E2F7: A MEMBER OF THE E2F FAMILY WITH A NOVEL MECHANISM OF TRANSCRIPTIONAL REPRESSION



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A thesis submitted for the degree of
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To my parents and sisters

Charalampos, Christina, Sophia and Danai

I love you more than words can say

Declaration of authentication

All the work presented in this thesis was performed entirely by me and in no way forms part of any other thesis in this or any other university. Where contributions have been made by others, this is acknowledged. The work was performed under the supervision of Prof. Nicholas B. La Thangue at the Laboratory of Cancer Biology, Department of Oncology, Division of Medical Sciences, University of Oxford.

Poli-Xenia Kesoglidou

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The mammalian E2F family of transcription factors plays a crucial role in the regulation of cellular proliferation, apoptosis and differentiation. E2F7 and E2F8 are the most recently identified family members and have unusual features that distinguish them from other members in the E2F family, including two distinct DNA-binding domains that bind to DNA in a DP-independent manner. E2F7 and E2F8 have been shown to be transcriptional repressors. However, the mechanism by which E2F7 represses E2F responsive gene expression remains to be elucidated.

The results presented here provide the first insight into the E2F7-mediated transcriptional mechanism. E2F7 was shown to contain a CtBP binding motif and form a complex with CtBP in both HeLa and MCF7 cells. An E2F7 deletion mutant lacking the CtBP binding motif was unable to form a complex with CtBP and repress the transcription of E2F target genes in luciferase assays, suggesting that this motif is essential for E2F7-dependent repression. Furthermore, the E2F7-CtBP complex was shown to be stable under different types of damage, such as following etoposide and UV treatment, and under different cell redox states. Interestingly, however, E2F7 was unable to repress the transcriptional activity of E2F target genes following treatment with the CtBP substrate MTOB. Moreover, E2F7 endogenous immunoprecipitations showed that E2F7 forms a complex with the chromatin-modifying enzymes HDAC1, HDAC2 and LSD1 and the co-repressor CoREST. Interestingly, via chromatin immunoprecipitations, E2F7 was shown to recruit these co-repressors to a subset of E2F-responsive promoters where they affect the activity of these promoters in a target gene-specific manner. Furthermore, results presented here suggest that CtBP could play a dual role in E2F7 function, not only being involved in E2F7-mediated repression but also in the repression of E2F7 itself as siRNA mediated CtBP depletion was shown to cause an upregulation of E2F7 protein levels. These results implicate a repertoire of co-repressors in a target gene-specific E2F7 repression mechanism, and as such define a new level of complexity in cell cycle control.

Table of contents

Dedication……

Acknowledgements……

Declaration of authentication……

Abstract……i

Table of Contents……ii

List of Figures……vii

List of Tables……x

Abbreviations……xi

CHAPTER 1. INTRODUCTION

1.1 Cancer and the major pathways involved in tumorigenesis……1

1.2 The mammalian cell cycle……3

1.2.1 The cyclin/Cdk network……4

1.2.2 Cell cycle progression……5

1.3 The Rb/E2F pathway……8

1.3.1 The Rb family of proteins……10

1.3.2 The E2F family of transcription factors……11

1.3.2.1 The activating E2Fs……13

1.3.2.2 The repressive E2Fs……15

1.3.3 The atypical E2F family members E2F7 and E2F8……17

1.3.3.1 E2F7……20

1.3.4 The DP family……22

1.4 Regulation of E2F activity……23

1.4.1 Cellular functions of E2F1……26

1.4.2 Role of E2F1 in proliferation.....28

1.4.3 Role of E2F1 in apoptosis induction.....28

1.4.3.1 E2F1 induces apoptosis through p53-dependent pathway.....29

1.4.3.2 E2F1 induces apoptosis through p53-independent mechanisms...30

1.4.3.3 E2F1 induces apoptosis by inhibition of survival signals.....31

1.5 Gene transcription.....34

1.5.1 The transcription initiation machinery.....35

1.5.2 Transcriptional activation and repression.....36

1.5.3 Transcriptional activation.....37

1.5.4 Transcriptional repression.....38

1.5.5 Co-repressor complexes and enzymatic activities.....40

1.5.5.1 The CtBP family of proteins.....42

1.5.5.1.1 The role of CtBP in repression.....43

1.5.5.1.2 Regulation of CtBP transcriptional activity by NAD(H) dinucleotides.....44

1.5.5.2 HDACs.....47

1.5.5.2.1 Class I HDACs.....48

1.5.5.3 CoREST.....50

1.5.5.4 LSD1.....51

1.6 Aims and objectives.....54

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell culture.....57

2.2 Compounds and drug treatments.....57

2.3 Transient transfections and expression vectors.....58

2.3.1 E2F7 mutant plasmid.....58
2.4 Oligonucleotide based siRNA.....59
2.5 Protein extraction, SDS-PAGE and western blotting.....60
2.6 Protein immunoprecipitation.....63
2.7 Immunofluorescence.....63
2.8 Flow cytometry.....64
2.9 Reporter assay.....65
2.10 Chomatin immunoprecipitation.....65
2.11 NAD^+/NADH quantification.....68

CHAPTER 3 IDENTIFICATION OF CtBP AS AN E2F7 CO-REPRESSOR

3.1 Introduction.....71
3.2 E2F7 is a transcriptional repressor of E2F target genes.....71
3.3 E2F7 contains a CtBP-binding motif and interacts with CtBP.....73
3.4 CtBP knock-down activates the transcription of certain E2F target genes.....76
3.5 E2F7 and CtBP are recruited to the promoters of E2F target genes.....76
3.6 E2F7 and CtBP co-exist at the promoters of E2F target genes.....80
3.7 Discussion.....82

CHAPTER 4 MUTATATIONAL ANALYSIS OF THE CtBP BINDING MOTIF AND INVESTIGATION OF THE E2F7-CtBP COMPLEX

4.1 Introduction.....87
4.2 Cellular localization of E2F7ΔCtBP.....88
4.3 E2F7ΔCtBP is unable to repress certain E2F target genes.....90
4.4 Investigation of the E2F7 – CtBP interaction under different types of stress....93

4.5 E2F7 can still interact with CtBP and repress transcription of E2F-responsive genes in the presence of E1A.....95

4.6 Altering the redox state does not affect the ability of E2F7 to repress.....100

4.7 MTOB treatment affects the E2F7-mediated repression.....105

4.8 Discussion.....107

CHAPTER 5 IDENTIFICATION OF HDACs IN THE E2F7 REPRESSION COMPLEX

5.1 Introduction.....115

5.2 E2F7 forms a complex with Class I histone deacetylases.....116

5.3 HDACs are involved in the repression of E2F responsive genes.....118

5.4 Promoter recruitment profiles of HDAC1 and HDAC2.....120

5.5 Discussion.....123

CHAPTER 6 IDENTIFICATION OF CoREST AND LSD1 AS COMPONENTS OF THE E2F7 REPRESSION COMPLEX

6.1 Introduction.....128

6.2 E2F7 forms a complex with CoREST.....128

6.3 Depletion of CoREST activates the transcription of certain E2F target genes129

6.4 E2F7 forms a complex with LSD1.....132

6.5 Depletion of LSD1 enhances the activity of certain E2F promoters.....134

6.6 Promoter recruitment profiles of CoREST and LSD1.....134

6.7 Consequences of CtBP siRNA treatment on E2F7.....139

6.8 Promoter recruitment profiles under E2F7 depletion.....141

6.7 Discussion.....143

CHAPTER 7 GENERAL DISCUSSION

7.1 CtBP is an E2F7 co-repressor that plays an important role in the repression of certain E2F target genes.....149

7.1.1 CtBP binds to the promoters of E2F target genes via E2F7 *in vivo*.....151

7.1.2 The CtBP binding motif is required for the E2F7-CtBP interaction and the repression of certain E2F-responsive promoters.....151

7.1.3 E2F7 and CtBP form a robust complex.....152

7.1.4 E2F7-mediated repression is compromised upon MTOB treatment.....153

7.2 HDAC1, HDAC2, CoREST and LSD1 are constituents of an E2F7 repression complex.....156

7.2.1 HDAC1, HDAC2, CoREST and LSD1 affect the activity of different E2F-responsive promoters.....157

7.2.2 HDAC1, HDAC2, CoREST and LSD1 are recruited to E2F-responsive promoters *in vivo* via E2F7.....158

7.2.3 E2F7-mediated repression of *E2F1*.....159

7.3 CtBP has a dual role in E2F7 function.....161

7.4 Conclusions.....163

REFERENCES.....165

List of figures

Figure 1.1: The cell cycle and its regulation by cyclins, Cdks, and CdkIs.....7

Figure 1.2: The retinoblastoma-E2F pathway.....9

Figure 1.3: The E2F family of transcription factors.....16

Figure 1.4: Regulation of E2F activity.....25

Figure 1.5: Mechanisms of E2F1-induced apoptosis.....33

Figure 1.6: The transcriptional repression mechanism.....39

Figure 1.7: Transcriptional repression mechanism by the CtBP dimer.....46

Figure 3.1: E2F7 represses E2F1, cdc6, cyclin E and p73 transcriptional activity.....73

Figure 3.2: E2F7 forms a repression complex with CtBP.....75

Figure 3.3: CtBP is a repressor of E2F target genes.....77

Figure 3.4: E2F7 and CtBP are recruited to E2F-responsive promoters.....79

Figure 3.5: E2F7 and CtBP co-exist on the E2F1, cdc6, cyclin E and p73 promoters.....81

Figure 3.6 Proposed model for E2F7-mediated repression of E2F target genes.....85

Figure 4.1: Cellular localisation of E2F7 and E2F7ΔCtBP.....89

Figure 4.2: Deletion of the E2F7 CtBP-binding motif affects the repression of certain E2F target genes.....91

Figure 4.3: Comparison of the E2F7ΔCtBP with the wild-type in CtBP interaction.....92

Figure 4.4: The E2F7-CtBP interaction under different types of stress.....94

Figure 4.5: Comparison of the E2F7-CtBP interaction in U2OS nad HEK293 cells.....96

Figure 4.6: E2F7 represses E2F1, cdc6, cyclin E and p73 transcriptional activity in HEK293 cells.....97

Figure 4.7: E2F7 can repress E2F1, cdc6, cyclin E and p73 transcriptional activity in the presence of E1A.....99

Figure 4.8: E2F7 interacts with CtBP under different cellular redox states.....102

Figure 4.9: E2F7 can repress the transcriptional activity of E2F1, cdc6, cyclin E and p73 under different cellular redox states.....104

Figure 4.10: E2F7 is unable to repress the E2F1, cdc6, cyclin E and p73 transcriptional activity under MTOB treatment.....106

Figure 4.11 Proposed model for the effect of CtBP binding to the MTOB substrate on E2F7-mediated repression of E2F target genes.....113

Figure 5.1: E2F7 forms a repression complex with HDAC1 and HDAC2.....117

Figure 5.2: Treatment with the HDAC inhibitor TSA activates certain E2F-responsive promoters.....119

Figure 5.3: HDAC1 and HDAC2 are recruited to E2F-responsive promoters.....121

Figure 5.4: E2F7 co-exists on the E2F1, cdc6, cyclin E and p73 promoters with HDAC1 and HDAC2.....122

Figure 5.5 Proposed model for the E2F7 repression complex.....126

Figure 6.1: E2F7 forms a repression complex with CoREST.....130

Figure 6.2: CoREST is a repressor of E2F target genes.....131

Figure 6.3: E2F7 forms a repression complex with LSD1.....133

Figure 6.4: LSD1 is a repressor of certain E2F target genes.....135

Figure 6.5: CoREST and LSD1 are recruited to E2F-responsive promoters.....137

Figure 6.6: E2F7 co-exists on the E2F1, cdc6, cyclin E and p73 promoters with CoREST and LSD1.....138

Figure 6.7: The effects of CtBP siRNA in E2F7 stability and cell cycle.....141

Figure 6.8: CtBP, HDAC1, HDAC2, LSD1 and CoREST are recruited to E2F-responsive promoters via E2F7.....142

Figure 6.9 Proposed model for the E2F7 repression mechanism.....147

Figure 7.1 Structure of CtBP ternary complex.....155

Figure 7.2 Proposed model for the E2F7-mediated repression of *E2F1*.....160

Figure 7.3 Hypothetical model for the E2F7 repression mechanism.....162

List of tables

Table 2.1: List of cell lines.....57

Table 2.2 List of siRNA oligonucleotides.....59

Table 2.3 List of antibodies.....62

Table 2.4 Oligonucleotide sequence of primers used for ChIP analysis.....67

Abbreviations

-/-	homozygous genotype
-/+	heterozygous genotype
^{o}C	Degrees Celsius
Apaf-1	Apoptosis protease-activating factor-1
APC	Anaphase promoting complex
APS	Ammonium persulphate
AR	Androgen receptor
ASPP	Apoptotic-stimulating protein of p53
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3-related
Bcl-2	B-cell leukaemia/ lymphoma-2
BH3	Bcl-2 homology 3
BIM	Bcl2-like 11
BRG1	Human brahma-related gene 1
BSA	Bovine serum albumin
CAK	CDK-activating kinase
CBP	CREB binding protein
Cdc	Cell division cycle
Cdh1	Cell division cycle 20 related 1
Cdk	Cyclin dependent kinase
CdkI	Cdk inhibitor
CDKN2A	Cyclin-dependent kinase inhibitor 2A
ChIP	Chromatin immunoprecipitation
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
Cip/Kip	Cdk-inhibitory protein/kinase-inhibitory protein
CoREST	Co-repressor for REST
C-terminal	Carboxy terminal
CtBP	C terminal binging protein
CTD	carboxy-terminal repeat domain
D2-HDH	D-isomer-specific 2-hydroxy acid dehydrogenases

DAPI	4,6-Diamidino-2-phenylindole
DBD	DNA binding domain
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DP	DRTF1 polypeptide
E1A	Adenovirus early protein 1A
E2F	E2A binding factor
EB	Elution buffer
EDTA	Ethylene diamine tetra-acetic acid
Etop	Etoposide
FACS	Florescence-activated cell sorting
FAD	Flavinadenine dinucleotide
G	Gap
G0	Quiescence
G1	Gap phase 1
G2	Gap phase 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione-S-transferase
GTF	General transcription initiation factor
HA	Haemagglutinin peptide
HAT	Histone acetyl transferase
HBRM	Human brahma protein
HDAC	Histone deacetylase
HDACi	HDAC inhibitor
HMT	Histone methyltrasferases
HP1	Heterochromatin 1
IgG	Imunoglobulin G
INK4	Inhibitor kinase 4
IP	Immunoprecipitation
JMY	Junction-mediating and regulatory protein
INR	Initiator

KDa	Kilodalton
KDM	Llysine demethylases
LCoR	Ligand-dependent nuclear receptor co-repressor
LSD1	Lysine specific demethylase 1
Luc	Luciferase
LZ	Leucine repeat sequence
M	Mitosis
m	Mouse
m	Milli
MAP3K5	Mitogen-activated protein kinase 5
Max	MYC associated factor X
MCL-1	Myeloid leukaemia cell differentiation protein 1
MB	Marked box
Mdm2	Murine double minute 2
MEF	Mouse embryonic fibroblasts
Mga	MAX gene associated
mRNA	Messenger RNA
MTase	Methyltransferase
MTOB	2-keto-4-methylthio-2-oxo butyrate
Myt1	Protein kinase, membrane associated tyrosine/threonine 1
NAD	Nicotinamide adenine dinucleotide
NES	Nuclear export signal
NF-kB	Necrosis factor kappa-B
NHFK	human foreskin keratinocytes
NLS	Nuclear localisation signal
NOXA	NADPH oxidase activator
NP-40	Igepal CA-630
NS	Non-specific
NT	Non-targeting
N-terminal	Amino-terminal
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PcG	Polycomb group
PCR	Polymerase chain reaction

PI	Propidium iodine
PIC	Phosphatase inhibitor cocktail
PIC	Pre-initiation complex
Pol	RNA polymerase
pRb	Retinoblastoma tumour suppressor protein
REST	Repressor element 1 silencing transcription factor
RNA	Ribonucleic acid
RP	Restriction point
S phase	Synthesis phase
SDS	Sodium dodecyl sulphate
siRNA	Short interfering RNA
TAF	TBP-associated factor
TBP	TATA-binding protein
TE	Tris EDTA solution
TEMED	Tetramethylethylenediamine
TF	Transcription factor
TNF	Tumour necrosis factor
TRAF2	TNF- receptor-associated factor 2
TSA	Trichostatin A
Tween-20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet light
WT	Wild-type
X	Any amino acid
β-gal	Beta-galactosidase
µ	Micro

CHAPTER 1

INTRODUCTION

1.1 Cancer and the major pathways involved in tumorigenesis

Neoplasia (a Greek word meaning "new growth") refers to new tissue composed of cells with the heritable capacity to grow beyond their normal confines (Halazonetis et al., 2008). Neoplasias can be benign or malignant. The former remain localised, whereas the latter, also referred to as cancer, invade the host tissues. Cancer is a diverse disease, characterised by the disruption of normal mechanisms that control proliferation. During tumour development cells sustain mutations that result in insensitivity to anti-growth signals and evasion of apoptosis, thereby gaining an unlimited replicative potential (Negrini et al., 2010, Fulda, 2010). Remarkably, the Rb-E2F and the p53-mdm2 pathways are both defective in most human tumours, which underscores the crucial role of these pathways in regulating cell cycle progression and viability (Polager and Ginsberg, 2009).

The p53 tumour suppressor is a nuclear transcription factor that affects cellular functions which include transcription, DNA synthesis and repair, cell cycle arrest and apoptosis (Coutts et al., 2009, Harris and Levine, 2005). Upon exposure to DNA damage, p53 is activated and transcriptionally regulates a variety of genes involved in cell survival, proliferation and death which have the potential to contribute to tumour suppression (Vousden and Prives, 2009, Lane and Levine, 2010). The critical importance of p53 function in suppressing tumorigenesis is highlighted by the fact that the 50% of human tumours contain p53 mutations that compromise p53 activity (Coutts et al., 2009, Vousden and Lane, 2007).

Another family of transcription factors that affect cell fate and cancer development is the E2F family (van den Heuvel and Dyson, 2008, Polager and Ginsberg, 2009,

Sherr and McCormick, 2002). The E2F transcription factors have a pivotal role in cell cycle control and are intimately regulated by the retinoblastoma (Rb) tumour suppressor (Yamasaki, 2006). Mutations of the retinoblastoma tumour suppressor gene or components regulating the Rb pathway have been identified is almost every human malignancy (Chen et al., 2009, Polager and Ginsberg, 2008, Sabado Alvarez, 2008). The tumorigenic potential resulting from deregulated E2F activity is not surprising, considering the wide range of E2F targets and functions. E2Fs both transactivate and repress gene expression in order to regulate various biological functions including cell cycle progression, DNA replication, mitosis, DNA damage checkpoints, DNA repair and apoptosis (Polager and Ginsberg, 2009, Hickman et al., 2002, Muller and Helin, 2000). Since E2F activity is essential in such a plethora of cell functions, elucidation of the mechanisms that control E2F regulation is highly significant. In addition to the further understanding of cell cycle progression and the aberrations that contribute to tumorigenesis, insights into these mechanisms may even provide new targets of therapeutic value.

1.2 The mammalian cell cycle

Cell growth and division are essential processes ensuring survival and propagation of a species through faithful division of a mother cell into two identical daughter cells with the same genetic complement. The cell cycle can be described as the set of successive transitions between four distinct cellular phases. A phase of cell growth (G1), followed by a phase of DNA synthesis and replication (S), another phase of cell growth and active synthesis of factors (G2) required for cell division or mitosis (M) (Kurzawa and Morris, 2010). A complete proliferative cycle entails sequential and regulated progression through G1, S, G2, and M phases (Moeller

and Sheaff, 2006). In the absence of growth factor signalling, normal cells may temporarily or permanently leave the cell cycle and enter a quiescent or arrested phase known as G0**.** Growth factor stimuli are necessary to initiate and maintain the transition through G1 to S phase. The point in G1 at which commitment occurs and the cell no longer requires growth-stimulating signals to complete the cell cycle is termed the restriction point (Blagosklonny, 2006, Foster et al., 2010).

1.2.1 The cyclin/Cdk network

The mammalian cell cycle is regulated by a subfamily of cyclin dependent kinases (Cdks), the activity of which is modulated by several activators (cyclins) and inhibitors (Ink4, Cip and Kip inhibitors). Cdks are the catalytic subunits of heteromeric serine/threonine protein family of kinases (Malumbres, 2011, Novak et al., 2010, Palmero and Peters, 1996). Active Cdks comprise a protein kinase subunit whose catalytic activity is dependent on association with a regulatory cyclin subunit (Suryadinata et al., 2010).

Cell cycle stage-dependent accumulation and proteolytic degradation of different cyclin subunits regulates their association with Cdks to control different stages of cell division. The cyclin-Cdk complexes involved in driving the cell cycle include three interphase Cdks (Cdk1, Cdk4 and Cdk6), a mitotic Cdk (Cdc2) and ten cyclins that belong to four different classes (the A, B, D and E type) (Gopinathan et al., 2011, Hu and Moscinski, 2011). Cyclins D and E are expressed during G1,cyclins E and A during S phase and cyclins A and B during the M phase (Echalier et al., 2010).

The kinase activity of cell cycle Cdks is also controlled by binding to negative regulators. The Cdk inhibitors comprise the Ink4 family and the Cip and Kip family. Ink4 proteins specifically bind to and inhibit monomeric Cdk4 and Cdk6 proteins, whereas the Cip and Kip proteins bind to cyclin-Cdk complexes blocking their kinase activity (Malumbres and Barbacid, 2005, Wang et al., 2011).

Activating and inhibitory phosphorylations and dephosphorylations provide an additional level of control for kinase activity. Wee1 and Myt1 kinases phosphorylate the Cdk subunit, thereby inhibiting the cyclin-Cdk activity (Tarn and Lai, 2011). The very same residues are dephosphorylated by the Cdc25 phosphatases in order to activate the cyclin-Cdk complex (Malumbres and Barbacid, 2009). In addition, Cdks must be phosphorylated within the activation loop of the kinase domain by a Cdk-activating kinase (CAK) to attain full activity (Wohlbold and Fisher, 2009).

1.2.2 Cell cycle progression

When G0 cells are stimulated by mitogenic signals to re-enter the cell division cycle, active cyclin D-Cdk4/6 complexes progressively accumulate as cells progress through G1 phase (Figure 1.1). Cyclin D-Cdk4/6 activation leads to the phosphorylation of the Rb protein family and subsequent release of E2F transcription factors (Blain, 2008, Medema and Macurek, 2011, Paternot et al., 2010). Transcriptional activation of E2F responsive genes such as the E and A type of cyclins is required for cell cycle progression. The availability of cyclin E during the cell cycle is tightly controlled and limited to the to the G1 to S phase transition (Sherr and Roberts, 2004). In late G1 phase, cyclin E binds to and activates Cdk2 in

order to further phosphorylate and completely inactivate Rb (Malumbres and Barbacid, 2009, Wong et al., 2011). Further transcriptional activation of E2F target genes leads to the passage through the G1/S phase boundary and to S phase initiation (Satyanarayana and Kaldis, 2009). At the onset of the S phase, cyclin E is degraded and it is the synthesis of cyclin A that activates Cdk2. Cyclin A-Cdk2 complexes phosphorylate proteins involved in DNA replication and are essential for proper completion and exit from the S phase (Coverley et al., 2000).

Towards the end of the S phase Cdk1 is activated by binding to A type cyclins. During G2, the A type cyclins are degraded while B type cyclins are synthesised leading to the association of Cdk1 with the latter thereby triggering mitosis (Malumbres and Barbacid, 2005). Cyclin B-Cdk1 complexes regulate several events during both the G2/M transition and progression to mitosis. In fact, inactivation of cyclin B-Cdk1 is required for proper exit from mitosis and is accomplished by cyclin B degradation by the anaphase promoting complex (APC) (Lindqvist et al., 2009).

The APC is a multi-subunit E3 ubiquitin ligase which ubiquitinates and thereby targets for degradation several cell cycle regulatory proteins. APC activity oscillates in response to the differential association of the APC with the activating subunits Cdc20 and Cdh1 (Sullivan and Morgan, 2007, McLean et al., 2011). APC-Cdc20 initiates the metaphase-anaphase transition by targeting for degradation cyclin B and securin, an inhibitor of the protease separase. Degradation of securin initiates chromosome segregation by activating separase which destroys sister chromatid cohesion (Queralt and Uhlmann, 2008). APC-Cdh1 conversely is

thought to prevent premature entry into S phase by limiting the accumulation of mitotic cyclins in G1 (Li and Zhang, 2009, Qiao et al., 2010).

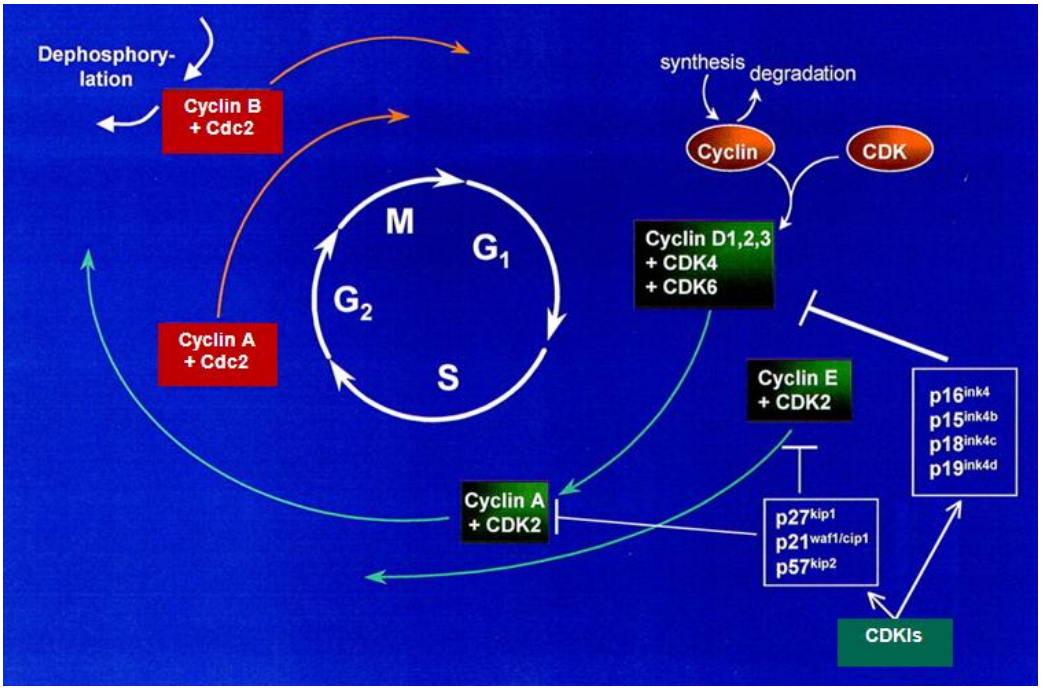


Figure 1.1: The cell cycle and its regulation by cyclins, Cdks, and CdkIs.

The cell cycle is divided into four distinct phases (G1, S, G2, and M). Cell cycle progression is promoted by Cdks, which in turn are positively and negatively regulated by cyclins and CdkIs, respectively. Cyclin D isoforms interact with Cdk4 and Cdk6 to drive the cell cycle progression through G1. Cyclin E-Cdk2 association is active at the G1/S transition in order to direct entry into S-phase. S-phase progression on the other hand is directed by the cyclin A/Cdk2 complex. The association of cyclin A with Cdc2 (also known as cdk1) is significant in G2. Cdc2-cyclin B is essential for the entry into mitosis. The Ink4 (for inhibitor of cdk4) class of CdkIs, such as p16lnk4a, p15lnk4b, p18lnk4c, and p191nk4d, bind and inhibit cyclin D-associated kinases (Cdk4 and Cdk6). Furthermore, the kinase inhibitor protein group of Cdk inhibitors such as p21waf1, p27kip1, and p57kip2, negatively

regulate cyclin E-Cdk2 and cyclin A-Cdk2 complexes. Adapted from (Shah and Schwartz, 2001).

1.3 The Rb/E2F pathway

The retinoblastoma protein (pRb) was the first tumour suppressor to be identified and is absent or mutated in at least one third of all human tumours (Trimarchi and Lees, 2002, Viatour and Sage, 2011). pRb has a crucial role in the G1 to S phase transition in mammalian cells. A key target in this process involves the E2F family of transcription factors, which regulate genes involved in cell proliferation and survival (Khidr and Chen, 2006, Du and Searle, 2009, van Deursen, 2007). In turn, the activity of pRb is regulated by post-translational modifications, primarily phosphorylation. Cyclin-Cdk complexes inactivate pRb by phosphorylation, resulting in the release of E2F and cell cycle progression (Figure 1.2) (Slansky and Farnham, 1996). Phosphorylation by cyclin D-Cdk5/6 and cyclin E-Cdk2 is essential for complete inactivation of pRb thereby leading to cell cycle progression (Poznic, 2009, Wong et al., 2011, Rizzolio et al., 2010). In addition to phosphorylation, other levels of post-translational regulation affect the activity of pRb. Methylation of pRb has been shown to be necessary for cell cycle arrest and transcriptional repression (Munro et al., 2010).

In targeting E2F, pRB uses a multiplicity of mechanisms to regulate transcription. The first general mechanism is based on the fact that the E2F transactivation domain and the pRb-binding domain physically overlap (Rizzolio et al., 2010, Seville et al., 2005). This enables pRb to hinder the interaction of E2F with the transcription apparatus. The second general mechanism relies on the interaction of

pRb with different chromatin modifying enzymes, including histone deacetylases and methyl-transferases (Markham et al., 2006). These interactions result in the formation of a dominantly repressing complex allowing pRb to enforce inactivation of transcriptional activity by regulating chromatin.

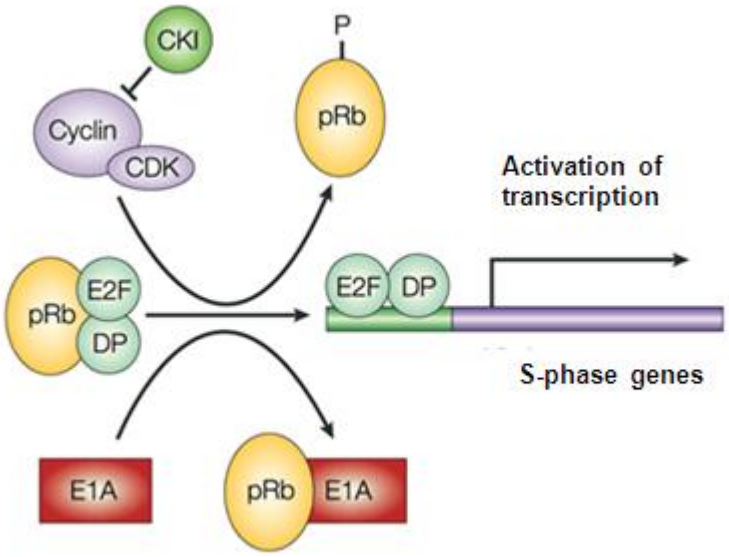


Figure 1.2: The retinoblastoma-E2F pathway.

In the G0/G1 phase of the cell cycle, the hypophosphorylated retinoblastoma protein (pRB) forms complexes with E2F/DP heterodimers, which thereby prevents their ability to activate transcription. Cell-cycle-dependent phosphorylation by cyclin–Cdk complexes releases E2F from pRB in order to activate transcription of target genes that are required for S phase entry. The interaction of Cdk inhibitors (CdkI) with the cyclin–Cdk complex blocks pRb phosphorylation. Additionally, expression of E1A overrides the normal cellular control of the pRb–E2F/DP interaction by binding to hypophosphorylated pRB and thus releasing E2F. Adapted from (Frisch and Mymryk, 2002).

1.3.1 The Rb family of proteins

The retinoblastoma family comprises of pRb and the related proteins p107 and p130, collectively referred to as 'pocket proteins'. This term derives from the conserved binding pocket region through which pRb, p107 and p130 bind viral oncoproteins and cellular transcription factors, including the E2F family of transcription factors (Giacinti and Giordano, 2006, Swiss and Casaccia, 2010, Dannenberg and te Riele, 2006). The pocket region comprises two sub-domains termed A and B which are separated by a highly conserved spacer region (Sun et al., 2007). Pocket proteins also contain a leucine-X-cysteine-X-glutamate (LXCXE) domain, X denoting any amino acid residue, to which a number of cellular partners can bind. The LXCXE domain is essential for the recruitment of the histone deacetylases HDAC1 and HDAC2 and the interaction with oncoproteins from DNA tumour viruses and the D- type cyclins (Chan et al., 2001b, Genovese et al., 2006).

Among the Rb family of proteins, p107 and p130 are more related to each other (about 50% amino acid identity) than either is related to pRb, both structurally and functionally. Although all proteins of the Rb family bind E2Fs, pRb contains a unique domain located within its C-terminus that specifically binds E2F1 (Burkhart and Sage, 2008). On the other hand, both p107 and p130 contain a cyclin-binding and a Cdk inhibitor domain that are absent form pRb (Wirt and Sage, 2010).

The Rb family proteins also differ in their binding to the E2F transcription factors throughout the cell cycle and act in a sequential manner to prevent entry to the S phase. Whereas pRb preferentially binds to the activator E2Fs (E2F1-3a), p107 and

p130 bind the repressors E2F4 and E2F5 and therefore regulate distinct E2F responsive genes (Macaluso et al., 2006, Zhu, 2005). Furthermore, while pRb is expressed in both proliferating and non-proliferating cells, p107 and p130 are most prominent in proliferating and arrested cells respectively (Cobrinik, 2005). During G0 and early G1, p130 is highly expressed and interacts mainly with E2F4 and to a less extent with E2F5, thereby exerting an inhibitory effect on the E2F target genes required for S phase entry (Sun et al., 2007). This complex recruits chromatin remodelling factors such as HDACs to E2F responsive promoters and therefore supports a state of transcriptional silence. As cells re-enter the cell cycle, E2F4 remains bound to p130 during early G1 until reaching mid to late G1 where p130 is replaced by p107. pRb levels remain stable throughout the cell cycle and the pRb association with the activating E2Fs prevents the transcriptional activation of the E2F responsive promoters (Poznic, 2009, Macleod, 1999).

Although the retinoblastoma family members have many similarities, they have additionally unique functions in arresting cells during G1. One major difference is that while pRb is commonly mutated in human cancers p107 and p130 are rarely directly inactivated. Instead, in the absence of pRb mutations in human cancers, it is the alteration of upstream regulators of all three pocket proteins that is evident (Burkhart and Sage, 2008).

1.3.2 The E2F family of transcription factors

The E2F family of transcription factors is the downstream effector of the retinoblastoma pathway and plays a pivotal role in the regulation of cellular proliferation (Reimer et al., 2006, Blais and Dynlacht, 2007, Fang and Han, 2006).

The E2 factor (E2F) was initially identified as a cellular component required for the early region transforming protein (E1A) of adenovirus in order to activate the viral E2 promoter (Yu et al., 2009). In cells, E2F activity arises from a family of heterodimeric transcription factors, each heterodimer consisting of an E2F family member bound to a member of the DP family (Stevens and La Thangue, 2003a).

Based upon sequence homology and functional properties, the eight members of the E2F family identified to date (E2F1-E2F8) can be subdivided into different functional groups: activating E2Fs (E2F1-3a) and repressing E2Fs (E2F3b-8) (Dimova and Dyson, 2005). All E2F family members share structural characteristics but also have distinctive differences reflecting their functional activities. E2F1-6 possess N-terminal DNA binding and DP dimerization domains, followed by a conserved region involved in dimerization and bending known as the marked box (Tsantoulis and Gorgoulis, 2005, Mundle and Saberwal, 2003). E2F1-E2F5 also share a C-terminal transactivation domain containing the pocket protein binging region.

Furthermore, E2F1-3 contain nuclear localisation signals (NLS) and cyclin A binding domains within the N-terminal region (Dimova and Dyson, 2005). Moreover, two E2F3 proteins (E2F3a and E2F3b) are produced from the E2F3 gene through the use of alternative promoters, E2F3b lacking the cyclin A binding domain (Trimarchi and Lees, 2002). E2F4 and E2F5 contain nuclear export signals (NES) and translocation to the nucleus depends on heterodimerization with the DP family members (Tsantoulis and Gorgoulis, 2005). On the other hand, E2F6 does not interact with either pocket proteins or DP proteins but has the ability to interact

with members of the mammalian polycomb complex (PcG) (Slack and McClellan, 2007). The most recently identified members of the E2F family, E2F7 and E2F8, are unique among the E2Fs in their ability to bind DNA in the absence of interaction with a DP subunit. Rather, they contain two DNA binding domains in place of the dimerisation domain (Lees and Iaquinta, 2007).

1.3.2.1 The activating E2Fs

Functionally, E2F1, E2F2 and E2F3a represent the group of the activating E2F transcription factors which are required for the transactivation of genes in the G1/S phase and redundantly promote cell proliferation (van den Heuvel and Dyson, 2008, Wenzel et al., 2011). Their expression is cell cycle regulated and peak expression coincides with G0. Over-expression of E2F1, E2F2, or E2F3a individually is sufficient to induce quiescent cells to re-enter the cell cycle and dominant negative mutants abolish the entry into S phase (Slack and McClellan, 2007). Activating E2Fs can also override the effect of growth arrest signals, including TGF-β and the Cdk inhibitors p16, p21 and p27 (Trimarchi and Lees, 2002). This ability is dependent on the dimerization, DNA-binding and transactivation domains, thereby suggesting that induction of E2F target gene transcription is required for S phase entry (Stevens and La Thangue, 2003a).

In normal cells, the activating E2Fs are regulated through pocket protein binding specifically with pRb but not with the related pocket proteins p107 and p130 (van den Heuvel and Dyson, 2008). The pocket protein binding domain is integrated with the activation domain, therefore pRb binding masks the activation domain and blocks the ability of E2F to communicate with the core transcriptional machinery

(Stevens and La Thangue, 2003a). Furthermore, pRb is able to mediate active repression by binding to the promoters of E2F target genes and assembling a dominantly acting repressor complex. Active repression is enforced through the recruitment of either histone deacetylases or the histone methyltransferase SUV39HP1. HDACs remove the acetyl group from lysine 9 of the histone H3 tail and therefore facilitate nucleosome packing, whereas SUV39H1 methylates the same lysine residue to create binding sites for HP1, leading to transcriptional silencing (Trimarchi and Lees, 2002).

In addition, pRb can recruit other proteins with chromatin modifying activity such as human brahma (HBRM) and human brahma-related gene 1 (BRG1), which are human homologues of the yeast SW12/SFN2 proteins possessing nucleosome remodelling activities (Nielsen et al., 2001, Zhang et al., 2000). HBRM and BRG1 affect the access of transcription factors to promoters by modifying nucleosomal structure and nucleosome position in a ATP hydrolysis dependent manner (Harbour and Dean, 2000).

As cells are stimulated to enter the cell cycle, the sequential activation of the cyclins and cyclin dependent kinases Cdk4/6-cyclin D and Cdk2-cyclin E leads to the phosphorylation of pRb and subsequent release of the E2Fs (Dimova and Dyson, 2005). The activating E2Fs, relieved of their pRb-mediated inhibition, can then drive cell cycle progression by binding to the promoters of their targets and activate transcription (Martinez et al., 2010).

1.3.2.2 The repressive E2Fs

The second subclass of the E2F family includes E2F3b, E2F4, E2F5, E2F6 and the most recently identified members E2F7 and E2F8. While E2F3a is an activating E2F with a cell cycle regulated expression pattern, E2F3b is a repressive E2F constitutively expressed comparable to E2F4 and E2F5 (Dimova and Dyson, 2005). Interestingly, significant levels of E2F4 and E2F5 are detected in quiescent cells, whereas the activating E2Fs are primarily restricted to actively dividing cells (Lees and Iaquinta, 2007). Furthermore, E2F4 and E2F5 are predominantly cytoplasmic and depend on pocket protein binding to be transported into nuclei, in contrast with the activating E2Fs which are constitutively nuclear (Trimarchi and Lees, 2002). In addition to pocket protein binding, association with DP-2 which contains an intrinsic NLS can result in the nuclear translocation of E2F4 and E2F5 (Stevens and La Thangue, 2003a).

In contrast to the other repressing E2Fs, E2F6, E2F7 and E2F8 lack the transactivation and pocket protein binding domains, yet are able to repress transcription via other mechanisms. The repression ability of E2F6 appears to be due to its ability to associate with Ring1 and YY1-binding protein (RYBP), members of the polycomb group (PcG) of transcriptional repressors (Giangrande et al., 2004). Primarily in quiescent cells, E2F6 recruits chromatin modifying proteins to E2F target genes including two MTases and the transcriptional repressor HP1γ (Ogawa et al., 2002). E2F7 and E2F8 are the more recent additions to the family of E2F transcription factors and the subclass of repressing E2Fs. They are particularly interesting members of the E2F family as they possess two distinct DNA binding domains, the integrity of both required for DNA binding activity (de Bruin et al.,

2003, Logan et al., 2004). The presence of the two DNA binding domains in addition to their ability to bind DNA in a DP-independent manner suggests that E2F7 and E2F8 perform a distinct role from the other E2Fs.

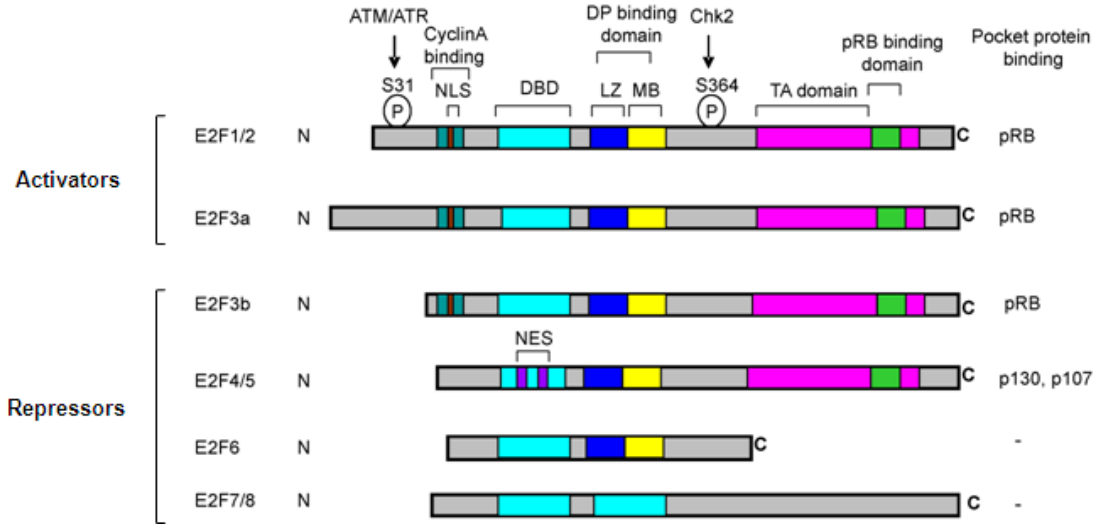


Figure 1.3: The E2F family of transcription factors.

The domain structures of the E2F family members. E2F1-E2F6 all contain a conserved DNA binding domain as well as a dimerization domain which encompasses two conserved motifs: a leucine repeat sequence (LZ) and the marked box motif (MB). E2F1-E2F5 members also share a transactivation and a pocket protein binding domain in their carboxy terminals. Furthermore, E2F1-E2F3a possess a nuclear localization signal at their amino terminal domains while E2F4 and E2F5 have a nuclear export signal (NES) instead. E2F7 and E2F8 contain two DNA binding domains but lack the transactivation and pocket protein binding domains. The serine residues 31 and 364, which are phorphorylated by ATM/ATR and Chk2 kinase respectively, are also indicated.

Adapted from (Wu et al., 2009)

1.3.3 The atypical E2F family members E2F7 and E2F8

The six classical E2F proteins, E2F1 to E2F6, have been shown to possess one N-terminally located DNA-binding domain (DBD) that is immediately followed by a dimerization domain, which allows interaction with a DP partner protein (Dimova and Dyson, 2005). The most recently identified members of the E2F family, E2F7 and E2F8, have distinctive molecular features and thus constitute the atypical family members.

The rather low overall sequence similarity between atypical and typical E2F proteins is suggestive of the important structural difference between the two classes. The most distinctive characteristic of atypical E2F members is the presence of two DNA-binding domains which comprises the DNA-binding as well as the dimerization residues. The typical E2F family members on the other hand possess only one such domain, while the second domain required for DNA binding is obtained by dimerization with a DP subunit (Lammens et al., 2009). Consistent with the presence of two DBDs, the atypical E2Fs recognize and bind E2F-binding sites of target genes independently of DP interaction. Furthermore, the integrity of both DBDs is required for DNA binding as mutation of either of the two DBDs completely abolishes binding to the target DNA (Di Stefano et al., 2003, Logan et al., 2004, Logan et al., 2005, Christensen et al., 2005). Three dimensional models of the atypical E2Fs suggest that the two DBDs structurally mimic the DNA-binding interface of the E2F-DP heterodimers (Logan et al., 2004, Maiti et al., 2005).

Interestingly, the duplicated DNA-binding domain of atypical E2F proteins also comprises the dimerization residues, which has been shown to make contact with the DP subunit in the E2F/DP heterodimer (Zheng et al., 1999). However, the atypical E2Fs do not interact with DP but rather form homodimers or E2F7/E2F8 heterodimers (Di Stefano et al., 2003, Logan et al., 2004, Li et al., 2008b). Detailed dimerization studies have shown that the integrity of the dimerization residues is required for E2F7/E2F8 dimerization (Zalmas et al., 2008). Moreover, E2F7 homodimers have been shown to be the preferential dimerisation state over E2F7/E2F8 heterodimers while E2F8 homodimers constitute the least preferred form (Li et al., 2008b).

Although the atypical E2Fs contain an extra DBD, they lack several features that are common in most typical E2Fs. In contrast to E2F activator proteins, E2F7 and E2F8 lack a transactivation domain. Knockout of atypical *E2F* genes leads to upregulation of certain E2F-regulated genes, thereby suggesting that atypical E2Fs function as transcriptional repressors (Ramirez-Parra et al., 2004, Vlieghe et al., 2005, Zalmas et al., 2008). Additionally, competition assays have shown that E2F7 and E2F8 are able to counteract the activating E2Fs, which is consistent with their function as transcriptional inhibitors (de Bruin et al., 2003, Logan et al., 2004, Logan et al., 2005). Interestingly, the repression effect of atypical E2Fs requires the integrity of both DBDs, including their dimerization residues (Maiti et al., 2005, Zalmas et al., 2008).

Although the expression of E2F activators peaks at the G1–to-S transition, the expression of the typical E2F repressors remains unchanged throughout the cell

cycle (Mariconti et al., 2002, Attwooll et al., 2004). However, unlike the typical E2F repressors, the E2F7 and E2F8 expression is cell cycle regulated. Transcription of the atypical E2Fs is induced at the G1-to-S transition, with mRNA levels peaking during S-to-G2 (Logan et al., 2004, Logan et al., 2005, Christensen et al., 2005). In accordance with the cell cycle expression pattern of E2F7 and E2F8 and their role as transcriptional repressors, E2F1, a target of E2F7/E2F8, shows a complementary expression profile. E2F1 expression has been shown to be disturbed upon double ablation of E2F7 and E2F8, particularly during the S to G2 phase of the cell cycle (Li et al., 2008b, Zalmas et al., 2008).

In terms of their function, data suggest that atypical E2Fs play a role in cell cycle control, mainly in proliferation. Molecular studies have shown that over-expression of E2F7 and E2F8 results in inhibition of cellular proliferation (DeGregori and Johnson, 2006). Atypical E2Fs are able to bind to the consensus E2F recognition sequence in order to repress expression from a subgroup of cell cycle-regulated E2F targets (Moon and Dyson, 2008). Furthermore, E2F7 and E2F8 were shown to be critical for mouse development. Although mice homozygous mutant for either E2F7 or E2F8 developed normally with no obvious developmental defects, the double-knockout mice failed to survive suggesting an extensive functional overlap between the atypical E2Fs (Li et al., 2008b). In addition, the atypical E2Fs were shown to play a role in the cellular response to DNA damage. Recent data suggested that E2F7 and E2F8 are DNA damage inducible and this induction coincides with their binding to the promoters of a certain subset of E2F target genes such as *E2F1* (Zalmas et al., 2008). Moreover, in addition to being transcriptional regulators of *E2F1*, E2F7 and E2F8 are themselves transcriptional targets of E2F1.

The data thus illustrate a negative feedback loop were *E2F7* and *E2F8* are activated by E2F1 at the G1 to S transition resulting in the repression of *E2F1* by E2F7 and E2F8 during S-G2 (Lammens et al., 2009). By regulating the activity of *E2F1* and thus cell viability, the atypical E2Fs play an important role in the E2F transcriptional network.

1.3.3.1 E2F7

Comparative analysis of protein sequences between different genomes including human, mouse, fly, nematode and *A.thaliana* revealed that the DNA binding domain of the E2F transcription factors is highly conserved (de Bruin et al., 2003). The same study utilized this feature in order to determine whether additional members exist and thus subsequent searches of the human and mouse genomes for genes sharing amino acid homology with the E2F DBD led to the identification of E2F7.

Inspection of the genomic locus sequence showed that the *E2F7* gene contains 13 introns and 14 exons and alternative splicing gives rise two E2F7 isoforms (Di Stefano et al., 2003). E2F7a is the shorter isoform lacking exon 12 that encodes a protein of 728 amino acids, while E2F7b is the longer isoform encoding a 911 amino acid protein. Cell line analysis of E2F7a and E2F7b expression has shown that E2F7b is the most abundant form. Furthermore, while E2F7a RNA levels are present at all stages of the cell cycle, E2F7b expression is cell cycle regulated with levels peaking during S phase. The latter is thought to be resulting from specifically targeted degradation of E2F7b RNA in early G1 (de Bruin et al., 2003). This thesis

is focused on the cell cycle regulated isoform E2F7b, which is termed E2F7 for simplicity.

Further analysis of the *E2F7* sequence has revealed the presence of four putative E2F binding sites in the promoter, which is directly regulated by E2F1 (de Bruin et al., 2003, Logan et al., 2004, Di Stefano et al., 2003). In turn, E2F7 was found to bind and transcriptional repress E2F regulated promoters. Interestingly, E2F7 binds and regulates specific E2F target genes such as *E2F1* and *cdc6*, but not cyclin A and cdc2 (Di Stefano et al., 2003). Further supporting the E2F7 target specificity, E2F7 depletion by RNA interference led to an increase in E2F1 and cdc6 levels whereas the expression levels of cyclin A and cdc2 remained constant. E2F7-mediated repression of genes required for S phase entry was shown to inhibit cell proliferation and ectopic E2F7 expression led to cells accumulating in G1 and a reduction of G2/M phase cells (Logan et al., 2004).

Furthermore, *E2F7* expression has been shown to be tissue specific with high expression observed in skin and thymus, moderate expression in spleen while low expression shown in intestine and testis (Lammens et al., 2009). A recent study showed that E2F7 can regulate proliferation, differentiation and apoptotic responses in human keratinocytes (Endo-Munoz et al., 2009). Moreover, the study suggested that disruption of E2F1 and E2F7 in keratinocytes could contribute to squamous cell carcinoma formation. Interestingly, another study investigating the clinical relevance of the E2F transcription factors in ovarian cancer showed that low E2F7 expression correlated with tumours resistant to platinum (Reimer et al., 2006). The study concluded that down-regulation of E2F7 could contribute to the mechanisms

underlying platinum resistance and calculation of the proliferation promoting E2F1 to E2F7 ratio may serve as a putative predictor of such resistance.

1.3.4 The DP family

With the exception of E2F7 and E2F8, all members of the E2F family exist and function as heterodimeric complexes formed with members of the DP family of transcription factors (Hitchens and Robbins, 2003). The DP family contains two well characterised members, DP-1 and DP-2 (murine DP-3), and the most recently characterised DP-4. Although DPs demonstrate little or no DNA binding activity, their association with E2F affects both the DNA binding and the transcriptional activity of E2F (Tian et al., 2007, Hitchens and Robbins, 2003).

Under normal physiological conditions DP-1 is the predominant subunit of the E2F-DP heterodimer (Bandara et al., 1994). DP-1 is encoded by an essential gene as *dp-1* $^{-/-}$ mice exhibit embryonic lethality, partly due to defective extra-embryonic tissue development (Kohn et al., 2003, Kohn et al., 2004). The association of the ubiquitously expressed DP-1 with E2F enhances both the DNA binding and the transcriptional activity of E2F (Hitchens and Robbins, 2003). Phosphorylation of DP-1 by cyclin A-cdk 2 blocks the DNA binding activity of the E2F-DP heterodimer, thereby assisting exit from S phase (Milton et al., 2006).

The second member of the DP family DP-2 also binds to E2F proteins and enhances their DNA binding and transcriptional function (Hitchens and Robbins, 2003). DP-1 and DP-2 although sharing high homology in the DNA binding-heterodimerisation domain they diverge from each other in the C terminus (Qiao et

al., 2007). Furthermore, DP-2 exists as multiple isoforms arising from tissue specific alternative splicing (Rogers et al., 1996).

DP-4, similarly to DP-1 and DP-2, also binds to the E2F site and forms heterodimers with E2F proteins. However, E2F/DP-4 heterodimers exhibit reduced DNA binding activity (Milton et al., 2006). Furthermore, DP-4 interferes with E2F1-dependent transcription and acts negatively on cell cycle progression (Milton et al., 2006). Additionally, DP-4 is DNA damage responsive and under damage conditions down-regulates E2F1 activity by creating an E2F1/DP-4 complex that is unable to bind to DNA (Ingram et al., 2011).

1.4 Regulation of E2F activity

E2F activity is dependent upon its dimerization with a DP subunit, as well as its interaction with the pocket proteins (Crosby and Almasan, 2004). However, in order to tightly regulate E2F activity during the cell cycle, multiple levels of control exist. Although pocket protein binding is the main mode of E2F regulation, protein interactions, post-translational modifications and functional interplay among E2F family members add several layers of complexity in E2F regulation.

An additional level of control occurs by the association between E2F1 and cyclin A-cdk 2 as cells enter the S phase (Tsantoulis and Gorgoulis, 2005). E2F1 interacts with cyclin A-cdk 2 via the cyclin A binding domain located near the amino terminus of activating E2Fs causing DP phosphorylation and subsequent loss of E2F DNA binding activity (Yu et al., 2009, Stevens and La Thangue, 2003b). This event, in addition to the rapid turnover of cyclin E via ubiquitin-dependent

proteolysis, is necessary for cell cycle exit from the S phase (Stevens and La Thangue, 2003a).

Targeted proteolysis and acetylation also play a role in the regulation of E2F activity. The F-box protein $p45^{Skp2}$, a cell cycle-regulated component of the ubiquitin-protein ligase SCF^{Skp2}, interacts with E2F1 causing the rapid degradation of E2F1 at the S to G2 transition (Hofman et al., 2001, Tsantoulis and Gorgoulis, 2005). Interestingly, $p45^{Skp2}$ binds to the N terminal region of E2F1 and therefore targets E2F1 for degradation following completion of the S phase, the duration of which is regulated by cyclin A (Stevens and La Thangue, 2003a).

Acetylation is another post-translational modification that affects E2F activity and involves the interaction with the p300/CBP family of proteins, which bind to a diverse array of transcription factors and interact directly with components of the basal transcriptional machinery (Stevens and La Thangue, 2003a). They acetylate lysine residues lying adjacent to the DNA binding domain of activating E2Fs, thereby increasing DNA binding activity, transcriptional activity and protein stability (Martinez-Balbas et al., 2000). Furthermore, acetylation also plays a role in pRb regulation as the adenovirus E1A binds to p300/CBP and stimulates pRb acetylation (Chan et al., 2001a).

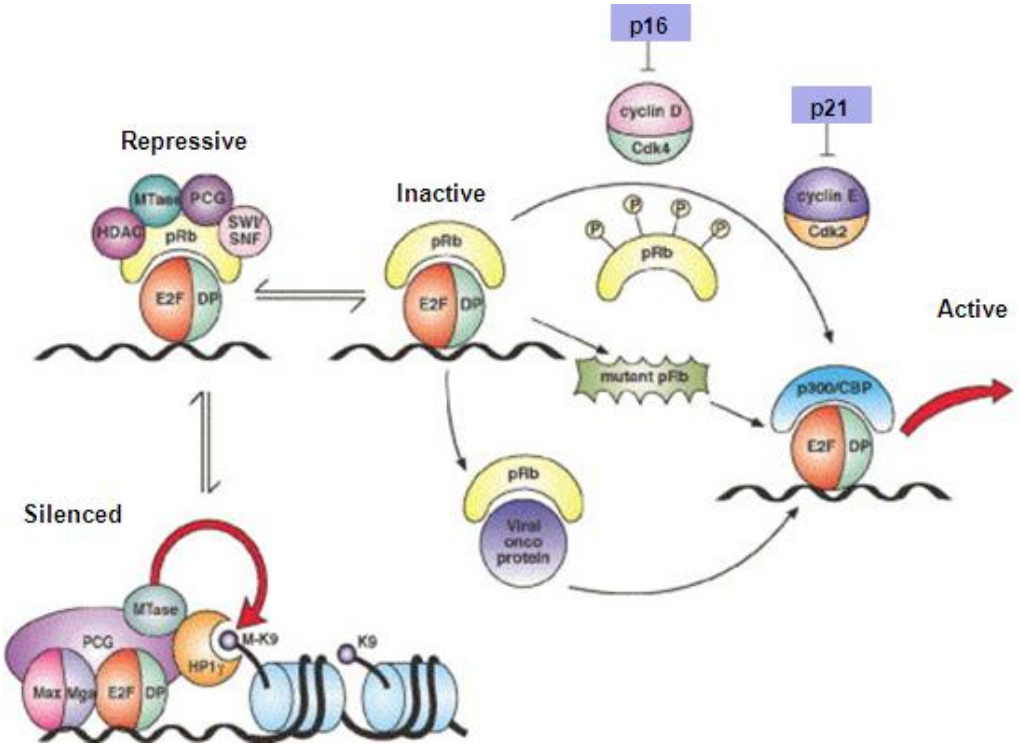


Figure 1.4: Regulation of E2F activity.

In non-proliferating cells, the E2F/DP heterodimer remains bound to pocket proteins such as pRb. During early cell cycle progression G1 cyclin-dependent kinase complexes phosphorylate pRb, thereby releasing E2F. E2F becomes transcriptionally active by interacting with transcription co-factors such as p300/CBP. In tumour cells on the other hand mutant pRb fails to bind E2F. Similarly, viral oncoproteins can bind pRb hindering its interaction with E2F. The pRb/E2F complex becomes an active transcriptional repressor by recruiting chromatin-modifying proteins, such as HDAC, MTase, PCG, and SWI/SNF. In quiescent G0 cells, E2F heterodimers can nucleate the assembly of another type of chromatin modifying complex containing MTase, PCG, and HP1 and involve other sequence-specific transcription factors such as Max and its partner Mga. By modifying the chromatin environment of target genes this complex coordinates long-term silencing of cell cycle-regulated genes.

Adapted from (Stevens and La Thangue, 2003a).

Furthermore, regulation of E2F activity involves the functional interplay among the E2F family members. The novel E2F family members E2F7 and E2F8 restrain the E2F1-p53 apoptotic axis and repress the E2F transcriptional network that is essential for embryonic development (Li et al., 2008a). Moreover, E2F7 and E2F8 are involved in the cellular response to DNA damage and suppress E2F target genes including *E2F1* (Zalmas et al., 2008). These findings underscore a critical role of the novel E2F family members in the regulation of E2F activity and thus cell fate determination.

1.4.1 Cellular functions of E2F1

E2F1, the founding member of the E2F family, has an essential role in cell cycle progression, proliferation and development (Dick and Dyson, 2003, La Thangue, 2003). Using DNA microarray analysis a diverse group of E2F1 target genes has been identified, including pro-proliferation genes as well as pro-apoptotic genes (Cress et al., 2002, Muller et al., 2001, Ishida et al., 2001, Polager et al., 2002). Interestingly, E2F1 can stimulate both proliferation and apoptosis and thus function as an oncogene or a tumour suppressor. The nature of this duality is based on the level of expression as well as the cellular environment (Crosby and Almasan, 2004).

The ability of E2F1 to function as an oncogene has been supported by cell culture-based transformation assays. An E2F1 mutant, which although being refractory to inhibition by Rb is capable to activate transcription, has been shown to have increased oncogenic potential (Johnson et al., 1994). This suggests that the oncogenic ability of E2F1 to transform cells is not due to Rb sequestration but

rather that Rb binding inhibits the oncogenic function of E2F1 (Johnson and DeGregori, 2006). However, under certain conditions, E2F1 has been shown to inhibit oncogenic transformation as well. In NIH3T3 cells, over-expression of E2F1 suppressed colony formation and inhibited focus formation (Lee and Farnham, 2000). Furthermore, in normal human foreskin keratinocytes (NHFK) expression of high levels of exogenous E2F1 reduced colony formation and immortalization mediated by the E6 and E7 human papillomavirus oncoproteins (Melillo et al., 1994). In contrast, when expressing a lower level of exogenous E2F1, instead of reducing colony formation E2F1 contributed to NHFK immortalization. Therefore, the levels of E2F1 expression and the cell type can dictate the oncogenic or tumour suppressor function of E2F1.

In addition to oncogenic transformation, E2F1 has been shown to have opposing effects in mouse models. *E2F1*$^{-/-}$ mice are predisposed to developing various tumours including lung adenocarcinomas and lymphomas (Yamasaki et al., 1996). Nevertheless, the tumour incidence in such mice is low in comparison to mice lacking other tumour suppressor genes but still significantly higher than the tumour incidence of wild type mice. Moreover, although absence of E2F1 protects *Rb*$^{+/-}$ mice from thyroid and pituitary tumours, the same mice often succumb to tumours that are commonly found in *E2F1* knockout mice (Yamasaki et al., 1998). These mouse models suggest that inactivation of *E2F1* can have either a positive or negative impact on tumour development depending on the tissue type.

1.4.2 Role of E2F1 in proliferation

Several lines of evidence demonstrate the ability of E2F1 to function as an oncogene, reflecting a positive role for this transcription factor in the promotion of cell proliferation. For example, the over-expression or untimely expression of E2F1 can result in cells progressing from the G0 to the S phase (Kowalik et al., 1995, Johnson et al., 1993, Sahin and Sladek, 2010).

The promoters of many cell-cycle regulated genes contain E2F binding sites and in a number of cases E2F1 has been shown to directly induce their expression. These E2F1 regulated genes include genes whose products regulate the cell cycle or play a role in DNA synthesis itself (Bell and Ryan, 2004, Kaelin, 2003). Thus, E2F1 can promote cell proliferation by activating the transcription of genes that regulate progression from the G1 to the S phase of the cell cycle, such as *E2F1* itself, *Cdk2* and *Cyclin E* (Phillips and Vousden, 2001, Hallstrom and Nevins, 2009). Furthermore, E2F1 induces the transcription of *dihydrofolate reductase (DHFR)*, *thymidine kinase* and *DNA polymerase α*, which are required for nucleotide and DNA synthesis (Phillips and Vousden, 2001).

1.4.3 Role of E2F1 in apoptosis induction

Despite the role of E2F1 in cell cycle progression, several studies demonstrate the ability of this transcription factor to function as a tumour suppressor (Pruschy et al., 1999, Meng et al., 1999). Under certain conditions, the activity of E2F1 is associated with events that determine cell fate by inducing apoptosis thereby protecting the organism from oncogenic transformation (Stanelle and Putzer, 2006, Wu and Yu, 2009, Adams and Kaelin, 1996).

A physiological role for E2F1 in apoptosis is suggested by the observation that ectopic expression of E2F1 in normal cells causes high levels of apoptosis (Qin et al., 1994). Furthermore, E2F1 deficient mice have been shown to have an excess of mature T cells caused by a defect in thymocyte apoptosis (Field et al., 1996). Moreover, aberrant apoptosis in embryos of Rb deficient mice was shown to be E2F1-dependend thereby suggesting the crucial role of E2F1 in apoptosis induction (Tsai et al., 1998).

1.4.3.1 E2F1 induces apoptosis through p53-dependent pathway

Different mechanisms have been described that play a role in the apoptosis mediated by the expression of E2F1. In the p53-dependent pathway, E2F1 expression leads to p53 accumulation through the activation of the cyclin-dependent kinase inhibitor 2A (CDKN2A) transcript *p14*^{ARF} (Knezevic and Brash, 2004, Bates and Vousden, 1999). In normal cells, p53 is negatively regulated by MDM2, an E3 ubiquitin ligase that targets p53 for proteosomal degradation (Vousden and Lu, 2002). p14^{ARF} binds to MDM2 and inhibits its ability to down-regulate p53, thus resulting in p53 accumulation and activation of the p53 target genes required for apoptosis (Wu et al., 2009).

However, E2F1 can induce apoptosis in a p14^{ARF} independent manner, thus indicating additional links between E2F1 and p53. E2F1-mediated apoptosis in the absence of p14^{ARF} was shown to correlate with the phosphorylarion of p53 at residues also phorsphorylated in response to DNA damage (Russell et al., 2002, Rogoff et al., 2002). The induction of apoptosis and p53 phosphorylation mediated by E2F1 is abolished by caffeine, an inhibitor of the protein kinases ATM and

ATR, suggesting that these kinases are implicated in E2F1-induced apoptosis (Rogoff et al., 2002, Ginsberg, 2002).

Furthermore, E2F1 can induce the expression of p53 apoptotic cofactors such as ASPP1 and ASPP2 which enhance the interaction of p53 with the pro-apoptotic target BAX (Wu et al., 2009). E2F1 can also up-regulate the expression of p53 apoptotic cofactors JMY and TP53INP1. JMY interacts with p300 to induce p53-mediated BAX expression, whereas TP53INP1 promotes p53 phosphorylation and apoptosis by activating the transcription of p53AIP1 (Coutts and La Thangue, 2006, Hershko et al., 2005).

1.4.3.2 E2F1 induces apoptosis through p53-independent mechanisms

The ability to mediate E2F1 induced apoptosis by inducing the transcription of p53 target genes is shared by another member of the p53 family. *p73* is an E2F1 responsive gene that is up-regulated upon ectopic or endogenous E2F1 expression (Irwin et al., 2000). Activation of p73 has been shown to lead to the transcriptional activation of p53-responsive genes and apoptosis in cells lacking p53 (Morris et al., 2006). On the other hand, disruption of p73 function causes the inhibition of E2F1-induced apoptosis (Stiewe and Putzer, 2000).

E2F1 can also directly activate the expression of the *Apaf-1* gene encoding for apoptosis protease-activating factor 1. Upon induction, Apaf-1 forms a complex with cytochrome c, a mitochondrial protein released in response to apoptotic signals, and activates procaspase 9 (Polager and Ginsberg, 2008). Activation of procaspase 9 leads to the activation of downstream effector caspases such as

caspase-3, caspase-6 and caspase-7, leading to apoptosis (Moroni et al., 2001, Furukawa et al., 2002). Apaf-1 is also targeted by p53 and is activated in response to DNA damage, suggesting that Apaf-1 could play role in mediating p53-dependent and E2F1-dependent apoptosis (Moroni et al., 2001).

Furthermore, E2F1 has been shown to induce the expression of pro-apoptotic Bcl-2 homology 3 (BH3)-only proteins, including Bcl-2 binding component 3 (PUMA), NADPH oxidase activator (NOXA) and Bcl2-like 11 (BIM) through a direct transcriptional mechanism (Hershko et al., 2005, Stanelle and Putzer, 2006). BH3-only proteins are members of the Bcl-2 protein family and trigger apoptosis by integrating diverse apoptotic stimuli into an apoptotic pathway governed by other Bcl-2 family members (Stanelle and Putzer, 2006).

In addition, E2F1 also transcriptionally activates other genes involved in apoptosis. These genes encode proteins that induce apoptosis by extracellular signals such as mitogen-activated protein kinase 5 (MAP3K5) as well as pro-apoptotic proteins including SIVA, Smac/DIABLO and the caspase family members caspase-3, caspase-7, caspase-8 and caspase-9 (Stanelle and Putzer, 2006, Wu et al., 2009).

1.4.3.3 E2F1 induces apoptosis by inhibition of survival signals

Another mechanism by which E2F1 induces apoptosis involves inhibition of survival signalling, including inhibition of necrosis factor κB (NF-κB) and anti-apoptotic Bcl-2 family members (Ginsberg, 2002). Tumour necrosis factor (TNF) receptors are activated in response to TNF-α, thereby resulting in the stimulation of NF-κB (Chen and Goeddel, 2002). Aberrant activation of NF-κB is responsible for tumourogenesis initiation by up-regulation of anti-apoptotic genes, such as *TNF*-

receptor-associated factor 2 (TRAF2) (Bracken et al., 2004, Putzer, 2007). Expression of E2F1 sensitizes cells to apoptosis by down-regulating TRAF2 protein levels and thereby inhibits anti-apoptotic NF-κB signalling (Phillips et al., 1999, Tanaka et al., 2002).

Furthermore, E2F1 can promote apoptosis by repressing the transcription of the anti-apoptotic family members Bcl-2 and myeloid leukaemia cell differentiation protein 1 (MCL-1) (Eischen et al., 2001, Croxton et al., 2002). Therefore, E2F1 can inhibit anti-apoptotic family members such as MCL-1 or induce the expression of pro-apoptotic proteins such as the BH3-only proteins and thereby disrupt the balance between apoptosis and survival signals, leading to programmed cell death. Thus, E2F1 can induce apoptosis via multiple mechanisms by transcriptionally activating or repressing its target genes.

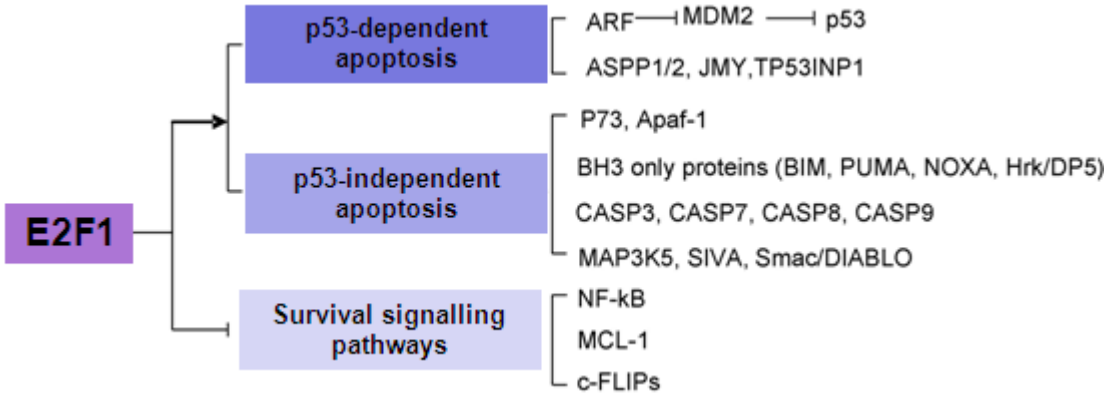


Figure 1.5: Mechanisms of E2F1-induced apoptosis.

E2F1 can induce apoptosis in a p53-dependent as well as a p53-independent manner. In the p53-dependent pathway, E2F1 induces apoptosis either by inducing p14ARF expression leading to p53 accumulation or by up-regulating the expression of p53 apoptotic co-factors such as ASPP1/2 and JMY. In the p53-independent pathway, E2F1 can induce numerous genes implicated in apoptosis such as p73. Furthermore, E2F1 activates the transcription of BH3-only proteins as well as several caspase family members. Additionally, E2F1 can also promote apoptosis through inhibition of survival signalling such as NF-κB and anti-apoptotic Bcl-2 family members. (Adapted from (Wu et al., 2009)

1.5 Gene transcription

The regulation of transcription, the synthesis of RNA from a DNA template, is one of the most significant steps in the control of cell growth and differentiation. The process of eukaryotic protein-coding gene transcription regulation is well-orchestrated, entailing the concerted functions of multiple proteins or transcription factors. These factors can be classified in three groups: (1) sequence-specific DNA-binding transcription regulators, (2) basal transcription factors and (3) transcription co-factors.

The DNA-binding transcription factors bind to proximal promoter elements and more distal regulatory sequences, such as enhancers and silencers, in order to modulate the rate of transcription of target genes in a tissue- and developmental stage-specific fashion or in response to environmental or physiological stimuli (Martinez, 2002). The basal transcription factors include RNA polymerase II (Pol II) and a set of general transcription initiation factors (GTFs), which bind to core promoter DNA elements allowing the specific recruitment of Pol II to the promoter of genes. The core promoter element consists of the initiator (INR) and the TATA box and is sufficient for directing initiation of transcription by the basal transcription machinery (Lee and Young, 2000). The TATA box is an AT-rich site that is located upstream of the start site and is the binding site for the TATA-binding protein (TBP) (Aso et al., 1994). On the other hand, the transcription co-factors include co-activators and co-repressors that interact with DNA-binding transcription factors and play important roles in mediating the effects of these factors on the basal transcription machinery via either direct interaction with GTFs or Pol II or indirectly by modifying the chromatin structure (Hahn, 2004).

Co-operative protein-protein and protein-DNA interactions play crucial roles in the precise binding of activating transcription factors to enhancers and in the specific assembly of GTFs and Pol II into a stable pre-initiation complex at the core promoter (Merika and Thanos, 2001, Orphanides et al., 1996).

1.5.1 The transcription initiation machinery

The basal transcription machinery is composed of Pol II and six GTFs: TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH. All GTFs, with the exception of TFIIB, are complexes formed by the stable association of multiple proteins. Pol II is also a large multi-subunit enzyme composed of 12 different proteins of which 5 (RPB5, RPB6, RPB8, RPB10 and RPB12) are shared with the other two RNA polymerases Pol I and Pol III. Pol I is involved in the synthesis of rRNAs whereas Pol II synthesizes small stable RNAs including the 5S rRNA and tRNAs.

Despite its complexity, Pol II is unable to recognise promoters and initiate transcription on its own. Recognition of core promoter elements by GTFs and the assembly of GTFs and Pol II into a pre-initiation complex (PIC) at the promoter are required for transcription initiation by Pol II (Hampsey, 1998). PIC assembly at a TATA-containing promoter is initiated by TFIID binding to the core promoter through both TBP interactions with the TATA box and TAF (TBP-associated factor) interactions with the initiator (INR) or other downstream promoter elements (Green, 2000). TFIIA binds to TBP and stabilises the TBP-TATA interactions contacting TBP and DNA upstream of the TATA element (Geiger et al., 1996). TFIIB further stabilises the TBP-TATA complex and allows the recruitment of TFIIF, Pol II, TFIIE, and TFIIH in either a sequential manner or as a preformed

holoenzyme in which Pol II is in also associated with the mediator co-activator components. Promoter melting is ATP-dependent and involves the ATPase activities in TFIIH. The nine-subunit TFIIH complex contains two helicases (XPB and XPD) involved in promoter melting, which are also important for Pol II promoter clearance (Dvir et al., 2001). TFIIE also stimulates the formation of the open complex that is competent to initiate transcription upon addition of ribonucleoside-triphosphate (NTPs). During initiation or early elongation the CTD (carboxy-terminal repeat domain) of Pol II becomes phosphorylated by Cdk7 kinase activity of TFIIH, allowing promoter clearance and transcription elongation by hyperphosphorylated Pol II in association with TFIIF (de Laat et al., 1999).

1.5.2 Transcriptional activation and repression

Starting from the first cell division and all the way to the complex integration of various signalling pathways in differentiated tissues, the multi-cellular organism must accurately regulate transcription in order to ensure correct gene expression. Therefore, gene activation and repression are tightly regulated mostly through changes in chromatin structure resulting from DNA methylation, chromatin remodelling as well as histone modifications. There is a variety of proteins involved in the regulation of chromatin structure including DNA methyltransferases, DNA-binding transcription factors and chromatin-remodelling complexes (Perissi et al., 2010).

Histone proteins are assembled on DNA as nucleosomes and pose a formidable intrinsic barrier that restricts access of transcription machinery, thereby reducing background transcriptional noise that could be generated by fortuitous interactions

(Struhl, 1999). The flexible tail regions of histones also provide a platform for elaboration of different modification marks of regulatory significance (Payankaulam et al., 2010). Chromatin-remodelling complexes utilize ATP hydrolysis in order to alter the chromatin structure. As a result, nucleosome structure is modified and subsequently the accessibility of DNA for transcription factors is modulated (Cairns, 2007). Co-activators and co-repressors play a crucial role in altering chromatin structure by modifying the core histone amino-terminal tails. These histone modifications include acetylation, methylation, phosphorylation, sumoylation, ubiquitylation and ADP ribosylation (Bhaumik et al., 2007, Kouzarides, 2007, Suganuma and Workman, 2008). The combination of such histone modifications that directs specific and distinct transcriptional programmes has been proposed to constitute a 'histone code' (Strahl and Allis, 2000).

1.5.3 Transcriptional activation

Gene-specific transcriptional activation is generally affected by the binding of activators to upstream activating sequences. Upon binding, the activators recruit and regulate the activities of chromatin-remodelling complexes and the transcription apparatus (Lee and Young, 2000). Transcriptional activators bind to DNA in a sequence-specific manner and mediate gene activation by recruiting co-activator proteins. Co-activators function in various ways and often contain enzymatic activities that alter chromatin structure from a quiescent state to a state that allows active gene transcription.

Co-activators can be broadly divided in three classes, the first class modifying histones in order to allow greater access of other proteins to DNA. This class includes p300 and CBP which are histone acetyltransferases (HATs), which function as co-activators for a variety of transcription factors (Hermanson et al., 2002). Members of the TRAP/ DRIP/Mediator/ARC complex constitute the second class of co-activators, which bind to transcription factors, recruit Pol II and interact with the general transcription apparatus. The last group comprises the protein complexes of the yeast SWI/SNF (mammalian homologues BRG1 or BRM) which contain ATP-dependent DNA unwinding activities that are necessary for efficient gene transcription (Spiegelman and Heinrich, 2004).

1.5.4 Transcriptional repression

Co-repressor complexes utilize mechanisms by which they drive repression events and the chromatin modifications that influence gene repression. Several modifications that are associated with repression, such as histone deacetylation and specific cases of methylation and ubiquitylation, are thought to act together to impose a higher order structure on chromatin in order to create an environment that is restrictive to gene transcription (Perissi et al., 2010). Nevertheless, the mode of action and specificity of co-repressors as well as the histone modifications they mediate have not been elucidated in great detail.

Recently, however, technical developments in the study of gene regulation on a genome-wide scale have provided a more integrated view of the dynamics involved in chromatin modifications and have thus increased our understanding of co-repressor actions. According to the original model of co-repressor activity, co-

repressors associate with repressed genes which are replaced by co-activators during gene activation (Hager et al., 2009). The recent genome-scale data resulted in a reassessment of this original model of co-repressor activity. The emerging concept suggests that long-term gene repression is maintained not by the constitutive presence of co-repressor complexes but rather by histone modifications which are maintained by intermittent co-repressor activity (Metivier et al., 2003, Degenhardt et al., 2009). The current models of co-repressor function provide a new viewpoint for the dynamics of the co-activator and co-repressor complexes, suggesting that they continually cycle on and off DNA.

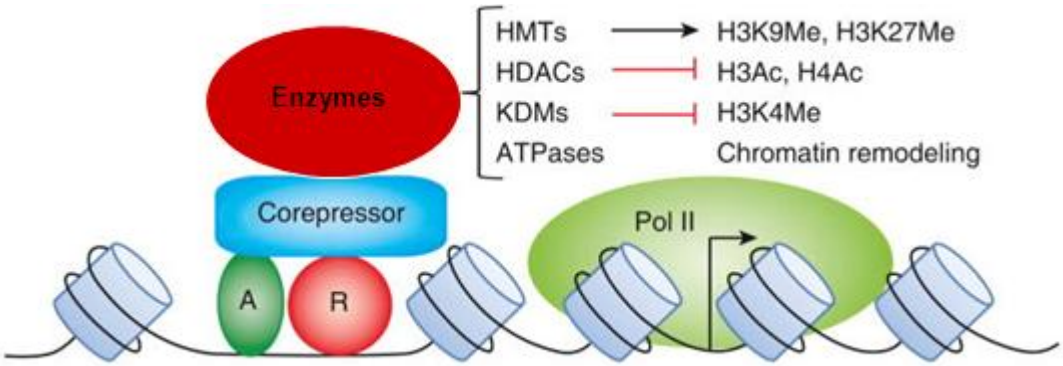


Figure 1.6: The transcriptional repression mechanism

Transcriptional repressors (R) recruit co-repressors and chromatin modifying enzymes such as histone methyltrasferases (HMTs), histone deacetylases (HDACs), lysine demethylases (KDMs) and chromatin remodelling factors that contain ATPase activity. In certain cases co-repressors interact directly with the basal machinery or the activators (A). (Adapted from (Payankaulam et al., 2010)

1.5.5 Co-repressor complexes and enzymatic activities

The term co-repressor refers to a single protein or a scaffolding protein that mediates the assembly of a multi-subunit complex which is recruited to a gene by transcription factors (Payankaulam et al., 2010). Co-repressor complexes can exhibit various enzymatic modifications of histone tails in order to mediate repression including histone lysine deacetylation, changes in the methylation status of certain lysine residues, arginine deamination and histone ubiquitination (Perissi et al., 2010, Kumar et al., 2004). These modifications are imposed by the recruitment of various enzymes to DNA by co-repressors.

Co-repressors complexes frequently contain histone deacetylases (HDACs), whose activity is important in stripping the activation marks from histone tails. Acetylation is associated with the relaxation of chromatin structure and thus with increased transcriptional activity. Removal of acetyl groups from histone tails results in a condensation of the DNA structure preventing gene transcription (Perissi et al., 2010). For example, HDAC activity is an essential part of the CoREST (co-repressor for repressor element 1 silencing transcription factor) complex which is recruited by the REST transcription factor in order to regulate neuronal gene expression. In addition to HDAC1 and HDAC2, the CoREST complex also contains lysine specific demethylase 1 (LSD1) thereby expanding its chromatin remodelling capacity with histone demethylase activity (Lee et al., 2005, Shi et al., 2005).

In addition to the CoREST complex, the C terminal binding protein (CtBP) co-repressor complex also exhibits histone deacetylase activity via recruitment of HDACs. Besides HDACs, the CtBP complex contains an array of enzymes that modify chromatin including histone methyltransferases and histone demethylases (Shi et al., 2003). This complex is involved in the regulation of various cellular processes including tumourogenesis, differentiation and apoptosis (Chinnadurai, 2003). Interestingly, CtBP is one of the co-repressors that link signalling pathways with transcription activity. Certain co-repressors are targeted by signalling pathways and could provide a unique communication link with the cellular environment. Co-repressors can be subject of post-translational modifications leading to a change in their subcellular localization or regulation of transcription factor binding. CtBP1 sumoylation is essential for its nuclear accumulation and subsequent repression of the E-cadherin promoter, while CtBP2 acetylation by p300 is necessary for nuclear retention and repression activity (Lin et al., 2003, Zhao et al., 2006).

Therefore, co-repressors are not only interchangeable levers that lower the nucleosome barrier but rather sensitive control points providing additional regulatory modulations via structural diversification, by sensing signalling cascades and context-dependent responses (Payankaulam et al., 2010). Furthermore, co-repressor complexes appear to contain a limited suite of chromatin modifying proteins but it is through their diversity and modifications that allow the fine-tuning of transcriptional output to the changing cellular environment.

1.5.5.1 The CtBP family of proteins

The CtBP family of proteins are highly conserved and unique to higher eukaryotes (Chinnadurai, 2007). The name CtBP derives from the discovery of the founding member of the family CtBP1 due to its interaction with the C-terminus of adenovirus E1A proteins (Boyd et al., 1993, Schaeper et al., 1995). The vertebrate genomes contain two *CtBP* genes, *CtBP1* and *CtBP2*, which exhibit redundant and unique functions during development (Hildebrand and Soriano, 2002, Turner and Crossley, 2001). CtBP1 and CtBP2 function as transcriptional co-repressors for a great number of repressors and are collectively referred as CtBP (Zhao and Chinnadurai, 2010).

Due to alternative RNA splicing there are two CtBP1 isoforms, CtBP-L and CtBP-S, both variants having transcriptional co-repressor and cytosolic functions (Chinnadurai, 2002). CtBP-S was identified as a Golgi-associated protein that mediates Golgi fission (Corda et al., 2006). CtBP1 associates with centrosomes during mitosis, suggesting a possible role of CtBP1 in Golgi maintenance during cell division (Spyer and Allday, 2006). Furthermore, CtBP1 contains a PDZ-binding motif at its C-terminus that is implicated in the nuclear export of CtBP1 in association with partners containing a PDZ domain (Riefler and Firestein, 2001).

The *CtBP2* locus on the other hand codes for three protein isoforms, two of those isoforms CtBP2-L and CtBP2-S being highly homologous to the CtBP1 isoforms (Verger et al., 2006, Chinnadurai, 2009). The third CtBP2 isoform designated as RIBEYE is generated from a different promoter than the CtPB2-L and CtBP2-S

promoter. RIBEYE contains a large N-terminal domain that is unrelated to other CtBPs and is exclusively cytosolic (Schmitz et al., 2000, tom Dieck et al., 2005).

1.5.5.1.1 The role of CtBP in repression

The CtBP family members function predominately as transcriptional co-repressors by forming a chromatin-remodelling complex recruited to promoters via DNA-binding transcription factors (Shi et al., 2003). Many proteins including E1A, transcription factors and class II HDACs bind to CtBP via a consensus PLDLS CtBP-binding motif (Chinnadurai, 2007, Bergman and Blaydes, 2006). In addition to the PLDLS-mediated binding, certain transcriptional repressors interact with CtBP via a second redundant binding motif, the RRT motif (Quinlan et al., 2006). Several DNA-binding proteins, that bind promoter elements directly, have been shown to interact with CtBP via the PLDLS and RRT motifs in analyses of the CtBP nuclear complex (Shi et al., 2003). In addition to DNA-binding proteins such as RREB-1, Znf217 and ZEB1/2 these analyses also showed that the CtBP complex contains enzymatic constituents that catalyze different histone modifications (Postigo and Dean, 1999, Thiagalingam et al., 1996, Cowger et al., 2007). These enzymes include class I and II HDACs, histone lysine methyl transferases and the lysine specific demethylase 1 (LSD1) (Chinnadurai, 2007). In contrast to class II, the class I HDACs do not contain apparent CtBP-binding motifs but are rather linked to CtBP via other co-repressors. These CtBP-associated co-repressors that interact individually with the class I HDACs include CoREST, LCoR (Ligand-dependent nuclear receptor co-repressor) and CDYL (Chromo Domain Y-Like) (Ballas et al., 2001, Caron et al., 2003, Fernandes et al., 2003).

CtBPs are able to function both in an HDAC-dependent and HDAC-independent manner in different promoter contexts (Shi et al., 2003, Dahiya et al., 2001). CtBP-mediated repression of the E-cadherin promoter was shown to be considerably relieved by the HDAC inhibitor TSA (Shi et al., 2003). The same study showed that siRNA depletion of HMTases impaired repression of the E-cadherin promoter. Although class I HDACs can target multiple lysine residues on histones, the HMTases were shown to mainly target lysine 9 of histone H3 (H3-K9), thus suggesting that these enzymes mediate CtBP-induced repression by coordinated methylation and deacetylation of H3-K9 (Tachibana et al., 2005). Additionally, the presence of LSD1 in the CtBP complex can further contribute in the CtBP-mediated transcriptional repression (Shi et al., 2005). LSD1 binds directly to CoREST, the interaction being essential for the demethylation of nuclesomal substrates by LSD1 (Lee et al., 2005, Yang et al., 2006). CoREST also binds directly to CtBP, thereby contributing to repression of some CtBP target genes (Cowger et al., 2007, Kuppuswamy et al., 2008). The presence of different chromatin-modifying enzymes in a common CtBP complex supports a model of coordinated deacetylation and demethylation of histone tails in order to generate a repressive chromatin structure (Lan et al., 2008).

1.5.5.1.2 Regulation of CtBP transcriptional activity by NAD(H) dinucleotides

CtBPs share striking amino acid and structural homology to D-isomer-specific 2-hydroxy acid dehydrogenases (D2-HDH) predicting a critical role for NAD(H) dinucleotides in CtBP activity (Kumar et al., 2002, Ghosh et al., 2010, Fjeld et al., 2003). However, the role of the dehydrogenase activity in CtBP-mediated repression remains controversial. The dinucleotides were shown to stimulate

dimerization and interaction of CtBP with certain PLDLS-containing target proteins (Nardini et al., 2003, Balasubramanian et al., 2003). On the other hand there are some reports based on analysis of CtBP mutants suggesting that NAD(H) binding may not be crucial for the CtBP repression activity (Grooteclaes et al., 2003, Marmorstein, 2002).

Although CtBP was shown to have a slow dehydrogenase activity, the cognate substrate of this activity remained obscure (Balasubramanian et al., 2003, Shi et al., 2003). Recently, 2-keto-4-methylthio-2-oxo butyrate (MTOB) an intermediate of the methionine salvage pathway was identified as a CtBP dehydrogenase substrate (Straza et al., 2010). The analysis of the CtBP protein complex constituents has lead to establishing a general mechanism of CtBP-mediated repression (Chinnadurai, 2007). Following recruitment to the target promoter by a DNA-binding transcriptional repressor, the CtBP dimer simultaneously interacts with a chromatin-modifying protein complex in order to suppress gene transcription. By structural and biochemical studies, a NAD(H)-binding domain in CtBP has been shown to be a redox sensor of cellular NAD^+/NADH ratio that alters CtBP conformation and dimerization thereby regulating CtBP interactions (Zhao and Chinnadurai, 2010).

In the CtBP structure, the PLDLS binding cleft and the substrate-binding domain are physically in close proximity to the dehydrogenase catalytic centre (Kumar et al., 2002, Nardini et al., 2003). Studies using MTOB suggest that substrate loading can exert a significant suppression on CtBP recruitment to target promoters (Straza et al., 2010). These results indicate that MTOB loading to the CtBP substrate-

binding domain could induce a conformational change in the PLDLS binding cleft that results in reduced interaction of CtBP with transcriptional repressors (Figure 1.7).

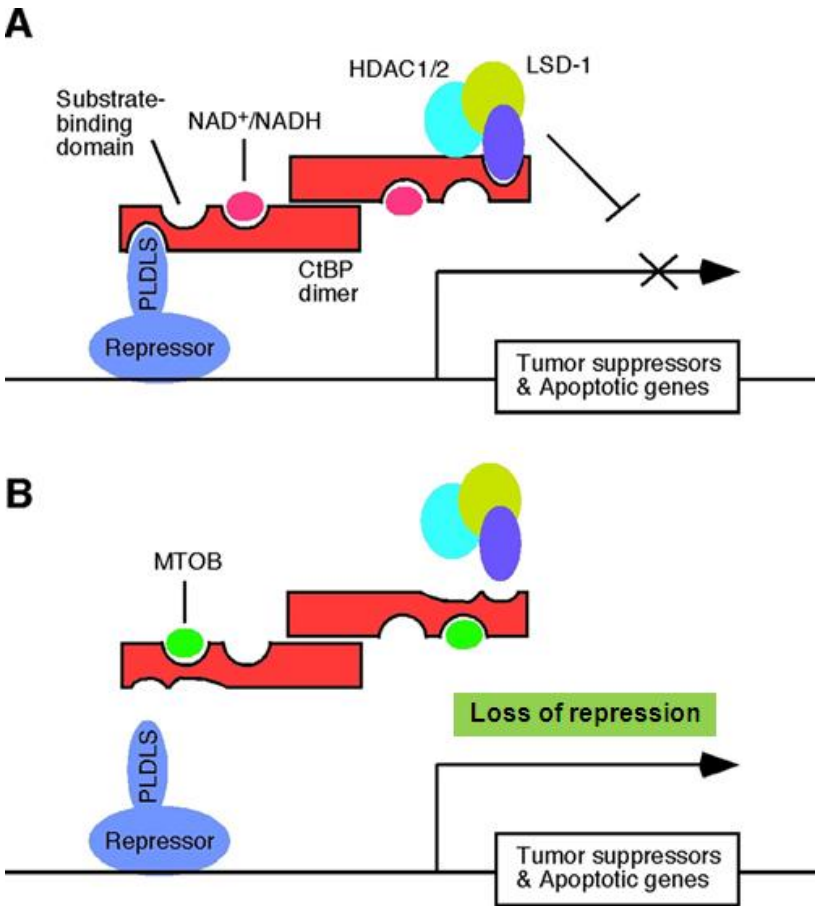


Figure 1.7: Transcriptional repression mechanism by the CtBP dimer.

(A) Under normal conditions CtBP mediates the transcriptional repression of tumour suppressors and apoptotic genes. One subunit of the CtBP dimer binds with the repressor while the second subunit interacts with the chromatin-modifying components of the repression complex. **(B)** Binding of MTOB to the substrate-binding domain of CtBP leads to the transcriptional activation of tumour suppressors and apoptotic genes. MTOB binding might induce conformational changes in the PLDLS-binding cleft causing the dissociation of the CtBP complex from the repressor, thereby resulting in de-repression of the target genes.

Adapted from (Zhao and Chinnadurai, 2010)

1.5.5.2 HDACs

Histone acetylation is a reversible process where histone and non-histone protein acetyltransferases transfer the acetyl moiety to lysines and HDACs remove the acetyl groups thereby re-establishing the positive charge in the proteins (Marks and Xu, 2009). In the past decade, the knowledge about lysine acetylation and deacetylation has grown exponentially with targets expanding from histones to transcription factors and other proteins including metabolic enzymes and signalling regulators in the cytoplasm (Peserico and Simone, 2011, Bertos et al., 2001). Furthermore, several protein properties are regulated by lysine acetylation such as DNA-protein interaction, transcriptional activity, subcellular localization and involvement in signalling pathways (Spange et al., 2009, Choudhary et al., 2009).

At the chromatin level, it has been extensively demonstrated that the balance between acetylation and deacetylation of histone and non-histone proteins plays a key role in the regulation of gene expression. The general model of transcription is based on the interaction among transcription factors, co-activators, co-repressors, as well as sequence-specific DNA-binding proteins, which confer tissue and signal-dependent specificity (Gregoretti et al., 2004). Several co-repressor complexes have been shown to contain a variety of chromatin-modifying enzymes, including HDACs.

HDACs can be grouped into four classes according to their phylogenetic conservation (Gregoretti et al., 2004). Class I, class II, and class IV HDACs are related to the yeast Rpd3, Had1, and Hos3 proteins respectively. These three classes encompass the classical family of zinc-dependent HDACs. On the other

hand class III HDACs consists of the NAD^+-dependent yeast Sir2 homologues comprising the sirtuin family (de Ruijter et al., 2003, Haigis and Guarente, 2006). Class III HDACs are not zinc dependent and generally not inhibited by compounds that inhibit the zinc dependent HDACs (Marks and Xu, 2009).

1.5.5.2.1 Class I HDACs

Class I HDACs consist of HDAC1, HDAC2, HDAC3 and HDAC8 which are ubiquitously expressed in all tissue types (Witt et al., 2009). HDAC8 plays a significant role in smooth muscle cells, where it associates with α-actin and is necessary for cell contractility (Waltregny et al., 2005). Recently, a relation has been described between the transcriptional over-expression of HDAC8 and advanced or metastasized stages of childhood neuroblastoma (Oehme et al., 2009).

HDAC3 on the other hand plays a role in cell cycle progression and DNA damage response (Bhaskara et al., 2008). Furthermore, HDAC3 has been shown to be involved in spindle assembly checkpoint and sister chromatid cohesion (Eot-Houllier et al., 2008). Studies have shown that germline deletion of HDAC3 is lethal, while conditional HDAC3 knockout in MEF cells plays a role in the transcription of a variety of genes involved in metabolism, apoptosis, cell cycle, development, and signal transduction (Bhaskara et al., 2008).

The other two members of the class I HDACs, HDAC1 and HDAC2, are strictly related proteins and have crucial roles in development and physiology (Haberland et al., 2009). HDAC1 and HDAC2 are highly homologous proteins that show redundant or specific roles in different cell types or in response to different stimuli

and signalling pathways (Segre and Chiocca, 2011). Additionally, they are deeply involved in cellular proliferation, cell cycle and apoptosis. HDAC1 has a key role in regulating the cyclin-dependent kinase inhibitor p21 (Zupkovitz et al., 2010). HDAC1 knockout mice are embryonic lethal as a result of strong impairment in cellular proliferation (Lagger et al., 2002). Depletion of HDAC1 causes cell cycle perturbation with loss of mitotic cells and increase in apoptosis in several human cancer cell lines (Senese et al., 2007).

HDAC1 and HDAC2 have been shown to be deregulated in many cancers and are therefore emerging as the main deacetylases implicated in aberrant pathways of tumor cells in humans (Segre and Chiocca, 2011). Over-expression of HDAC1 has been reported for gastric, pancreatic, colorectal, hepatocellular and prostatic carcinomas (Weichert et al., 2008a, Miyake et al., 2008, Weichert et al., 2008b). HDAC2 has been shown to play a major role in APC-colon cancer, as well as in cervical dysplasia and invasive carcinomas (Mariadason, 2008). Furthermore, high expression levels of both HDAC1 and HDAC2 have been associated with advanced disease stages in prostate, gastric and colorectal cancers (Weichert et al., 2008c). Due to the involvement of deacetylases in cancers, HDACs have been targeted as promising anticancer drugs. Pharmacological manipulation of acetylation has been possible following the identification of small molecule HDAC inhibitors (HDACi) (Stimson and La Thangue, 2009). HDACi have been shown to affect tumour cell growth and survival by inducing cell cycle arrest, blocking angiogenesis, increasing antigenicity of tumour cells and induction of apoptosis (Marks et al., 2001).

1.5.5.3 CoREST

The CoREST (co-repressor for element-1-silencing transcription factor) complex was first identified by its association with the repressor for element-1 silencing transcription factor (REST). The CoREST complex is a chromatin-modifying co-repressor complex that associates with REST in order to regulate neuronal gene expression and neuronal stem cell fate (Lakowski et al., 2006). Although the CoREST complex is best characterized for its role in the regulation of REST target gene expression, it is also recruited by additional transcription factors and thus has additional biological roles (Qureshi et al., 2010).

The CoREST protein contains two SANT domains which are commonly found in components of transcriptional repressor complexes. SANT domains were originally identified in SW13, ADA3, NCoP and TFIIIB and thus named SANT (Aasland et al., 1996). These are conserved domains that resemble the DNA-binding domains of Myb-related DNA-binding proteins and have been implicated in binding to histone H3 tails thereby helping to present these tails to associated chromatin-modifying enzymes (Boyer et al., 2004). CoREST also contains an ELM2 domain, a small domain that might be involved in DNA-binding or protein-protein interactions which is often found N-terminally to a SANT domain (Lakowski et al., 2006).

CoREST appears to be the scaffold protein on which the co-repressor complex assembles. Components of this complex include the chromatin-modifying proteins HDAC1, HDAC2 and LSD1. The N-terminal 179 amino acids of CoREST, that encompass the ELM2 and SANT1 domains has been shown to interact with HDAC1 (You et al., 2001). Furthermore, CoREST has been shown to contain two

distinct repressor domains that seem to act cooperatively. The first repressor domain is an N-terminal domain spanning amino acids 102 and 195 (the ELM2 and SANT1 domains) while the second repressor domain is a C-terminal domain between amino acids 321–442, including the SANT2 domain (Ballas et al., 2005). On the other hand, LSD1 binds to CoREST through a domain mapping between the two SANT domains. However, the presence of the SANT2 domain has been shown to be required for the CoREST-mediated stimulation of LSD1 activity (Shi et al., 2005).

1.5.5.4 LSD1

Lysine-specific histone demethylase 1 (LSD1) is a polyamine oxidase-like protein. It contains an N-terminal SWIRM (Swi3p Rsc8, Moira) domain, which is a small α-helical 85 amino acid domain that might act as a protein-protein interaction domain in chromatin-protein complexes (Aravind and Iyer, 2002). It is therefore likely that LSD1 associates with the CoREST complex through its SWIRM domain. Most of the protein C-terminal to the SWIRM domain shows high similarity to flavin-dependent polyamine oxidases and therefore the ability of LSD1 to bind riboflavin was expected (Humphrey et al., 2001). Furthermore, the flavinadenine dinucleotide (FAD)-binding pocket as well as the catalytic domains of the protein family seem to be conserved in LSD1 (Aravind and Iyer, 2002). LSD1 has histone lysine demethylase activity and has been shown to be able to remove activating mono- and di- methyl marks on lysine 4 of histone H3 (H3 K4) (Shi et al., 2004). The demethylase activity of LSD1 is stimulated by CoREST association (Lee et al., 2005; Shi et al., 2005), which is required for the demethylation of nucleosomes (Lee et al., 2005). FAD binding is essential for

LSD1 activity and the first 21 N terminal amino acids of histone H3 are required for LSD1 high demethylase activity (Forneris et al., 2005b). Furthermore, acetylation of H3 K9 reduces LSD1 activity whereas phosphorylation of H3 serine 10 abolishes the demethylase activity (Forneris et al., 2005a). Therefore it is suggested that LSD1 can recognize the modification state of the amino terminus of histone H3 which affects its activity.

The initial characterization of the LSD1–CoREST–HDAC complex came from the study of the molecular mechanism by which the transcription factor repressor REST mediates long-term repression of neuronal genes in non-neuronal cells and in neuronal precursors (Ooi and Wood, 2007, Battaglioli et al., 2002, Ballas et al., 2005). Recruitment of the LSD1–CoREST–HDAC core leads to the repression of REST target genes, followed by lysine deacetylation on H3 and H4 and demethylation of H3K4. Interference with any of these chromatin-modifying events results in the loss of REST mediated repression as well as ectopic reactivation of neuronal genes (Lunyak et al., 2002). In particular, LSD1 knockdown is accompanied by the reappearance of H3K4 methylation marks in the proximity promoters targeted by REST (Shi et al., 2005). Thus, the physiological activity of LSD1 in repressing REST is consistent with its histone demethylase activity.

Whereas the role of LSD1 as a transcriptional co-repressor has been well characterized, more debated is its ability to activate transcription. Recent studies have shown that activation of androgen receptor (AR) target genes requires histone H3K9 demethylation by LSD1 (Metzger et al., 2005). In particular, following hormone treatment, AR and LSD1 were shown to co-localize on promoters and

stimulate H3K9 demethylation without altering the H3K4 methylation status. Further establishing its role in the activation of AR target genes, LSD1 knockdown resulted in decreased activation of AR-responsive promoters.

However, the mechanism by which LSD1 demethylation specificity is altered from H3K4 to H3K9 in the AR-dependent gene regulation context remains unknown. It is possible that this activity results from a conformational change induced by either a protein–protein interaction or from a post-translational modification. Alternatively, another possibility is that H3K9 demethylation might result from an H3K9-specific histone demethylase recruited by LSD1 or by an LSD1- associated protein, rather than being catalyzed directly by LSD1. The latter hypothesis is corroborated by the observation that certain chromatin-remodelling complexes contain both LSD1 and an H3K9 demethylase of the Jumonji-containing class (Wissmann et al., 2007).

1.6 Aims and objectives

The E2F family of transcription factors plays a crucial role in the regulation of cellular proliferation, apoptosis, and differentiation. The most recently identified family members E2F7 and E2F8 have unusual features that distinguish them from other members in the E2F family. These atypical E2Fs contain two distinct DNA-binding domains and bind to DNA in a DP-independent manner. Studies investigating the role of E2F7 have shown that E2F7 is a transcriptional repressor of certain E2F target genes including *E2F1*. However, the mechanism by which E2F7 represses E2F responsive gene expression is unknown.

As E2F7 plays a crucial role in dictating the effect of E2F1 in cells, identification of the mechanisms involved in E2F7-dependent repression is of great importance. The purpose of this study is to attempt to elucidate the mechanism by which E2F7 can repress transcription. The chapters of this thesis aim to:

Chapter 3

- Verify the role of E2F7 as a transcriptional repressor and identify the target genes at which E2F7 exerts its repressive role
- Identify components involved in the E2F7 repression mechanism
- Following identification of CtBP as an E2F7 interacting protein, assess the importance of this co-repressor in the repression of E2F target genes
- Investigate the recruitment and co-recruitment of E2F7 and CtBP to E2F responsive promoters

Chapter 4

- Assess the importance of the E2F7 CtBP-binding motif via mutational analysis
- Investigate the E2F7-CtBP interaction under different conditions such as: different types of cell stress, presence of other proteins containing a CtBP-binding motif and different cellular redox states
- Examine the effect of the recently identified CtBP substrate MTOB on E2F7-mediated transcriptional repression

Chapter 5

- Following the identification of CtBP in an E2F7 repression complex, investigate the presence of chromatin modifying enzymes known to be components of CtBP repression complexes, such as HDAC1 and HDAC2
- Assess the effect of the histone deacelylase inhibitor TSA on the transcriptional activity of E2F target genes
- Investigate the recruitment and co-recruitment with E2F7 of HDAC1 and HDAC2 to the promoters of E2F target genes

Chapter 6

- Identify additional co-repressors or chromatin modifying enzymes that form a complex with E2F7
- Following identification of CoREST and LSD1 as E2F7 interacting proteins, investigate their importance in the transcriptional repression of E2F target genes via siRNA
- Examine the promoter recruitment profiles of CoREST and LSD1
- Investigate the recruitment of all co-repressors and chromatin modifying enzymes to E2F responsive promoters upon E2F7 siRNA

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

Table 2.1: List of cell lines

Cell Line	Origin	Information
HEK293	Embryonic kidney	Transformed with adenovirus DNA
HeLa	Cervical carcinoma	pRb inactivated, p53 degraded
MCF7	Breast adenocarcinoma	Caspase-3 deletion, wild-type p53
U2OS	Osteosarcoma	Hyperphosphorylated pRb, wild-type p53

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 5% foetal calf serum (FCS) in the presence of 5% (v/v) penicillin and streptomycin (Invitrogen). Cells were grown at 37 ºC in a humified incubator with 5% carbon dioxine (CO_2) and were regularly passaged at 80-90% confluency. For this, cells were rinsed once with Phosphate Buffered Saline (PBS) and incubated with trypsin/EDTA solution (Lonza) until they detached from the flask. Trypsin was quashed by addition of equal volume of full medium and cells were maintained in fresh growth medium or seeded for subsequent manipulations.

2.2 Compounds and drug treatments

Cells were damaged where indicated, with 10 µM etoposide (Sigma-Aldrich) or 50 J/m^2 UV, and harvested 2-16 hours later. Cells were also treated with 2-Deoxy-D-glucose (2-DG), sodium azide (NaN_3) or 4-methylthio-2-oxobutyric acid (MTOB) (all Sigma-Aldrich) at the indicated concentrations and for the indicated times before harvesting.

2.3 Transient transfections and expression vectors

Cells were transfected with 1-3 μg of total DNA at 30-50% total confluency, using GeneJuice (Merck) according with the manufacturer's protocol. Untransfected and mock controls were routinely used and pcDNA3.1 vector (Invitrogen) was used to equalize the amount of DNA, in the case of co-transfection. Cells were harvested 24-48 hours after transfection.

The following plasmids were previously described (Logan et al., 2004): pCMV HA-E2F7, pCMV HA-E2F1, pCMV HA-DP1, pCMV β-galactosidase, pLuc E2F1, pLuc cdc6, pLuc cyclin E and pLuc p73. The E1A and E1AΔCID plasmids (Johansson et al., 2005) were a gift from Dr Catharina Svensson. Plasmid DNA was extracted from bacterial cultures using the Plasmid Maxi Kit (Qiagen) according to manufacturer's protocol. DNA purity and yield were assessed by spectrometry.

2.3.1 E2F7 mutant plasmid

Stratagene's QuikChange Multi Site-Directed Mutagenesis kit was used to generate the pCMV HA-E2F7 deletion mutant lacking the CtBP binding motif (E2F7Δ) using the following primers: forward: caaagtagggaatatgaagacggcatgcccaag and reverse: cttgggcatgccgtcttcatattccctactttg (Sigma Genosys). The resulting construct was verified by sequencing.

2.4 Oligonucleotide based siRNA

Short interfering RNA (siRNA) was performed using synthesized 21 base pair (bp) oligonucleotides. The siRNAs were designed to either contained a 19 bp target sequence to which a 3' dTdT or UU was added, or consisted of a 21 bp target sequence starting with AA. The siRNA duplexes were (Table 2.2) were diluted in siRNA buffer (100 mM KCl, 30 mM HEPES pH7.5, 1 mM $MgCl_2$) and used at 20-50 nM final concentration. An equal concentration of non-targeting (NT) siRNA was transfected as a control. Cells were transfected at 20-40 % confluency with Oligofectamine (Invitrogen), according to manufacturer's protocol. Cells were incubated with the siRNA for 48-96 hours prior to further treatments and harvesting.

Table 2.2 List of siRNA oligonucleotides

siRNA	SEQUENCE	SOURCE
E2F7.1	5'- AAA GGT ACG CCT CTA TGA -3'	Dharmacon
E2F7.2	5'- AAC AGA AGA GCG AGG TCG TAA -3'	Dharmacon
CtBP	5'- GGG AGG ACCU GGA GAAG UU -3'	Dharmacon
LSD1	5'- GGC CUA GAC AUU AAA CUG ATT -3'	Dharmacon
Co-REST.1	5'- GGA AUU GGU UUC AGU CAA AUU -3'	Dharmacon
Co-REST.2	5'- CGA CGC CGC UUC AAC AUA GUU -3'	Dharmacon
Non-targeting	5'- UAA GGC UAU GAA GAG AUA CUU -3'	Dharmacon

2.5 Protein extraction, SDS-PAGE and western blotting

Cells were washed and harvested in phosphate buffered saline (PBS) and pelleted by centrifugation for 10 minutes at 0.8 *g* at 4 ºC. The supernatant was discarded and the cell pellet was lysed in TNN lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM ethylene diamine tetra-acetic acid (EDTA) pH 8.0, 150 mM NaCl, 0.5 % Igepal (NP-40), 10 mM NaF, 1 mM $NaVO_4$ and protease inhibitor cocktail (PIC) on ice for 20 minutes and then centrifuged at 16,000 g for 10 minutes at 4 ºC. Protein concentration was measured using the Bradford method (BioRad).

In order to measure transfection efficiency β-galactosidase was transfected into cells, and then the β-galactosidase activity was measured by mixing 15 µl of cell extract with an equal amount of 2x ONPG buffer (20 mM sodium phosphate buffer pH 7.3, 2 mM $MgCl_2$, 1.33 mg/ml ONPG (Sigma) and 100 mM β-mercaptoethanol) in a cuvette. The mixture was incubated at 37 ºC until a yellow colour was detected. 770 ml of water was then added to the cuvette and the absorbance was read at OD 420 nm. All ectopically expressed proteins were loaded by the ratio of total protein concentration/β-galactosidase activity.

Protein extracts were routinely fractionated by SDS-PAGE using the Mini Protean III system (BioRad). Briefly, samples were diluted accordingly into Laemmli sample buffer (0.125 M Tris pH 6.8, 2 % SDS, 10% glycerol, 10 % β-mecraproethanol, 0.1 % bromophenol blue), heat denatured at 100 ºC for 5 minutes and subsequently run on 10 % SDS gels (resolving gel: 10 % acrylamide: bisacrylamide (Sigma), 375 mM Tris-HCl pH 8.8, 0.1 % SDS (Sigma), 0.1 % tetramethylethylenediamine (TEMED) (Sigma), 0.1 % ammonium persulphate

(APS) (Singma), stacking gel: 5 % acrylamide: bisacrylamide (Sigma), 125 mM Tris-HCl pH 6.8, 0.1 % SDS (Sigma), 0.1 % TEMED (Sigma), 0.1 % APS (Sigma)). Electrophoresis was performed in Laemmli running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS) at 200 V, alongside Rainbow molecular weight marker (GE Healtcare), to estimate the elecrophoretic mobility of proteins.

Proteins were next electro-transferred overnight at 150 V onto Protran nitrocellulose membrane (Whatman) in modified Laemmli transfer buffer pH 10 (192 mM Tris-HCl, 25 mM glycine, 10 % methanol, 1% SDS) in a BioRad mini-gel Western transfer cell. For immunoblotting membranes were blocked in 5 % (w/v) milk solution in 0.1 % Tween-20 in PSB (0.1 % PBST) for 20 minutes-1 hour and washed once in PBST washing buffer. Protein levels were detected with primary antibodies at dilutions 1:500-1:1000 in 5 % milk 0.1% PBST, and incubated for either 1 hour at room temperature or overnight at 4 ºC. Horseradish peroxidase (HRP)-conjugated secondary antibodies were diluted in 1:10,000 in 5 % milk 0.1 % PBST and incubated for 45 minutes-1 hour. HPR signals were detected with SuperSignal West Pico Substrate (Pierce) by exposure to X-ray film (Kodac) in a fast speed cassette. Exposed X-ray films were developed and fixed in an automatic developer.

Table 2.3 List of antibodies

Antibody	Species/Description	Source
cdc6	Mouse monoclonal	Santa Cruz
CtBP	Mouse monoclonal (E-12)	Santa Cruz
Co-REST	Rabbit Polyclonal	Upstate
Cyclin E	Mouse monoclonal (E-4)	Santa Cruz
E1A	Mouse monoclonal	Santa Cruz
E2F1	Mouse monoclonal (KH95)	Santa Cruz
E2F7	Goat polyclonal (N-20)	Santa Cruz
GAPDH	Goat polyclonal (V-18)	Santa Cruz
HA	Mouse monoclonal (HA.11)	Eurogentec
HDAC1	Mouse monoclonal (H-11)	Santacruz
HDAC2	Mouse monoclonal (H-54)	Santacruz
LSD1	Rabbit polyclonal (H-220)	Cell Signalling
p73	Rebbit polyclonal (H-79)	Santa Cruz
α-lg-Alexa Fluor 488	Fluorescein conjugated	Molecular Probes
α-mouse/ Rabbit IgG	HRP conjugated	DAKO
α-rabbit/ Mouse IgG	HRP conjugated	DAKO
α-rabbit/ Goat IgG	HRP conjugated	Calbiochem
α-mouse IgG True blot	HRP conjugated	E-bioscience

2.6 Protein immunoprecipitation

Cells were harvested and lysed as previously described. Protein A-agarose and G-agarose (Roche) were combined and washed three times in equal volumes of TNN buffer. The cell extract was precleared by incubating with protein A and G agarose for 1 hour at 4ºC rotating with IgG. The supernatant lysate was then incubated with 1µg of specific antibody and 30µl of protein A and G-agarose mix at a rotating wheel overnight at 4 ºC. The agarose beads were collected by centrifugation for 1 minute at 0.8 *g* and washed three times in the TNN buffer. The immunocomplexes were eluted by the addition of SDS loading buffer and boiled at 100 ºC for 5 minutes prior to loading onto a gel.

2.7 Immunofluorescence

Cells seeded on coverslips were washed twice in PBS and then fixed in 3.7 % formaldehyde in PBS, and incubated for 10 minutes at room temperature. Cells were washed twice in PBS followed by permeabilisation in 0.5 % Triton X-100 in PBS for 10 minutes at room temperature. After washing twice in PBS, cells were blocked in 5 % FCS in PBS for 20 minutes at room temperature, and then washed again in PBS. Primary antibodies (Table 2.3) were diluter 1:500 in PBS and incubated for 30 minutes at room temperature. The cells were then washed four times in 0.025 % PBST. Secondary antibodies were diluted 1:500 in PSB and incubated in the dark at room temperature for 15-20 minutes. Cells were washed four times in 0.025 % PBST. Coverslips were mounted on glass slides using Vectashield containing DAPI (4,6-diamino-2-phenylindole) to stain the DNA (Vector Laboratories). The slides were examined using a fluorescent microscope

using 60 X 1.25 oil immersion lens (Olympus, Melville, NY). Images were taken with a digital CCD camera (Hamamatsu) using OpenLab software (Improvision).

2.8 Flow cytometry

Cells were plated at approximately 50,000 cells per 60 cm plate before treatments for appropriate time as indicated. For ectopic flow cytometry cells were co-transfected with 500 ng pBB14 which served as an internal marker for transfected cells. Media containing floating cells was collected and the remaining cells were harvested in Trypsin-EDTA (Lonza) until they detached from the plates, followed by addition of 5 % FCS in DMEM. Cells were washed in PBS, centrifuged and fixed by addition of 1 ml of ice-cold 50 % ethanol/PBS dropwise on the cell pellet while vortexing , followed by incubation at 4 ºC overnight. Fixed cells were washed in PBS and re-suspended in 25 U/ml RNAse A (Sigma-Aldrich) and 50 µg/ml propidium iodine (PI) (Sigma-Aldrich) and incubated for 30 minutes on ice in the dark. Cells were collected on a FACScan flow cytometer (Becton-Dickinson) and analysed using the Cell Quest software package (Becton-Dickinson). The cell populations were gated to allow only events with FL2-W < 400 to allow acceptance of only single cells in the analysis of PI staining as a representation of DNA content within each single cell. These cells were then gated relative to total DNA content as measured by FL2-A to determine the percentage of cells in each phase of the cell cycle. All experiments were performed in triplicate and between $1\text{-}2x10^4$ events were collected per sample.

2.9 Reporter assay

Cells on 60mm dishes were transfected with the indicated amount of plasmid DNA together with 200ng of pCMV-βgalactosidase to control for plasmid efficiency. The total volume of DNA transfected was kept constant using pcDNA3.1 empty vector. For reporter assays under siRNA, the 50nM of the appropriate siRNA was transfected 24 hours before transfecting 200ng of pCMV-βgalactosidase. Cells were washed with ice-cold PBS on the dishes before addition of 300µl 1x reporter lysis buffer (Promega). Cells were harvested into eppendorf tubes, vortexed and freeze thawed. The samples were then centrifugated for 2 min at 15.7 *g* to remove cell debris. Expression levels of transfected plasmids were analyzed via immunoblotting with the appropriate antibodies. For luciferase activity 50µl of cell lysate was mixed with luciferase reagent (Promega) and measured using a luminometer. Luciferase activity was recorded and relative activity calculated according to the transfection efficiency indicated by β-galactosidase activity. All assays were performed in triplicate.

2.10 Chomatin immunoprecipitation

Cells were treated as indicated. Cells were cross-linked with formaldehyde to a final concentration of 1% for 10 min at room temperature. Crosslinking was halted by washing cells with 125 mM glycine in PBS. Cells were then lysed in lysis buffer (5 mM Tris-Cl pH 8.0, 85 mM KCI, 0.5% Igepal, 1 x PIC) for 20 minutes on ice to remove cytoplasmic fractions. The remaining nuclei were lysed in RIPA lysis buffer (10 mM Tris-HCI pH 7.5, 150 mM NaCI, 1mM EDTA, 1% Igepal, 1% DOC, 0.1% SDS, 1 x PIC) for 10 minutes on ice. The lysates were sonicated 10 times at 25% power for 10 seconds to shear the chromatin to 200-1000 bp

fragments, and then centrifuged at 15.7 *g* for 15 minutes. The chromatin was then pre-cleared overnight by rotation with Protein A and G-agarose, which had been previously blocked for 16 hours with 1 mg/ml bovine serum albumin (BSA) and salmon sperm DNA. The Protein A and G-agarose beads were discarded and the supernatants were added to either 2 µg anti-E2F7, anti-CtBP, anti-HDAC1, anti-HDAC2, anti-LSD1, anti-CoREST or the appropriate control goat, mouse or rabbit IgG antibodies, and rotated overnight at 4 ºC.

Immunoprecipitations were performed by the addition of pre-blocked Protein A and G-agarose beads to the incubated chromatin and antibodies, which was then rotated at 4ºC for 4 hours. Immunocomplexes were washed twice in 20 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA, 0.1 % SDS, 1 % Triton X-100, 1 x PIC, three times in Tris-HCl pH 8, 500 mM NaCl, 2 mM EDTA, 0.1 % SDS, 1 % Triton X-100, 1 x PIC, three times in 10 mM Tris-HCl pH8, 250 mM LiCl, 1 mM EDTA, 1 % igepal, 1% DOC, 1 x PIC and once in TE (10 mM Tris-HCl pH8, 1mM EDTA, 1 x PIC). All washes were rotated for 5 minutes at 4 ºC, and the eppendorf tubes were replaced with fresh tubes between different buffers. Immunocomplexes were twice eluted from Protein A and G-agarose beads with 1 % SDS, 0.1 M $NaHCO_3$, by rotation for 15 minutes at room temperature. Eluates were reverse-crosslinked in 200 mM NaCI, 10 mM EDTA, 40 mM Tris-HCl pH 6.5, 40 ng/ml Proteinase K, 20 ng/ml RNaseA for 3 hours at 55 ºC and then overnight at 65ºC.

Reverse-crosslinked DNA was purified using the Qiaquick PCR purification kit (Qiagen), following the protocol of the manufacturer. DNA was eluted in 50 µl buffer EB (10 mM Tris). PCR was performed in GC cDNA PCR buffer (40 mM

Tricine-KOH pH 9.2, 15 mM KOAc, 3.5 mM $Mg(OA_c)_2$, 5 % DMSO, 3.75 µg/ml BSA) containing 0.5 µM of the forward and reverse DNA primers (Sigma), 0.5 mM dNTPs (Promega), 5 % GC melt (BD biosciences), 17 ng/ml Advantage-GC cDNA Polymerase mix (BD Biosciences), using 1/100 of the obtained immunoprecipitated DNA as the template. See Table 2.4 for specific promoter sequences. PCR settings were as follows: initial denaturation at 95 ºC for 3 minutes, followed by 30 cycles of; 95 ºC for 30 seconds, 58-65 ºC for 1 minute, and 72 ºC for 1 minute and a final elongation at 72 ºC for 5 minutes. Products were visualised on a 1 % agarose gel with 0.5 µ/ml ethidium bromide.

Table 2.4 Oligonucleotide sequence of primers used for ChIP analysis

Name	Sequence	Source
albumin forward	5'-TACATTGACAAGGTCTTGTGGAG-3'	Sigma-Genosys
albumin reverse	5'-TACATTGACAAGGTCTTGTGGAG-3'	Sigma-Genosys
cdc6 forward	5'-GGCCTCACAGCGACTCTAAGA-3'	Sigma-Genosys
cdc6 reverse	5'-CTCGGACTCACCACAAGC-3'	Sigma-Genosys
cyclin E forward	5'-GACGGGCTCTGGGTCCCGCGC-3'	Sigma-Genosys
cyclin E reverse	5'-CGCCTGCCCCCTACACCGCGC -3'	Sigma-Genosys
E2F1 forward	5'-AGGAACCGCCGCCGTTGTTCCCGT-3'	Sigma-Genosys
E2F1 reverse	5'-AGGAACCGCCGCCGTTGTTCCCGT-3'	Sigma-Genosys
p73 forward	5'- TGAGCCATGAAGATGTGCGAG-3'	Sigma-Genosys
p73 reverse	5'-GCTGCTTATGGTCTGATGCTTATG-3'	Sigma-Genosys

2.11 NAD^+/NADH quantification

Cells were treated as indicated and then were washed with cold PBS. 2 x 10^5 cells were pelleted for each assay by centrifugation at 0.4 *g* for 5 minutes. Cells were extracted with 400 µl of NADH/NAD extraction buffer (Biovision) by two freeze/thaw cycles; 20 minutes on dry ice then 10 minutes at room temperature. The extraction was vortexed for 10 seconds and the sample centrifuged at 18.407 *g* for 5 minutes. The extracted NADH/NAD supernatant was then transferred into a fresh eppendorf tube.

In order to have a standard curve, 10 µl of 1 nmol/µl NADH standard (Biovision) were diluted with 990 µl of NADH/NAD extraction buffer to generate 10 pmol/ µl standard NADH. 0, 2, 4, 6, 8, and 10 µl of the diluted NADH standard were added into a 96-well plate in duplicate to generate 0, 20, 40, 80 and 100 pmol/ well standard. The final volume in each well was 50 µl by adding the appropriate amount of NADH/NAD extraction buffer.

In order to detect total NADt (NADH and NAD), 50 µl of each extracted sample were transferred into the 96-well plate in duplicates. To detect NADH, NAD had to be decomposed before the reaction. To decompose NAD, 200 µl of the extracted samples were aliquoted into eppendorf tubes and heated at 60ºC for 30 minutes in a heating block. Under this condition all NAD was decomposed, while NADH remained intact. Samples were then cooled on ice and centrifuged briefly to remove precipitates. 50 µl of NAD decomposed samples were transferred into the 96-well plate in duplicates. The NAD cycling mix was prepared by mixing 100 µl NAD cycling buffer (Biovision) with 2 µl of NAD cycling enzyme mix (Biovision). 100

µl of this mix was added into each well of NADH standard and samples. The contents of each well were mixed and then the plate incubated at room temperature for 5 minutes to convert NAD to NADH. 10 µl of NADH developer (Biovision) were added into each well. The reaction was let to cycle at room temperature for 1-4 hours depending on the reading of $OD_{450nm.}$ The plate was read using a plate reader at OD_{450nm}. The sample readings were applied to the NADH standard curve. The amount of NADt and NADH in each sample was calculated and then divided by the sample amount. The NAD/NADH ratio was calculated as: NADt-NADH/NADH.

CHAPTER 3

IDENTIFICATION OF CtBP AS AN E2F7 CO-REPRESSOR

3.1 Introduction

E2F7 is a novel E2F family member that has been shown to be critical for embryonic development and control of the E2F1-p53 apoptotic axis (Li et al., 2008b). Several studies have shown that E2F7 functions as a transcriptional repressor that regulates the transcription of a subset of E2F target genes, such as E2F1, whose products are required for cell cycle progression (Logan et al., 2004, Di Stefano et al., 2003, de Bruin et al., 2003). Furthermore, E2F7 contains two DNA binding domains which is a unique structural characteristic that distinguishes E2F7 from the typical E2F proteins. Together, the structural and functional properties of E2F7 imply a unique role in regulating cell cycle progression and cell proliferation. However, the mechanism by which E2F7 mediates transcriptional repression remains unknown. Here, CtBP was identified as an E2F7 co-repressor and the involvement of CtBP in the transcriptional repression of E2F target genes was investigated.

3.2 E2F7 is a transcriptional repressor of E2F target genes

Initially, it was important to verify the ability of E2F7 to repress the transcription of certain E2F target genes (Figure 3.1). Previously this has been demonstrated in luciferase assays showing that E2F7 can repress E2F responsive promoters such as *E2F1* and *Apaf-1* (Di Stefano et al., 2003, Logan et al., 2004). In order to assess the ability of E2F7 to repress E2F target genes, luciferase reporter assays were carried out on E2F responsive promoters including E2F1 and the impact of ectopic E2F7 expression on these promoters was explored.

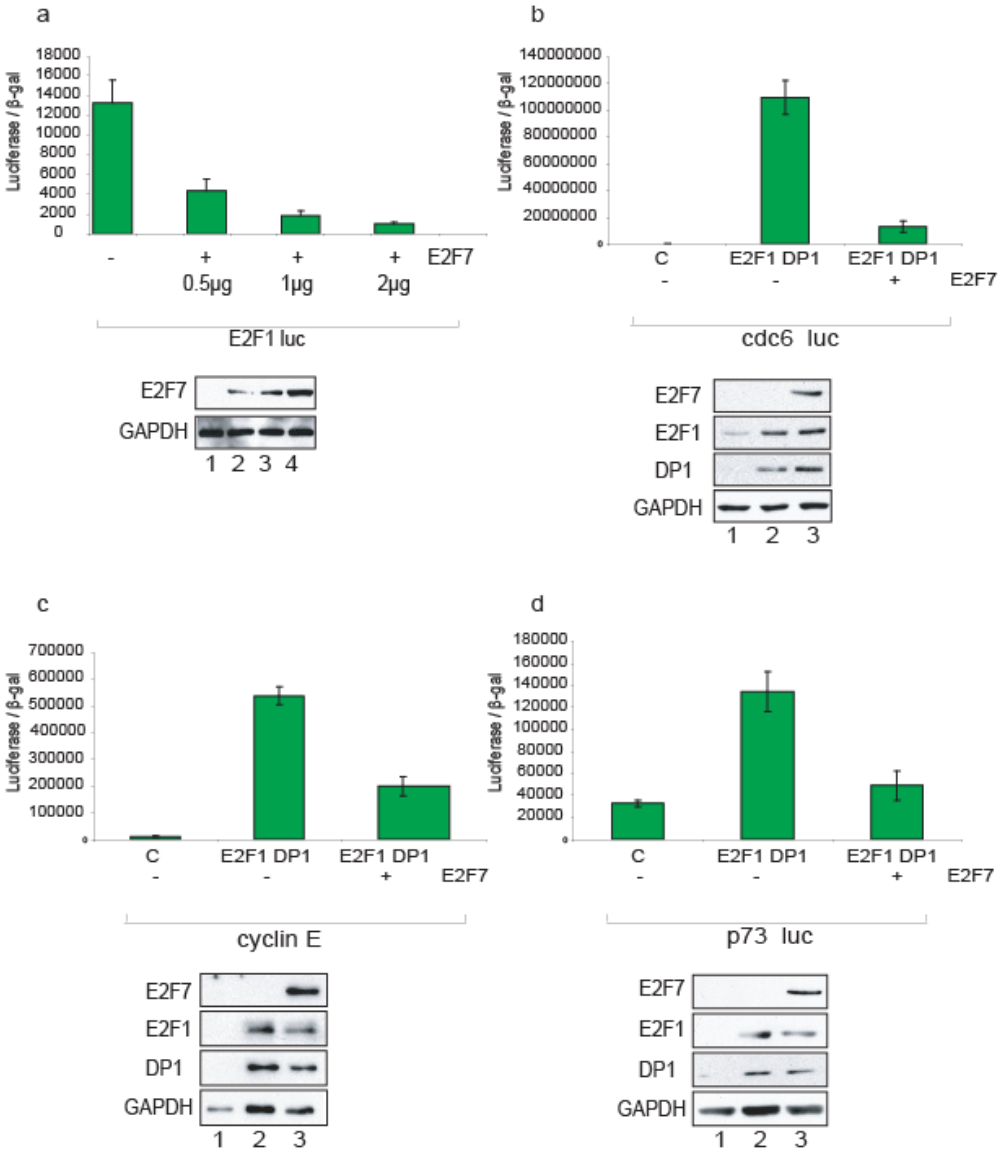


Figure 3.1: E2F7 represses E2F1, cyclin E, cdc6 and p73 transcriptional activity. (a) U2OS cells were transfected with the reporter construct pE2F1-luc alone and with increasing amounts of the expression vector encoding E2F7. (b), (c) and (d) U2OS cells were transfected with pcdc6-luc (b), pcyclin E-luc (c) or pp73-luc (d) alone and together with expression vectors encoding E2F1 and DP1 of E2F1, DP1 and E2F7. All were co-transfected with β-gal. The results represent the ratio of luciferase to β-gal activity. Values in all graphs represent the mean values of triplicate experiments and the error bars represent standard deviations. Expression levels of the different proteins used in the reporter assays were analysed by blotting with the appropriate antibodies as indicated. (n=4)

The *E2F1* promoter was activated and expression of ectopic E2F7 resulted in a significant suppression of promoter activity. Furthermore, the reduction of the *E2F1* promoter activity was shown to result in an E2F7 dose-dependent manner (Figure 3.1a). The ability of E2F7 to repress the *E2F1* promoter was shown previously (Di Stefano et al., 2003) and therefore this experiment also served as a positive control.

Following verification of E2F7 as a transcriptional repressor of the *E2F1* promoter, it was of great interest to investigate the effect of ectopic E2F7 expression on the promoters of other E2F responsive genes. The *cyclin E*, *cdc6* and *p73* promoters were activated in the presence of ectopically expressed E2F1/DP1, and co-expression of ectopic E2F7 significantly suppressed this activation (Figure 3.1b, c and d).

Taken together, these data show that E2F7 is a transcriptional repressor of E2F responsive genes, which can exert its repressive function on the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters.

3.3 E2F7 contains a CtBP-binding motif and forms a complex with CtBP

As E2F7 was shown to function as a transcriptional repressor of E2F responsive genes, the mechanism by which E2F7 exerts its repressive role was explored. In order to gain an insight of the repression mechanism the E2F7 sequence was analysed.

Previous analysis of the E2F7 sequence in the laboratory revealed the presence of a CtBP (C-terminal binding protein) binding motif at amino acid positions 621-625 (Figure 3.2a). This was performed by Rebacca Fisher, a past member of the cancer biology laboratory, who analysed the E2F7 sequence in order to identify binding motifs and provided the information regarding the CtBP-binding motif via personal communication (unpublished observation). As discussed earlier (Section 1.5.2), CtBP is recruited to promoters by a large number of DNA-binding transcription factors to mediate sequence-specific transcriptional repression (Shi et al., 2003). Therefore, the presence of the binding motif suggested that E2F7 could recruit CtBP in a repression complex.

To explore the possibility that E2F7 might interact with CtBP, immunoprecipitations were performed under normal and DNA damage conditions, as E2F7 has been previously shown to play a role in the DNA damage response (Zalmas et al., 2008). In order to induce DNA damage cells were treated with etoposide, an anti-tumour agent that inhibits topoisomerase II and induces apoptosis in normal and tumour cell lines by activating the DNA damage response (Stevens and La Thangue, 2003a). Endogenous E2F7 immunoprecipitations showed that E2F7 forms a complex with CtBP both under normal conditions and under DNA damage, in both MCF7 breast adenocarcinoma and HeLa cervical carcinoma cells (Figure 3.2b and c).

These results therefore show that E2F7 forms a complex with CtBP suggesting that CtBP could be a part of an E2F7 repression complex and thus play a role in the transcriptional repression of E2F target genes.

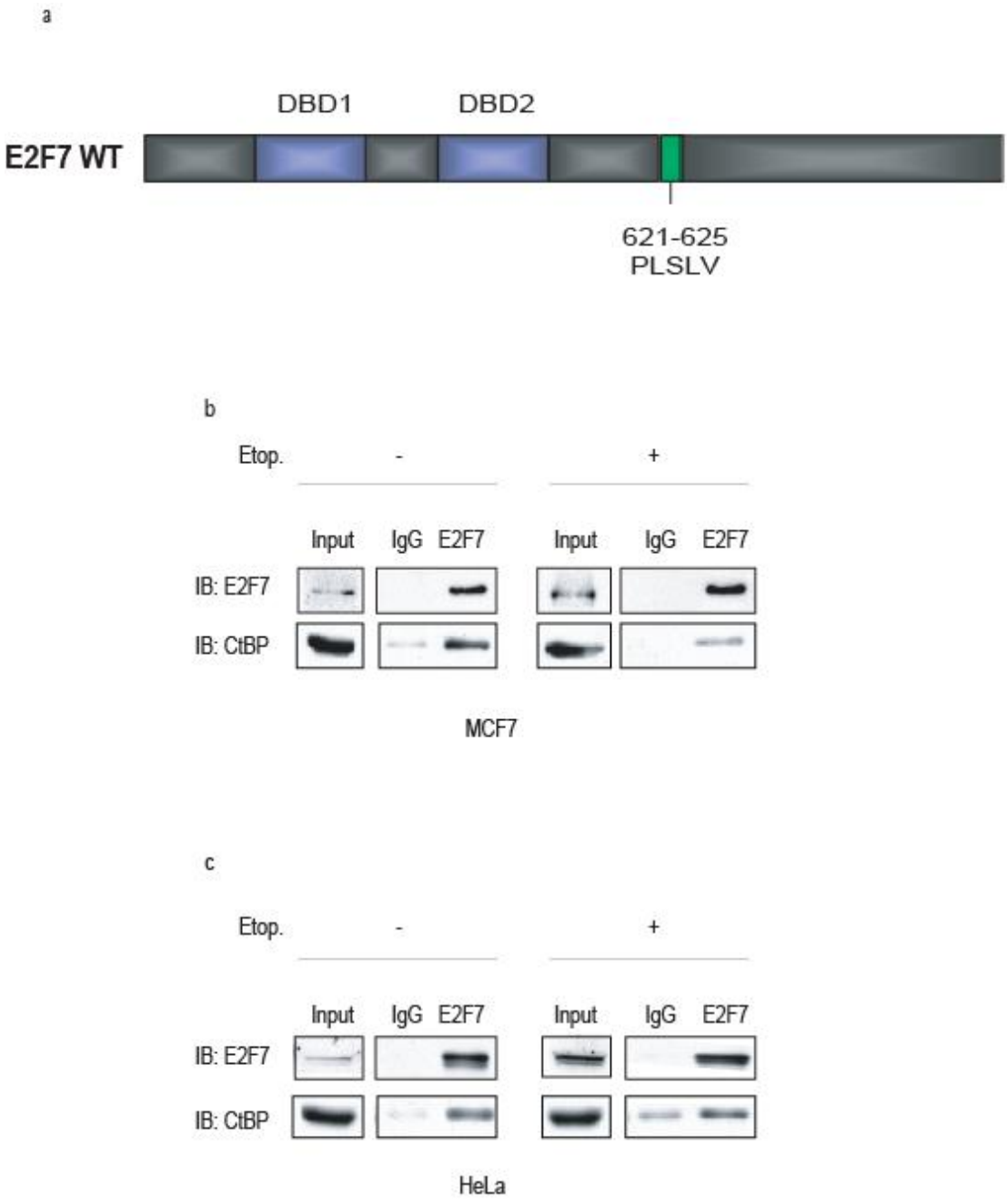


Figure 3.2: E2F7 forms a repression complex with CtBP.
(a) Schematic representation of the E2F7 protein showing the DNA binding domains (DBD) (blue) and the PLSLV motif (green). (b) and (c) Cell extracts were obtained from MCF7 (b) and HeLa (c) cells and used for endogenous E2F7 immunoprecipitations under normal conditions and after treatment with 10µM etoposide for 4 hours. The anti-E2F7 and anti-CtBP antibodies were used to detect the endogenous E2F7 and CtBP proteins respectively. (n=5)

3.4 CtBP knock-down activates the transcription of certain E2F target genes

Following the identification of CtBP as a component of an E2F7 complex, it was of interest to investigate whether CtBP is involved in the transcriptional repression of E2F target genes. In order to determine this, the impact of depleting CtBP on the activity of E2F responsive promoters was explored. CtBP levels were depleted by means of specific CtBP siRNA treatment, whilst non-targeting siRNA served as a negative control.

Depletion of CtBP resulted in significantly enhanced activity of the *E2F1*, *cdc6* and *cyclin E* promoters (Figure 3.3a, b and c), suggesting that CtBP plays a role in the repression of E2F target genes. Interestingly however, CtBP depletion had no effect on the activity of the *p73* promoter (Figure 3.3d) thereby suggesting that CtBP is involved in the transcriptional repression of certain E2F responsive genes.

Taken together, these data indicate that CtBP plays a role in the transcriptional repression of a subset of E2F target genes and suggest that CtBP could be involved in E2F7-mediated repression.

3.5 E2F7 and CtBP are recruited to the promoters of E2F target genes

E2F7 has been reported to bind promoters containing E2F DNA-binding consensus sites under normal conditions (de Bruin et al., 2003, Di Stefano et al., 2003) and during the DNA damage response (Zalmas et al., 2008). Previous work has shown that E2F7 is recruited to the E2F1 promoter both under normal conditions and under DNA damage (Zalmas et al., 2008). Therefore, recruitment of E2F7 to other E2F-responsive promoters was investigated.

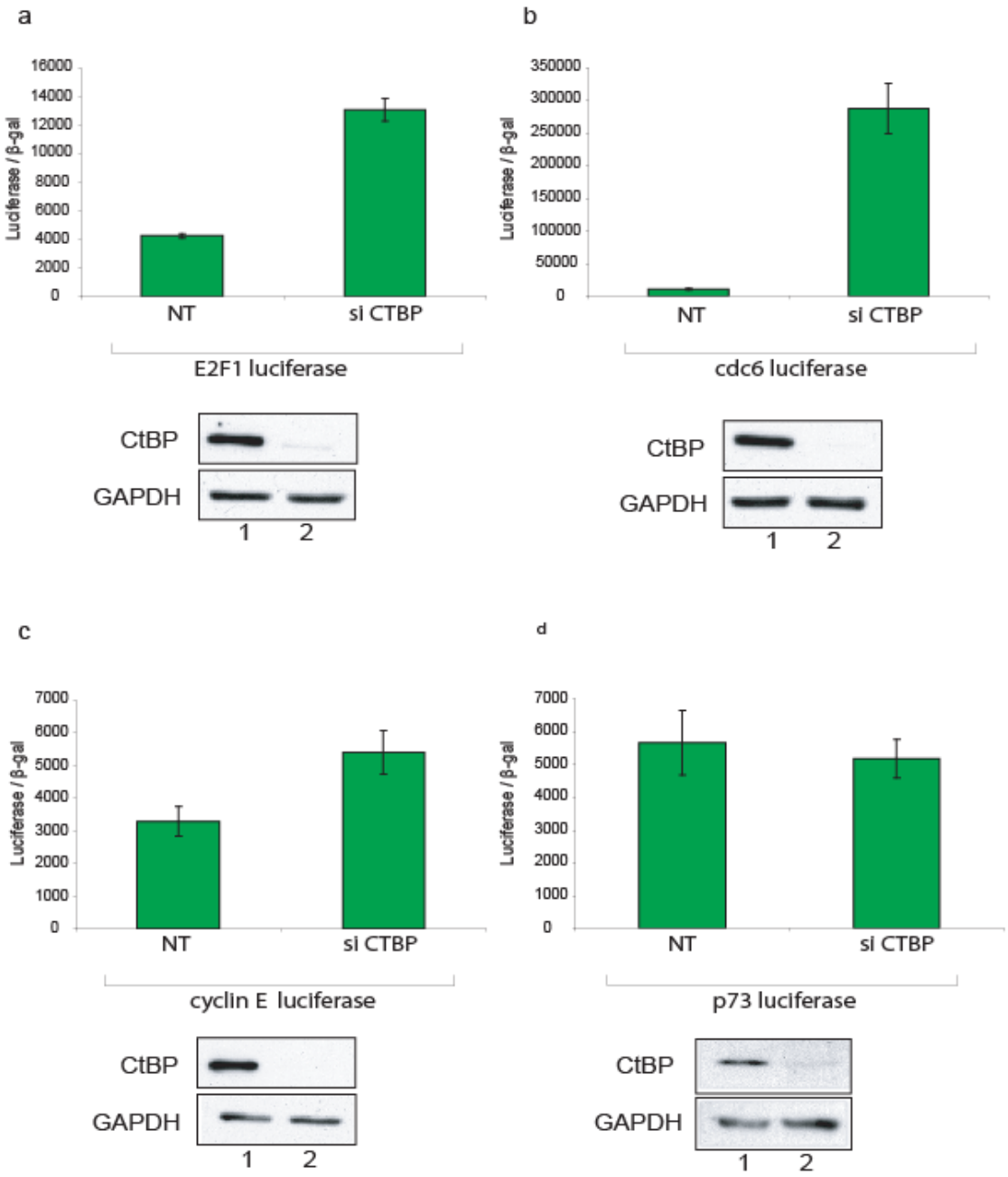


Figure 3.3: CtBP is a repressor of E2F target genes.
Luciferase assays using E2F target gene promoters. U2OS cells were transfected with the reporter construct pE2F1-luc (a), pcdc6-luc (b), pcyclin E-luc (c) or pp73-luc (d) under non targeting (NT) or CtBP siRNA. All were co-transfected with β-gal as a transfection control. The results represent the ratio of luciferase to β-gal activity. Values in all graphs represent the mean values of triplicate experiments and the error bars represent standard deviations. The CtBP levels were analysed by Western blotting with the appropriate antibodies as indicated. (n=4)

Furthermore, following the identification of CtBP as a component of an E2F7 complex that plays a role in the repression of E2F responsive promoters, the recruitment of CtBP on these promoters was also explored.

By chromatin immunoprecipitation (ChIP), the binding of E2F7 and CtBP to the *E2F1* promoter was investigated with specific antibodies to each protein. Non-specific (NS) binding was assessed by use of control IgG antibodies and binding of E2F7 to the *E2F1* promoter served as a positive control. E2F7 and CtBP were shown to occupy the *E2F1* promoter under normal conditions and under DNA damage, whereas the *albumin* promoter was not occupied by either of the two proteins (Figure 3.4).

The DNA binding ability of E2F7 and CtBP was then tested on promoters of known E2F target genes including *cdc6*, *cyclin E* and *p73*. Promoter occupancy was assessed by PCR with an array of primers that specifically amplify the E2F site containing regions of these promoters. The *albumin* promoter was used as a negative control. E2F7 and CtBP were shown to occupy the *cdc6*, *cyclin E* and *p73* promoters both under normal conditions and under DNA damage conditions (Figure 3.4).

Overall, these data show that E2F7 binds to E2F-recognition sites on the *E2F1*, *cdc6*, *cyclin E* and *p73*, *in vivo*. Also, CtBP was also recruited to the same promoters suggesting that CtBP could be involved in their repression.

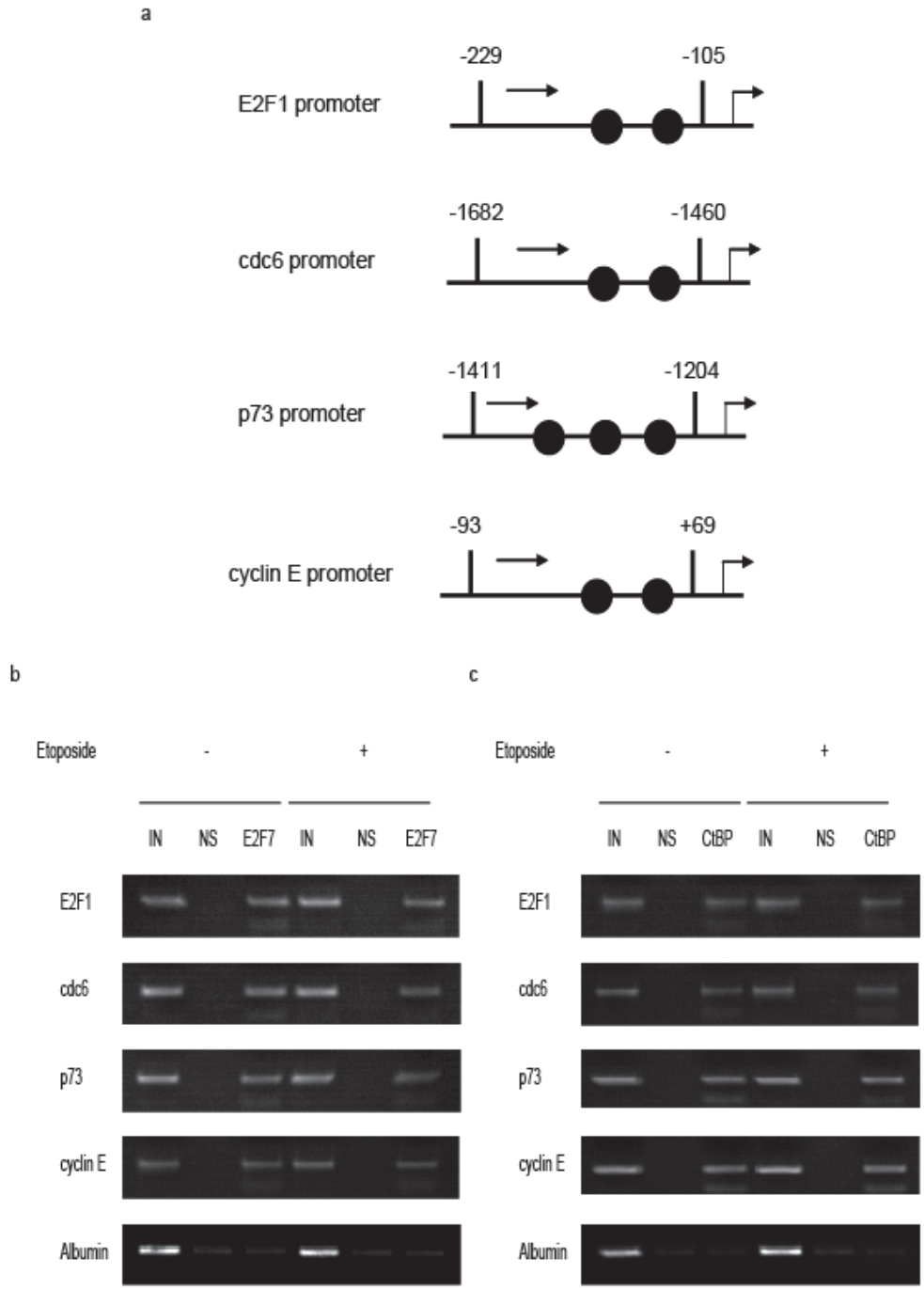


Figure 3.4: E2F7 and CtBP are recruited to E2F-responsive promoters.
(a) Summary of the E2F1, cdc6, p73 and cyclin E promoters: the circles indicate the location of the E2F sites and the arrows indicate the position of the primers used in the ChIP analysis. Chromatin immunoprecipitation analysis on MCF7 cells carried out using E2F7 (b) or CtBP (c) and control antibodies under normal conditions and after treatment with 10µM etoposide for 4h. PCR was performed against the promoters indicated. (n=4)

3.6 E2F7 and CtBP co-exist at the promoters of E2F target genes

The ability of E2F7 to form a complex with CtBP in cells as well as the presence of both E2F7 and CtBP on E2F responsive promoters led to the hypothesis that these proteins could be found in a DNA binding complex on target promoters *in vivo*. Chromatin complexes were immunoprecipitated with E2F7 antibody from extracts of MCF7 breast adenocarcinoma cells. The resulting E2F7-bound chromatin complexes were isolated and subjected to a second round of immunoprecipitation with CtBP antibody. This enriched the immunoprecipitated chromatin with DNA fragments bound by both E2F7 and CtBP.

Consistent with previous observations (Figure 3.4), E2F7 was shown to be recruited on the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters. Interestingly, as shown by the secondary immunoprecipitation CtBP exhibited a similar recruitment pattern on those promoters (Figure 3.5).

The data here suggest that E2F7 and CtBP could be present in a DNA binding complex on a number of E2F responsive promoters including the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters.

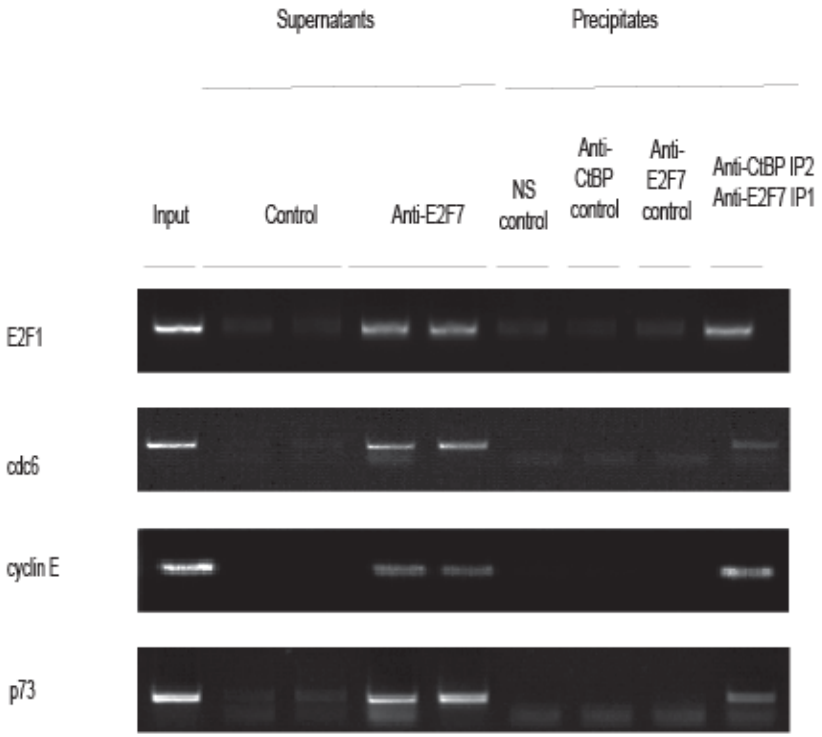


Figure 3.5: E2F7 and CtBP co-exist on the E2F1, cdc6, cyclin E and p73 promoters. Chromatin complexes were immunoprecipitated with the E2F7 antibody from extracts of MCF7 breast adenocarcinoma cells. The resulting E2F7-bound chromatin complexes were isolated and subjected to a second round of immunoprecipitation with CtBP antibody. PCR was performed against the promoters indicated. (n=3)

3.7 Discussion

E2F7 has been previously suggested to function as a transcriptional repressor but the repression mechanism remained unknown (de Bruin et al., 2003, Di Stefano et al., 2003, Logan et al., 2004). The work described here verifies the repression ability of E2F7 and increases our understanding on the mechanism by which E2F7 can repress the transcription of E2F target genes. In addition to the previously described repressive effect of E2F7 on the *E2F1* promoter, this investigation shows that additional E2F responsive promoters are targeted by E2F7. Furthermore, E2F7 was shown to form a complex with CtBP providing a first line of information regarding the E2F7-mediated transcriptional repression. The results presented here also suggest that CtBP could play an important role in the repression of E2F target genes.

Previous studies have shown that E2F7 has the ability to repress transcription and delay cell cycle progression (de Bruin et al., 2003, Zalmas et al., 2008). The results of this study verify that E2F7 is a potent repressor of *E2F1* and also demonstrates the ability of E2F7 to repress a subset of E2F responsive promoters including *cdc6*, *cyclin E* and *p73* (Figure 3.1). Interestingly, the repressive function of E2F7 on the *E2F1* promoter was shown to be dose dependent (Figure 3.1a).

In order to gain insight into the mechanism of E2F7-mediated repression the E2F7 sequence was analysed leading the identification of a CtBP-binding motif, residues 621-625 (Figure 3.2a). Subsequently, the possibility of an E2F7-CtBP complex was investigated by endogenous E2F7 immunoprecipitations under normal conditions as well as under DNA damage. E2F7 was shown to form a complex with

endogenous CtBP in both MCF7 breast adenocarcinoma and HeLa cervical carcinoma cells under both normal and DNA damage conditions (Figure 3.2b and c), thereby suggesting that CtBP could play a role in the E2F7-mediated repression of E2F target genes.

In order to investigate the importance of CtBP in mediating repression of E2F responsive promoters, luciferase assays were carried out and the effect of siRNA depletion of CtBP was examined. CtBP knock-down was shown to lead to significant activation of the *E2F1*, *cdc6* and *cyclin E* promoters (Figure 3.3a, b and c) as compared to the non-targeting control, indicating that CtBP plays a role in the transcriptional repression of certain E2F responsive promoters. However, CtBP depletion had no effect on the activity of the *p73* promoter (Figure3.3d) as promoter activity levels were comparable to promoter activity under control siRNA, suggesting that the CtBP is involved in the repression of a subset of E2F responsive genes.

Since it has been previously described that E2F7 binds promoters containing E2F DNA-binding consensus sites under normal conditions (de Bruin et al., 2003, Di Stefano et al., 2003) as well as under the DNA damage response (Zalmas et al., 2008), it was of interest to investigate the recruitment of E2F7 on other E2F responsive promoters. By chromatin immunoprecipitation E2F7 was shown to bind the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters both under normal conditions and under DNA damage (Figure 3.4), thus extending the repertoire of promoters targeted by E2F7. Binding of CtBP on the same promoter was then investigated in order to assess whether there is a correlation between the recruitment of CtBP and

E2F7 to certain promoters. Endogenous CtBP chromatin immunoprecipitations showed that CtBP is also recruited to the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters under normal conditions as well as under DNA damage (Figure 3.4).

The correlation between the promoter recruitment pattern of E2F7 and CtBP led to the speculation that E2F7 and CtBP could be co-recruited at these sites. In order to assess whether E2F7 and CtBP co-exist in a DNA binding complex on target promoters *in vivo*, a double chromatin immunoprecipitation was performed as analysed in section 3.6. Consistent with previous chromatin immunoprecipitations (Figure 3.4), E2F7 was shown to be recruited on the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters. Additionally, the secondary immunoprecipitation showed that CtBP co-co-occupies these promoters with E2F7 (Figure 3.5), thereby further indicating a role for CtBP in E2F7-mediated repression.

E2F7 functions as transcriptional repressor of a subset of E2F responsive genes which are implicated in the control of several processes including cell cycle progression, and apoptosis. The data presented here indicate that E2F7 mediates the transcriptional repression of a number of E2F target genes, and as such plays an important role in dictating cell fate. Furthermore, the identification of CtBP as a component of an E2F7 complex provides the first insight into the mechanism of E2F7 mediated repression (Figure 3.6). The implication of CtBP in the repression of E2F target genes as well as its co-recruitment with E2F7 on E2F responsive promoters suggests that CtBP could possibly play an important role in the repression function of E2F7. Moreover, the ability of E2F7 to form a complex with CtBP also suggests that E2F7 could repress transcription by recruiting a co-

repressor complex to DNA the constituents of which are investigated later in this thesis.

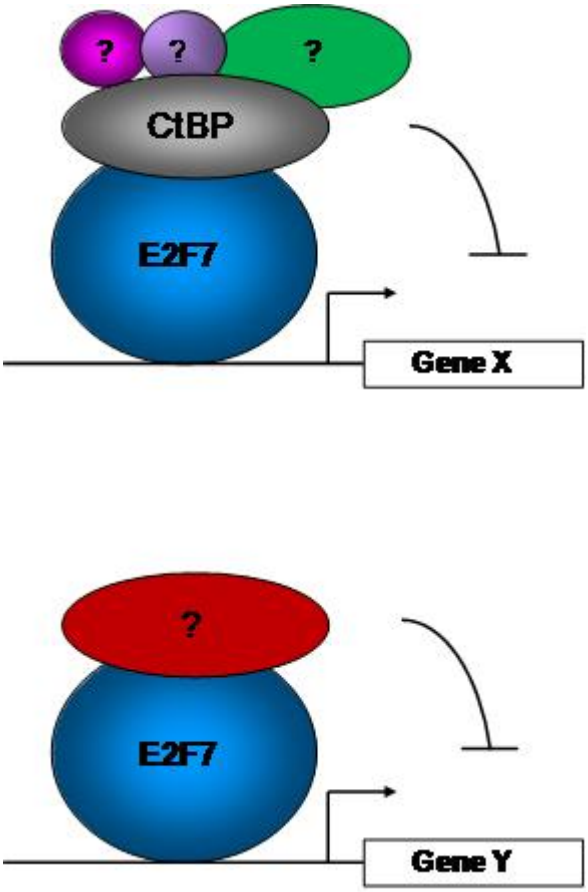


Figure 3.6 Proposed model for E2F7-mediated repression of E2F target genes. E2F7 forms a repression complex with CtBP. CtBP is involved in the repression of certain E2F target genes and recruited to E2F-responsive promoters, and thus could play a role in E2F7-mediated transcriptional repression of a subset of E2F targets.

CHAPTER 4

MUTATIONAL ANALYSIS OF THE CtBP BINDING MOTIF AND INVESTIGATION OF THE E2F7-CtBP COMPLEX

4.1 Introduction

CtBP has been shown to function as a transcriptional co-repressor for a large number of repressors (Zhao and Chinnadurai, 2010). Certain DNA-binding transcriptional repressors recruit CtBP to target promoters through a PLDLS motif which was first identified in E1A (Kuppuswamy et al., 2008, Chinnadurai, 2002). Furthermore, CtBP has been show to contain a NAD(H) binding domain with the cellular NAD^+/NADH ratio altering CtBP conformation and thus regulate CtBP interaction with certain repressors (Chinnadurai, 2007).

As discussed in chapter 3, E2F7 contains a CtBP-binding motif and forms a complex with CtBP in both MCF7 breast adenocarcinoma and HeLa cervical carcinoma cells under both normal and DNA damage conditions. It was therefore of interest to investigate the importance of the CtBP-binding motif in E2F7-mediated transcriptional repression by mutational analysis. Also of interest was to investigate the E2F7-CtBP complex under different types of stress as well as under different cell redox states.

Furthermore, in order to further explore the significance of CtBP in E2F7-induced repression the recently identified CtBP substrate MTOB was used in functional assays. As discussed in chapter one, MTOB loading to the CtBP substrate-binding domain was suggested to induce a conformational change in the PLDLS binding cleft resulting in reduced interaction of CtBP with transcriptional repressors (Straza et al., 2010). Therefore, MTOB treatment was used as a tool to explore the ability of E2F7 to induce transcriptional repression to previously identified target genes following binding of CtBP to its substrate.

4.2 Cellular localization of E2F7ΔCtBP

In order to explore the significance of the CtBP-binding motif in E2F7-mediated repression an E2F7 deletion mutant was produced by site-directed mutagenesis. The resulting E2F7 mutant lacking amino acids at positions 621-625 was verified by sequencing and will be referred to as E2F7ΔCtBP. As discussed in chapter 1, E2F7 contains several nuclear localisation signals (NLSs) and previous studies have demonstrated that ectopically expressed E2F7 localises in the nucleus (Logan et al., 2004, Zalmas et al., 2008). The cellular localisation of E2F7ΔCtBP was thus explored in order to investigate whether deletion of the CtBP-binding motif affects the E2F7 nuclear localisation.

U2OS cells were fixed and stained with α-HA antibody against the ectopically expressed E2F7 and E2F7ΔCtBP proteins as indicated (Figure 4.1). As E2F7 was previously shown to localise in the nucleus, E2F7 immunofluorescence also served as a positive control. Comparison between E2F7 and E2F7ΔCtBP staining with DAPI, a DNA specific fluorescent probe, show that both the wild-type and the mutant E2F7 are localised to the nucleus.

Thus, these results indicate that deletion of the CtBP-binding motif does not affect the cellular localisation of E2F7. E2F7ΔCtBP can be therefore be used in assays where the functional consequences of the CtBP-motif deletion are not attributed to changes in the E2F7 conformation that affect the NLSs and thus nuclear localisation.

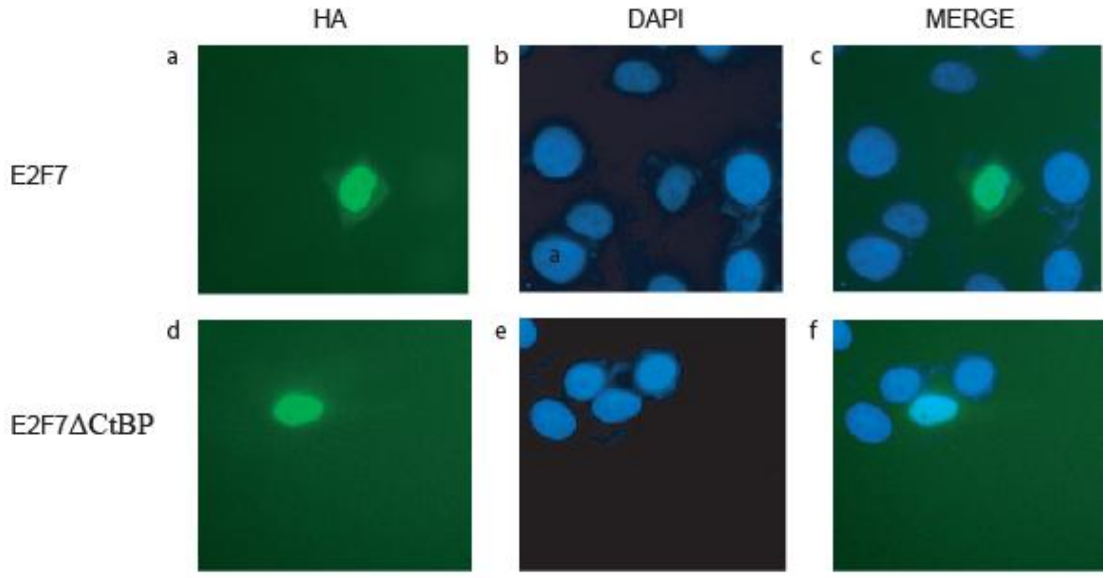


Figure 4.1: Cellular localization of E2F7 and E2F7ΔCtBP.
U2OS cells were transfected with HA-E2F7 (a, b, c) of HA-E2F7ΔCtBP (d, e, f) and immunostained using anti-HA antibody. DAPI staining was used to visualise nuclei. (n=3)

4.3 E2F7ΔCtBP is unable to repress certain E2F target genes

The E2F7ΔCtBP was used to investigate the importance of the CtBP-binding motif in the E2F7-mediated transcriptional repression of E2F target genes. In order to determine this, the impact of E2F7ΔCtBP on the activity of E2F responsive promoters was explored as compared to the effect of wild-type E2F7 to the same promoters.

E2F7ΔCtBP was shown to be unable to repress the activity of the *E2F1*, *cdc6* and *cyclin E* promoters (Figure 4.2a, b and c) as compared to the repressive effect exerted by wild-type E2F7 on the same promoters, suggesting that the CtBP-binding motif plays a role in the ability of E2F7 to repress E2F target genes. Interestingly, E2F7ΔCtBP was not only unable to confer a repression effect to the *cdc6* and *cyclin E* promoters but rather enhanced the activity of these promoters as compared to the empty vector control. On the other hand, deletion of the CtBP-binding motif had no effect on the ability of E2F7 to repress the activity of the *p73* promoter (Figure 4.2d) which correlates with the previous observation that siRNA-mediated CtBP depletion has no effect on the activity of the *p73* promoter (Figure 3.3d).

Taken together, the data presented here indicate that the CtBP-binding motif plays an important role in the E2F7-induced transcriptional repression of a subset of E2F target genes.

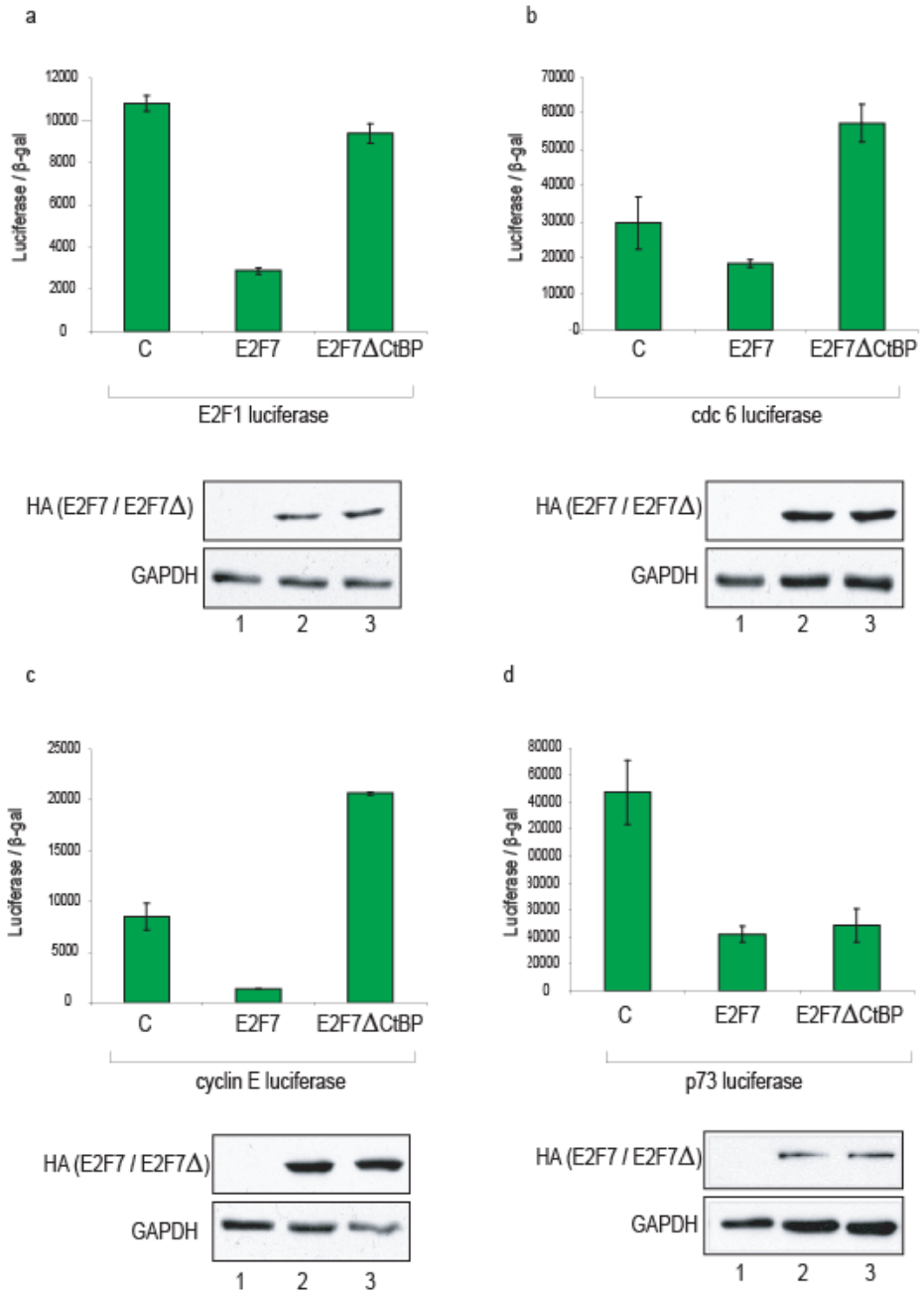


Figure 4.2: Deletion of the E2F7 CtBP-binding motif affects the repression of certain E2F target genes. Luciferase reporter assays on E2F target gene promoters. U2OS cells were transfected with the reporter construct pE2F1-luc (a), pcdc6-luc (b), pcyclin E-luc (c) or pp73-luc (d) alone and together with expression vectors encoding pcDNA3 (control), E2F7 or E2F7ΔCtBP. All were co-transfected with β-gal as a transfection control. The results represent the ratio of luciferase to β-gal activity. Values in all graphs represent the mean values of triplicate experiments and the error bars represent standard deviations. Expression levels of the different constructs used in the reporter assays were analysed by Western blotting with the appropriate antibodies as indicated. (n=4)

As deletion of the CtBP-binding motif was shown to have a negative impact on the repression effect of E2F7 on certain E2F target genes, it was therefore of interest to investigate whether absence of the motif affects the ability of E2F7 to interact with CtBP. Endogenous CtBP was immunoprecipitated in the presence of ectopically expressed E2F7 or E2F7ΔCtBP showing that lack of the CtBP-binding motif abolishes the E2F7-CtBP interaction (Figure 4.3). Immunoprecipitation of endogenous CtBP in the presence of ectopic empty vector was used as a negative control.

There results therefore indicate that the CtBP-binding motif is essential for the E2F7-CtBP interaction and deletion of the motif disrupts the E2F7-CtBP complex, leading to loss of E2F7-induced transcriptional repression on a subset of E2F target genes.

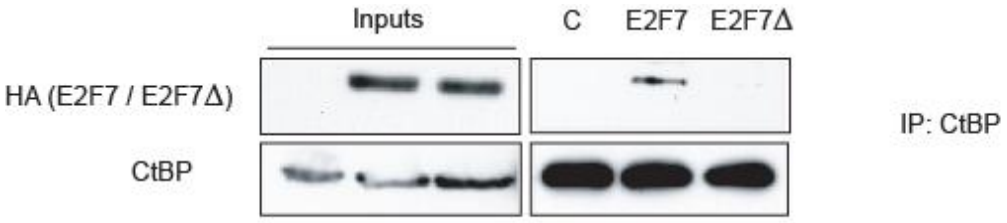


Figure 4.3: Comparison of the E2F7ΔCtBP with the wild type in CtBP interaction. MCF7 cells were transfected with either HA-E2F7 or HA-E2F7ΔCtBP and immunoprecipitated using CtBP antibody. Anti-HA antibody was used to detect E2F7 and E2F7ΔCtBP, and anti-CtBP antibody was used to detect endogenous CtBP. (n=3)

4.4 Investigation of the E2F7 – CtBP interaction under different types of stress

Having shown that E2F7 can interact with CtBP under normal and DNA damage conditions following treatment with etoposide and given that E2F7 has been shown to play a role in the DNA damage response (Zalmas et al., 2008), this interaction was explored under different types of cell stress. To investigate this, cells were either irradiated with UV for 10 seconds and allowed to recover overnight or heat shocked at 42ºC for 30 minutes followed by a 4 hour recovery at 37ºC.

Endogenous E2F7 immunoprecipitations showed that E2F7 interacts with CtBP under normal conditions as previously demonstrated (Figure 3.2) and that the interaction was also stable following cell stress induced by either UV or heat shock (Figure 4.4a and b). In order to show that the interaction is specific to E2F7, immunoprecipitations using goat IgG were used as a negative control.

These results therefore show that the E2F7-CtBP interaction is robust under normal conditions as well as under different types of cell stress, including UV radiation and heat shock. Furthermore, the presence of CtBP in the E2F7 repression complex under different conditions further suggest that CtBP is an important E2F7-interacting protein playing a role in E2F7-mediated transcriptional repression of certain E2F-responsive genes.

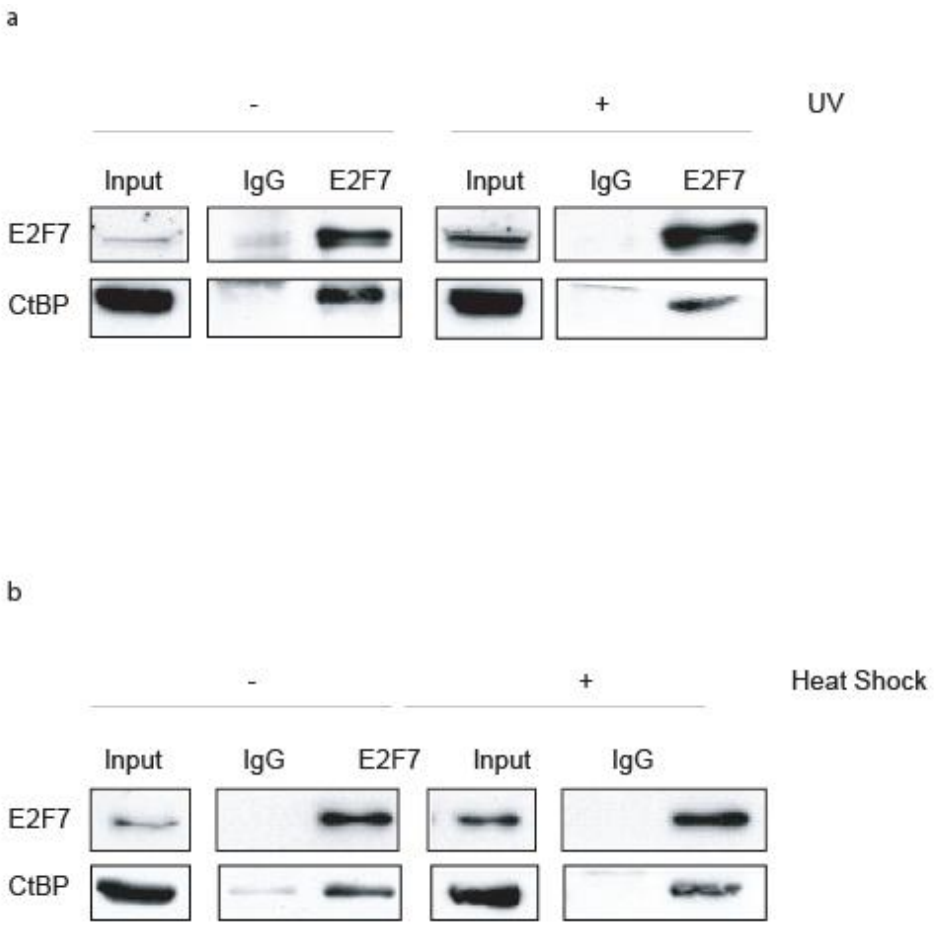


Figure 4.4: The E2F7-CtBP interaction under different types of stress.
Cell extracts were obtained from MCF7 cells untreated or following either UV treatment (50J/m2, followed by 16 hours of recovery) or heat shock treatment (30 minutes at 42ºC followed by 4 hours of recovery at 37ºC) and immunoprecipitated using anti-E2F7 antibody. Anti-E2F7 and anti-CtBP antibodies were used to detect the endogenous E2F7 and CtBP proteins respectively. (n=3)

4.5 E2F7 can still interact with CtBP and repress transcription of E2F-responsive genes in the presence of E1A

E1A was the first protein that was shown to interact with CtBP via the PLDLS motif (Chinnadurai, 2007). E2F7 was shown to interact with CtBP via the CtBP-binding motif and that this interaction is robust under different conditions. The E2F7-CtBP interaction was explored in the presence of E1A in order to address whether E1A binding to CtBP can affect binding of E2F7.

To explore this, immunoprecipitations were performed using the HEK293 embryonic kidney cells, which stably express E1A (Seifried et al., 2008). Endogenous E2F7 was immunoprecipitated with CtBP, thereby showing that E2F7 can still interact with CtBP in cells expressing E1A (Figure 4.5). Immunoprecipitation of E2F7 in U2OS cells served as a positive control.

Although the presence of E1A did not show to have an effect on the E2F7-CtBP interaction, an effect on the ability of E2F7 to repress the transcription of target genes could not be ruled out. In order to address this, functional assays in HEK293 cells were performed and the impact of ectopic E2F7 expression on E2F regulated promoters was investigated.

The *E2F1*, *cyclin E*, *cdc6* and *p73* promoters were previously shown to be repressed by E2F7 (Figure 3.1). Ectopic expression of E2F7 was shown to significantly repress the activity of these E2F-responsive promoters in HEK293 as compared to the empty vector control (Figure 4.6), thus suggesting that the repression ability of E2F7 is not influenced by E1A.

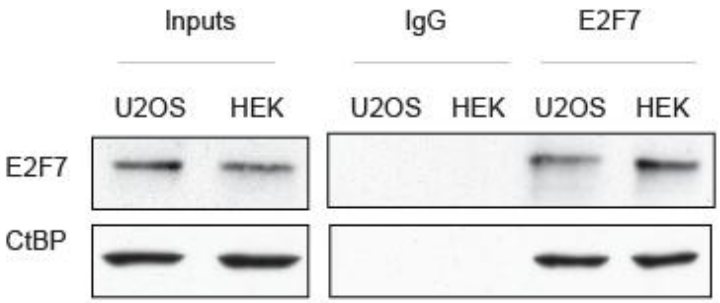


Figure 4.5: Comparison of the E2F7-CtBP interaction in U2OS and HEK293 cells. U2OS and HEK293 cells (as indicated) were immunoprecipitated using anti-E2F7 antibody. Anti-E2F7 and anti-CtBP antibodies were used to detect E2F7 and CtBP respectively. Immunoprecipitations using igG served as a negative control. (n=3)

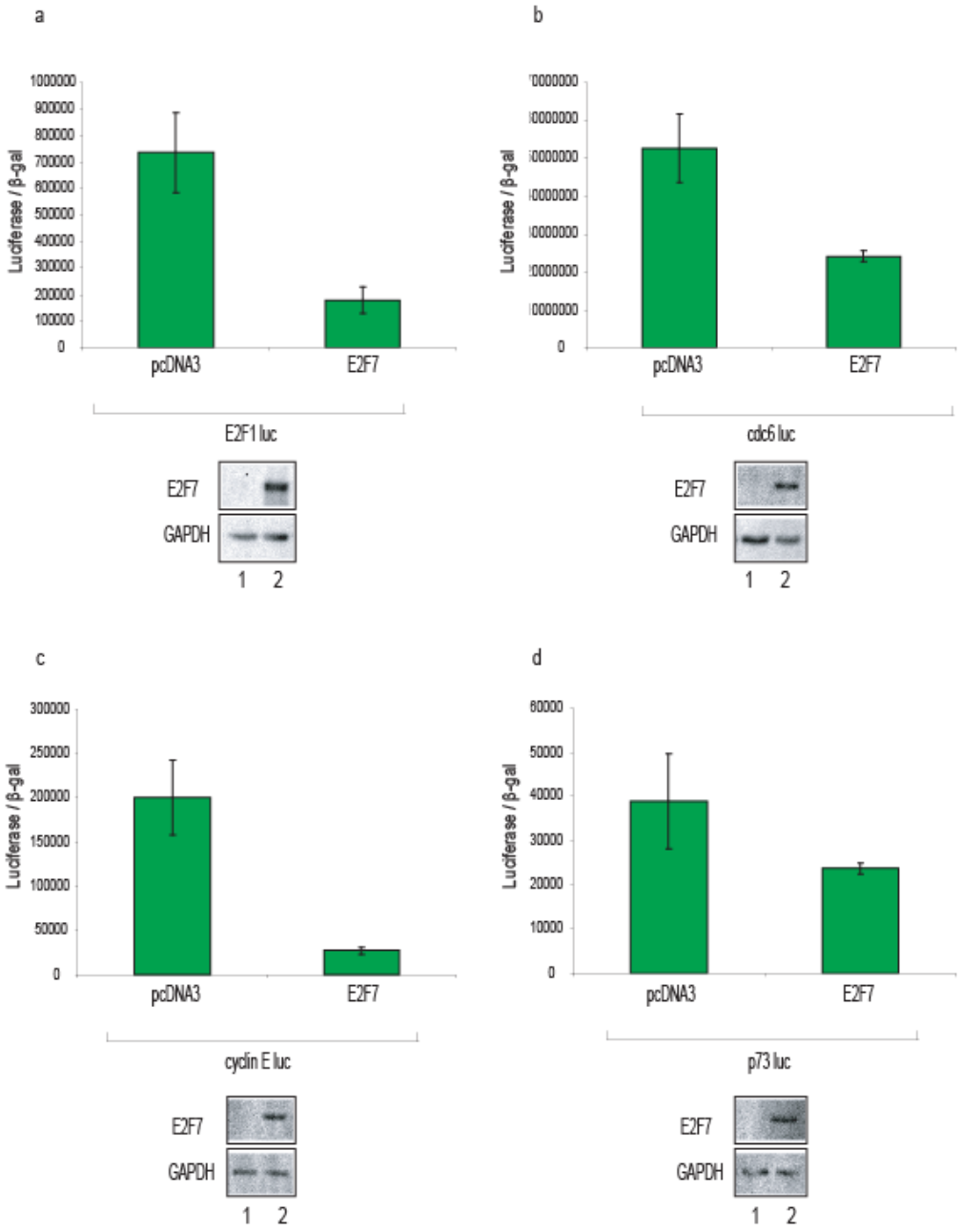


Figure 4.6:E2F7 represses E2F1, cdc6, cyclin E and p73 transcriptional activity in HEK293 cells. HEK293 cells were transfected with the reporter construct pE2F1-luc (a), pcdc6-luc (b), pcyclin E-luc (c) or pp73-luc (d) alone and together with expression vectors encoding pcDNA3 (control) or E2F7. All were co-transfected with β-gal as a transfection control. The results represent the ratio of luciferase to β-gal activity. Values in all graphs represent the mean values of triplicate experiments and the error bars represent standard deviations. Expression levels of the different constructs used in the reporter assays were analysed by Western blotting with the appropriate antibodies as indicated. (n=4)

In order to further verify that E1A expression is unable to affect E2F7-mediated transcriptional repression, luciferase assays were performed in U2OS cells in the presence of ectopically expressed E1A. To ensure that any effect of E1A to the repression function of E2F7 is attributed to the ability of E1A to interact with CtBP, E1AΔ was used as a control. E1AΔ is an E1A deletion mutant lacking 14 amino acids spanning the CtBP interacting domain (amino acids 225–238) and thus unable to interact with CtBP (Johansson et al., 2005).

It has been previously shown that E1A interacts with members of the pRb family, inhibiting their interaction with the E2F transcription factors and thus inducing cell cycle progression (Pelka et al., 2011). As it was therefore expected, ectopic expression of E1A enhanced the activity of E2F-responsive promoters including *E2F1*, *cdc6*, *cyclin E* and *p73* (Figure 4.7). Interestingly, while expression of E1A activated E2F-responsive promoters, co-expression of E2F7 significantly reduced the activity of the promoters (Figure 4.7). Ectopic expression of E2F7 had a repressive effect on the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters in the presence of either E1A or E1AΔ. Repression of the promoters by E2F7 expression as compared to expression of the empty vector was used as a control.

Therefore, the results here suggest that the E2F7-CtBP interaction is robust and unaffected by E1A. Although E1A is known to interact with CtBP via the CtBP-binding motif, the presence of either endogenous E1A or ectopic E1A and E1AΔ were shown to be unable to affect on the repression ability of E2F7 on E2F target genes.

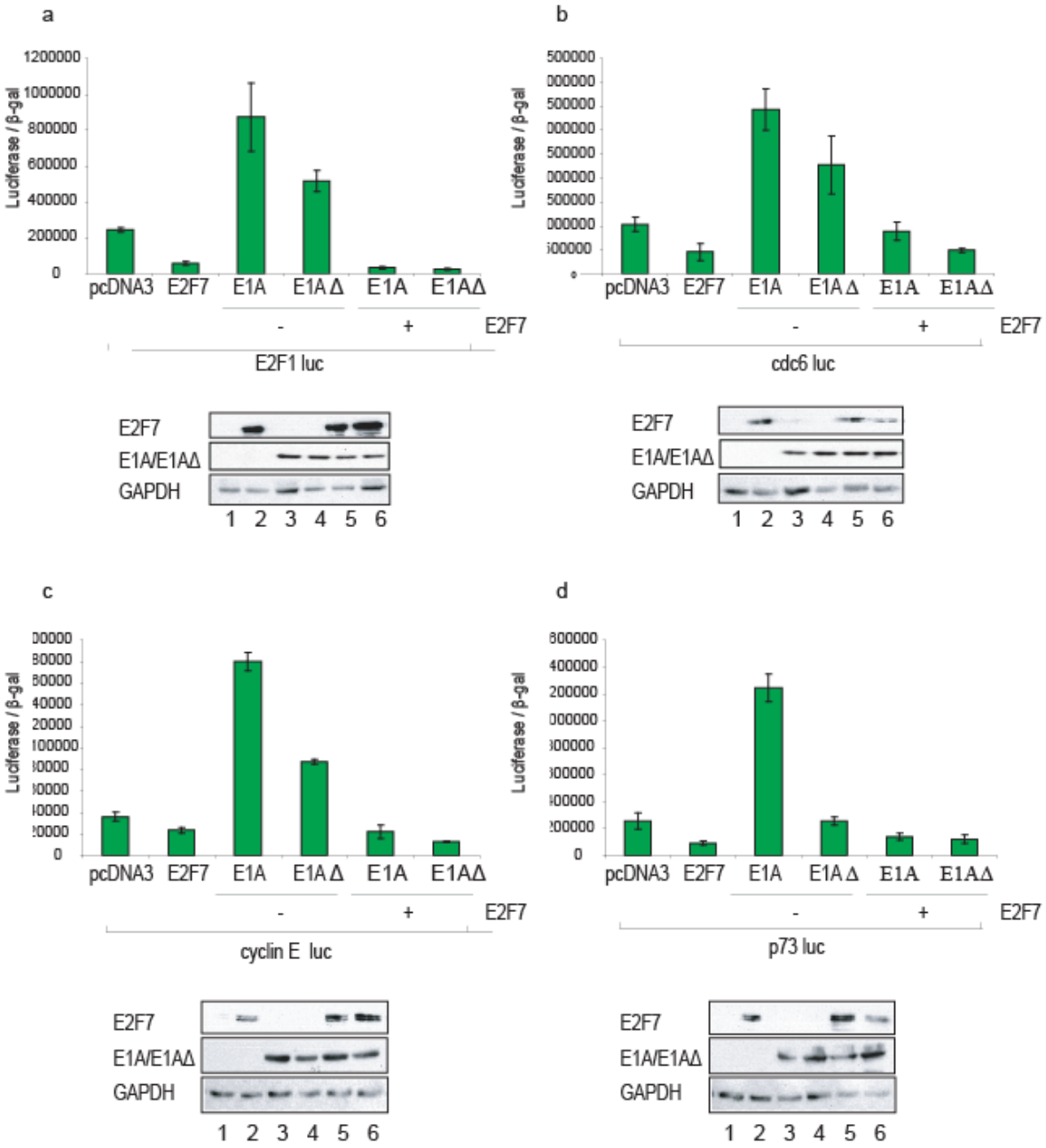


Figure 4.7: E2F7 can repress E2F1, cyclin E, cdc6 and p73 transcriptional activity in the presence of E1A. U2OS cells were transfected with the reporter construct pE2F1-luc (a), pcdc6-luc (b), pcyclin E-luc (c) or pp73-luc (d) alone and together with expression vectors encoding pcDNA3 (control), E2F7, E1A, E1AΔ, E1A and E2F7 or E1AΔ and E2F7. All were co-transfected with β-gal as a transfection control. The results represent the ratio of luciferase to β-gal activity. Values in all graphs represent the mean values of triplicate experiments and the error bars represent standard deviations. Expression levels of the different constructs used in the reporter assays were analysed by Western blotting with the appropriate antibodies as indicated. (n=3)

4.6 Altering the redox state does not affect the ability of E2F7 to repress

As discussed in chapter 1, CtBP shares compelling sequence and structural homology with D-isomer-specific 2-hydroxy acid dehydrogenases (D2-HDH) predicting a critical role for NAD(H) dinucleotides in CtBP activity (Kumar et al., 2002, Ghosh et al., 2010). The dinucleotides have been shown to stimulate dimerization and interaction of CtBP with certain PLDLS-containing target proteins (Zhang et al., 2002, Balasubramanian et al., 2003). On the other hand, however, there are some reports based on analysis of CtBP mutants suggesting that NAD(H) binding may not be crucial for the CtBP repression activity (Marmorstein, 2002, Grooteclaes et al., 2003).

It was therefore of interest to explore whether the NAD(H) dinucleotides can affect the CtBP-E2F7 interaction and thus CtBP activity in E2F7-mediated repression. In order to assess the effect of these dinucleotides in CtBP activity, cells were subject to different treatments with agents that alter the NAD^+/NADH ratio. To perturb the cellular redox state, cells were treated with 10mM sodium azide (NaN_3) which has been shown to cause a decrease in the NAD^+/NADH ratio (Zhang et al., 2002). On the other hand, the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) was used to treat cells at a 10mM final concentration thereby inducing an increase in the cellular NAD^+/NADH ratio (Garriga-Canut et al., 2006, Zhang et al., 2007). Untreated cells were used as a control.

To investigate whether altering the cellular redox state affects the E2F7-CtBP interaction, endogenous immunoprecipitations were performed under different NAD^+/NADH ratios. Decreasing the NAD^+/NADH ratio was previously shown to

increase the interaction of ectopic CtBP with endogenous E1A and enhance CtBP-mediated repression of the E-cadherin promoter due to an increase of the CtBP interaction with the ZEB repressor (Zhang et al., 2002). However, decreasing the NAD^+/NADH ratio showed an opposite effect on the Hdm2 interaction with CtBP (Mirnezami et al., 2003). On the other hand, increasing this ratio by 2-DG treatment was shown to decrease the association of CtBP with HIC1 (Zhang et al., 2007). Interestingly, another study has shown 2-DG treatment to increase the interaction of the neuronal repressor NRSF/REST with CtBP (Garriga-Canut et al., 2006).

Untreated cells or cells treated with NaN_3 or 2-DG cells were used for endogenous E2F7 immunoprecipitations. In order to confirm that these treatments caused the expected change in the cellular redox state, the NAD^+/NADH ratio was quantified subsequent to NaN_3 and 2-DG treatments and compared to the ratio of the untreated cells as discussed in chapter 2. As expected, treating cells with NaN_3 caused a decrease in the NAD^+/NADH ratio, whereas 2-DG treatment significantly increased the ratio (Figure 4.8a).

Following quantification of the NAD^+/NADH ratio confirming the expected changes in the cellular redox state, endogenous E2F7 immunoprecipitations were performed to investigate whether altering the redox state can impact the E2F-CtBP interaction. E2F7 and CtBP were shown to interact under normal redox conditions, as well as under different redox states (Figure 4.8b). Increasing the NAD^+/NADH ratio by 2-DG treatment was shown to cause a marginal increase in the levels of CtBP interacting with E2F7. It was thus investigated whether this marginal increase

in the E2F7-CtBP interaction could affect the E2F7-mediated repression of E2F target genes.

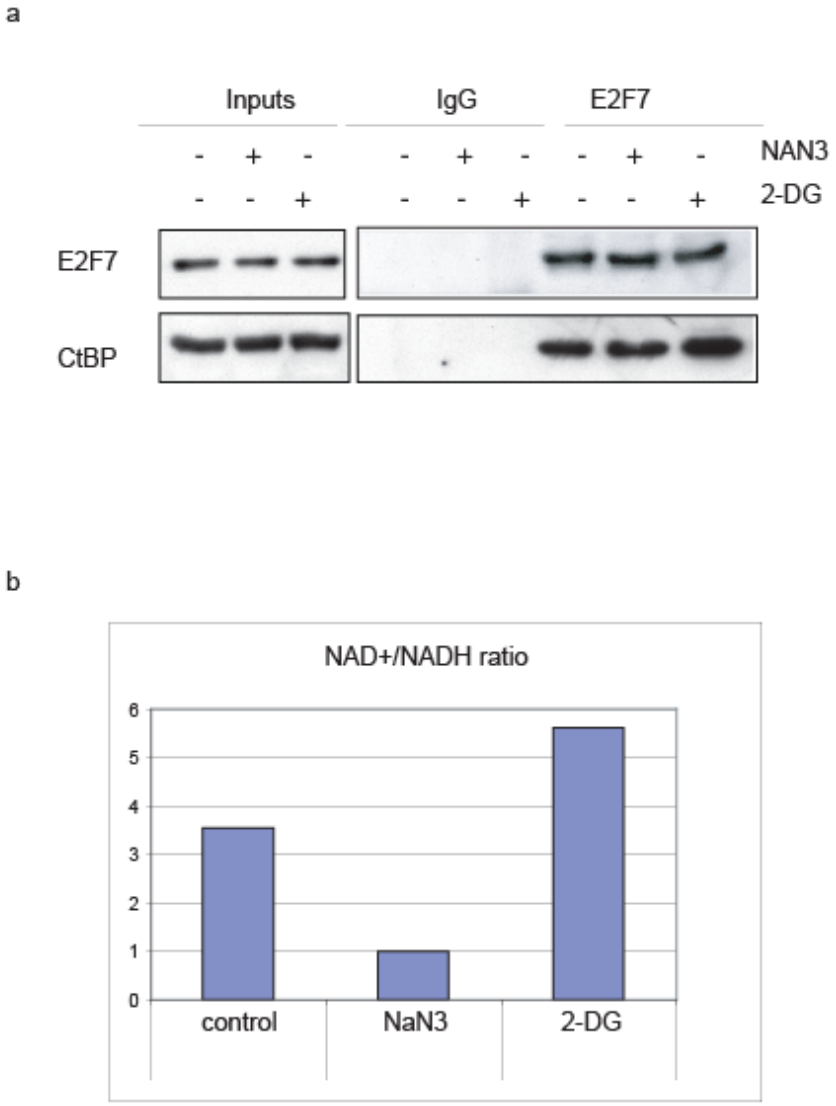


Figure 4.8: E2F7 interacts with CtBP under different cellular redox states.
MCF7 cells were untreated or undergone treatment with NaN3 or 2-DG as indicated. (a) Cells were immunoprecipitated using anti-E2F7 antibody. Anti-E2F7 and anti-CtBP antibodies were used to detect the endogenous E2F7 and CtBP proteins respectively. (b) NAD+ and total NAD levels were measured in MCF7 cells that were either untreated (control) or following treatment with 10 mM NaN3 or 5mM 2-DG. The bar chart represents the quantification of the NAD+/NADH ratio of untreated and treated samples, as indicated. (n=3)

In order to address this, the effect of ectopic E2F7 expression on the activity of E2F-responsive promoters was explored under normal conditions and under conditions different redox states. Cells expressing either ectopic E2F7 or empty vector remained untreated or subjected to treatment with NaN_3 or 2-DG and the resulting changes on the NAD^+/NADH were quantified.

Following confirmation of the expected NAD^+/NADH ratios subsequent to each treatment the effect of these treatments on the ability of E2F7 to repress the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters was investigated. E2F7 was shown to repress these promoters as compared to the empty vector control under normal conditions, as well as under different cellular redox states (Figure 4.9). Although cells expressing the empty vector showed different levels of promoter activity following treatment with NaN_3 or 2-DG, ectopic expression of E2F7 caused repression of the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters under all redox states.

Therefore, the results here suggest that altering the cellular redox state does not have a significant impact on the ability of CtBP to interact with E2F7. Furthermore, neither increasing nor decreasing the NAD^+/NADH ratio can compromise the repression effect of E2F7 on E2F-regulated promoters.

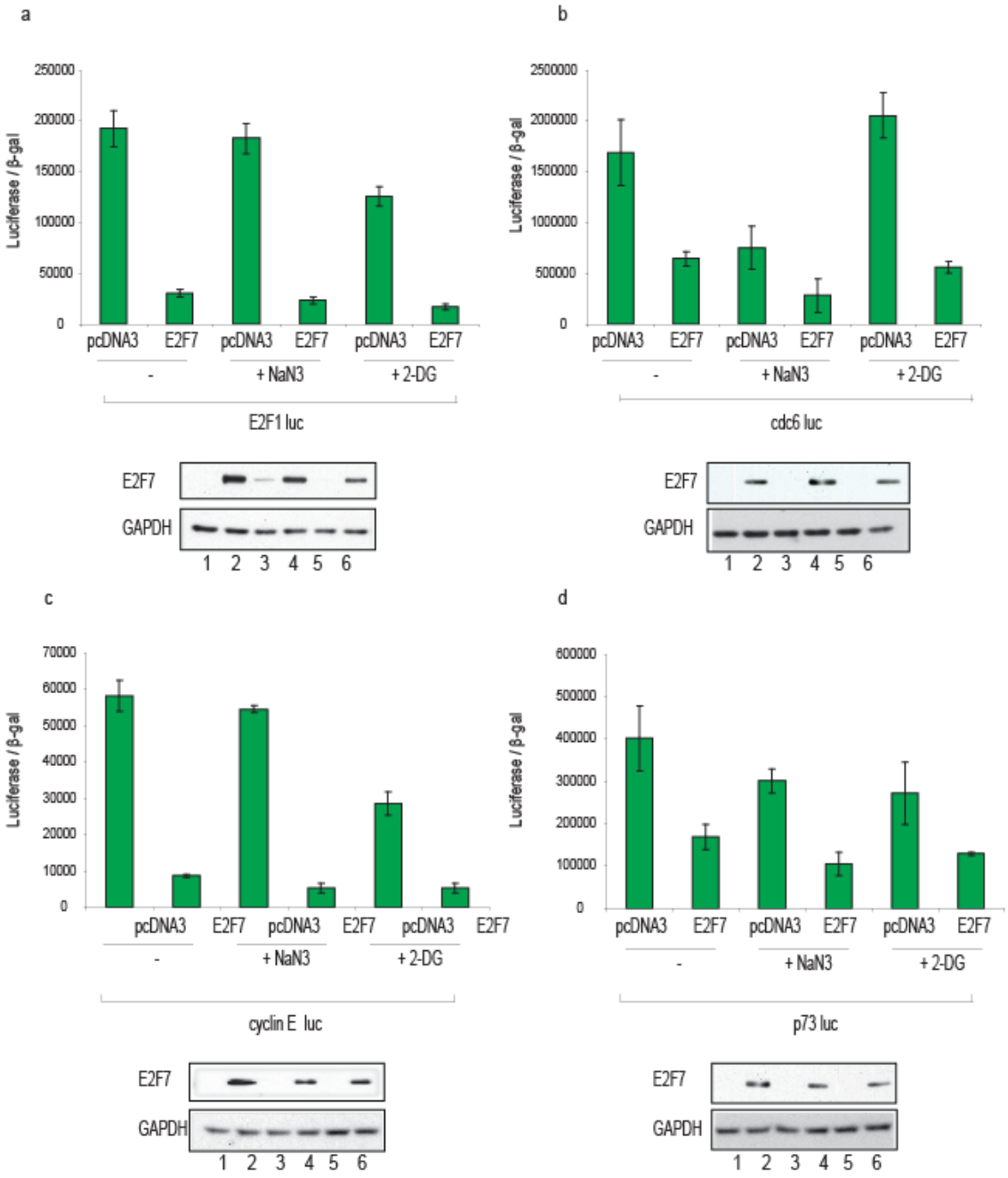


Figure 4.9: E2F7 can repress the transcriptional activity of the E2F1, cyclin E, cdc6 and p73 promoters under different redox states. U2OS cells were transfected with the reporter construct pE2F1-luc (a), pcdc6-luc (b), pcyclin E-luc (c) or pp73-luc (d) alone and together with expression vectors encoding pcDNA3 (control) or E2F7 under no treatment or following treatment with 10mM NaN3 or 5mM 2-DG as indicated. All were co-transfected with β-gal as a transfection control. The results represent the ratio of luciferase to β-gal activity. Values in all graphs represent the mean values of triplicate experiments and the error bars represent standard deviations. Expression levels of the different constructs used in the reporter assays were analysed by Western blotting with the appropriate antibodies as indicated. (n=3)

4.7 MTOB treatment affects the E2F7-mediated repression

The ability of E2F7 to repress the transcription of E2F target genes was shown to be unaffected by neither E1A expression nor changes in the cellular redox state. Although changes in the cellular NAD^+/NADH ratio did not have an effect on E2F7-mediated transcriptional repression, it was possible that CtBP binding to a substrate could have an effect on the E2F7 repression function.

As discussed in chapter 1, the PLDLS binding cleft and the substrate-binding domain are physically in close proximity to the CtBP dehydrogenase catalytic centre (Kumar et al., 2002, Nardini et al., 2003). Studies using the CtBP substrate MTOB indicate that substrate loading can exert a significant suppression on CtBP recruitment to target promoters (Straza et al., 2010, Achouri et al., 2007). Furthermore the same study suggested that MTOB loading to the CtBP substrate-binding domain could induce a conformational change in the PLDLS binding cleft resulting in reduced interaction of CtBP with transcriptional repressors. It was therefore reasonable to speculate that CtBP binding to a substrate could affect the E2F7-mediated transcriptional repression.

In order to assess the consequence of CtBP substrate binding on the ability of E2F7 to repress E2F-responsive genes, the repressive effect of ectopic E2F7 expression was investigated following MTOB treatment. Under normal conditions, ectopic expression of E2F7 reduced the activity of the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters as compared to expression of the empty vector control. However, under MTOB treatment E2F7 was unable to confer a repression effect to any of these E2F-responsive promoters (Figure 4.10). Following MTOB treatment, the activity

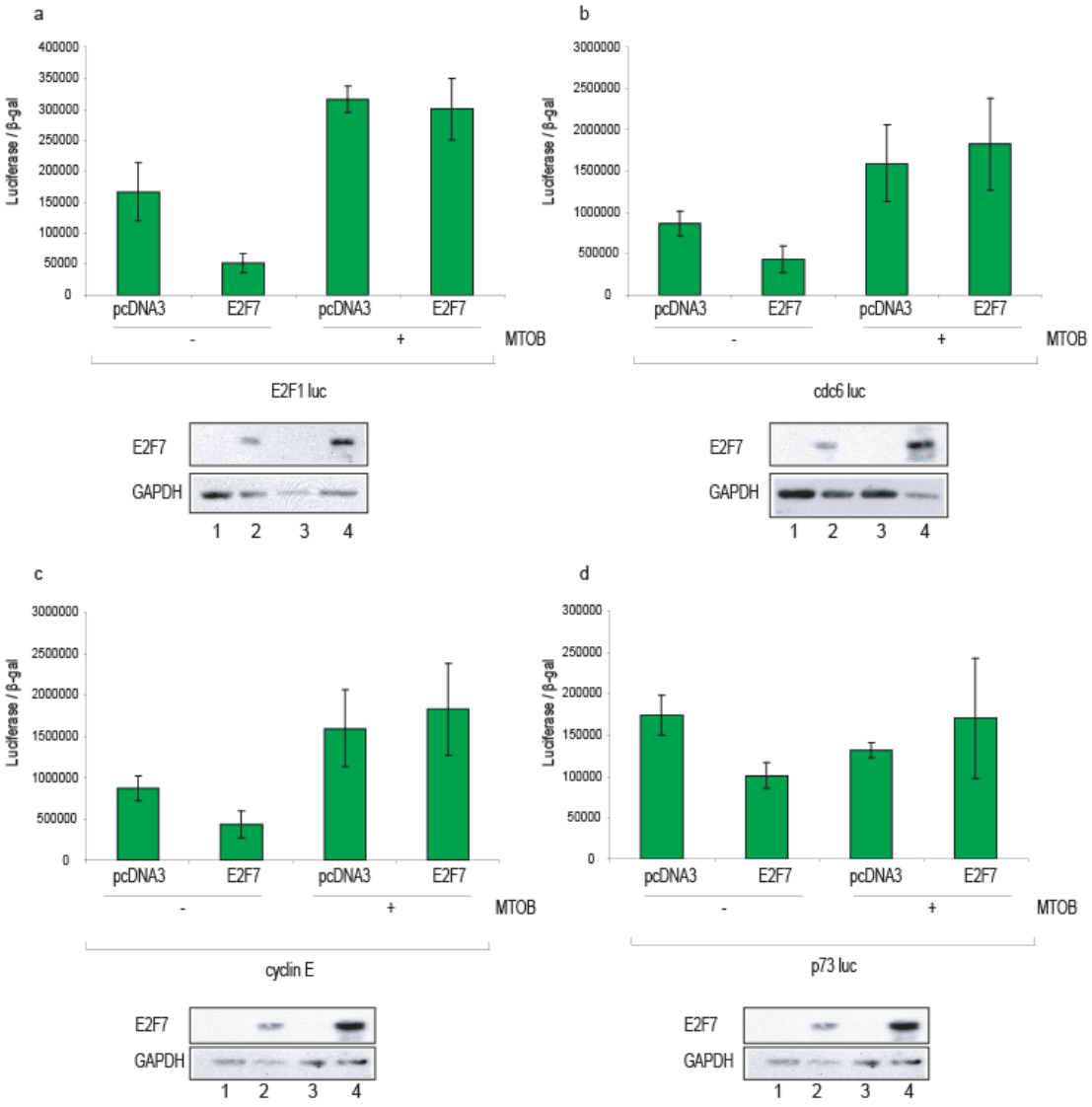


Figure 4.10: E2F7 is unable to repress the E2F1, cyclin E, cdc6 and p73 transcriptional activity under MTOB treatment. U2OS cells were transfected with the reporter construct pE2F1-luc (a), pcdc6-luc (b), pcyclin E-luc (c) or pp73-luc (d) alone and together with expression vectors encoding pcDNA3 (control) and E2F7 under no treatment and following treatment with 4mM MTOB as indicated. All were co-transfected with β-gal as a transfection control. The results represent the ratio of luciferase to β-gal activity. Values in all graphs represent the mean values of triplicate experiments and the error bars represent standard deviations. Expression levels of the different constructs used in the reporter assays were analysed by Western blotting with the appropriate antibodies as indicated. (n=3)

of the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters under ectopic empty vector expression was comparable with promoter activity under E2F7 expression.

These results therefore suggest that in the presence of MTOB, the ability of E2F7 to confer repression to E2F-responsive genes is abolished. Thus, binding of CtBP to its substrate could affect the function of CtBP as a co-repressor. Interestingly, complete loss of E2F7-mediated repression under MTOB treatment indicates that CtBP plays an important role in the ability of E2F7 to repress the transcription of E2F-responsive genes.

4.8 Discussion

CtBP is a well-established transcriptional co-repressor that has been shown to associate with sequence specific DNA binding transcriptional repressors (Chinnadurai, 2007). Following the identification of CtBP as an E2F7-interacting protein and the involvement of CtBP in the repression of E2F-responsive genes, it was reasonable to speculate that CtBP could play an important role in E2F7-mediated transcriptional repression. Thus, it was of great interest to investigate the importance of the E2F7 CtBP-binding motif, explore the E2F7-CtBP interaction under different conditions and establish which conditions that affect CtBP have an impact on the ability of E2F7 to repress E2F target genes.

Initially, in order to establish whether the CtBP interaction is significant in E2F7-mediated transcriptional repression, an E2F7 mutant was produced lacking the CtBP-binding motif. Previous studies have demonstrated that ectopically expressed E2F7 localises in the nucleus (Logan et al., 2004, Zalmas et al., 2008).

Investigation of the cellular localisation of E2F7 deletion mutant E2F7ΔCtBP by immunofluorescence demonstrated that deletion of the CtBP-binding motif does not affect the nuclear localisation (Figure 4.1). E2F7ΔCtBP was therefore utilized in assays where the functional consequences of the CtBP-motif deletion are not attributed to changes in the E2F7 conformation that affect nuclear localisation.

The E2F7ΔCtBP was then used to investigate the importance of the CtBP-binding motif in the E2F7-mediated transcriptional repression of E2F target genes. E2F7ΔCtBP was shown to be unable to confer repression tof the *E2F1*, *cdc6* and *cyclin E* promoters (Figure 4.2a, b and c) as compared to the repressive effect exerted by wild-type E2F7 on the same promoters, thus suggesting that the CtBP-binding motif plays an important role in the ability of E2F7 to repress E2F target genes. Interestingly, E2F7ΔCtBP was not only unable to repress the *cdc6* and *cyclin E* promoters but rather enhanced the activity of these promoters as compared to the empty vector control. Increased promoter activity as conferred by the E2F7 mutant raises the possibility that under certain conditions E2F7 might be able to play an activating rather than a repressive role to certain E2F target genes. On the other hand, deletion of the CtBP-binding motif had no effect on the E2F7-mediated repression of the *p73* promoter (Figure 4.2d) correlating with the previous observation that siRNA-mediated CtBP depletion has no effect on the activity of the *p73* promoter (Figure 3.3d) and thereby suggesting that CtBP plays a role in the E2F7-mediated repression of a subset of E2F target genes.

As these results indicated the importance of the CtBP-binding motif in the ability of E2F7 to repress its target genes, it was of interest to further investigate the

conditions under which E2F7 interacts with CtBP. Having shown in chapter 3 that E2F7 can interact with CtBP under normal and DNA damage conditions following treatment with etoposide and given that E2F7 has been shown to play a role in the DNA damage response (Zalmas et al., 2008), the E2F7-CtBP interaction was explored different types of cell stress. Endogenous E2F7 immunoprecipitations showed that E2F7 interacts with CtBP under normal conditions as shown previously (Figure 3.2) and that the interaction was also robust following cell stress induced by either UV or heat shock (Figure 4.4a and b). The ability of E2F7 to interact with CtBP under different conditions further highlights an important role for CtBP in E2F7-mediated transcriptional repression of E2F-responsive genes.

As the E2F7-CtBP interaction was shown to be robust under different stress conditions, the effect of other factors on this interaction was explored. The inability of E2F7ΔCtBP to interact with CtBP led to the speculation that the presence of other proteins interacting with CtBP via the CtBP-binding motif could possibly perturb the E2F7-CtBO interaction. Since E1A is an established protein interacting with CtBP via the PLDLS motif, the E2F7-CtBP interaction was explored in the E1A expressing HEK293 embryonic kidney cells. Endogenoun E2F7 was shown to interact with CtBP in HEK293 cells, thereby indicating that the E2F7-CtBP interaction is not perturbed by the presence of E1A (Figure 4.5).

Although the presence of E1A did confer an effect on the E2F7-CtBP interaction, an effect on the E2F7-mediated transcriptional transcription of target genes could not be ruled out. In order to address this, functional assays in HEK293 cells were performed and the impact of ectopic E2F7 expression on E2F target genes was

investigated. Ectopic expression of E2F7 was shown to significantly repress the activity of the *E2F1*, *cyclin E*, *cdc6* and *p73* promoters in HEK293 as compared to the empty vector control (Figure 4.6), thus indicating that the repression ability of E2F7 is not influenced by E1A.

In order to further verify this observation, functional assays were also performed in U2OS cells in the presence of ectopically expressed E1A. Again, ectopic expression of E2F7 resulted in the repression of the E2F-responsive promoters and this repression was unaffected by the expression of ectopic E1A. Therefore, these results suggest that neither the E2F7-CtBP interaction not the repression ability of E2F7 are affected by the expression of E1A and thus indicate that this interaction is robust in the presence of another CtBP-interacting protein.

Although the presence of E1A was unable to affect the E2F7-CtBP interaction, other factors affecting CtBP interactions were still to be investigated. The NAD(H) dinucleotides have been previously shown to stimulate dimerization and interaction of CtBP with certain PLDLS-containing target proteins (Zhang et al., 2002, Balasubramanian et al., 2003). In order to explore whether altering the cellular redox state affects the E2F7-CtBP interaction, endogenous immunoprecipitations were performed under different NAD^+/NADH ratios. E2F7 and CtBP were shown to interact under normal redox conditions, as well as under different redox states (Figure 4.8b). Interestingly, increasing the NAD^+/NADH ratio by 2-DG treatment was shown to cause a marginal increase in the levels of CtBP interacting with E2F7.

In order to address whether this marginal increase in the E2F7-CtBP interaction affects the E2F7-mediated repression of E2F target genes, the effect of ectopic E2F7 expression on the activity of E2F-responsive promoters was explored under normal conditions and under conditions different redox states. E2F7 was shown to repress the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters as compared to the empty vector control under normal conditions, as well as under different cellular redox states (Figure 4.9). Therefore, these results here indicate that altering the cellular redox state does not have a significant impact on the E2F7-CtBP interaction. Moreover, any changes in the NAD^+/NADH ratio do not compromise the repression effect of E2F7 on E2F target genes.

Interestingly, neither E1A expression nor changes in the cellular redox state were shown to affect the repression function exerted by E2F7 to E2F target genes However, it was possible that CtBP binding to a substrate could have an effect on the E2F7 repression ability. Studies using the CtBP substrate MTOB suggested that MTOB loading to the CtBP substrate-binding domain could induce a conformational change in the PLDLS binding cleft and thus result in reduced interaction of CtBP with transcriptional repressors. It was therefore of great interest to explore whether CtBP binding to a substrate could affect the ability of E2F7 to repress transcription.

Although under normal conditions ectopic expression of E2F7 exerted a repression effect on the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters this was not the case under MTOB treatment. Following MTOB treatment, the activity of the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters under ectopic empty vector expression was comparable

with promoter activity under E2F7 expression (Figure 4.10). These results thus indicate that binding of CtBP to a substrate significantly compromises the function of E2F7 as a transcriptional repressor. Furthermore, MTOB treatment was shown to abolish E2F7-mediated repression indicates thus suggesting that CtBP plays an important role in the function of E2F7 to repress the transcription of E2F-responsive genes.

The results presented here demonstrate that the E2F7 CtBP-binding motif is not only important for the E2F7-CtBP interaction but for the ability of E2F7 to repress transcription. This interaction was shown to be robust under different types of cell stress, as well as under conditions that affect certain CtBP interactions including presence of E1A and different redox states. Interestingly, however, E2F7 was shown to be unable to confer any repression effect to E2F target genes under treatment with the CtBP substrate MTOB. As MTOB has been shown to function as a CtBP inhibitor (Straza et al., 2010), loss of E2F7 repression under MTOB treatment suggests that CtBP could be essential for E2F7 repression function.

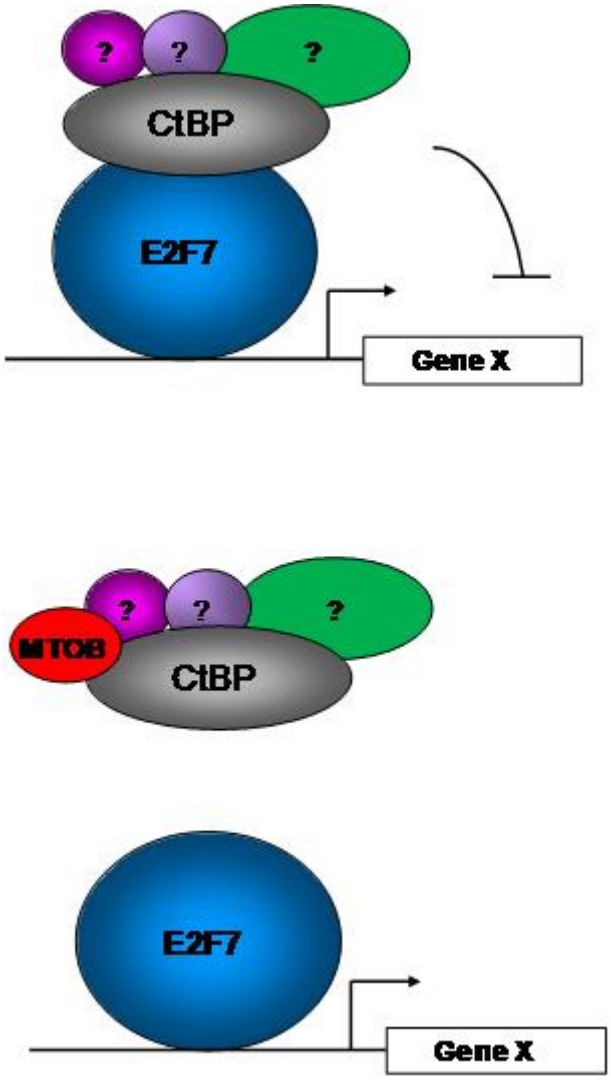


Figure 4.11 Proposed model for the effect of CtBP binding to the MTOB substrate on E2F7-mediated repression of E2F target genes.

Binding with the MTOB substrate induces conformational changes to the CtBP affecting the CtBP-binding motif and thus interaction with E2F7. As a result, in the absence of CtBP interaction, E2F7 is unable to repress E2F target genes.

CHAPTER 5

IDENTIFICATION OF HDACs IN THE E2F7 REPRESSION COMPLEX

5.1 Introduction

CtBP functions as a transcriptional repressor in association with sequence-specific DNA binding transcription factors and recruits other co-repressors and chromatin remodelling proteins to target promoters (Chinnadurai, 2007). Proteomic analysis of the CtBP repressor complex has revealed the presence of numerous CtBP co-factors (Shi et al., 2003). In addition to DNA-binding transcriptional repressors, the CtBP complex was shown to contain enzymatic constituents catalyzing different histone modifications. These chromatin remodelling enzymes included Class I histone deacetylases, histone lysine methyl transferases and a histone lysine-specific demethylase (Tachibana et al., 2005, Shi et al., 2004). Furthermore, the CtBP complex also contained certain co-repressors such as CoREST (Andres et al., 1999).

As the results presented in chapter 3 showed that CtBP is an E2F7 co-repressor, it was reasonable to speculate that the E2F7 repression complex could contain chromatin-modifying enzymes. In order to identify the enzymatic constituents of the E2F7 complex, endogenous E2F7 immunoprecipitations were performed. Furthermore, the role if the E2F7-interacting proteins in the repression of E2F target genes was addressed by functional assays as well as by investigating their occupancy on E2F regulated promoters. Overall, these studies were focused on identifying constituents of the E2F repression complex and establishing their involvement in the repression of E2F target genes.

5.2 E2F7 forms a complex with Class I histone deacetylases

Following the identification of CtBP an E2F7 interacting protein, it was reasonable to speculate that the E2F7 repression complex could contain chromatin-modifying enzymes. The mammalian CtBP appears to mediate transcriptional repression in either a histone deacetylase-dependent or -independent manner, depending on the context of the promoter (Subramanian and Chinnadurai, 2003, Koipally and Georgopoulos, 2000). It was therefore explored whether HDACs are components of the E2F7 repression complex and thus play a role in E2F7-mediated transcriptional repression.

To investigate the possibility that E2F7 might form a complex with HDACs, immunoprecipitations were performed under normal conditions as well as under DNA damage as induced by etoposide treatment. Endogenous E2F7 immunoprecipitations showed that E2F7 forms a complex with HDAC1 and HDAC2 under normal and DNA damage conditions. Non-specific (NS) binding was assessed by use of control IgG antibodies. The presence of HDAC1 and HDAC2 in an E2F7 complex was evident in both MCF7 breast adenocarcinoma and HeLa cervical carcinoma cells (Figure 5.1a and b).

These results therefore demonstrate that HDAC1 and HDAC2 are components of an E2F7 repression complex and could thus play a role in the transcriptional repression of E2F target genes.

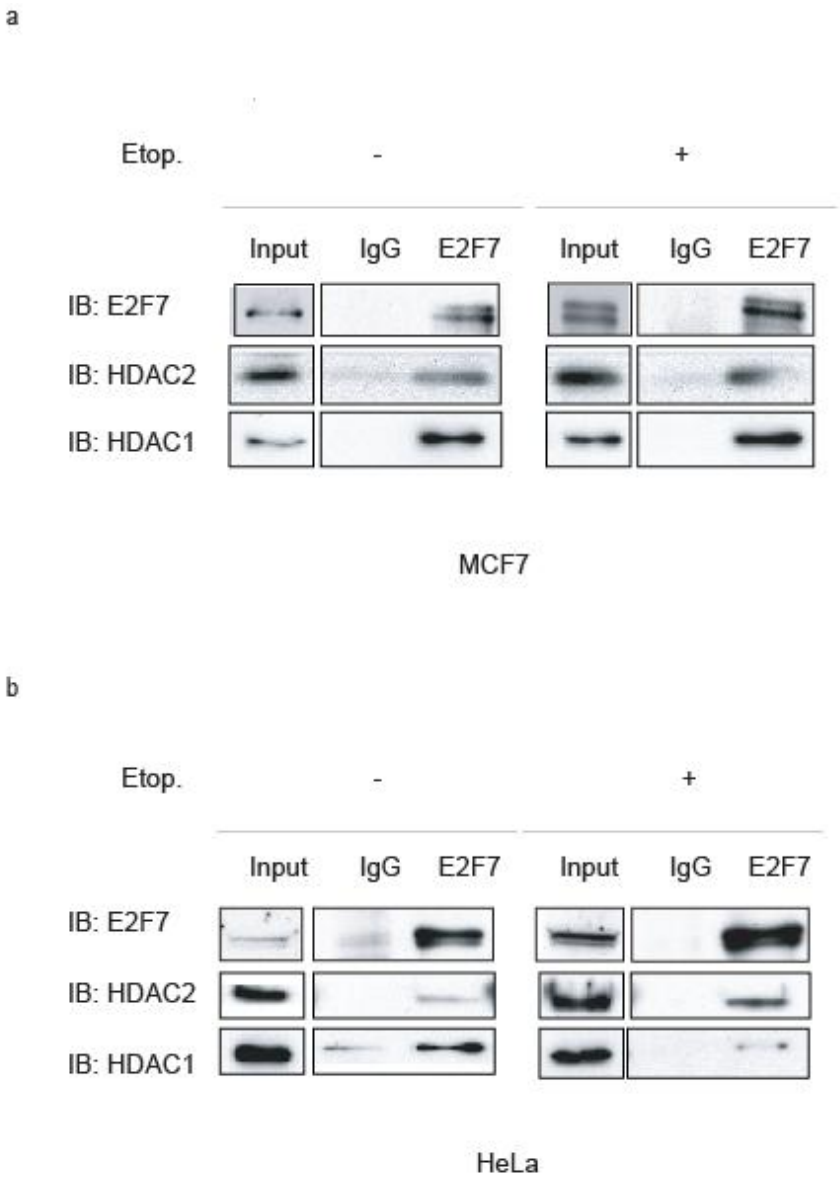


Figure 5.1: E2F7 forms a complex with HDAC1 and HDAC2.
Cell extracts were obtained from MCF7 (a) and HeLa (b) cells and used for endogenous E2F7 immunoprecipitations under normal conditions and following treatment with 10µM etoposide for 4 hours. The anti-E2F7, anti-HDAC1 and anti-HDAC2 antibodies were used to detect the endogenous E2F7, HDAC1 and HDAC2 proteins respectively. (n=4)

5.3 HDACs are involved in the repression of E2F responsive genes

Following the identification of HDAC1 and HDAC2 as constituents of an E2F7 repression complex, it was important to assess the involvement of these chromatin-modifying enzymes in the repression of E2F-regulated promoters. In order to address this, functional assays were performed in the presence of the HDAC inhibitor trichostatin A (TSA).

The activity of E2F responsive promoters was explored in cells treated with TSA as compared to treatment with dimethyl sulfoxide (DMSO) control. The levels of acetylated histone H3 of DMSO treated cells were compared with the corresponding levels following TSA treatment to confirm effectiveness of the HDAC inhibitor. Treatment with TSA resulted in significantly enhanced activity of the *E2F1* and *cdc6* promoters (Figure 5.2a and b), suggesting that HDACs play a role in the repression of E2F target genes. On the other hand, TSA treatment did not exert an effect on the activity of the *cyclin E* and *p73* promoters (Figure 5.2c and d) thereby suggesting that HDACs are involved in the transcriptional repression of a subset E2F responsive genes.

Taken together, the results presented here indicate that HDAC1 and HDAC2 play a role in the repression of E2F target genes and suggest that HDACs could be involved in E2F7-mediated repression.

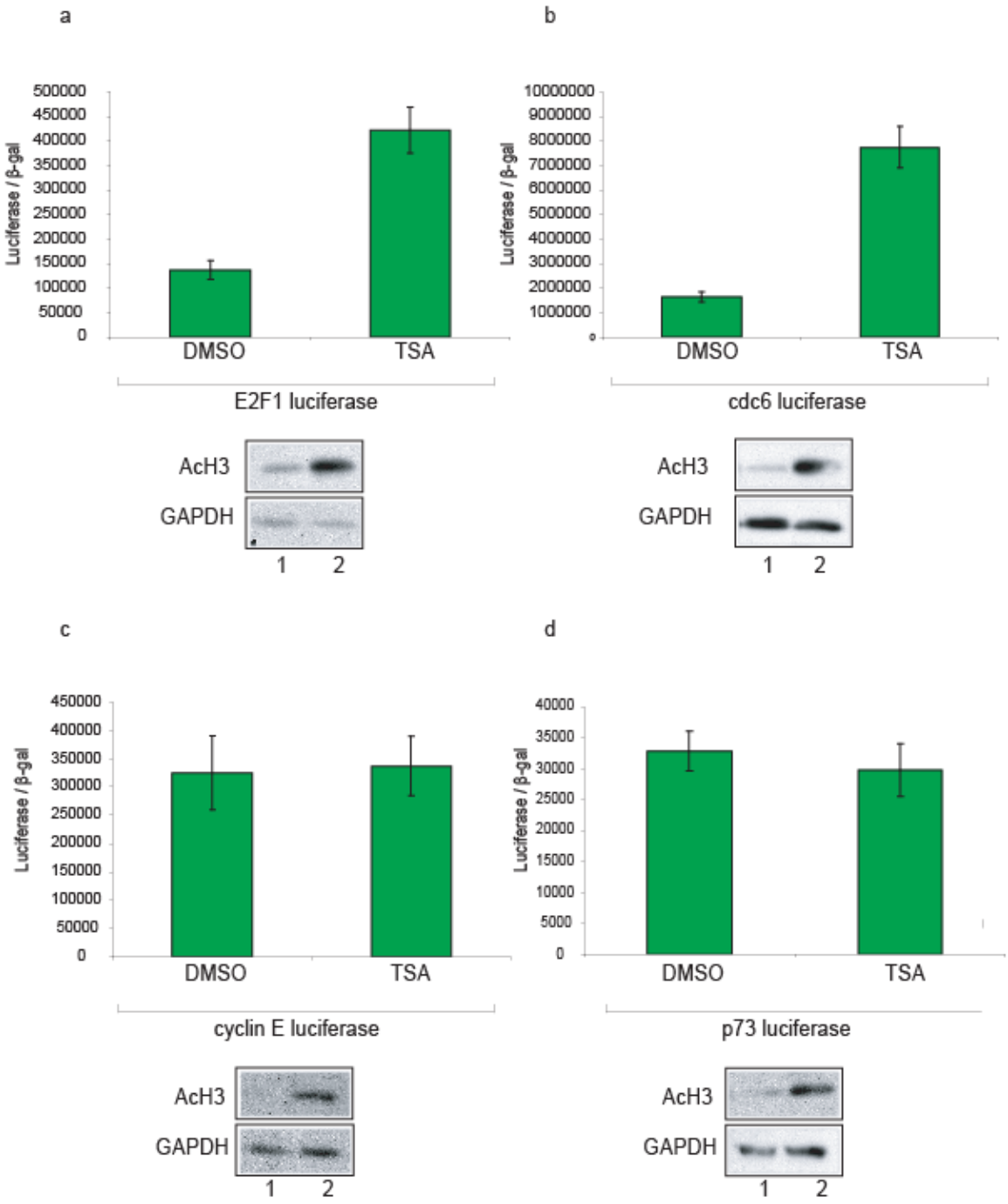


Figure 5.2: Treatment with the HDAC inhibitor TSA activates certain E2F-responsive promoters. U2OS cells were transfected with the reporter construct pE2F1-luc (a), pcdc6-luc (b), pcyclin E-luc (c) or pp73-luc (d) and treated with DMSO control or 5μM TSA for 16 hours. All were co-transfected with β-gal as a transfection control. The results represent the ratio of luciferase to β-gal activity. Values in all graphs represent the mean values of triplicate experiments and the error bars represent standard deviations. Expression levels of acetylated histone 3 and GAPDH loading control were analysed by Western blotting with the appropriate antibodies as indicated. (n=4)

5.4 Promoter recruitment profiles of HDAC1 and HDAC2

Having demonstrated that E2F7 and CtBP both bind E2F responsive promoters under normal conditions as well as under DNA damage (chapter 3), the recruitment of HDAC1 and HDAC2 to the same promoters was explored. By chromatin immunoprecipitation (ChIP), the binding of HDAC1 and HDAC2 to the *E2F1, cdc6, cyclin E* and *p73* promoters was investigated with specific antibodies to each protein. Non-specific (NS) binding was assessed by use of control IgG antibodies.

HDAC1 and HDAC2 were shown to occupy the *E2F1, cdc6, cyclin E* and *p73* promoters under normal conditions and under DNA damage (Figure 5.3). The *albumin* promoter, which was used as a negative control, was not occupied by either of the two proteins. Therefore, these data show that HDAC1 and HDAC2 are recruited to the same E2F-recognition sites as E2F7 and CtBP on the *E2F1, cdc6, cyclin E* and *p73, in vivo.*

The presence of HDAC1 and HDAC2 in an E2F7 complex and their recruitment to the same E2F responsive promoters as E2F7 led to the hypothesis that these proteins could be found in a DNA binding complex on target promoters *in vivo.* To address this, chromatin complexes were immunoprecipitated with E2F7 antibody from extracts of MCF7 breast adenocarcinoma cells. The resulting E2F7-bound chromatin complexes were then isolated and subjected to a second round of immunoprecipitation with either the HDAC1 or the HDAC2 antibody. This enriched the immunoprecipitated chromatin with DNA fragments bound by both E2F7 and each HDAC.

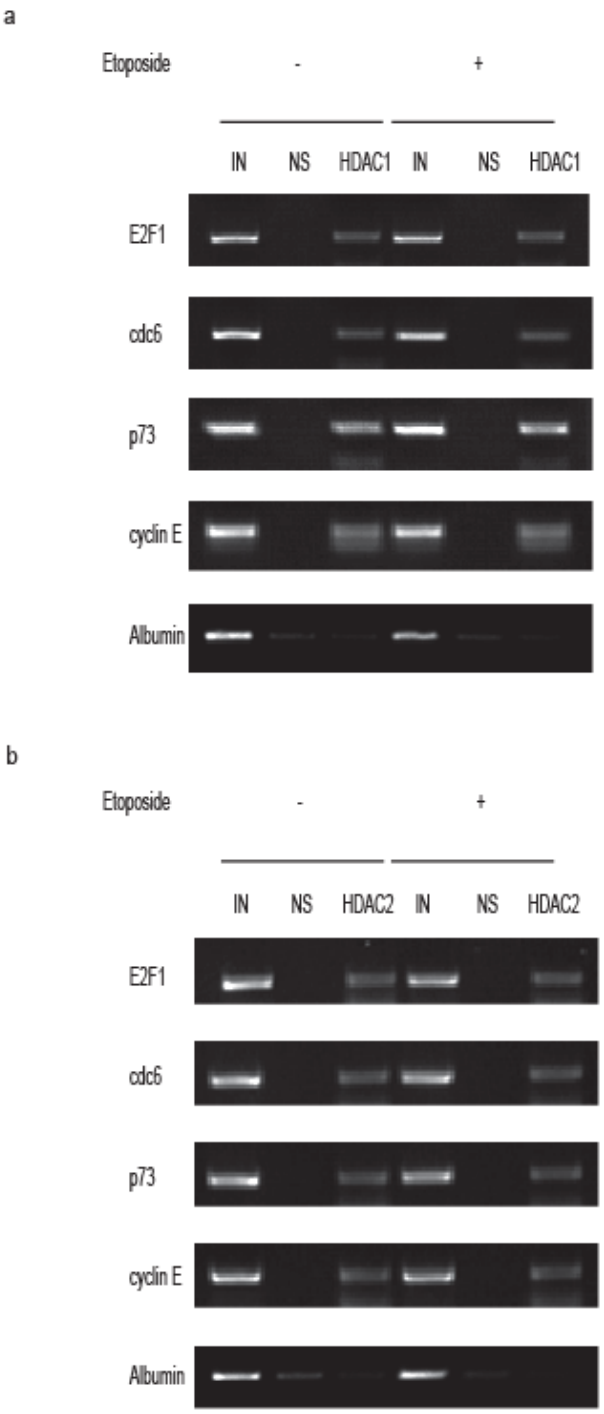


Figure 5.3: HDAC1 and HDAC2 are recruited to E2F-responsive promoters. Chromatin immunoprecipitation analysis on MCF7 cells carried out using HDAC1 (a) or HDAC2 (b) and control antibodies under normal conditions and after treatment with 10μM etoposide for 4h. PCR was performed against the promoters indicated. (n=4)

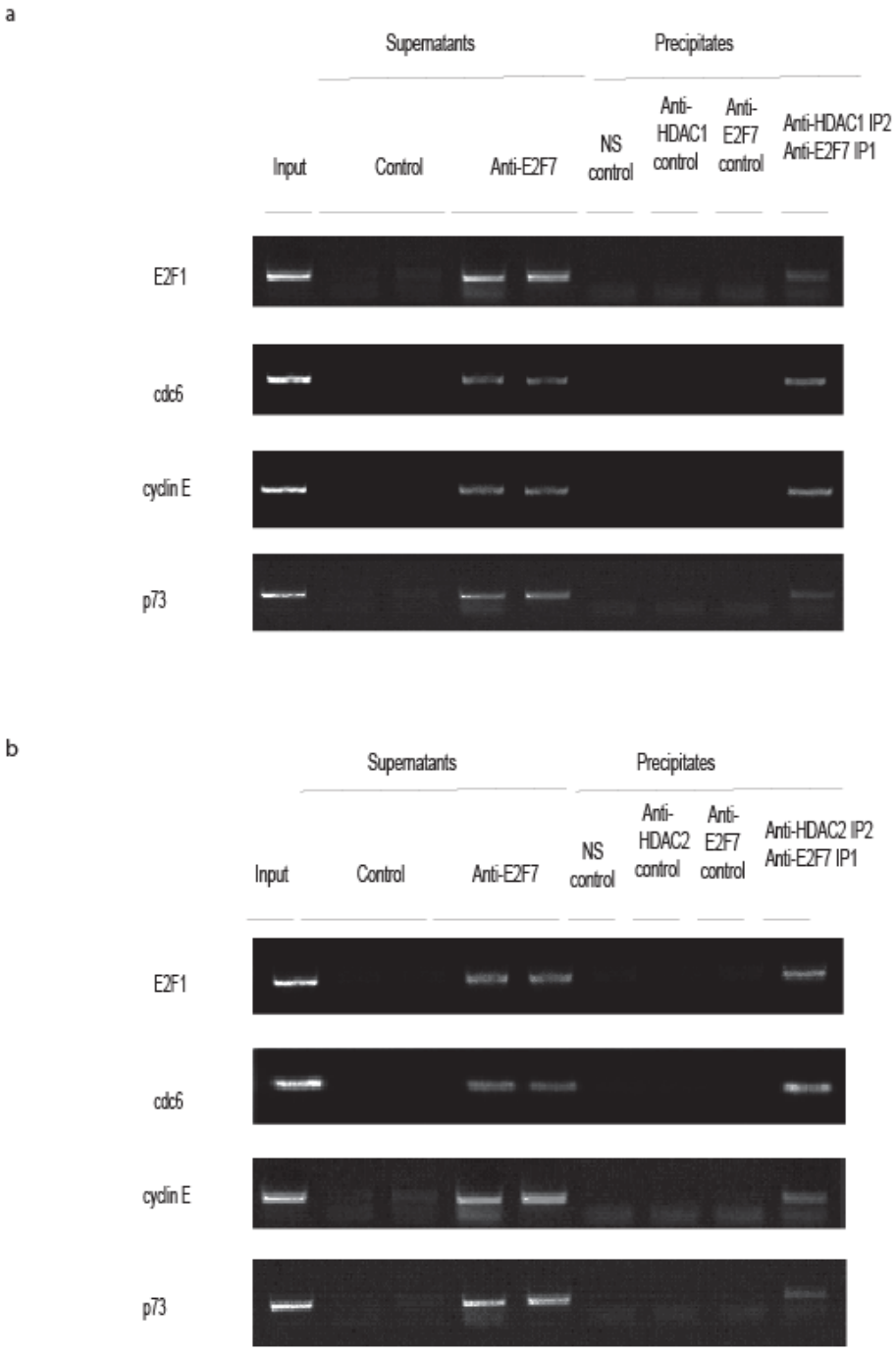


Figure 5.4: E2F7 co-exists on the E2F1, cdc6, cyclin E and p73 promoters with HDAC1 and HDAC2. Chromatin complexes were immunoprecipitated with the E2F7 antibody from extracts of MCF7 breast adenocarcinoma cells. The resulting E2F7-bound chromatin complexes were isolated and subjected to a second round of immunoprecipitation with either HDAC1 (a) or HDAC2 (b) antibodies. PCR was performed against the promoters indicated. (n=3)

Consistent with previous observations (Figure 3.4), E2F7 was shown to be recruited on the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters. Interestingly, as shown by the secondary immunoprecipitations HDAC1 and HDAC2 both exhibited a similar recruitment pattern on those promoters (Figure 5.4).

The data therefore suggest that HDAC1 and HDAC2 could be present in a DNA binding complex with E2F7 on a number of E2F responsive promoters including the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters.

5.5 Discussion

CtBP functions as a co-repressor of a wide array of DNA-binding transcriptional repressors. Following the identification of CtBP as an E2F7-interacting protein that plays a role in the transcriptional repression of E2F target genes, it was of interest to investigate whether chromatin-remodelling enzymes that are present in CtBP repression complexes are also constituents of the E2F7 complex. The mammalian CtBPs has been shown to mediate transcriptional repression in a histone deacetylase-dependent or -independent manner, depending on the context of the promoter (Subramanian and Chinnadurai, 2003). In this chapter the E2F7 repression complex was explored in order to investigate whether HDACs are present in the E2F7 complex and assess their involvement in the repression of E2F-responsive promoters.

To explore whether HDACs are components of the E2F7 repressor complex, E2F7-containing protein complexes were isolated from the nuclear extracts prepared from MCF7 breast adenocarcinoma and HeLa cervical carcinoma cells.

These endogenous immunoprecipitations showed that both HDAC1 and HDAC2 are components of an E2F7 complex under normal conditions as well as under DNA damage conditions (Figure 5.1a and b). The identification of HDAC1 and HDAC2 as constituents of an E2F7 repression complex suggested that these chromatin-modifying enzymes could play a role in the repression of E2F target genes.

In order to assess the involvement of these chromatin-modifying enzymes in the repression of E2F-regulated promoters, the HDAC inhibitor TSA was used in functional assays. The activity of E2F responsive promoters was explored in cells treated with TSA as compared to treatment with dimethyl sulfoxide (DMSO) control. TSA treatment significantly enhanced the activity of both *E2F1* and *cdc6* promoters (Figure 5.2a and b), thereby indicating that HDACs are involved in the repression of E2F target genes. In contrast, TSA treatment did not exert the same effect on the activity of the *cyclin E* and *p73* promoters (Figure 5.2c and d), as following treatment the activity of these promoters was unchanged. These results therefore suggest that HDACs are involved in the transcriptional repression of a subset of E2F responsive genes.

As HDACs were shown to be involved in the repression of E2F-responsive genes, it was of interest to explore whether they are recruited to E2F promoters in a manner comparable to the recruitment profile exhibited by E2F7 and CtBP. This was addressed by chromatin immunoprecititations, where HDAC1 and HDAC2 were shown to bind the *E2F1, cdc6, cyclin E* and *p73* promoters under normal conditions and under DNA damage (Figure 5.3). These results thus indicate that

HDAC1 and HDAC2 are recruited to the same E2F-recognition sites as E2F7 and CtBP on the *E2F1, cdc6, cyclin E* and *p73, in vivo* leading to the hypothesis that these proteins could be found in a DNA binding complex on target promoters. This was investigated by subjecting chromatin complexes immunoprecipitated with E2F7 antibody to a second round of immunoprecipitation with either the HDAC1 or the HDAC2 antibody. As shown in chapter 3, E2F7 was recruited to the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters. Interestingly, HDAC1 and HDAC2 both exhibited a similar recruitment pattern on the same promoters (Figure 5.4) thus suggesting that HDAC1 and HDAC2 could be present in a DNA binding complex with E2F7 on a number of E2F responsive promoters *in vivo*.

The results presented here demonstrate that HDAC1 and HDAC2 play a role in the repression of E2F-target genes. The ability of HDAC1 and HDAC2 to form a complex with E2F7 as well as their co-existence on the same E2F- responsive promoters with E2F7 and CtBP suggest that these HDACs are involved in E2F7-mediated transcriptional repression (Figure 5.5).

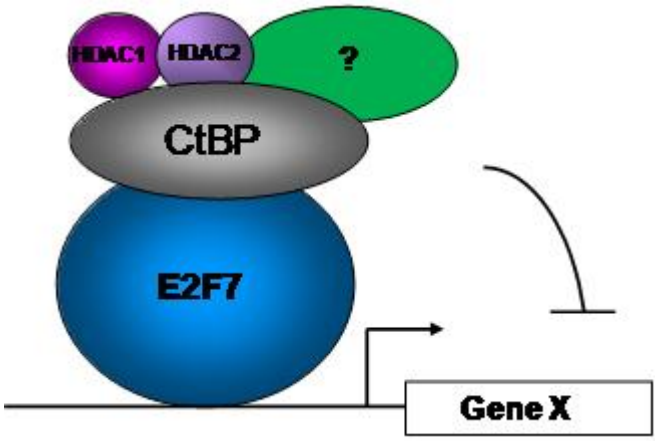


Figure 5.5 Proposed model for the E2F7 repression complex.

E2F7 forms a repression complex with CtBP, HDAC1 and HDAC2, which are involved in the repression of certain E2F target genes and recruited to E2F-responsive promoters. Therefore, there co-repressors could play a role in E2F7-mediated transcriptional repression of a subset of E2F targets.

CHAPTER 6

IDENTIFICATION OF CoREST AND LSD1 AS COMPONENTS OF THE E2F7 REPRESSION COMPLEX

6.1 Introduction

As discussed previously, proteomic analysis of the CtBP repressor complex has revealed the presence of numerous CtBP co-factors (Shi et al., 2003). In addition to DNA-binding transcriptional repressors and chromatin-modifying enzymes (Tachibana et al., 2005, Shi et al., 2004), the CtBP complex was also shown to contain certain co-repressors such as CoREST (Andres et al., 1999).

The ability of E2F7 to form a complex with CtBP, HDAC1 and HDAC2 and the function of CtBP to recruit HDACs to repression complexes led to the hypothesis that these HDACs could be linked to E2F7 via CtBP. However, the Class I HDACs do not process any apparent CtBP-binding motifs such as the PLDLS motif. Studies have suggested that HDACs are linked to CtBP through various co-repressors, such as CoREST, which have been shown to interact individually with these HDACs (Ballas et al., 2001, You et al., 2001).

In this chapter, the E2F7 repression complex was explored in order to identify other constituents of this complex and their involvement in the repression of E2F target genes was investigated.

6.2 E2F7 forms a complex with CoREST

Following the identification of CtBP, HDAC1 and HDAC2 as constituents of the E2F7 repression complex, it was of interest to explore other co-repressors that could be part of this complex and thus be involved in the repression of E2F target genes. In order to investigate whether CoREST is a component of the E2F7 repression complex, E2F7 immunoprecipitations were performed under normal

conditions as well as under DNA damage. Endogenous E2F7 immunoprecipitations showed that E2F7 forms a complex with CoREST under normal and DNA damage conditions. Non-specific (NS) binding was assessed by use of control IgG antibodies. The E2F7-CoREST complex was evident in both MCF7 breast adenocarcinoma and HeLa cervical carcinoma cells (Figure 6.1a and b).

These results therefore demonstrate that, in addition to CtBP, HDAC1 and HDAC2, CoREST is also a component of the E2F7 repression complex and as such could play a role in the repression of E2F-responsive genes.

6.3 Depletion of CoREST activates the transcription of certain E2F target genes

The ability of CoREST to form a complex with E2F7 suggested that CoREST could be involved in the repression of E2F-responsive promoters. In order to determine this, the impact of CoREST siRNA deletion on the activity of E2F responsive promoters was explored as compared to the effect of non-targeting siRNA.

Upon CoREST depletion, the activity of the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters (Figure 6.2a, b, c and d) was enhanced as compared to the activity of these promoters under non-targeting siRNA. Interestingly, the activity of all four E2F-responsive promoters was increased by CoREST siRNA whereas only the *E2F1* and *cdcd6* promoters were affected by HDAC inhibition thereby suggesting that CoREST could recruit other chromatin-modifying enzymes in order to repress E2F target genes.

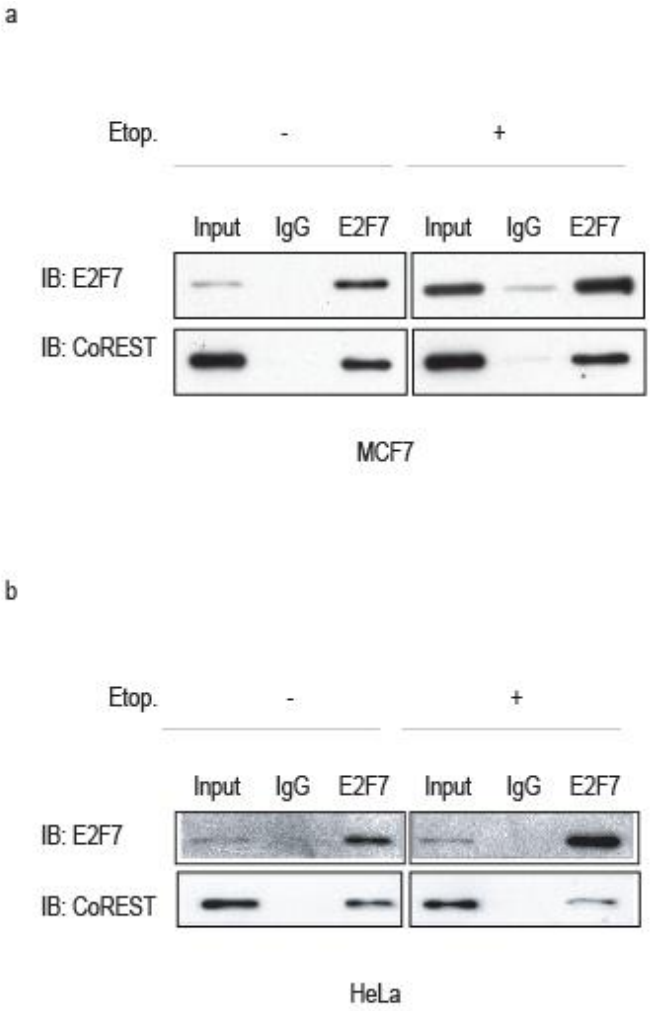


Figure 6.1: E2F7 forms a repression complex with CoREST.
Cell extracts were obtained from MCF7 (a) and HeLa (b) cells and used for endogenous E2F7 immunoprecipitations under normal conditions and following treatment with 10μM etoposide for 4 hours. The anti-E2F7 and anti-CoREST antibodies were used to detect the endogenous E2F7 and CoREST proteins respectively. (n=4)

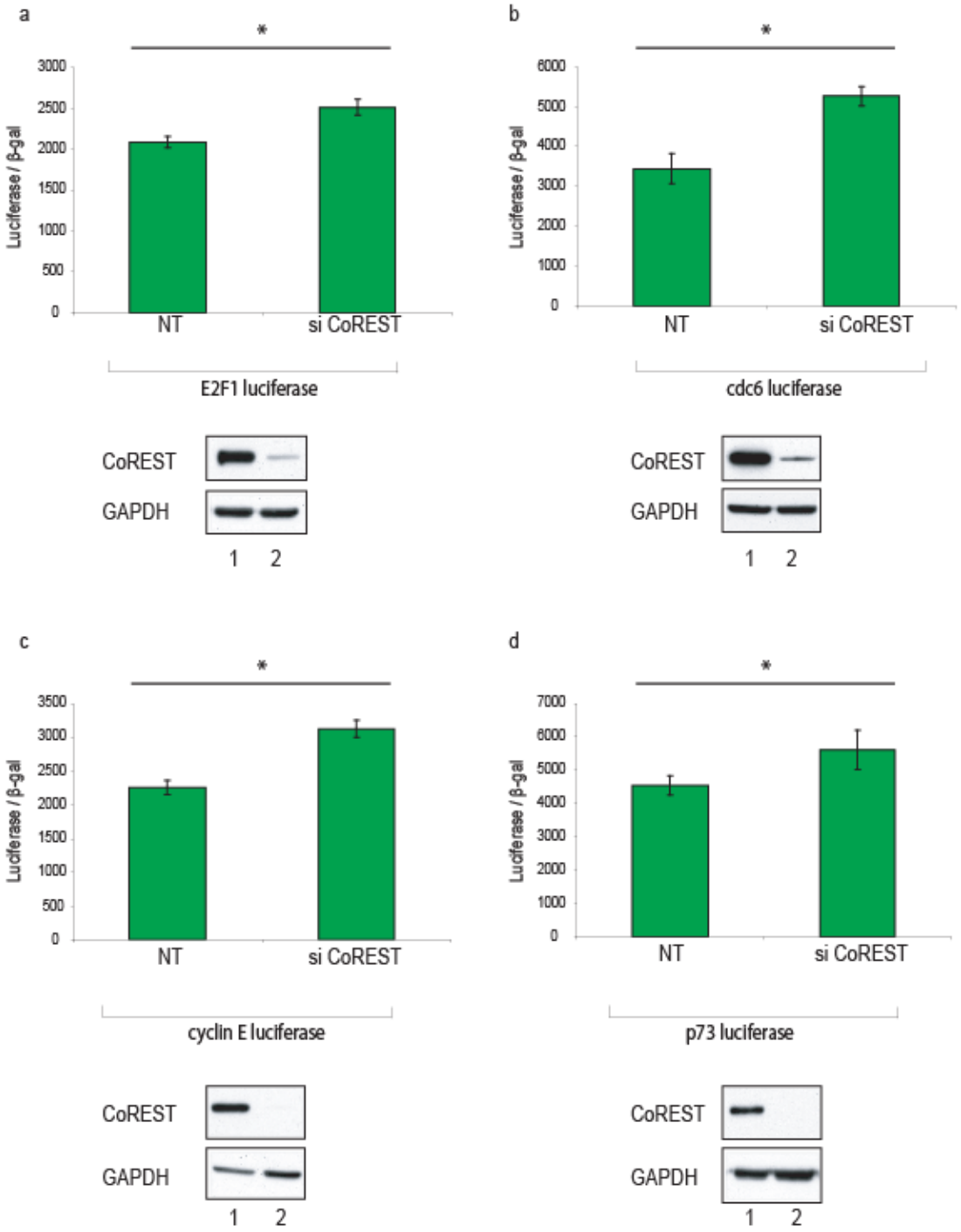


Figure 6.2: CoREST is a repressor of E2F target genes.
Luciferase assays using E2F target gene promoters. U2OS cells were transfected with the reporter construct pE2F1-luc (a), pcdc6-luc (b), pcyclin E-luc (c) or pp73-luc (d) under non targeting (NT) or CoREST siRNA. All were co-transfected with β-gal as a transfection control. The results represent the ratio of luciferase to β-gal activity. Values in all graphs represent the mean values of triplicate experiments and the error bars represent standard deviations. The difference in transcriptional activity of NT and si CoREST treated cells is statistically significant. The asterisk (*) indicates statistically significant difference (p-value 0.05). The CoREST levels were analysed by Western blotting with the appropriate antibodies as indicated. (n=4)

Taken together, the data presented here indicate that the CoREST is involved in the repression of E2F-responsive genes. Furthermore, the enhanced activity of the *E2F1*, *cdc6, cyclin E* and *p73* promoters as a result of CoREST siRNA depletion suggest that CoREST could recruit chromatin-modifying enzymes to the target promoters.

6.4 E2F7 forms a complex with LSD1

In addition to HDAC1 and HDAC2, CoREST has also been shown to often associate with the lysine-specific demethylase 1 (LSD1) (Hou and Yu, 2010). CoREST binding to LSD1 enhances both the stability and enzymatic activity of LSD1 (Forneris et al., 2005b, Mosammaparast and Shi, 2010). As the E2F7 repression complex was shown to contain histone deacetylase activity by the presence of HDACs, it was of interest to explore whether the repression complex also contains histone demethylase activity conferred by LSD1.

In order to address this, endogenous E2F7 immunoprecipitations were performed under normal condition and under DNA damage. Interestingly, E2F7 was shown to form a complex with LSD1 in both MCF7 breast adenocarcinoma and HeLa cervical carcinoma cells (Figure 6.3). Again, non-specific (NS) binding was assessed by use of control IgG antibodies.

The results thus indicate that LSD1 is also a constituent of the E2F7 repression complex thereby adding histone demethylation in the enzymatic abilities of the complex. Furthermore, it is possible that LSD1 could also play a role in the repression of E2F target genes.

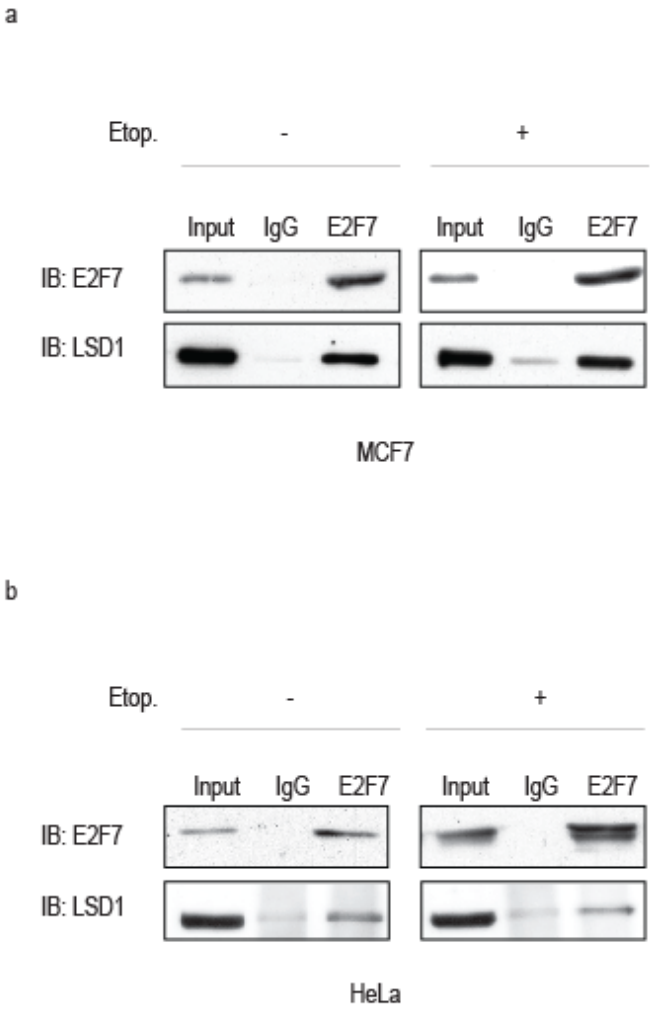


Figure 6.3: E2F7 forms a complex with LSD1.
Cell extracts were obtained from MCF7 (a) and HeLa (b) cells and used for endogenous E2F7 immunoprecipitations under normal conditions and following treatment with 10µM etoposide for 4 hours. The anti-E2F7 and anti-LSD1 antibodies were used to detect the endogenous E2F7 and LSD1 proteins respectively. (n=4)

6.5 Depletion of LSD1 enhances the activity of certain E2F promoters

As LSD1 was identified as a constituent of the E2F7 repression complex, it was reasonable to speculate that LSD1 is involved in the repression of E2F target genes. Functional assays under LSD1 depletion by siRNA were thus performed to investigate the effect of such depletion on the activity of E2F-repsonsive promoters, as compared to the activity levels of these promoters under non-targeting siRNA.

LSD1 depletion by siRNA treatment resulted in increased activity of the *E2F1*, *cdc6* and *p73* promoters (Figure 6.4a, b and d) as compared to the activity of these promoters under non-targeting siRNA. Interestingly, however, LSD1 depletion had a repressive effect on the activity of the cyclin E promoter (Figure 6.4c), thus suggesting that LSD1 is involved in the transcriptional repression of certain E2F-responsive promoters.

The results present here indicate that LSD1 is implicated in the repression of certain E2F target genes. In addition to CtBP, HDAC1, HDAC2 and CoREST, the E2F7 repression complex also contains LSD1 thereby increasing the complexity of E2F7-mediated transcriptional repression.

6.6 Promoter recruitment profiles of CoREST and LSD1

Having demonstrated that CoREST and LSD1 are both constituents of an E2F7 complex under normal conditions as well as under DNA damage, their recruitment to E2F-responsive promoters was investigated. As E2F7, CtBP, HDAC1 and HDAC2 were shown previously to be recruited to certain E2F-target promoters, the recruitment profile of CoREST and LSD1 to the same promoters was explored.

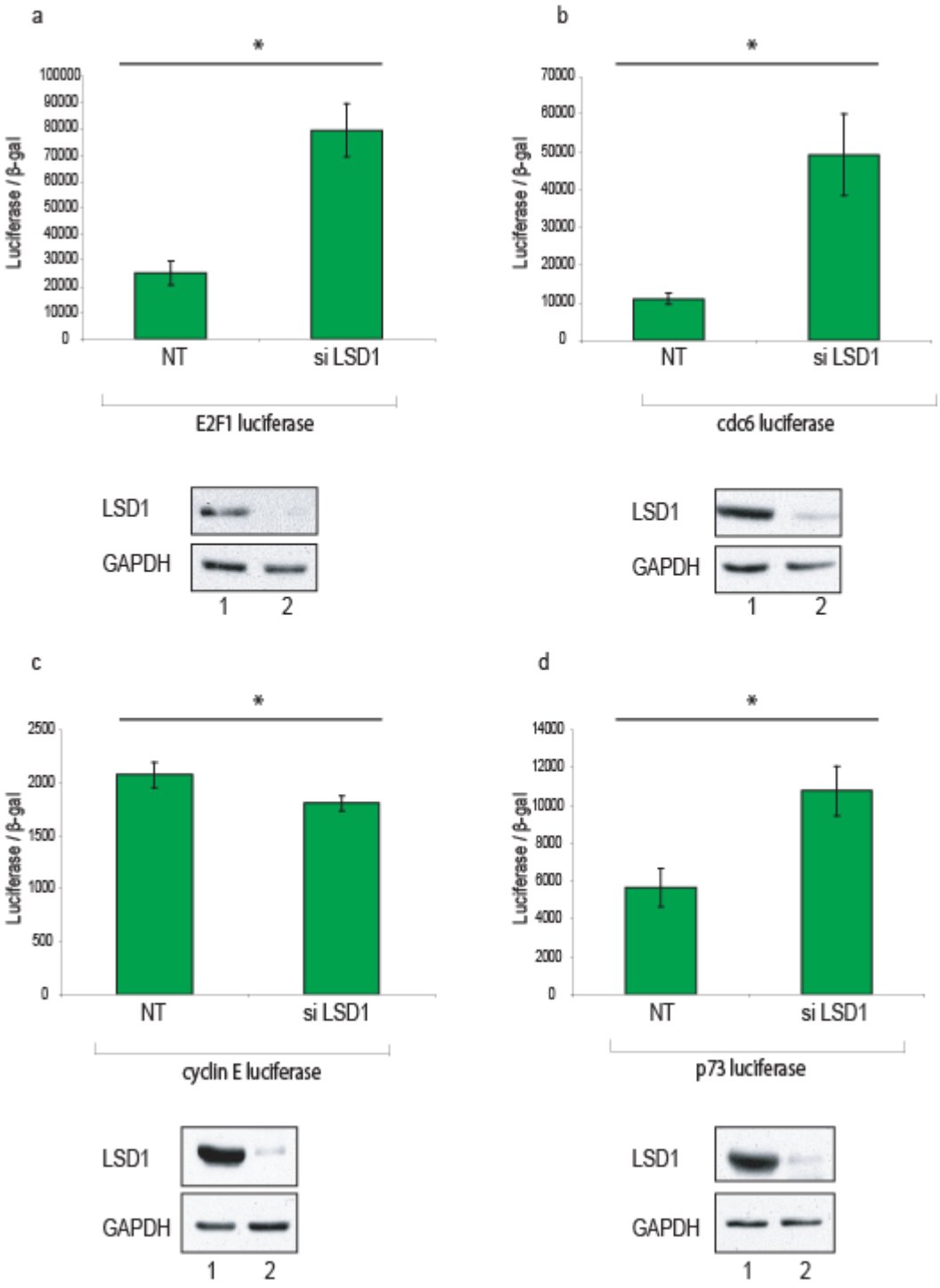


Figure 6.4: LSD1 is a repressor of certain E2F target genes.
Luciferase assays using E2F target gene promoters. U2OS cells were transfected with the reporter construct pE2F1-luc (a), pcdc6-luc (b), pcyclin E-luc (c) or pp73-luc (d) under non targeting (NT) or LSD1 siRNA. All were co-transfected with β-gal as a transfection control. The results represent the ratio of luciferase to β-gal activity. Values in all graphs represent the mean values of triplicate experiments and the error bars represent standard deviations. The difference in transcriptional activity of NT and siLSD1 treated cells is statistically significant. The asterisk (*) indicates statistically significant difference (p-value 0.05). The LSD1 levels were analysed by Western blotting with the appropriate antibodies as indicated. (n=4)

By chromatin immunoprecipitation (ChIP), binding of CoREST and LSD1 to the *E2F1, cdc6, cyclin E and p73* promoters was investigated with specific antibodies to each protein. Non-specific (NS) binding was assessed by use of control IgG antibodies, as before. Both CoREST and LSD1 were shown to occupy these E2F-responsive promoters under normal conditions as well as under DNA damage (Figure 6.5). In contrast, the *albumin* promoter which served as a negative control, was not occupied by either of the two proteins.

Therefore, these results presented here indicate that CoREST and LSD1 are recruited to the same E2F-recognition sites as E2F7, CtBP, HDAC1 and HDAC2 on the *E2F1, cdc6, cyclin E* and *p73, in vivo.*

As CoREST and LSD1 were shown to form a complex with E2F7 and be recruited to the same E2F responsive promoters as E2F7, it was reasonable to speculate that these proteins could be found in a DNA binding complex on target promoters *in vivo*. In order to explore this possibility, chromatin complexes were immunoprecipitated with E2F7 antibody and the resulting E2F7-bound chromatin complexes were then subjected to a second immunoprecipitation with antibodies specific to either CoREST or LSD1. Therefore, this enriched the immunoprecipitated chromatin with DNA fragments bound by either E2F7 and CoREST or E2F7 and LSD1.

As previously shown, E2F7 was recruited to the *E2F1, cdc6, cyclin E* and *p73* promoters. Interestingly, following the secondary immunoprecipitations CoREST and LSD1 were both shown to be recruited to these promoters (Figure 6.6).

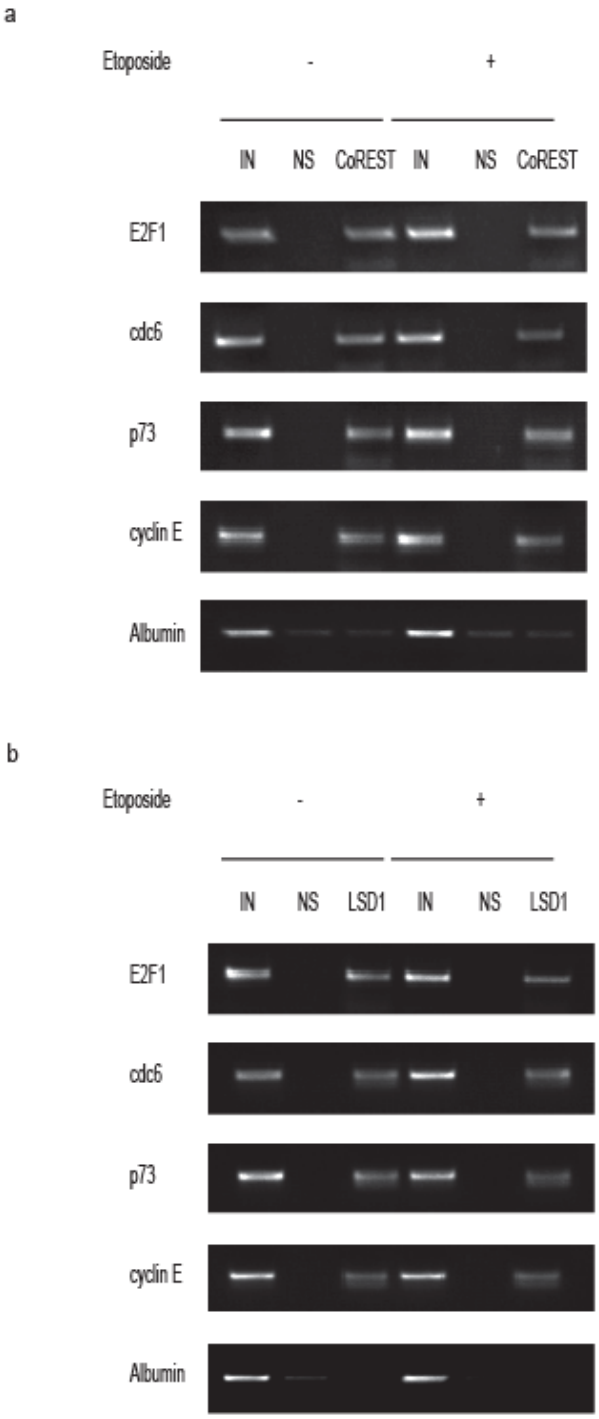


Figure 6.5: CoREST and LSD1 are recruited to E2F-responsive promoters. Chromatin immunoprecipitation analysis on MCF7 cells carried out using CoREST (a) or LSD1 (b) and control antibodies under normal conditions and after treatment with 10μM etoposide for 4h. PCR was performed against the promoters indicated. (n=3)

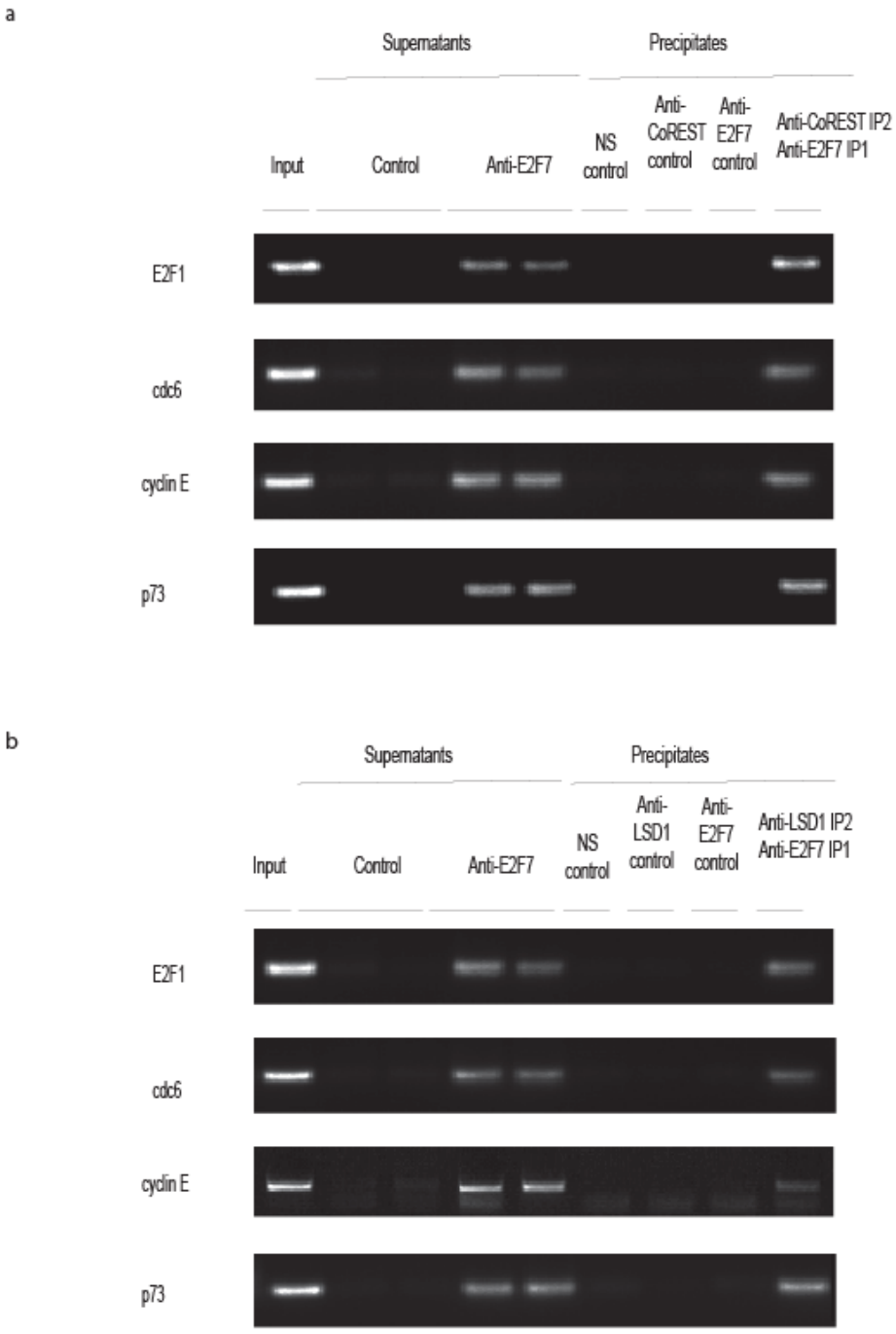


Figure 6.6: E2F7 co-exists on the E2F1, cdc6, cyclin E and p73 promoters with CoREST and LSD1. Chromatin complexes were immunoprecipitated with the E2F7 antibody from extracts of MCF7 breast adenocarcinoma cells. The resulting E2F7-bound chromatin complexes were isolated and subjected to a second round of immunoprecipitation with either CoREST (a) or LSD1 (b) antibodies. PCR was performed against the promoters indicated. (n=3)

These results therefore indicate that CoREST and LSD1 could be present in a DNA binding complex with E2F7 on E2F responsive promoters such as the *E2F1, cdc6, cyclin E* and *p73* promoters. Furthermore, the co-existence of E2F7 on the same promoters with CoREST and LSD1 suggests that these co-repressors could play an important role in the E2F7 repression complex and thus E2F7-mediated repression.

6.7 Consequences of CtBP siRNA treatment on E2F7

CtBP, HDAC1, HDAC2, CoREST and LSD1 were shown to form a complex with E2F7, and play a role in the repression of certain E2F target genes. Furthermore, these co-repressors were shown to occupy E2F-responsive promoters and co-exist on the same promoters with E2F7. As CtBP was previously shown to associate with DNA-binding transcriptional repressors and recruit chromatin modifying enzymes to target promoters (Chinnadurai, 2007), it was of interest to investigate the effect of CtBP depletion on the recruitment of E2F7 complex components.

In order to address this, endogenous E2F7 immunoprecipitations were performed under non-targeting siRNA and CtBP siRNA conditions. Following non-targeting siRNA treatment, E2F7 was shown to interact with CtBP as compared to the control immuneprecipitation. Interestingly, under CtBP siRNA E2F7 levels were increased and as a result there was an increase in immunoprecipitated E2F7. Therefore, although there was an siRNA-mediated reduction of CtBP levels as shown by the immunoprecipitation inputs, the increase in E2F7 levels resulted in the immunoprecipitated CtBP levels to be comparable under non-targeting and CtBP siRNA conditions (Figure 6.7a).

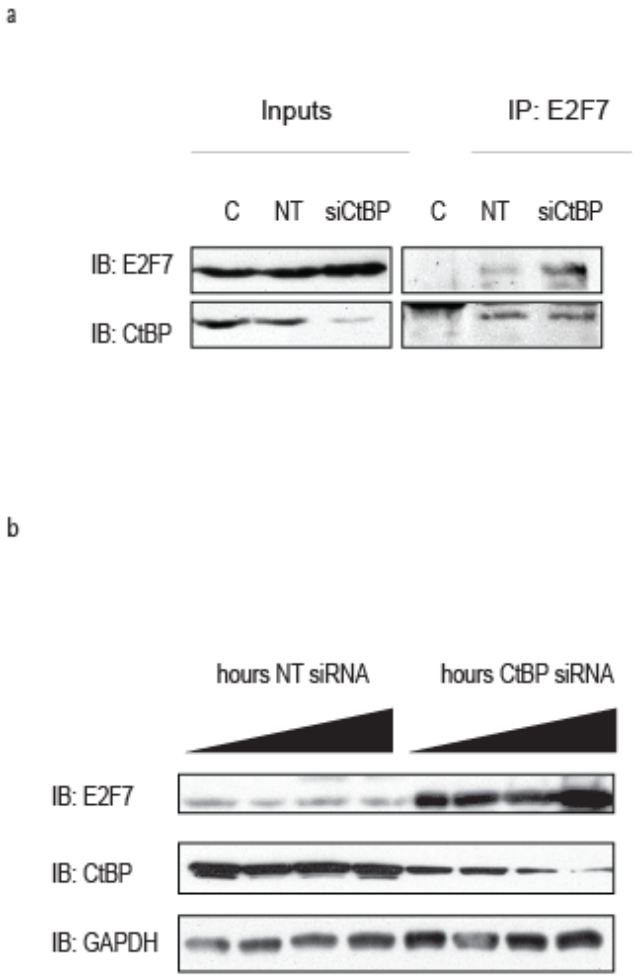


Figure 6.7: The effects of CtBP siRNA in E2F7 stability.
Endogenous E2F7 immunoprecipitation of cells either treated with non-targeting siRNA (NT) or CtBP siRNA. (b) E2F7 levels under non-targeting siRNA (NT) or following 12, 24 and 48 hours of CtBP siRNA treatment. The anti-E2F7 and anti-CtBP antibodies were used to detect the endogenous E2F7 and CtBP protein levels respectively, as indicated. (n=3)

Although the stabilization of E2F7 levels upon CtBP depletion did not allow exploring the effects on the recruitment of the E2F7 complex components resulting from a decrease in E2F7-CtBP interaction, it led to the hypothesis that CtBP is not only recruited by E2F7 to repress the transcription of E2F target genes but could also be recruited by other transcriptional repressors that target *E2F7*. Interestingly, by means of a CtBP siRNA time-course, E2F7 levels were shown to increase in a CtBP-level dependent manner (Figure 6.7b).

These results therefore suggest that CtBP could have opposing roles on E2F7, being involved in E2F7-mediated repression but also impact on the repression of *E2F7* itself. Hence, CtBP could play an important role in E2F7-mediated transcriptional repression.

6.8 Promoter recruitment profiles under E2F7 depletion

Furthermore, it was essential to assess the hypothesis that the constituents of the E2F7 repression complex that were shown to repress the activity of certain E2F-responsive promoters were recruited to these promoters by E2F7 and thus mediate E2F7-induced repression.

The promoter recruitment of CtBP, HDAC1, HDAC2, LSD1 and CoREST was explored under non-targeting siRNA treatment as well as under E2F7 siRNA conditions. Due to limitations of the E2F7 siRNA, upon siRNA treatment there was a decrease in E2F7 levels but E2F7 was still detectable (Figure 6.8b). Following E2F7 siRNA treatment, binding of E2F7 to the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters was reduced as compared to the control (Figure 6.8). Furthermore,

recruitment of all constituents of the E2F7 repression complex was shown to be reduced under E2F7 siRNA compared to the non-targeting control, thus indicating that these co-repressors are recruited to the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters via E2F7 (Figure 6.8a).

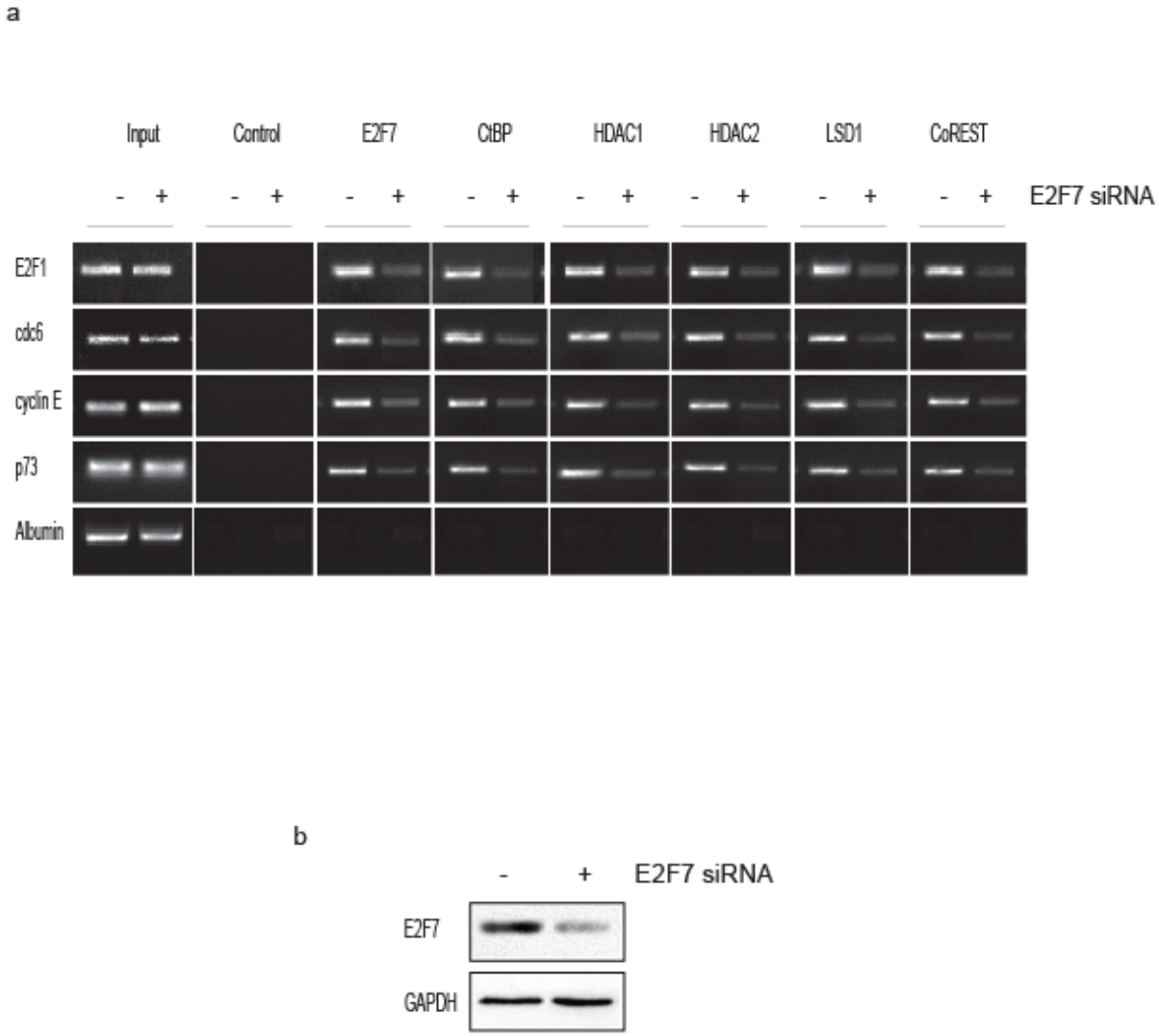


Figure 6.8: CtBP, HDAC1, HDAC2, LSD1 and CoREST are recruited to E2F responsive promoters via E2F7. (a) Chromatin complexes were immunoprecipitated with control IgG, E2F7, CtBP, HDAC1, HDAC2, LSD1 or CoREST antibodies as indicated from extracts of MCF7 breast adenocarcinoma cells following treatment with either non-targeting (NT) or E2F7 siRNA. PCR was performed against the promoters as indicated. (b) Western blotting analysis of the corresponding input levels. The anti-E2F7 and anti-GAPDH antibodies were used to detect the endogenous E2F7 and GAPDH protein levels respectively, as indicated. (n=3)

6.7 Discussion

Following the ability of CtBP, HDAC1 and HDAC2 to form a complex with E2F7, in this chapter CoREST and LSD1 were also identified as components of the E2F7 repression complex. CoREST has been shown to function as a scaffold protein for co-repressor complexes recruiting chromatin-modifying protein such as HDAC1, HDAC2 and LSD1. Interestingly, the demethylase activity of LSD1 is stimulated by CoREST association (Lee et al., 2005; Shi et al., 2005), which is required for the demethylation of nucleosomes (Lee et al., 2005). Recruitment of the CoREST-LSD1–HDAC core has been shown to lead to the repression of several target genes such as REST.

In order to investigate whether CoREST and LSD1 are components of the E2F7 repression complex, endogenous E2F7 immunoprecipitations were performed. E2F7 was shown to form a complex with CoREST and LSD1 under normal conditions and under DNA damage conditions in both MCF7 and HeLa cells (Figures 6.1 and 6.3), suggesting that these proteins are components of the E2F7 repression complex and could play a role in E2F7-mediated repression. This identification thus led to the speculation that CoREST and LSD1 are involved in the repression of E2F responsive genes.

This hypothesis was addressed by functional assays where the activity of E2F-responsive promoters was investigated upon depletion of either CoREST or LSD1. Depletion of CoREST by siRNA led to increased activity of the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters (Figure 6.2a, b, c and d) as compared to non-targeting siRNA control. Interestingly, LSD1 depletion resulted in an increase in the activity of the

E2F1, *cdc6* and *p73* promoters (Figure 6.4a, b and d) but had the opposite effect on the activity of the cyclin E promoter (Figure 6.4c). These results therefore indicate that CoREST and LSD1 are implicated in the repression of certain E2F target genes.

The ability of CoREST and LSD1 to form a complex with E2F7 as well as their implication in the repression of E2F-responsive suggested that these proteins could be recruited to E2F-responsive promoters. As E2F7, CtBP, HDAC1 and HDAC2 were shown previously to be recruited to certain E2F-target promoters, the recruitment profile of CoREST and LSD1 to the same promoters was thus explored.

By chromatin immunoprecipitation (ChIP), both CoREST and LSD1 were shown to be recruited to the *E2F1, cdc6, cyclin E and p73* promoters under normal conditions and under DNA damage (Figure 6.5). In contrast, the *albumin* promoter which served as a negative control was not occupied by either of the two proteins. Recruitment of CoREST and LSD1 to E2F-responsive promoters as well as their presence in an E2F7 complex raised the possibility that these proteins could be found in a DNA binding complex with E2F7 on target promoters *in vivo*. In order to address this, chromatin complexes were immunoprecipitated first with E2F7 antibody and the resulting E2F7-bound chromatin complexes were then subjected to a second immunoprecipitation with antibodies specific to either CoREST or LSD1. Therefore, the resulting chromatin was enriched with DNA fragments bound by either E2F7 and CoREST or E2F7 and LSD1. Interestingly, following the secondary immunoprecipitations CoREST and LSD1 were both shown to co-exist

to E2F-responsive promoters with E2F7 (Figure 6.6). These results therefore suggest that CoREST and LSD1 could be present in a DNA binding complex with E2F7 on E2F responsive promoters such as the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters.

The results presented here suggest that CoREST and LSD1 are components of the E2F7 repression complex and play a role in the repression function exerted by E2F7, thus adding a new level of complexity to the E2F7-mediated repression.

Following the identification of HDAC1, HDAC2, CoREST and LSD1 as components of an E2F7 complex that play a role in the repression of certain E2F target genes, E2F7 immunoprecipitations under CtBP siRNA were performed in order to investigate whether these co-repressors bind to E2F7 via CtBP. However, CtBP depletion resulted in the up-regulation of endogenous levels and thus indicated that CtBP could play a dual role in E2F7 function (Figure 6.7a). Furthermore, E2F7 levels were shown to increase in a CtBP-dependent manner when assessed under a CtBP siRNA time-course (Figure 6.7b). These results therefore indicated that CtBP could be recruited by E2F7 to repress target genes and also associate with other transcription factors that target *E2F7*.

As investigation of E2F7 interactions under CtBP siRNA was not possible due to the subsequent increase of E2F7 levels, it was of interest to assess the promoter recruitment profiles of CtBP, HDAC1, HDAC2, LSD1 and CoREST upon E2F7 depletion. Following treatment with E2F7 siRNA, recruitment of E2F7 to the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters was reduced as compared to the siRNA

control. The recruitment of E2F7 complex components to these promoters was also shown to be reduced after E2F7 siRNA treatment, thus indicating that these co-repressors are recruited to the E2F-responsive promoters via E2F7 (Figure 6.8).

These results therefore suggest that the E2F7 transcriptional mechanism has multiple components that play different roles in E2F7 mediated transcriptional repression. E2F7 forms a complex with CtBP, HDAC1, HDAC2, CoREST and LSD1 and each co-repressor affects the transcription of certain E2F target genes. Chromatin immunoprecipitations under E2F7 siRNA suggested that all constituents of the E2F7 repression complex are recruited to E2F-repsonsive promoters via E2F7. Adding a new level of complexity, the results indicate that CtBP plays a dual role in E2F7 function by associating with E2F7 to repress target genes but is also recruited by other transcription factor(s) that target *E2F7* (Figure 6.9).

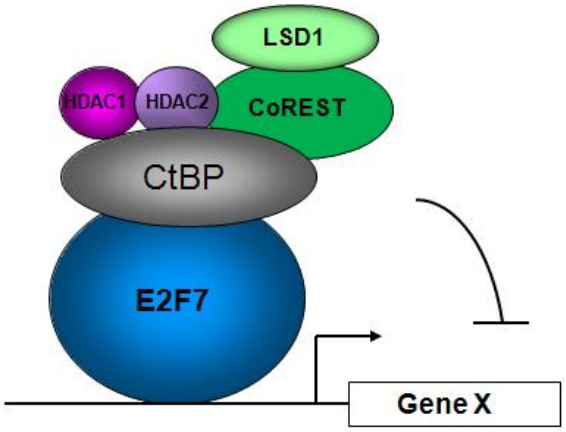


Figure 6.9 Proposed model for the E2F7 repression mechanism.

E2F7 forms a repression complex with CtBP, HDAC1, HDAC2, CoREST and LSD1 which play a role in the repression of certain E2F target genes and recruited to E2F-responsive promoters via E2F7. CtBP also plays a role in the repression of E2F7, adding a new level of complexity to the E2F7 repression mechanism.

CHAPTER 7

GENERAL DISCUSSION

7.1 CtBP is an E2F7 co-repressor that plays an important role in the repression of E2F target genes

The main aim of this project was to identify the mechanism by which E2F7 mediates the transcriptional repression of E2F target genes. E2F7 has been previously shown to function as a transcriptional repressor, albeit the mechanism by which this function is exerted remained to be elucidated (Logan et al., 2004, de Bruin et al., 2003, Di Stefano et al., 2003). In this study, the repression function of E2F7 was confirmed by functional assays were E2F7 was shown to repress the activity of the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters. Analysis of the E2F7 sequence revealed the presence of a CtBP binding motif at amino acid positions 621-625 and thus suggested that CtBP could function as an E2F7 co-repressor. Indeed, E2F7 was shown to form a complex with CtBP under normal as well as DNA damage conditions in different cancer cell lines (Chapter 3), further suggesting that CtBP could play a role in the repression of E2F target genes.

Functional assays under CtBP siRNA treatment indicated that CtBP is involved in the repression of certain E2F target genes. Interestingly, however, depletion of CtBP led to increased activity of the *E2F1*, *cdc6* and *cyclin E* promoters but had no effect on the activity of the *p73* promoter (Chapter 3). This promoter specificity suggested that CtBP could associate with E2F7 in order to repress the activity of a subset of E2F responsive genes. Moreover, since E2F7 could efficiently repress *p73* promoter activity, this suggests that E2F7 can mediate the repression of *p73* in a CtBP-independent mechanism. These results were thus indicative of an E2F7 transcriptional mechanism where different co-repressors are involved in the repression of different promoters.

7.1.1 CtBP binds to the promoters of E2F target genes via E2F7 *in vivo*

Considering the distinct effects of CtBP depletion in the promoter activity of different E2F-responsive genes, it was reasonable to consider that it could correlate with differential promoter recruitment. E2F7 was shown to be recruited to the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters under normal and DNA damage conditions (Chapter 3). Although depletion of CtBP had no effect on the *p73* promoter activity, CtBP was also shown to occupy the same promoters as E2F7 including *p73*. These results thus suggested that CtBP could be present in a DNA binding complex with E2F7 on E2F responsive promoters.

Further investigation of the recruitment profiles of E2F7 and CtBP indicated that E2F7 and CtBP co-occupy E2F consensus binding sites on the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters *in vivo* (Chapter 3). Moreover, reduced promoter binding by E2F7 due to E2F7 siRNA resulted in a reduction of CtBP binding to the promoters (Chapter 6), thus indicating that CtBP is recruited to E2F-responsive promoters via E2F7.

CtBP is an established co-repressor known to associate with various DNA binding transcription factors and represses gene transcription by recruiting histone modifying enzymes (Straza et al., 2010). The ability of CtBP to form a complex with E2F7, affect the activity of E2F-responsive promoters and bind to these promoters via E2F7 indicates that CtBP plays an important role in E2F7-mediated transcriptional repression thus providing the first insight into the repression mechanism exerted by E2F7.

7.1.2 The CtBP binding motif is required for the E2F7-CtBP interaction and the repression of certain E2F-responsive promoters

The presence of the CtBP-binding motif, the involvement of CtBP in the repression of E2F target genes as well as binding of CtBP to E2F responsive promoters suggested an important role for CtBP in E2F7-mediated repression. It was therefore reasonable to hypothesize that the CtBP-binding motif could be significant in the E2F7-CtBP interaction and the ability of E2F7 to repress target genes.

The importance of the CtBP-binding motif in E2F7-mediated repression was investigated using E2F7ΔCtBP, an E2F7 deletion mutation lacking the motif. Indeed, deletion of the motif affected the interaction with CtBP, as E2F7ΔCtBP was unable to interact with CtBP (Chapter 4). Furthermore, loss of interaction with CtBP was shown to have an impact on the repression effect of E2F7, as E2F7ΔCtBP was unable to repress the *E2F1*, *cdc6* and *cyclin E* promoters as compared to the wild-type. Albeit loss of the repression ability of E2F7 on these promoters, the deletion mutant could still repress the *p73* promoter correlating with the previous observation that CtBP siRNA treatment has no effect on the activity of this promoter.

These findings thus indicated that the presence of the CtBP-binding motif is significant for E2F7-mediated repression of certain E2F responsive genes. Moreover, the involvement of CtBP in a subset of promoters targeted by E2F7 was suggestive of a differential E2F7 repression mechanism in a promoter-specific manner.

7.1.3 E2F7 and CtBP form a robust complex

The interaction of E2F7 with CtBP was shown to play an important role in the ability of E2F7 to repress certain target genes. The E2F7-CtBP interaction was shown to occur under normal conditions as well as under DNA damage conditions induced by etoposide (Chapter 3). Furthermore, E2F7 was shown to interact with CtBP under DNA damage induced by UV and also under heat shock thus indicating that this is a stable interaction (Chapter 4).

Considering that the E2F7-CtBP interaction is stable under different types of stress, it was of interest to investigate whether certain conditions previously shown to affect certain CtBP interactions could also affect the interaction with E2F7. As E2F7 was shown to interact with CtBP via the CtBP-binding motif, it was reasonable to speculate that co-expression of E1A which is known to interact with CtBP via a PLDLS motif could compete for the CtBP interaction and have an impact on the E2F7-CtBP interaction and thus E2F7-mediated repression of certain target genes. Interestingly, the ability of E2F7 to associate with CtBP and repress E2F-responsive promoters was unaffected in cells expressing E1A. Moreover, ectopic expression of E1A was unable to compromise the repression ability of E2F7, further supporting that the E2F7-CtBP association is robust.

As the presence of another PLDLS-binding protein was unable to influence the E2F7-CtBP interaction and the ability of E2F7 to repress, E2F7-mediated repression was investigated under different conditions that are known to affect CtBP interactions such as changes in the cell redox states. CtBP shares compelling sequence and structural homology with D-isomer-specific 2-hydroxy acid

dehydrogenases (D2-HDH) and NAD(H) dinucleotides that have been shown to affect dimerization and interaction of CtBP with certain PLDLS-containing target proteins (Zhang et al., 2002, Balasubramanian et al., 2003). Altering the redox state exhibited no significant effect on the E2F7-CtBP interaction and the ability of E2F7 to repress the activity of E2F responsive promoters (Chapter 4).

7.1.4 E2F7-mediated repression is compromised upon MTOB treatment

The ability of E2F7 to repress the transcription of E2F target genes was shown to be unaffected by neither E1A expression nor changes in the cellular redox state. As the PLDLS binding cleft and the substrate-binding domain are physically in close proximity (Kumar et al., 2002, Nardini et al., 2003) and E2F7 was shown to interact with CtBP via this motif, the repression ability of E2F7 was assessed upon treatment with a CtBP substrate.

Studies using the CtBP substrate MTOB indicate that MTOB functions as a CtBP inhibitor affecting CtBP recruitment to target promoters (Straza et al., 2010, Achouri et al., 2007). A possible explanation for the inhibitory effect of MTOB on CtBP function is that, due to the close proximity of the PLDLS cleft and the substrate-binding domain, MTOB binding to CtBP induces a conformational change that affects the PLDLS binding cleft and thus CtBP interaction. Loss of repression ability upon substrate binding suggested that MTOB loading to the CtBP substrate-binding domain could induce a conformational change in the PLDLS binding cleft resulting in reduced interaction of CtBP with transcriptional repressors. Considering the effect of MTOB treatment on CtBP function, E2F7-mediated repression was explored under MTOB treatment.

Interestingly, albeit unaffected by either E1A presence or different redox states, the repression ability of E2F7 was compromised upon MTOB treatment (Chapter 4). Although under normal conditions ectopic expression of E2F7 reduced the activity of the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters, under MTOB treatment E2F7 was unable to confer a repression effect to any of these E2F-responsive promoters. The effect of MTOB treatment on *p73* promoter activity was quite surprising considering that CtBP siRNA treatment had no effect on the activity of this promoter. One possible explanation is that, although treatment with CtBP siRNA reduced the CtBP protein levels, there was still sufficient CtBP to repress the *p73* promoter. On the other hand, binding of CtBP with the MTOB substrate could affect the PLDLS binding cleft and thus E2F7-mediated repression of the *p73* promoter. These results thus suggest that the effect of substrate binding to CtBP compromises E2F7-mediated transcriptional repression.

A previous study has demonstrated that MTOB can function as a CtBP inhibitor and, at high concentrations, MTOB is cytotoxic to cells (Straza et al., 2010). Interestingly the same study showed that MTOB-induced apoptosis was rescued by CtBP overexpression. It would thus be interesting to investigate the effect of MTOB on E2F7-mediated transcriptional repression under conditions of CtBP overexpression and determne whether such overexpression is sufficient for E2F7 to exert its repressive function under MTOB treatment.

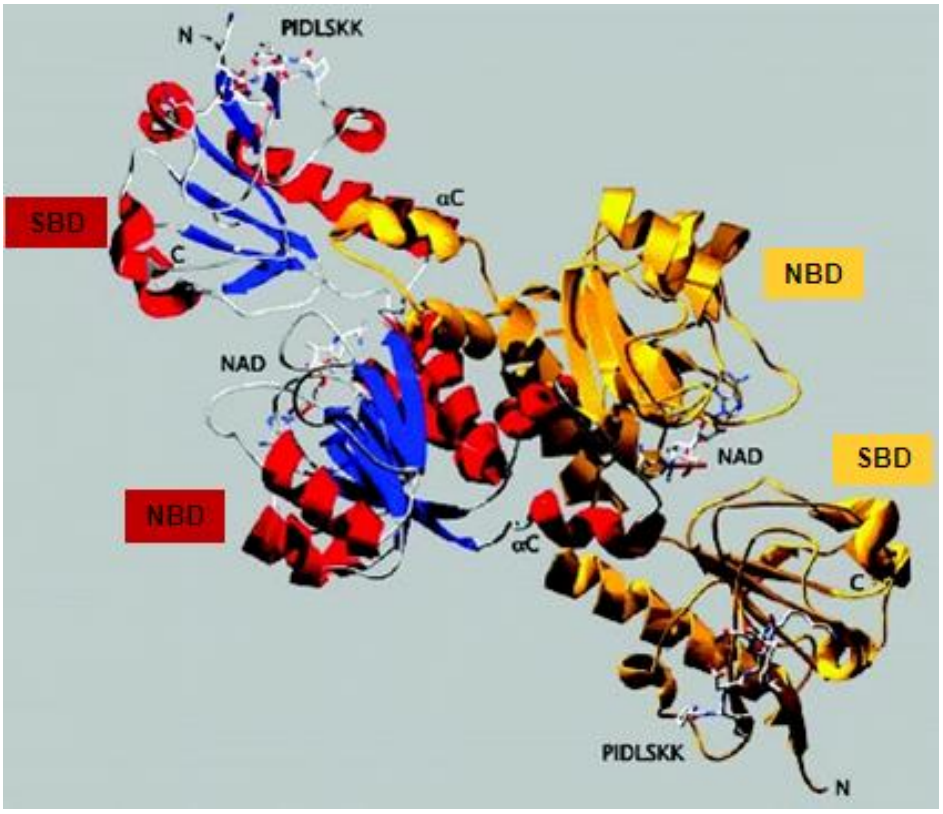


Figure 7.1 Structure of CtBP ternary complex.

A ribbon diagram of rat CtBP (residues 15-345) bound to NAD, in complex with a model heptamer peptide corresponding to a consensus binding sequence (PIDLSKK) showing a 2-fold crystallographic axis between the two monomers. Blue ribbons represent β-strand, and red ribbons α-helix secondary structure. One of the dimer pair is represented in gold for clarity. The structures of NAD and PIDLSKK peptide bound to each monomer are indicated in stick representation. The substrate-binding domain (SBD), the nucleotide-binding domain (NBD), and amino- (N) and carboxyl-terminal (C) residues are indicated.

Adapted from (Lundblad, JR 2000)

7.2 HDAC1, HDAC2, CoREST and LSD1 are constituents of an E2F7 repression complex

CtBP association with E2F7 as well as its role in E2F7-mediated repression led to the speculation that the E2F7 repression complex could also contain chromatin remodelling enzymes known to be recruited by CtBP. CtBP components of the histone modification machinery include the histone deacetylases HDAC1 and HDAC2, as well as the histone demethylase LSD1 (Chinnadurai, 2007). In addition to DNA-binding transcriptional repressors and chromatin-modifying enzymes (Tachibana et al., 2005, Shi et al., 2004), the CtBP complex is also known to contain certain co-repressors such as CoREST (Andres et al., 1999). Several studies have suggested that HDACs are linked to CtBP through CoREST, which has been shown to interact individually with the HDACs (Ballas et al., 2001, You et al., 2001).

Indeed, HDAC1, HDAC2, CoREST and LSD1 were shown to associate with E2F7 in both MCF7 (breast adenocarcinoma) and HeLa (cervical carcinoma) cells under normal and DNA damage conditions (Chapters 5 and 6). As components of an E2F7 complex, if was reasonable to speculate that these co-repressors could play a role in E2F7-mediated transcriptional repression.

It is thus apparent that E2F7 forms a repression complex with different co-repressors and chromatin modifying enzymes. However, whether this is an E2F7 multi-subunit complex or different E2F7 complexes containing different components remains to be elucidated. This could be addressed in future experiments by mass spectrometry. Following endogenous E2F7

immunoprecipitation, the eluted E2F7 complexes could be separated by using an HPLC size exclusion column. Due to the limitations of the E2F7 antibody and the high protein requirement for this experiment, the immunoprecipitations could be performed with ectopic E2F7. The separated E2F7 complexes could be then subjected to trypsin digestion and the samples prepared for mass spectrometry in order to identify the components of each E2F7 complex.

7.2.1 HDAC1, HDAC2, CoREST and LSD1 affect the activity of different E2F-responsive promoters

Interestingly, although all these co-repressors were shown to form a complex with E2F7, each affected the activity of different E2F-responsive genes. HDAC inhibitors increased the activity of the *E2F1* and *cdc6* promoters but had no effect on the *cyclin E* and *p73* promoters. On the other hand, under conditions of LSD1 knockdown the activity of the *E2F1*, *cdc6* and *p73* promoters was enhanced while the *cyclin E* promoter activity was reduced. In contrast, siRNA-mediated depletion of CoREST resulted in increased activity of *E2F1*, *cdc6*, *cyclin E* and *p73* promoters.

These findings thus suggest that HDAC1, HDAC2, LSD1 and CoREST are components of an E2F7 repression complex and indicate that each co-repressor affects the activity of target genes in a promoter-dependent manner.

However, although LSD1 siRNA resulted in the increased activity of certain E2F responsive promoters, the observation that LSD1 knockdown reduced the activity of the *cyclin E* promoter raises certain questions (Figure 6.4). LSD1 has been

shown to play a role in transcriptional repression by removing mono- and di-methyl marks on lysine 4 of histone H3 (H3K4) (Shi et al., 2004). On the other hand, LSD1-mediaded demethylation of H3K9 was shown to be required for the activation of androgen receptor (AR) target genes and LSD1 knockdown resulted in decreased activation of AR-responsive promoters (Metzger et al., 2005). It would be therefore of interest to investigate whether LSD1 plays a role in the transcriptional activation of the *cyclin E* promoter by mediating H3K9 demetylation. By using methylation specific antibodies, the effect of LSD1 on H3K4 and H3K9 demethylation of E2F target genes could be investigated.

7.2.2 HDAC1, HDAC2, CoREST and LSD1 are recruited to E2F-responsive promoters *in vivo* via E2F7

The ability of HDAC1, HDAC2, CoREST and LSD1 to form a complex with E2F7 as well as their involvement in the repression of E2F target genes, led to the hypothesis that these co-repressors could exist in a DNA binding complex with E2F7 on E2F-responsive promoters *in vivo*. Initially, HDAC1, HDAC2, CoREST and LSD1 were shown to occupy E2F sites on the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters under normal and DNA damage conditions (Chapters 5 and 6). Furthermore, all co-repressors were shown to co-occupy these E2F sites with E2F7 further supporting the hypothesis that they bind to the E2F responsive promoters in an E2F7 DNA binding complex.

However, it was important to establish that HDAC1, HDAC2, CoREST and LSD1 bind to E2F-responsive promoters via E2F7 and thus affect the transcriptional ability of target genes in an E2F7-mediated manner. Indeed, reduced binding of

E2F7 to the *E2F1*, *cdc6*, *cyclin E* and *p73* promoters due to E2F7 siRNA treatment resulted in the reduced recruitment of all the co-repressors to the same E2F sites. These results thus indicated that HDAC1, HDAC2, CoREST and LSD1 bind to E2F-responsive promoters via E2F7, suggesting that they are recruited to target genes in order to exert E2F7-mediated transcriptional repression.

7.2.3 E2F7-mediated repression of *E2F1*

Although different co-repressors and chromatin-modifying enzymes were shown to affects the activity of different subsets of E2F-responsive promoters, all components of the E2F complex were shown to bind to the *E2F1* promoter via E2F7 and affect its activity (Figure 7.2). E2F1 is an interesting member of the E2F family as it has been implicated in regulating cell cycle progression and apoptosis (Hallstrom and Nevins, 2003, Lazzerini Denchi and Helin, 2005, DeGregori et al., 1997). Studies have suggested the E2F1 protein levels influence the cellular consequence of E2F1 activity, thus accounting for the context-dependent physiological roles exerted by E2F1 (Trimarchi and Lees, 2002, La Thangue, 2003, Matsumura et al., 2003). E2F1 levels above a certain threshold have been shown to promote apoptosis, whereas lower levels of E2F1 have been suggested to affect cell cycle progression (Blattner et al., 1999, Lin et al., 2001, Stevens and La Thangue, 2004). The importance of E2F1 levels in dictating E2F1 activity and thus cell viability, suggest that it is critical for E2F1 levels to be finely regulated.

It has been previously shown that E2F7 mediates E2F1 repression with E2F7 depletion resulting in an up-regulation of E2F1 protein levels (Zalmas et al., 2008). All E2F7 co-repressors were shown to bind to the E2F1 promoter and affect its

activity, indicating that E2F7-mediated recruitment of co-repressors on the *E2F1* promoter plays an important role in *E2F1* transcription and thus affects the outcome of E2F1 activity. Therefore, these results suggest that E2F7 could have an important role in regulating cell fate by mediating the recruitment of different co-repressors and chromatin-modifying enzymes to the *E2F1* promoter.

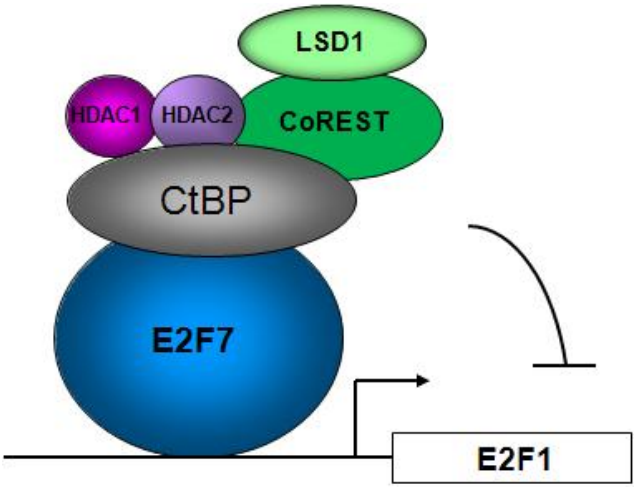


Figure 7.2 Proposed model for the E2F7-mediated repression of *E2F1*.

E2F7 recruits CtBP, HDAC1, HDAC2, CoREST and LSD1 to the *E2F1* promoter. All co-repressors and chromatin-modifying enzymes bind the *E2F1* promoter via E2F7 affecting its activity and thus playing a role in the E2F7-mediated transcriptional repression of *E2F1*.

7.3 CtBP has a dual role in E2F7 function

Adding to the complexity of the E2F7 repression mechanism, CtBP was shown to have a dual role in E2F7 function (Chapter 6). The results presented here suggest that CtBP plays an important role in E2F7-mediated transcriptional repression. In addition to its role in the repression of E2F7 target genes, however, CtBP was also shown to affect E2F7 itself. Upon siRNA-mediated CtBP depletion E2F7 protein levels were shown to increase. It is therefore possible that CtBP is not only an E2F7 co-repressor but could also function as a co-repressor targeting E2F7.

This is perhaps not surprising as previous studies have shown that the *E2F7* promoter sequence contains four E2F binding sites (Di Stefano et al., 2003). Furthermore, the same study provided strong evidence that E2F1 occupies the *E2F7* promoter and E2F1 activation increases *E2F7* mRNA levels, thus indicating that *E2F7* is an E2F target gene. As CtBP was shown here to bind and affect the activity of a subset of E2F responsive promoters, *E2F7* could be another E2F target gene affected by CtBP. As E2F4 has also been shown to bind the *E2F7* promoter, it is reasonable to hypothesise that this repressive E2F could potentially repress *E2F7* transcription by recruiting CtBP and chromatin-modifying enzymes to the *E2F7* promoter (Figure 7.3). Therefore, according to this hypothesis, E2F7 not only regulates the transcription of E2F family members but could also be regulated itself by members of the same family.

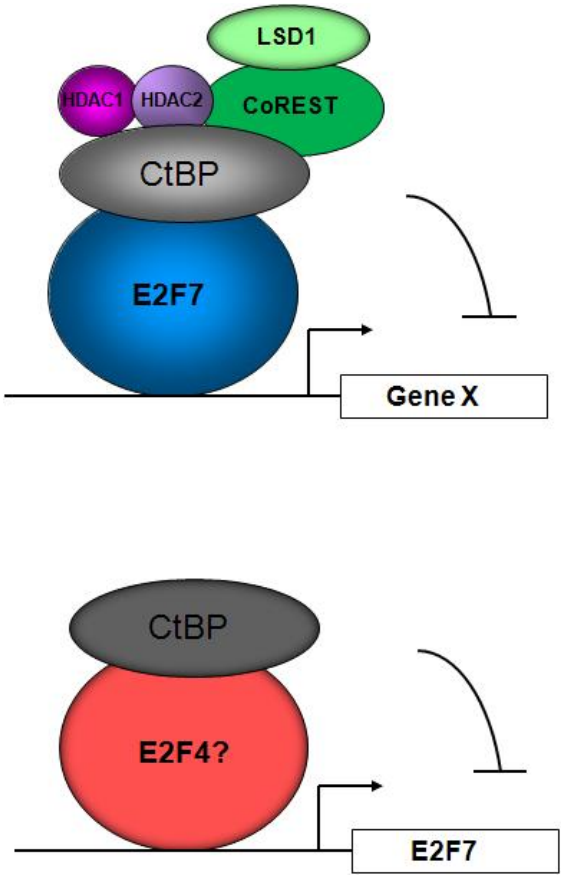


Figure 7.3 Hypothetical model for the E2F7 repression mechanism.

E2F7 forms a repression complex with CtBP, HDAC1, HDAC2, CoREST and LSD1 which play a role in the repression of certain E2F target genes and is recruited to E2F-responsive promoters via E2F7. CtBP also plays a role in *E2F7* repression, by associating with other transcription factors. The ability of E2F4 to bind the E2F7 promoter suggests that this repressive E2F could possibly recruit CtBP in order to mediate *E2F7* repression.

7.4 Conclusions

The E2F family of transcription factors plays a pivotal role in the regulation of cell cycle progression and cellular proliferation. The work presented in this thesis provides the first insight into the repression mechanism exerted by E2F7, one of the more recently identified atypical E2F family members. E2F7 was shown to play a significant role in the repression of E2F target genes by mediating the formation of a repression complex on E2F responsive promoters. In addition, the identification of a repertoire of co-repressors and chromatin-modifying enzymes recruited via E2F7 in order to mediate the repression of different target genes, illustrates a complex target gene-specific E2F7 repression mechanism. Finally, this study also indicates that CtBP could have a dual role in E2F7 function by associating with E2F7 to repress E2F target genes but also play a role in the repression of E2F7 itself.

This study adds a new level of complexity in the function of the E2F family in regulating transcriptional activity. Exploration of the E2F8 mechanism in transcriptional repression is essential in order to decipher the function of atypical E2Fs in the regulation of E2F target genes. In addition, further investigation of the interplay among the E2F transcription factors is required in order to elucidate the E2F family dynamics. Gaining a further understanding in the regulation of these pathways could provide new insights into the mechanisms underlying cancer initiation and progression and could thus lead to the development of new therapeutic approaches.

However, in order to obtain a complete understanding of the role of E2F7 in cell cycle control, identification of the full range of E2F7 target genes is required. An issue that might arise in determining the role of individual E2Fs is that loss of an E2F family member could lead to compensation by another. In the case of atypical E2Fs, previous studies have shown that long-term loss of E2F7 results to compensatory function by E2F8 and vice versa in order to ensure cell viability and organism survival (Li et al., 2008b). Therefore, an approach of ChIP in combination with sequencing (ChIP-seq) would allow the identification of *in vivo* E2F7 binding sites without affecting the ratios of the atypical E2Fs. Identification of these sites and validation of the E2F7 targets in order to determine their functional significance will generate a new basis upon which the role of E2F7 in cell cycle control can be further investigated.

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