **24**(14): p. 2634-45.
- [73] Linares, L.K., et al., *Intrinsic ubiquitination activity of PCAF controls the stability of the oncoprotein Hdm2*. *Nat Cell Biol*, 2007.

- [74] Perissi, V., et al., *Factor-specific modulation of CREB-binding protein acetyltransferase activity*. Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3652-7.
- [75] Sterner, D.E. and S.L. Berger, *Acetylation of histones and transcription-related factors*. Microbiol Mol Biol Rev, 2000. **64**(2): p. 435-59.
- [76] Torchia, J., et al., *The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function*. Nature, 1997. **387**(6634): p. 677-84.
- [77] Kurokawa, R., et al., *Differential use of CREB binding protein-coactivator complexes*. Science, 1998. **279**(5351): p. 700-3.
- [78] Radhakrishnan, I., et al., *Solution structure of the KIX domain of CBP bound to the transactivation domain of CREB: a model for activator:coactivator interactions*. Cell, 1997. **91**(6): p. 741-52.
- [79] Shi, Y., et al., *Direct regulation of CREB transcriptional activity by ATM in response to genotoxic stress*. Proc Natl Acad Sci U S A, 2004. **101**(16): p. 5898-903.
- [80] Yang, X.J., et al., *A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A*. Nature, 1996. **382**(6589): p. 319-24.
- [81] Shen, W.F., et al., *The HOX homeodomain proteins block CBP histone acetyltransferase activity*. Mol Cell Biol, 2001. **21**(21): p. 7509-22.
- [82] Merienne, K., et al., *Mitogen-regulated RSK2-CBP interaction controls their kinase and acetylase activities*. Mol Cell Biol, 2001. **21**(20): p. 7089-96.
- [83] Kazantsev, A., et al., *Insoluble detergent-resistant aggregates form between pathological and nonpathological lengths of polyglutamine in mammalian cells*. Proc Natl Acad Sci U S A, 1999. **96**(20): p. 11404-9.
- [84] Steffan, J.S., et al., *Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila*. Nature, 2001. **413**(6857): p. 739-43.
- [85] Ikura, T., et al., *Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis*. Cell, 2000. **102**(4): p. 463-73.
- [86] Brenner, S., *RNA, ribosomes, and protein synthesis*. Cold Spring Harb Symp Quant Biol, 1961. **26**: p. 101-10.
- [87] Busch, H., et al., *SnRNAs, SnRNPs, and RNA processing*. Annu Rev Biochem, 1982. **51**: p. 617-54.

- [88] Costa, F.F., *Non-coding RNAs: Lost in translation?* Gene, 2007. **386**(1-2): p. 1-10.
- [89] Eddy, S.R., *Noncoding RNA genes*. Curr Opin Genet Dev, 1999. **9**(6): p. 695-9.
- [90] Eddy, S.R., *Non-coding RNA genes and the modern RNA world*. Nat Rev Genet, 2001. **2**(12): p. 919-29.
- [91] Erdmann, V.A., et al., *Regulatory RNAs*. Cell Mol Life Sci, 2001. **58**(7): p. 960-77.
- [92] Erdmann, V.A., et al., *The non-coding RNAs as riboregulators*. Nucleic Acids Res, 2001. **29**(1): p. 189-93.
- [93] Goodrich, J.A. and J.F. Kugel, *Non-coding-RNA regulators of RNA polymerase II transcription*. Nat Rev Mol Cell Biol, 2006. **7**(8): p. 612-6.
- [94] Gottesman, S., *Micros for microbes: non-coding regulatory RNAs in bacteria*. Trends Genet, 2005. **21**(7): p. 399-404.
- [95] Gregory, R.I., et al., *The Microprocessor complex mediates the genesis of microRNAs*. Nature, 2004. **432**(7014): p. 235-40.
- [96] Mattick, J.S. and I.V. Makunin, *Non-coding RNA*. Hum Mol Genet, 2006. **15 Spec No 1**: p. R17-29.
- [97] Lander, E.S., et al., *Initial sequencing and analysis of the human genome*. Nature, 2001. **409**(6822): p. 860-921.
- [98] Frith, M.C., M. Pheasant, and J.S. Mattick, *The amazing complexity of the human transcriptome*. Eur J Hum Genet, 2005. **13**(8): p. 894-7.
- [99] MacIntosh, G.C., C. Wilkerson, and P.J. Green, *Identification and analysis of Arabidopsis expressed sequence tags characteristic of non-coding RNAs*. Plant Physiol, 2001. **127**(3): p. 765-76.
- [100] Prabhakar, S., et al., *Accelerated evolution of conserved noncoding sequences in humans*. Science, 2006. **314**(5800): p. 786.
- [101] Venkatesh, B., et al., *Ancient noncoding elements conserved in the human genome*. Science, 2006. **314**(5807): p. 1892.
- [102] Waterston, R.H., et al., *Initial sequencing and comparative analysis of the mouse genome*. Nature, 2002. **420**(6915): p. 520-62.

- [103] Woolfe, A., et al., *Highly conserved non-coding sequences are associated with vertebrate development*. PLoS Biol, 2005. **3**(1): p. e7.
- [104] Zieve, G.W., *Two groups of small stable RNAs*. Cell, 1981. **25**(2): p. 296-7.
- [105] Tarn, W.Y. and J.A. Steitz, *Highly diverged U4 and U6 small nuclear RNAs required for splicing rare AT-AC introns*. Science, 1996. **273**(5283): p. 1824-32.
- [106] Sharp, P.A. and C.B. Burge, *Classification of introns: U2-type or U12-type*. Cell, 1997. **91**(7): p. 875-9.
- [107] Eliceiri, G.L., *Small nucleolar RNAs*. Cell Mol Life Sci, 1999. **56**(1-2): p. 22-31.
- [108] Cavaille, J., et al., *Identification of brain-specific and imprinted small nucleolar RNA genes exhibiting an unusual genomic organization*. Proc Natl Acad Sci U S A, 2000. **97**(26): p. 14311-6.
- [109] Meguro, M., et al., *Large-scale evaluation of imprinting status in the Prader-Willi syndrome region: an imprinted direct repeat cluster resembling small nucleolar RNA genes*. Hum Mol Genet, 2001. **10**(4): p. 383-94.
- [110] Franke, A. and B.S. Baker, *Dosage compensation rox!* Curr Opin Cell Biol, 2000. **12**(3): p. 351-4.
- [111] Brannan, C.I. and M.S. Bartolomei, *Mechanisms of genomic imprinting*. Curr Opin Genet Dev, 1999. **9**(2): p. 164-70.
- [112] Avner, P. and E. Heard, *X-chromosome inactivation: counting, choice and initiation*. Nat Rev Genet, 2001. **2**(1): p. 59-67.
- [113] Kelley, R.L. and M.I. Kuroda, *Noncoding RNA genes in dosage compensation and imprinting*. Cell, 2000. **103**(1): p. 9-12.
- [114] Tilghman, S.M., *The sins of the fathers and mothers: genomic imprinting in mammalian development*. Cell, 1999. **96**(2): p. 185-93.
- [115] Feng, J., et al., *The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator*. Genes Dev, 2006. **20**(11): p. 1470-84.
- [116] Martens, J.A., L. Laprade, and F. Winston, *Intergenic transcription is required to repress the Saccharomyces cerevisiae SER3 gene*. Nature, 2004. **429**(6991): p. 571-4.

- [117] Martianov, I., et al., *Repression of the human dihydrofolate reductase gene by a non-coding interfering transcript*. Nature, 2007.
- [118] O'Gorman, W., et al., *Non-coding RNA in transcription initiation*. Biochem Soc Symp, 2006(73): p. 131-40.
- [119] Petruk, S., et al., *Transcription of bxd noncoding RNAs promoted by trithorax represses Ubx in cis by transcriptional interference*. Cell, 2006. **127**(6): p. 1209-21.
- [120] Sanchez-Elsner, T., et al., *Noncoding RNAs of trithorax response elements recruit Drosophila Ash1 to Ultrabithorax*. Science, 2006. **311**(5764): p. 1118-23.
- [121] Szymanski, M., et al., *A new frontier for molecular medicine: noncoding RNAs*. Biochim Biophys Acta, 2005. **1756**(1): p. 65-75.
- [122] Nemes, J.P., et al., *The SCA8 transcript is an antisense RNA to a brain-specific transcript encoding a novel actin-binding protein (KLHL1)*. Hum Mol Genet, 2000. **9**(10): p. 1543-51.
- [123] Sutherland, H.F., et al., *Identification of a novel transcript disrupted by a balanced translocation associated with DiGeorge syndrome*. Am J Hum Genet, 1996. **59**(1): p. 23-31.
- [124] Lee, M.P., et al., *Loss of imprinting of a paternally expressed transcript, with antisense orientation to KVLQTI, occurs frequently in Beckwith-Wiedemann syndrome and is independent of insulin-like growth factor II imprinting*. Proc Natl Acad Sci U S A, 1999. **96**(9): p. 5203-8.
- [125] Lanz, R.B., et al., *A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex*. Cell, 1999. **97**(1): p. 17-27.
- [126] Cassiday, L.A., et al., *Binding stoichiometry of an RNA aptamer and its transcription factor target*. Anal Biochem, 2002. **306**(2): p. 290-7.
- [127] Lanz, R.B., et al., *Steroid receptor RNA activator stimulates proliferation as well as apoptosis in vivo*. Mol Cell Biol, 2003. **23**(20): p. 7163-76.
- [128] Lanz, R.B., et al., *Distinct RNA motifs are important for coactivation of steroid hormone receptors by steroid receptor RNA activator (SRA)*. Proc Natl Acad Sci U S A, 2002. **99**(25): p. 16081-6.
- [129] Chooniedass-Kothari, S., et al., *The steroid receptor RNA activator is the first functional RNA encoding a protein*. FEBS Lett, 2004. **566**(1-3): p. 43-7.



- [130] Kawashima, H., et al., *A novel steroid receptor co-activator protein (SRAP) as an alternative form of steroid receptor RNA-activator gene: expression in prostate cancer cells and enhancement of androgen receptor activity*. *Biochem J*, 2003. **369**(Pt 1): p. 163-71.
- [131] Murphy, L.C., et al., *Altered expression of estrogen receptor coregulators during human breast tumorigenesis*. *Cancer Res*, 2000. **60**(22): p. 6266-71.
- [132] Shi, Y., et al., *Sharp, an inducible cofactor that integrates nuclear receptor repression and activation*. *Genes Dev*, 2001. **15**(9): p. 1140-51.
- [133] Hatchell, E.C., et al., *SLIRP, a small SRA binding protein, is a nuclear receptor corepressor*. *Mol Cell*, 2006. **22**(5): p. 657-68.
- [134] Cobos, I., et al., *Mice lacking *Dlx1* show subtype-specific loss of interneurons, reduced inhibition and epilepsy*. *Nat Neurosci*, 2005. **8**(8): p. 1059-68.
- [135] Acampora, D., et al., *Craniofacial, vestibular and bone defects in mice lacking the *Distal-less-related* gene *Dlx5**. *Development*, 1999. **126**(17): p. 3795-809.
- [136] Anderson, S.A., et al., *Interneuron migration from basal forebrain to neocortex: dependence on *Dlx* genes*. *Science*, 1997. **278**(5337): p. 474-6.
- [137] Anderson, S.A., et al., *Mutations of the homeobox genes *Dlx-1* and *Dlx-2* disrupt the striatal subventricular zone and differentiation of late born striatal neurons*. *Neuron*, 1997. **19**(1): p. 27-37.
- [138] Depew, M.J., et al., **Dlx5* regulates regional development of the branchial arches and sensory capsules*. *Development*, 1999. **126**(17): p. 3831-46.
- [139] Robledo, R.F., et al., *The *Dlx5* and *Dlx6* homeobox genes are essential for craniofacial, axial, and appendicular skeletal development*. *Genes Dev*, 2002. **16**(9): p. 1089-101.
- [140] Zerucha, T., et al., *A highly conserved enhancer in the *Dlx5/Dlx6* intergenic region is the site of cross-regulatory interactions between *Dlx* genes in the embryonic forebrain*. *J Neurosci*, 2000. **20**(2): p. 709-21.
- [141] Ghanem, N., et al., *Regulatory roles of conserved intergenic domains in vertebrate *Dlx* bigene clusters*. *Genome Res*, 2003. **13**(4): p. 533-43.
- [142] Kohtz, J.D. and G. Fishell, *Developmental regulation of *EVF-1*, a novel non-coding RNA transcribed upstream of the mouse *Dlx6* gene*. *Gene Expr Patterns*, 2004. **4**(4): p. 407-12.

- [143] Iler, N., et al., *A single homeodomain binding site restricts spatial expression of Wnt-1 in the developing brain*. Mech Dev, 1995. **53**(1): p. 87-96.
- [144] Cumberledge, S., A. Zaratzian, and S. Sakonju, *Characterization of two RNAs transcribed from the cis-regulatory region of the abd-A domain within the Drosophila bithorax complex*. Proc Natl Acad Sci U S A, 1990. **87**(9): p. 3259-63.
- [145] Lipshitz, H.D., D.A. Peattie, and D.S. Hogness, *Novel transcripts from the Ultrabithorax domain of the bithorax complex*. Genes Dev, 1987. **1**(3): p. 307-22.
- [146] Sanchez-Herrero, E. and M. Akam, *Spatially ordered transcription of regulatory DNA in the bithorax complex of Drosophila*. Development, 1989. **107**(2): p. 321-9.
- [147] Bender, W. and D.P. Fitzgerald, *Transcription activates repressed domains in the Drosophila bithorax complex*. Development, 2002. **129**(21): p. 4923-30.
- [148] Hodgson, J.W., B. Argiropoulos, and H.W. Brock, *Site-specific recognition of a 70-base-pair element containing d(GA)(n) repeats mediates bithoraxoid polycomb group response element-dependent silencing*. Mol Cell Biol, 2001. **21**(14): p. 4528-43.
- [149] Rank, G., M. Prestel, and R. Paro, *Transcription through intergenic chromosomal memory elements of the Drosophila bithorax complex correlates with an epigenetic switch*. Mol Cell Biol, 2002. **22**(22): p. 8026-34.
- [150] Hogga, I. and F. Karch, *Transcription through the iab-7 cis-regulatory domain of the bithorax complex interferes with maintenance of Polycomb-mediated silencing*. Development, 2002. **129**(21): p. 4915-22.
- [151] Grimaud, C., N. Negre, and G. Cavalli, *From genetics to epigenetics: the tale of Polycomb group and trithorax group genes*. Chromosome Res, 2006. **14**(4): p. 363-75.
- [152] Schmitt, S., M. Prestel, and R. Paro, *Intergenic transcription through a polycomb group response element counteracts silencing*. Genes Dev, 2005. **19**(6): p. 697-708.
- [153] Tillib, S., et al., *Trithorax- and Polycomb-group response elements within an Ultrabithorax transcription maintenance unit consist of closely situated but separable sequences*. Mol Cell Biol, 1999. **19**(7): p. 5189-202.
- [154] Pirrotta, V., et al., *Distinct parasegmental and imaginal enhancers and the establishment of the expression pattern of the Ubx gene*. Genetics, 1995. **141**(4): p. 1439-50.

- [155] Blume, S.W., et al., *The 5'-untranslated RNA of the human dhfr minor transcript alters transcription pre-initiation complex assembly at the major (core) promoter.* J Cell Biochem, 2003. **88**(1): p. 165-80.
- [156] Masters, J.N. and G. Attardi, *Discrete human dihydrofolate reductase gene transcripts present in polysomal RNA map with their 5' ends several hundred nucleotides upstream of the main mRNA start site.* Mol Cell Biol, 1985. **5**(3): p. 493-500.
- [157] Letai, A.G., et al., *Specificity in formation of triple-stranded nucleic acid helical complexes: studies with agarose-linked polyribonucleotide affinity columns.* Biochemistry, 1988. **27**(26): p. 9108-12.
- [158] Marck, C. and D. Thiele, *Poly(dG).poly(dC) at neutral and alkaline pH: the formation of triple stranded poly(dG).poly(dG).poly(dC).* Nucleic Acids Res, 1978. **5**(3): p. 1017-28.
- [159] Morgan, A.R. and R.D. Wells, *Specificity of the three-stranded complex formation between double-stranded DNA and single-stranded RNA containing repeating nucleotide sequences.* J Mol Biol, 1968. **37**(1): p. 63-80.
- [160] Erdjument-Bromage, H., et al., *Examination of micro-tip reversed-phase liquid chromatographic extraction of peptide pools for mass spectrometric analysis.* J Chromatogr A, 1998. **826**(2): p. 167-81.
- [161] Sebastiaan Winkler, G., et al., *Isolation and mass spectrometry of transcription factor complexes.* Methods, 2002. **26**(3): p. 260-9.
- [162] Whitlock, J.P., Jr. and R.T. Simpson, *Preparation and physical characterization of a homogeneous population of monomeric nucleosomes from HeLa cells.* Nucleic Acids Res, 1976. **3**(9): p. 2255-66.
- [163] Dignam, J.D., R.M. Lebovitz, and R.G. Roeder, *Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei.* Nucleic Acids Res, 1983. **11**(5): p. 1475-89.
- [164] Niranjanakumari, S., et al., *Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions in vivo.* Methods, 2002. **26**(2): p. 182-90.
- [165] Seo, S.B., et al., *Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein.* Cell, 2001. **104**(1): p. 119-30.

- [166] Impey, S., et al., *Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions*. Cell, 2004. **119**(7): p. 1041-54.
- [167] Zhang, X., et al., *Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues*. Proc Natl Acad Sci U S A, 2005. **102**(12): p. 4459-64.
- [168] Jacob, F. and J. Monod, *Genetic regulatory mechanisms in the synthesis of proteins*. J Mol Biol, 1961. **3**: p. 318-56.
- [169] Cassiday, L.A. and L.J. Maher, 3rd, *Having it both ways: transcription factors that bind DNA and RNA*. Nucleic Acids Res, 2002. **30**(19): p. 4118-26.
- [170] Dubnau, J. and G. Struhl, *RNA recognition and translational regulation by a homeodomain protein*. Nature, 1996. **379**(6567): p. 694-9.
- [171] Bernstein, E., et al., *Role for a bidentate ribonuclease in the initiation step of RNA interference*. Nature, 2001. **409**(6818): p. 363-6.
- [172] Borchert, G.M., W. Lanier, and B.L. Davidson, *RNA polymerase III transcribes human microRNAs*. Nat Struct Mol Biol, 2006. **13**(12): p. 1097-101.
- [173] Borsani, O., et al., *Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis*. Cell, 2005. **123**(7): p. 1279-91.
- [174] Chen, K. and N. Rajewsky, *The evolution of gene regulation by transcription factors and microRNAs*. Nat Rev Genet, 2007. **8**(2): p. 93-103.
- [175] Raponi, M. and G.M. Arndt, *Double-stranded RNA-mediated gene silencing in fission yeast*. Nucleic Acids Res, 2003. **31**(15): p. 4481-9.
- [176] Morris, K.V., et al., *Small interfering RNA-induced transcriptional gene silencing in human cells*. Science, 2004. **305**(5688): p. 1289-92.
- [177] Mette, M.F., et al., *Transcriptional silencing and promoter methylation triggered by double-stranded RNA*. Embo J, 2000. **19**(19): p. 5194-201.
- [178] Ting, A.H., et al., *Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation*. Nat Genet, 2005. **37**(8): p. 906-10.
- [179] Li, L.C., et al., *Small dsRNAs induce transcriptional activation in human cells*. Proc Natl Acad Sci U S A, 2006. **103**(46): p. 17337-42.

- [180] Miyakawa, Y. and H. Matsushime, *Rapid downregulation of cyclin D1 mRNA and protein levels by ultraviolet irradiation in murine macrophage cells*. *Biochem Biophys Res Commun*, 2001. **284**(1): p. 71-6.
- [181] Lee, Y., et al., *The nuclear RNase III Droscha initiates microRNA processing*. *Nature*, 2003. **425**(6956): p. 415-9.
- [182] Moumen, A., et al., *hnRNP K: an HDM2 target and transcriptional coactivator of p53 in response to DNA damage*. *Cell*, 2005. **123**(6): p. 1065-78.
- [183] Alex, D. and K.A. Lee, *RGG-boxes of the EWS oncoprotein repress a range of transcriptional activation domains*. *Nucleic Acids Res*, 2005. **33**(4): p. 1323-31.
- [184] Deloulme, J.C., et al., *The prooncoprotein EWS binds calmodulin and is phosphorylated by protein kinase C through an IQ domain*. *J Biol Chem*, 1997. **272**(43): p. 27369-77.
- [185] Felsch, J.S., W.S. Lane, and E.G. Peralta, *Tyrosine kinase Pyk2 mediates G-protein-coupled receptor regulation of the Ewing sarcoma RNA-binding protein EWS*. *Curr Biol*, 1999. **9**(9): p. 485-8.
- [186] Guinamard, R., M. Fougereau, and P. Seckinger, *The SH3 domain of Bruton's tyrosine kinase interacts with Vav, Sam68 and EWS*. *Scand J Immunol*, 1997. **45**(6): p. 587-95.
- [187] Boland, M.P., *DNA damage signalling and NF-kappaB: implications for survival and death in mammalian cells*. *Biochem Soc Trans*, 2001. **29**(Pt 6): p. 674-8.
- [188] Einhorn, L.H., et al., *Phase II study of paclitaxel plus gemcitabine salvage chemotherapy for germ cell tumors after progression following high-dose chemotherapy with tandem transplant*. *J Clin Oncol*, 2007. **25**(5): p. 513-6.