

Regulation of E2F-1 by Methylation and NEDDylation

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Abstract

E2F-1 has a central role in cell cycle orchestration, and its activity is tightly regulated. One of the ways E2F-1 activity is controlled is by direct modification by post translational modifications such as acetylation, ubiquitination and phosphorylation. Here it was demonstrated that E2F-1 is targeted by two novel modifications, namely methylation by Set7/9 and NEDDylation, both within the DNA binding and heterodimerisation domain of the protein. NEDDylation and methylation of E2F-1 both decrease the stability and diminish the transcriptional activity of E2F-1. Lysine residues in E2F-1 involved in NEDDylation are also targeted by methylation, allowing the potential for interplay between these modifications. Methylation of E2F-1 was demonstrated to be a prerequisite for its NEDDylation and the multi-domain protein UHRF1 implicated in mediating this effect. The results define a new level of control on E2F-1 and suggest a protein code with pleiotropic effects involved in E2F-1 regulation.

Publications and Posters

Publication

Loftus SJ, Liu G, Carr SM, Munro S, La Thangue NB (2012) NEDDylation regulates E2F-1-dependent transcription. *EMBO Rep* **13**(9)**:** 811-818

Poster

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List of Abbreviations

Proteins

Abl	Abelson murine leukaemia viral oncogene homolog 1
ACTR	Activator of thyroid and retinoid receptors
AIB1	Amplified in breast 1
AMP	Adenosine monophosphate
Apaf-1	Apoptotic protease activating factor 1
APC/C	Anaphase-promoting complex/cyclosome
APP-BP1	Amyloid precursor protein binding protein
Atg8	Autophagy
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM- and Rad3-related
BAX	Bcl-2-associated X protein
BCA3	Breast cancer associated protein 3
Bcl-2	B-cell lymphoma 2
Bik	Bcl-2-interacting killer
BRCA1	Breast cancer associated gene 1
BRIT1	BRCT-repeat inhibitor of hTERT expression
BTB	Bric-a-brac/Tramtrack/Broad complex
cAMP	Cyclic adenosine monophosphate
CAND1	Cullin-associated and NEDDylation-dissociated 1
CBP	CREB binding protein
c-Cbl	Cellular casitas B-lineage lymphoma
Cdc	Cell division cycle
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
Cdt1	Chromatin licensing and DNA replication factor 1
CEBPα	CAAT enhancer/binding protein-α
Chk	Checkpoint kinase
CIP	Cdk-inhibitor protein
CKI	CDK inhibitor
COP9	Constitutive photomorphogenesis 9

CREB	cAMP-response-element binding protein
CRL	Cullin RING ligase
CSN	COP9 signalosome
Cul	Cullin
CW domain	Conserved cysteine and tryptophan residues domain
DBD	DNA binding domain
DCN1	Defective in Cul NEDDylation 1
DEAF-1	Deformed epidermal autoregulatory factor-1
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DNMT	DNA methyltransferase
DP	Dimerisation partner
drICE	*Drosophila* interleukin 1-β-converting enzyme
E2A	Adenovirus Early DNA - Binding Protein 2A
E2F	E2A binding factor (human)
E2f	E2A binding factor (mouse)
ECV	Elongin B/C–Cul2–VHL
EGFR	Epidermal growth factor receptor
Emi1	Early mitotic inhibitor
ER	Oestrogen receptor α
ERA1	Early retinoic acid 1
ERF1	Eukaryotic release factor 1
E(z)	Enhancer of zeste
EZH2	Enhancer of zeste homologue 2
FAT10	Human leukocyte antigen F-associated
FBXO11	F-box protein 11
FUB1	Fau ubiquitin-like protein
G_0	Growth arrest
$G_{1/2}$	Gap phases 1/2
GSK3β	Glycogen synthase kinase 3 beta
HAT	Histone acetylase
H3K4/9	Histone 3 lysine 4/9
HDAC	Histone deacetylase
HECT	Homology to E6AP carboxyl terminus

HIF1α	Hypoxia inducible factor α
HIV	Human immunodeficiency virus
HP1	Heterochromatin protein 1
HPRT	Hypoxanthine guanine phosphoribosyl transferase
hTERT	Human telomerase reverse transcriptase
ICBP90	Inverted CCAAT box-binding protein of 90kDa
IKKγ	Inhibitor of κB kinase (IKK) gamma
ISG15	Interferon-induced 15 kDa protein
JAK-STAT	The janus kinase -signal transducer and activator of transcription
KIP	Kinase inhibitor protein
LSD1	Lysine-specific demethylase 1
LUBAC	Linear ubiquitin chain assembly complex
Mad2	Mitotic arrest deficient 2
MAPK	Mitogen-activated protein kinase
MBT domain	Malignant brain tumour domain
Mcm	Mini-chromosome maintenance
MCPH1	Microcephalin-1
MDC1	Mediator of DNA damage checkpoint 1
MDM2	Murine double minute 2
MEF	Mouse embryonic fibroblast
MINT	Msx2-interacting nuclear target protein
MLL1	Mixed lineage leukaemia 1
M-phase	Mitotic phase
MRN	Mre11–Rad50–Nbs1
mRNA	Messenger RNA
Msh	MutS homologue
Msx	Msh homeobox 2
MYND	Myeloid, Nervy, and DEAF-1
NAE	NEDD8 activating enzyme
Nbs1	Nijmegen breakage syndrome 1
NEDD8	Neural precursor cell-expressed developmentally downregulated protein 8
NEDP1	NEDD8 specific protease 1
NEMO	NF-κB essential modulator
NER	nucleotide excision repair

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
Np95	Nuclear protein 95kDa
NUB1	NEDD8 ultimate buster 1
NuSAP	Nucleolar and spindle-associated protein
ORC	Origin replication complex
PARC	Parkin-like Cytoplasmic Protein
P/CAF	p300/CBP-associated factor
PCNA	Proliferating cell nuclear antigen
PHD	Plant homeodomain
PHF20L1	PHD finger protein 20-like 1
PIG3	p53-induced gene 3
PI(3)K	Phosphatidylinositol-3-OH kinase
PIKK	PI(3)K-like kinases
PSA	Prostate-specific antigen
PTM	Post translational modification
PUMA	p53 upregulated modulator of apoptosis
PUP	Prokaryotic ubiquitin-like protein
PWWP domain	Conserved Pro-Trp-Trp-Pro domain
Rb	Retinoblastoma
RBX1	RING box protein 1
RING	Really interesting new/novel gene
RIZ	Retinoblastoma protein-interacting zinc finger gene
RNA	Ribonucleic acid
RNAi	RNA interference
RNF8/168	RING finger protein 8/168
SAMP	Small archaeal modifier proteins
SANT domain	SWI3-ADA2-N-CoR-TFIIIB domain
SET	Identified in Su(var)3-9, E(z) and Trx,
SCF	Skp1–cullin1–F-box
SIRT	Sirtuin
Skp	S-phase kinase-associated protein
SMYD	SET and MYND domain
S-phase	Synthesis phase

SRA domain	SET- and RING finger-associated
SUMO	Small ubiquitin-like modifier
SUV39H1	Suppressor of variegation 39H1
SWIRM	SWI3p, Rsc8p and Moira
TAF10	TBP associated factor-10
TBP	TATA-box binding protein
TFIID	Transcription factor II D
Tip60	Tat-interactive protein 60 kDa
TK	Thymidine kinase
TNF	Tumour necrosis factor
TopBP1	DNA topoisomeraseIIβ binding protein 1
TRAF2	TNF receptor-associated factor 2
TRAP	Thyroid hormone receptor-associated protein
TRIM 40	Tripartite motif 40
Trx	Trithorax
UBA3	Ubiquitin-like modifier activating enzyme 3
UBAN	Ubiquitin-binding in ABIN and NEMO
UBE2C/2S	Ubiquitin conjugating enzyme 2C/2S
UCHL3	Ubiquitin carboxyl-terminal hydrolase isozyme L3
UFM1	Ubiquitin-fold modifier
UHRF1	Ubiquitin-like, containing PHD and RING finger domains 1
URM1	Ubiquitin-related modifier
VHL	von Hippel–Lindau
WAF1	Wild-type p53 activated fragment 1
WDPMP	Well differentiated papillary mesothelioma of the peritoneum
XIAP	X-linked inhibitor of apoptosis protein
XPA/C	Xeroderma pigmentosum group A/C

Chemicals

AEBSF	4-(2-Aminoethyl)benzenesulfonyl fluoride
APS	Ammonium persulphate
CO_2	Carbon dioxide

DOC	Deoxycholic acid sodium salt
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ENU	Ethylnitrosourea
GST	Glutathione-S-transferase
HRP	Horseradish peroxidase
hyg	Hygromycin
IPTG	isopropyl-1-thio-β-D-galactopyranoside
KCl	Potassium chloride
LiCl	Lithium chloride
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Monosodium phosphate
NaF	Sodium fluoride
Na_2PO_4	Sodium phosphate
ONPG	*ortho*-Nitrophenyl-β-galactoside
PBS	Phosphate buffered saline
RNAse A	Ribonuclease A
SDS	Sodium dodecyl sulphate
TEMED	Tetramethylethylenediamine
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloride

Methodologies and Figures

-/-	Homozygous genotype
-/+	Heterozygous genotype
AdoHcy	*S*-adenosyl-L-homocysteine
AdoMet	*S*-adenosyl-L-methionine
AQUA	Absolute quantification
CADOR	Chromatin-associated domain array
ChIP	Chromatin immunoprecipitation
CHX	Cyclohexamide

COSMIC	Catalogue of Somatic Mutations in Cancer
E. Coli	*Escherichia coli*
EMSA	Electrophoretic mobility shift assay
In	Input
iTRAQ	Isobaric tags for relative and absolute quantification
LTQ MS	Linear trap quadrupole mass spectrometry
Nd8	NEDD8
OD	Optical density
QTOF MS	Quadrupole time-of-flight mass spectrometry
SAM	*S*-adenosyl-L-methionine
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SILAC	Stable isotope labelling by amino acids in cell culture
siRNA	Small interfering ribonucleic acid
TAP-tag	Tandem affinity purification-tag
UV	Ultraviolet
WT	Wild-type

Units

^{o}C	Degrees celcius
hr	Hours
kDa	Kilodaltons
µCi	Microcurie
ml	Millilitre
M, mM	Molar, millimolar
ng, mg, µg	Nanogram, milligram, microgram
rpm	Rotations per minute

Sequences of protein tags

GST	Glutathione-S-transferase protein (220 amino acids)
3x Flag	DYKDHDGDYKDHDIDYKDDDDK
HA	TMYPYDVPDYA
His	HHHHHH

Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine

Nucleotides

G	Guanine
A	Adenine
T	Thymine
C	Cytosine
U	Uracil

Chapter 1

Introduction

1.1 Cancer in 2012

The global cancer burden is continually growing as a result of an aging and ever growing population, as well as an increase in the number of people in the developed world pursuing cancer promoting lifestyles that include smoking, physical inactivity, and ‘‘westernized’’ diets (Jemal *et al.,* 2011). In 2008 alone, 12.7 million cancer cases and 7.6 million cancer deaths are predicted to have occurred worldwide. Cancer is the leading cause of death in economically developed countries and the second biggest cause of death in the developing world (Jemal *et al.*, 2011).

Cancer occurs when cell proliferation becomes dysregulated resulting in the overproduction of cells. Hanahan and Weinberg famously proposed “The Hallmarks of Cancer” describing how a normal cell becomes cancerous by acquiring six essential alterations in cell physiology: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis (blood vessel growth), and tissue invasion and metastasis, each of which represents the breeching of an anticancer defence mechanism (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011).

Despite decades of research, the most effective chemotherapeutic agents used to treat cancer are general DNA damaging agents designed to inhibit the cell division of rapidly dividing cells. To do this, they exploit the fact that tumour cells have defects in checkpoint control, periods during a cell’s normal division cycle when DNA damage is detected and DNA replication and cell division halted until the damage is repaired, or the cell undergoes programmed cell death (cell cycle and checkpoint control are

explained in more detail in the following section). Faulty checkpoint mechanisms in tumour cells mean that DNA damage is ignored and cells continue to divide, rapidly acquiring more and more damage until it causes death of the cell. Many of these drugs have been used for decades and their generic nature means they target not only cancer cells, but other rapidly dividing cells such as those lining the gut, bone marrow cells and hair follicle cells, leading to the nausea, immunosuppression, anaemia and hair loss characteristic of chemotherapy treatment (Kastan & Bartek, 2004). Deterioration in renal function is also common following chemotherapy treatment (Inai *et al.*, 2012). As our understanding of cellular signalling pathways grows, the field of cancer research looks towards a targeted or stratified therapy approach in which the chemotherapeutic regime reflects the combination of biomarkers the patient's tumour presents. Biomarkers are identifiable signatures of a tumour, for example over-expression of a growth factor receptor, that can be used for accurate diagnosis and to predict response to treatments with certain chemotherapeutic drugs and overall prognosis. This stratified therapeutic approach looks to improve the efficiency of the drug by targeting specific cellular pathways, thereby avoiding some of the side effects associated with the commonest chemotherapeutic agents used today (Shaw & Johnson, 2012). To date some of the most effective examples of this approach include the treatment of epithelial tumours with kinase inhibitors such as gefitinib and erlotinib, which target the epidermal growth factor receptor (EGFR). This receptor acts at the start of many important signalling pathways and is found to be either overexpressed or mutated in a subset of tumours (Shaw & Johnson, 2012). The stratified medicine concept has led to the development of screening techniques such as the MammaPrint 70-gene signature which was designed to identify 10-15% of breast cancer patients for whom conventional chemotherapy would not be beneficial (Bueno-de-Mesquita *et al.*, 2007).

The move towards stratified medicine is still at an infantile stage and the hunt for effective biomarkers to detect the presence of disease, the prognosis and likely response to certain treatments is ongoing. The current lack of definitive biomarkers is a particular problem for the most commonly diagnosed cancer in the western world, prostate cancer (Schroder *et al.*, 2009). At present, prostate cancer is diagnosed when prostate-specific antigen (PSA) is detected in the serum. However, of the patients for whom PSA is detected, 76% will be false positives leading to unnecessary, costly and traumatic further investigations (Schroder *et al.*, 2009). Of the estimated 650,000 men to be diagnosed with the disease this year (Jemal *et al.*, 2011), only a small fraction will have tumours capable of metastasising and causing death (Gulzar *et al.*, 2012). The difficulty in identifying the more serious tumours means that prostate cancer is overtreated, with a recent study suggesting that with the current treatment strategy, of every 48 men treated, only one will benefit from the treatment, since the remaining 47 have either indolent tumours or tumours resistant to treatment (Schroder *et al.*, 2009).

There are still as yet many shortcomings of the stratified medicine approach. For example, the strategy depends on the "oncogene addiction" theory in which a cancer cell depends on a single transforming mutation in a gene for its survival and that therapeutic targeting of the aberrant protein product of this gene will result in death of the cancer (Weinstein & Joe, 2006). In reality, tumours can adapt to become resistant to chemotherapy by mutation, epigenetic reprogramming, or remodelling of the stromal microenvironment (Hanahan & Weinberg, 2011). As we get a better understanding of the molecular pathways involved in cancer and a stratified approach to medicine becomes more widespread, we will hopefully become better equipped with the knowledge enabling us to overcome chemotherapy resistance.

1.2 Cell cycle control by cyclins and CDKs

Dividing cells are governed by the cell cycle. Cells progress through four main stages with intervening checkpoints so the cell can monitor whether the previous stage has been completed correctly and it is safe and appropriate to progress onto the next stage (Hartwell & Weinert, 1989). The main stages of the cell cycle are synthesis (S-) phase where the DNA in the cell is duplicated in anticipation of cell division and mitotic (M-) phase, where a cell divides into two daughter cells. These are sandwiched between two gap (G-) phases G_1 ad G_2 (Figure 1.1). During G_1, cells are very sensitive to growth regulating signals and once past the G_1 restriction point, cells are committed to progression though the whole cell cycle (Williams & Stoeber, 2012). If the cell does not divide, for example in response to nutrient deprivation or high cell density, it may be instructed to leave the cell cycle, where it is said to have entered G_0. Cells are driven through the cell cycle by the cyclin-dependent kinase (CDK) family of serine/threonine kinases which are themselves regulated by cyclins, a family of proteins that, as their name suggests, cycle through periods of activity and inactivity governed by cyclical rounds of cyclin expression and degradation. The various cyclins bind and activate different CDKs. Since different cyclins are produced at various stages of the cell cycle, individual CDKs are also active at different stages. For example, the G_1-S-phase transition is mediated by cyclin D-CDK4, cyclin D-CDK6 and cyclin E-CDK2, S- to G_2-phase promoted by cyclin A-CDK2 and G_2- to M-phase initiated by cyclin B-CDK1 (Figure 1.1) (Williams & Stoeber, 2012). One of the primary roles of the cyclin-CDK complexes is the phosphorylation of pocket proteins which results in the release of E2F, a family of transcription factors, crucial to the orchestration of the cell cycle (discussed later). If during a checkpoint, aberrant cellular events are detected such as DNA damage, the cell cycle can be arrested until the problem is remedied. To do this, CDK

inhibitors (CKIs) are employed, such as the INK4 family, that inhibit CDK4 and CDK6 or CIP/KIP family members that inhibit CDK2 activity.

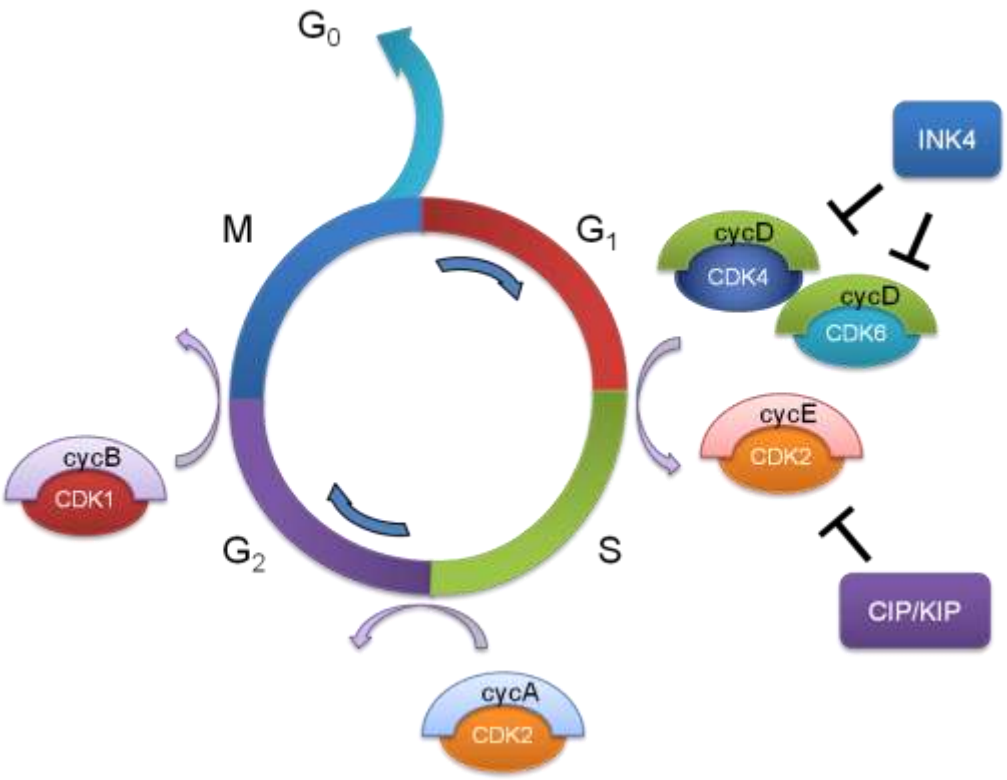


Figure 1.1: The cell cycle

A cell cycles through DNA synthesis (S) and Mitotic (M) phases with two intervening growth (G) phases. Cell cycle progression is mediated by the activity of cyclins (cycA-E) and cyclin-dependent kinases (CDKs) whose activity it inhibited by the activity of INK or CIP/KIP family members.

DNA replication is initiated at specific origins of replication. In order to ensure replication only occurs once during a cell cycle, a complex of proteins known as licensing proteins, namely the origin replication complex (ORC), Cdc6, Cdt1 and Mcm2–7 assemble onto the pre-replicative complex during late M- and early G_1-phases (Williams & Stoeber, 2012). The six Mcm proteins function as a helicase to unwind the DNA template, with Cdc6 and Cdt1 acting as "clamp loaders" for this heterohexameric complex. Following the G_1- to S-phase transition, where the cell becomes committed to

progression through the cell cycle, the replication origin is instructed to begin DNA synthesis by the action of Cdc7 which phosphorylates minichromosome maintenance proteins (Mcm)-2, -4 and -6. This results in a conformational change, leading to its helicase activity and recruitment of the DNA polymerase complex to the single stranded template. In order to prevent a second round of DNA replication before cell division, the licensing complex is disassembled by a combination of cell cycle dependent proteolysis of Cdt1 and suppression of any residual Cdt1 by its binding to the regulatory protein geminin which is expressed at high levels during S-, G_2- and M-phases (Williams & Stoeber, 2012).

1.3 DNA damage and checkpoint controls

In normal cells, response to DNA damage involves the detection of the lesion at one of three checkpoints, G_1/S, intra-S or G_2/M, followed by activation of effectors such as ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-related) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), all members of the PI(3)K (phosphatidylinositol-3-OH kinase)-like kinases (PIKKs) family (Langerak & Russell, 2011). This ultimately leads to cell cycle arrest (Ishikawa *et al.*, 2006; Kastan & Bartek, 2004). ATM kinase is activated primarily following DNA double strand breaks where it is recruited by the MRN complex (comprising Mre11–Rad50–Nbs1) which recognises and localises to these sites of damage (Langerak & Russell, 2011). Double stranded breaks are also detected and bound by the Ku heterodimer which recruits DNA-PKcs, leading to DNA repair by non-homologous end joining (NHEJ) (Langerak & Russell, 2011). In contrast, ATR kinase is recruited to sites of single stranded DNA (for example

where there is an arrested replication fork) by replication binding protein A (RPA) which detects this type of damage (Kastan & Bartek, 2004).

The DNA damage response is propagated by the cooperation of ATR and ATM with the transducer kinases Chk1 and Chk2, respectively, which cause phosphorylation of substrates involved in cell cycle regulation (including E2F), DNA repair and cell death (Kastan & Bartek, 2004). Although a cell is committed to a full round of DNA replication and cell division following the G_1- to S-phase transition, the ATM/ATR–Chk2/Chk1-controlled checkpoint network can transiently delay cell cycle progression in G_1-, S- or G_2-phases to allow time for DNA damage repair.

To halt cells in G_1, ATM/ATR and Chk2/Chk1 phosphorylate their targets, including p53 and its suppressor MDM2, which results in the stabilisation and increased transcriptional activity of the p53 tumour suppressor (Ishikawa *et al.*, 2006). Amongst the genes targeted by p53 is *p21*/CIP*1/WAF1* which mediates the inhibition of cyclin E/CDK2. This also retains the Rb-E2F pathway, a major regulator of the G_1-S-phase transition, in its growth suppressing mode. Cell division is inhibited further by phosphorylation and downregulation of cell cycle activator Cdc25a by ATR/Chk1 (Kastan & Bartek, 2004).

The G_2 checkpoint prevents cells from progressing into mitosis when DNA damage has gone unrepaired during previous G_1/S-phases or if DNA damage has occurred during G_2-phase. The main target for this checkpoint is cyclin B/CDK1 whose activity is

inhibited by phosphorylation by either ATM/ATR, Chk1/Chk2 or p38 kinase and inhibition of Cdc25a by various upstream mechanisms (Malumbres & Barbacid, 2007).

1.4 Regulation of protein level by ubiquitination

Control of the levels of cell cycle regulators is fundamental to proper cell division or response to stress. This can be achieved both by the fine tuning of *de novo* synthesis of the protein by transcription factors, or by regulating protein degradation. Proteins are targeted to the proteasome for degradation by ubiquitination. Ubiquitin is added in a three step enzymatic cascade involving E1 (activating), E2 (conjugating) and E3 (ligating) enzymes, culminating in the formation of an isopeptide bond between a substrate lysine residue and the carboxy-terminus of ubiquitin (Mocciaro & Rape, 2012). Further ubiquitin molecules can then be added to form a poly-ubiquitin chain. When the chain is connected through lysine (K) 48 of ubiquitin the chain targets the substrate for degradation, whereas when it is linked through K63, it is involved in kinase activation or DNA repair. The ubiquitin E3 ligases SCF (Skp1–cullin1–F-box) and APC/C (anaphase-promoting complex/cyclosome) are central to cell cycle regulation since they trigger the degradation of cyclins, Cdc25 phosphatases and cyclin-dependent kinase inhibitors. SCF activity towards its substrates is regulated by the necessity for its targets to be phosphorylated. In contrast, APC/C activity is controlled at various stages of the cell cycle by its own phosphorylation or by its binding to inhibitors (Mocciaro & Rape, 2012). Rather than forming K48-linked chains for the proteasomal targeting of its substrates, APC/C mediates the formation of unique K11-linked chains by employing a dedicated pair of E2 enzymes, UBE2C for chain initiation, and UBE2S for chain elongation (Ye & Rape, 2009). A fourth more recently discovered type of

ubiquitin chain involves linkage at the amino-terminus of one ubiquitin to the carboxy-terminus of another ubiquitin to form a linear chain. To date the only known substrate for these chains is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-essential modulator (NEMO) and the chain is added by the oligomeric E3 LUBAC (linear ubiquitin chain assembly complex), ultimately leading to NF-κB-dependent transcription and activation (Gerlach *et al.*, 2011). These linear chains are recognised by specific domains such as the ubiquitin-binding in ABIN and NEMO (UBAN) domain of NEMO itself (Rahighi *et al.*, 2009).

1.5 Cell cycle control by pocket proteins

Integral to the control of the cell cycle are the pocket protein family of transcriptional repressors, which consists of retinoblastoma (Rb), p107 and p130. The family name stems from the fact that all family members have a "pocket" which binds and inhibits the activity of the E2F family of transcription factors (discussed more thoroughly in the next section). Pocket proteins are required at all stages of the cell cycle (Henley & Dick, 2012). p130 expression predominates in G_0 where it acts in conjunction with Rb (expressed at low levels) to suppress ribosomal and tRNA transcription. The pocket protein family members are differentially expressed during G_1 with p130 being the predominant member in early G_1 (complexed with E2F-4), p107 replacing it at E2F responsive promoters in mid to late G_1 and Rb levels become most abundant at late G_1. All three pocket proteins work together to prevent the transcription of E2F regulated genes and control cell cycle progression (Henley & Dick, 2012).

Binding to E2F is dependent on the pocket protein being in a hypophosphorylated form. Therefore CDK activity must be kept low during G_1 (Henley & Dick, 2012). This is achieved through the activity of CKI proteins as well as through CKI activity of pocket proteins themselves (p107 and p130 bind and inhibit cyclin/CDK complexes directly and Rb impedes ubiquitination of $p27^{KIP1}$, a CKI that plays a crucial role in preventing the onset of S-phase through its inhibition of cyclin E/CDK2) (Besson *et al.*, 2008). As well as binding to E2Fs, pocket proteins also repress gene activity by recruiting chromatin remodelling proteins such as histone deacetylase 1 (HDAC1) and SUV39H1, which mediate nucleosome remodelling, histone acetylation/deacetylation and methylation, respectively (DeGregori & Johnson, 2006; Giacinti & Giordano, 2006).

The G_1- to S-phase transition is tightly regulated as it is the point where a cell commits to a full round of DNA synthesis and mechanisms are in place to ensure linear progression though the cell cycle after this point. Mitogenic stimuli promote phosphorylation of pocket proteins at their carboxy-termini and displacement of HDAC bound to the pocket. This allows further phosphorylation, this time in the pocket domain itself by cyclin E/CDK2 (p107 and p130 can additionally be phosphorylated by cyclin A/CDK2) (Giacinti & Giordano, 2006). Pocket protein phosphorylation results in release and activation of E2F transcription factors. A surge of cyclin E/CDK2 activity follows, promoted by phosphorylation and inactivation of the CKI $p27^{KIP1}$ and a positive feedback loop in which E2F activity promotes further cyclin E expression (Henley & Dick, 2012). Once committed to S-phase, the cyclin E/CDK2 complex phosphorylates unbound cyclin E subunits targeting them for degradation, thereby ending the surge in cyclin E/CDK2 activity.

Rb is also required for controlling the expression of mitotic checkpoint genes such as Emi1 and Mad2, E2F target genes whose over-expression upon Rb deficiency leads to the generation of binucleated, aneuploid and polyploid cells (Henley & Dick, 2012). At the end of mitosis, pocket proteins are dephosphorylated by protein phosphatase-1 (Rb only) and protein phosphatase-2 (all three pocket proteins) so that they can once again suppress E2F activity (Hirschi *et al.*, 2010). Following DNA double strand breaks, increased levels of tumour suppressor p53 leads to upregulation of the CKI $p21^{CIP1}$ so that pocket proteins remain in their E2F-suppressing hypophosphorylated state and growth arrest ensues. If damage occurs in S-phase, phosphatases are required to activate pocket proteins so they can bind E2F transcription factors (Henley & Dick, 2012).

1.6 The E2F family

E2F was originally identified as a factor that binds to the adenoviral E2 promoter, but was later implicated in cancer upon the discovery that it associates with and is regulated by Rb (DeGregori & Johnson, 2006). In the last decade it has become clear that E2F family members are key components of the regulatory machinery for cell growth and proliferation (Sahin & Sladek, 2010), following studies showing that E2F controls the transcription of cell cycle regulators such as cyclin E, cyclin A, Cdc2, Cdc25A and enzymes involved in nucleotide biosynthesis such as dihydrofolate reductase, thymidylate synthetase and thymidine kinase as well as the DNA-replication machinery such as Cdc6, ORC1 and the Mcm proteins (Trimarchi & Lees, 2002). There are eight known members of the E2F family, E2F-1 through to E2F-8, all of which contain a conserved DNA binding domain (Figure 1.2). In addition, two E2F-3 proteins exist, E2F-3a and E2F-3b, which are transcribed from the E2F-3 gene using different

promoters and differ only in E2F-3b's lack of an amino-terminal region (DeGregori & Johnson, 2006; Trimarchi & Lees, 2002). E2F family members regulate distinct but overlapping sets of promoters, with their specificity determined by promoter DNA sequence and cofactors bound to regulatory elements neighbouring the DNA binding domain. E2F-1 to E2F-6 require dimerisation to one of three dimerisation partner (DP) proteins (DP1, DP2/3, DP4) for their ability to bind DNA, whereas E2F-7 and E2F-8 function independently of DP proteins, and instead have two DNA binding domains (Dimova & Dyson, 2005). As described in the previous section, the activity of E2F proteins (E2F-1-5) is regulated by pocket proteins. The binding of these pocket proteins to E2F masks the transactivation domain of E2F and prevents recruitment of transcription factor TFIID and transcriptional coactivators such as p300/CBP (CREB binding protein), GCN5, TRAP, Tip60 and ACTR/AIB1. Rb can additionally regulate E2F-1 activity by a second interaction that inhibits its DNA binding ability (DeGregori & Johnson, 2006). E2F binding is also mediated by the specific combination of E2F and DP family member and whether it is bound to a pocket protein (Tao *et al.*, 1997). By substituting E2F binding sites in promoters it was shown that individual consensus sequences associated with each heteromeric combination directs distinct patterns of cell cycle-regulated transcription (Tao *et al.*, 1997).

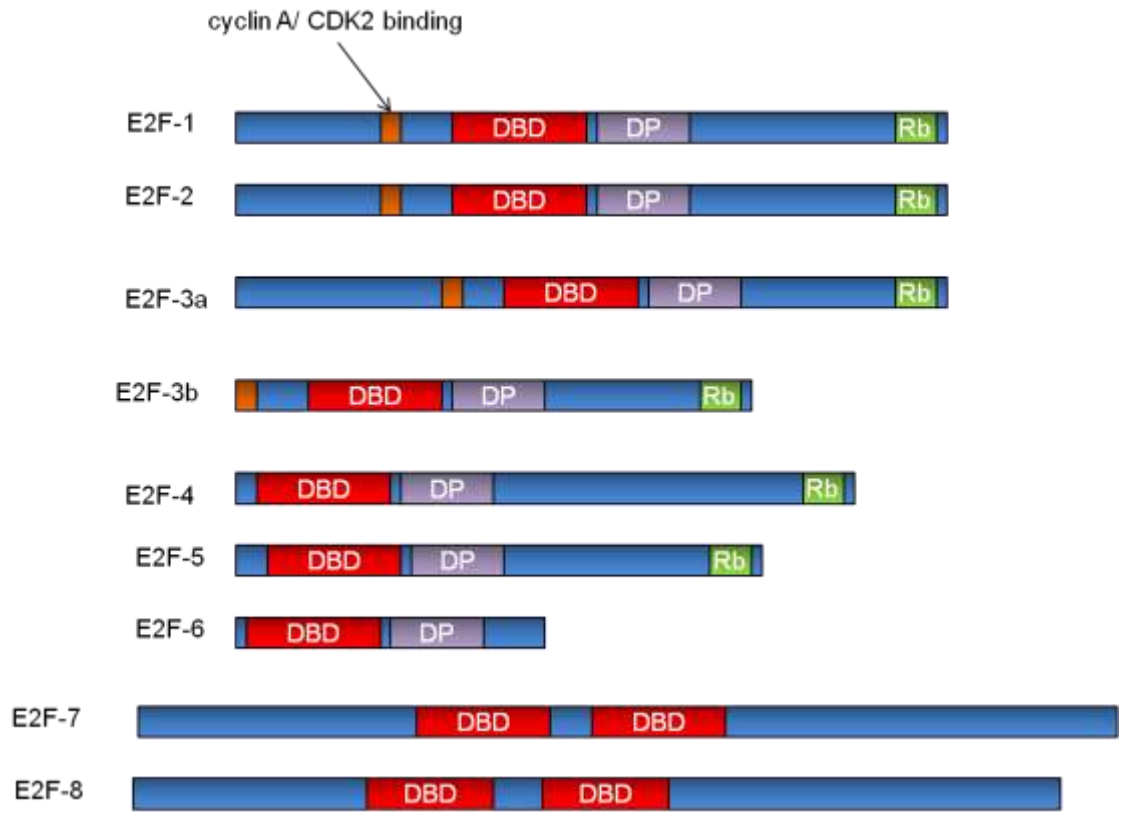


Figure 1.2: The E2F family

All family members contain a DNA binding domain (DBD) but only E2F-1 to E2F-6 have a DP binding domain (DP). E2F-1 to E2F-5 have domains for binding to Rb family members (Rb). E2F-7 and E2F-8 each have two DNA binding domains. As the DP binding domain is required for DNA binding and the DBD seems to be required for dimerisation in those family members that have both domains, from this point on, DP and DBD are shown as combined (DeGregori & Johnson, 2006).

In a simplistic model, E2F-1, E2F-2 and E2F-3a are referred to as "activator E2Fs" as they bind and activate gene promoters such as that of *cyclin E* and are expressed and activated primarily at the G_1 to S-phase transition to promote cell division following their release from Rb (DeGregori & Johnson, 2006; Trimarchi & Lees, 2002). Indeed, the combined mutation of E2F-1, E2F-2 and E2F-3 results in complete ablation of cellular proliferation (Wu *et al.*, 2001). Each of the activating E2Fs plays a unique role in cell cycle control and also in development. For example, $E2f3^{-/-}$ mice mostly die *in utero*, and those that survive die prematurely of congestive heart failure. However $E2f\text{-}1^{-/-}$ are viable and fertile but have a defect in the negative selection of T cells. They

also develop testicular atrophy at 9 to 12 months of age and develop a broad spectrum of tumours between 8 and 18 months of age (Trimarchi & Lees, 2002).

Originally cloned as a result of their association with p107 and p130, E2F-4 and E2F-5 are referred to as "repressor E2Fs" as they are predominantly involved in transcriptional repression at gene promoters where they remain bound to pocket protein family members (Figure 1.3). Repressor E2Fs are associated with growth suppression and exit from the cell cycle (DeGregori & Johnson, 2006; Dimova & Dyson, 2005). They are expressed constitutively but are mainly active in G_0 and early G_1-phase. In contrast to the activating E2Fs which are constitutively nuclear due to a nuclear localisation signal at their amino terminus, E2F-4 and E2F-5 are predominantly cytoplasmic. Association with a pocket protein is sufficient to induce the nuclear localisation of these E2Fs, where they bring about transcriptional repression (Trimarchi & Lees, 2002). Cells lacking repressor E2Fs fail to respond to certain cell cycle arrest signals (Dimova & Dyson, 2005).

E2F-6, E2F-7 and E2F-8 are also transcriptional repressors, but act independently of pocket proteins (Logan *et al.*, 2004; Logan *et al.*, 2005). Transcriptional repression by E2F-6 may involve recruitment of the polycomb complex of proteins (Trimarchi & Lees, 2002). E2F-7 and E2F-8 have a second DNA binding domain instead of a DP binding domain which allows the proteins to form homo- or heterodimers with a second E2F-7 or E2F-8 protein (Li *et al.*, 2008; Logan *et al.*, 2004; Logan *et al.*, 2005). These "atypical E2Fs" are expressed at S- to G_2-phase, later than other members of the family, and directly repress transcription of E2F-1 (Li *et al.*, 2008). E2F-7 and E2F-8 have a

large degree of functional redundancy since loss of either one of them has no significant effect on development, but the double knockout mouse results in embryonic lethality (Li *et al.*, 2008). E2F-3b shares properties of both the activator E2Fs and the repressor E2Fs in that it is constitutively expressed similar to the repressor E2Fs, but it lacks the nuclear export signal characteristic of repressor E2Fs meaning that it remains in the nucleus after dissociation from pocket proteins. E2F-3 also associates with Rb rather than p107 and p130 (similar to activator E2Fs) (Danielian *et al.*, 2008). There is some functional redundancy amongst the E2Fs. With the exception of E2F-3, knockout of any of the E2F family members has only subtle developmental effects (Li *et al.*, 2008).

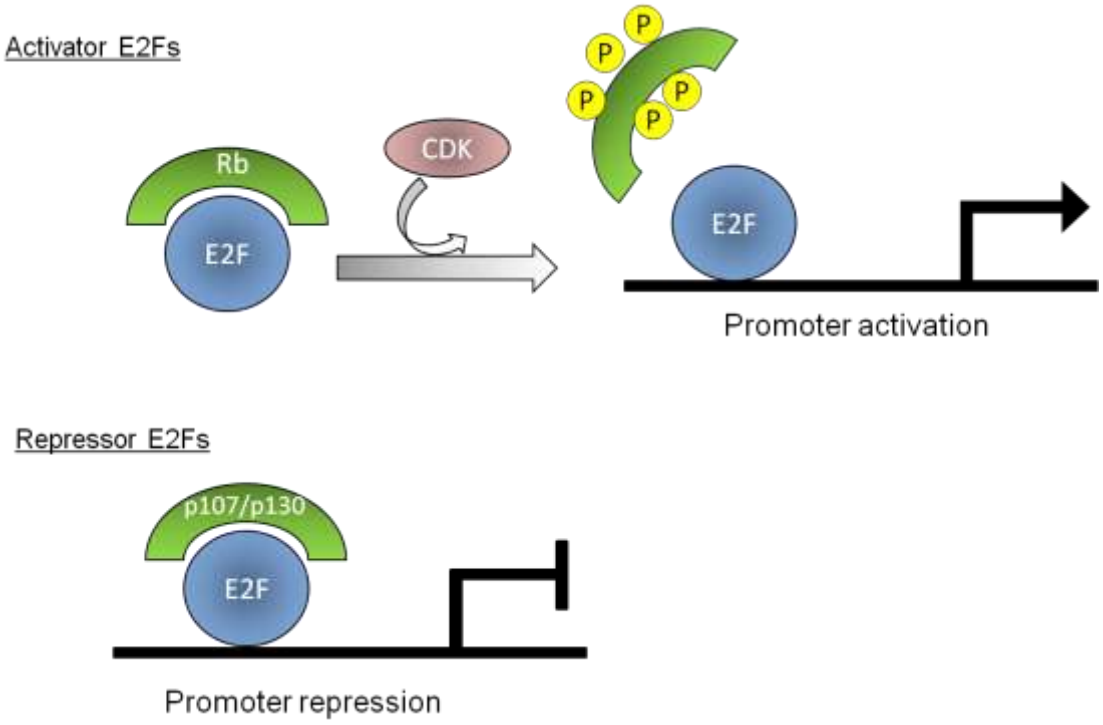


Figure 1.3: Regulation of E2Fs by pocket protein family members

Activator E2Fs are unable to associate with their target promoters when bound to the pocket protein Rb. When Rb becomes hyperphosphorylated through the action of cyclin-dependent kinases (CDKs), the activator E2Fs dissociate from the pocket protein and promote transcription. Repressor E2Fs are able to bind the promoters of their responsive genes whilst bound to pocket proteins (p107 or p130) where they inhibit transcription.

It is now known that transcriptional regulation by the E2Fs is more complex than this simple division of the E2F family into activator or repressor, as some of the E2Fs have the ability to activate or repress promoter activity depending on the promoter itself and the recruitment of specific cofactors (DeGregori & Johnson, 2006). The array of genes regulated by the E2Fs extends much further than those involved in cell cycle checkpoints. For example genes involved in DNA synthesis, apoptosis, DNA repair, differentiation and development are also E2F regulated. E2Fs additionally regulate transcription of their own regulatory proteins such as pocket protein family members (DeGregori & Johnson, 2006). Furthermore, studies have identified E2F-1 at gene promoters that do not contain the E2F consensus site of TTTSSCGC (where S = C or G) where it is proposed that E2F-1 is recruited by transcription factors such as NF-κβ, mYC56 and CAAT enhancer/binding protein-α (CEBPα) (Bieda *et al*., 2006; Chen *et al*., 2009). In an additional layer of complexity, there are documented examples of CDK-independent regulation of the pocket protein-E2F interaction (Dimova & Dyson, 2005). In some cases the same E2F complexes were displaced from a set of promoters associated with one stage of the cell cycle, but remained at the promoters of genes associated with the next stage, or at apoptotic promoters. p38MAPK has additionally been shown to regulate the stability of the pocket protein-E2F complex by inactivating Rb independently of CDKs (Dimova & Dyson, 2005).

1.7 E2F-1: oncogene or tumour suppressor?

The best-characterised member of the E2F family, E2F-1, regulates around 1,000 genes (Sahin & Sladek, 2010). E2F-1 behaves as both an oncogene and a tumour suppressor, and a wealth of literature exists that documents studies attempting to understand its

complex regulation and its bipolar nature. For example, at the *DHFR, b-myb, thymidine kinase (TK)* and *cdc2* promoters E2F-1 forms part of the repression complex with Rb in cycling cells but acts as an activator of the same promoters upon re-entry into the cell cycle (Sahin & Sladek, 2010). Additionally, free E2F-1 (unbound to Rb) is a repressor of Rb expression at G_1 but is an activator of the same gene at S-phase of the cell cycle (Sahin & Sladek, 2010). Therefore, it seems the role of E2F-1 in the cell is very much dependent on the cellular context, taking into account factors such as the stage of the cell cycle and whether cells have received an insult such as DNA damage (Stevens & La Thangue, 2004). The nature of this DNA damage is also important. For example, E2F-1 is acetylated in response to DNA damaging agents that cause double stranded breaks, such as ionising radiation, which targets E2F-1 to the *p73* promoter and promotes apoptosis. However, in response to damage by ultraviolet radiation (UV), which instead causes the generation of pyrimidine dimers, E2F-1 suppresses apoptosis and promotes cell survival (Johnson & Degregori, 2006). Conversely, it was recently reported that upon treatment with the DNA damaging agent doxorubicin, E2F-1 is induced, binds to p53 and suppresses transcription from p53-responsive promoters (Yoshihara *et al.*, 2012) possibly so that E2F-1 may mediate a non-apoptotic response, such as DNA repair.

1.8 E2F-1 and the DNA damage response

E2F-1 activity is crucial in governing the cellular response to DNA damage (Lin *et al.*, 2001) conditions under which E2F-1 stability increases following phosphorylation by the DNA damage sensors ATM/ATR kinases (Stevens & La Thangue, 2004). This phosphorylation may inhibit ubiquitination and proteasomal degradation of E2F-1 by

preventing binding of the ubiquitin E3 ligases $p45^{SKP2}$ and MDM2. This response is unique to E2F-1 as there is no induction of other E2F family members under DNA damage (Stevens & La Thangue, 2004).

Following its stabilisation E2F-1 is able to mediate apoptosis by both p53-dependent and p53-independent mechanisms. In the p53-dependent mechanism, E2F-1 activates transcription of the ARF tumour suppressor, which sequesters the p53 inhibitor MDM2 into the nucleolus. p53 is therefore no longer suppressed by MDM2 and p53-dependent transcription ensues (Stevens & La Thangue, 2004). E2F-1 may also be able to induce apoptosis independently of ARF by directly regulating the transcription of ATM (Stevens & La Thangue, 2004). In another ARF independent mechanism it is thought that E2F-1 can stabilise p53 by either binding it directly via its cyclin A-binding domain or by promoting its phosphorylation (Dimova & Dyson, 2005). These ARF-independent events are independent of the ability of E2F-1 to activate transcription, demonstrating its multi-functional properties.

E2F-1 can induce apoptosis independently of p53 by several mechanisms. For example, E2F-1 regulates the transcription of the p53 family member p73, as well as Apaf-1, a protein that mediates release of cytochrome C from the mitochondria leading to caspase cleavage (Stevens & La Thangue, 2004). E2F-1 additionally directly regulates expression of caspases such as caspase 7 (Nahle *et al.*, 2002). Furthermore, E2F-1 can promote apoptosis by inhibiting NFκB activity, a transcription factor that induces expression of various anti-apoptotic genes. It can do this both by preventing NFκB from

binding to DNA and by inhibiting the NFκB regulator, TRAF-2 (Stevens & La Thangue, 2004).

Two distinct E2F-1-Rb complexes seem to exist in response to DNA damage; a repressive complex containing HDAC1, which inhibits expression of cell cycle progression genes such as *cyclin A2*, and an activating complex containing P/CAF which activates proapoptotic genes such as *p73* and *caspase-7* (Biswas & Johnson, 2012). Conversely, DNA damage can promote E2F-1 binding both to microcephalin (MCPH1/BRIT1) which enhances E2F-1 transcriptional activity at apoptotic promoters and its binding to DNA topoisomeraseIIβ binding protein 1 (TopBP1) an inhibitor of E2F-1 activity. As these proteins bind the same region of E2F-1, they are likely to be mutually exclusive. E2F-1 response to DNA damage may therefore be governed by DNA damage induced serine (S) 31 phosphorylation which is required for TopBP1 binding but not for MCPH1 binding (Biswas & Johnson, 2012).

As well as controlling cell cycle progression and apoptosis, E2F-1 is involved in the recruitment of the MRN complex at sites of DNA double stranded breaks as well as proteins involved in nucleotide excision repair (NER) such as XPA and XPC to sites of UV damage (Biswas & Johnson, 2012). It also regulates other proteins involved in the DNA damage response such as BRCA1, Msh2 and Msh6, DNA replication factor C, Chk1 and PCNA (Polager *et al.*, 2002)

1.9 E2F-1 and cancer

The importance of the E2F and pocket protein family is reflected in its conservation throughout evolution, in that homologues have been identified in flies, worms, frogs and even several plant species (Dimova & Dyson, 2005). A ChIP-based genomic screen identified E2F-1 as having the potential to bind the promoters of 25-35% of genes in the human genome, further demonstrating the importance of this transcription factor (Bieda *et al.*, 2006). It is clear that E2F-1 is integral to cell cycle control and is therefore likely to be highly relevant to our understanding of cancer. Indeed, at least a third of cancer cells have defects in the pocket proteins that regulate E2F activity. Of those positive for Rb, almost all have either activating mutations in cyclin D1 or CDK4, which promote E2F release from Rb or inactivating mutations in the CDK4 inhibitor, p16 (Trimarchi & Lees, 2002). Although mutations in the E2F-1 gene itself are rare, abnormalities in E2F-1 expression and activity are observed in many types of cancer such as hepatocellular carcinoma, bladder cancer, retinoblastoma, liposarcoma, glioblastoma and lung, ovarian, breast, gastric and colon cancer (Chen *et al.*, 2009). Large chromosomal deletions in E2F-1, E2F-2 and E2F-3 genes have been identified in some cases of neuroblastoma, thyroid cancer and pancreatic cancer (Chen *et al.*, 2009). Some of the earlier E2F-1 studies demonstrated that E2F-1 over-expression can induce cellular transformation and tumours (Johnson *et al.*, 1994; Yang & Sladek, 1995). Respective studies have indicated that increased E2F-1 expression is associated with high-grade tumours or metastases and unfavourable patient prognosis (Engelmann & Putzer, 2012). It has also been shown that high E2F-1 expression correlates with the transition from superficial to metastatic bladder cancer (Lee *et al.*, 2010). Recently E2F-1 was shown to drive the expression of the nucleolar and spindle-associated protein (NuSAP), a protein involved in binding DNA to the mitotic spindle. NuSAP was shown to be a potential

biomarker for prostate cancer likely to recur following prostatectomy (Gulzar *et al.*, 2012). NuSAP may cooperate with the androgen receptor, whose levels are similarly increased by the elevated E2F-1 levels, to produce a more aggressive form of prostate cancer.

Conversely, E2F-1 deficiency has been shown to induce tumours in mouse models (Yamasaki *et al.*, 1996). The first somatic mutation within the E2F-1 gene was recently identified in well differentiated papillary mesothelioma of the peritoneum (WDPMP), a rare variant of epithelial mesothelioma that has low malignancy potential (Yu *et al.*, 2011). The mutation was a single arginine to histidine mutation at residue 166 which is in the DNA binding domain of E2F-1, and consequently diminishes the DNA binding ability of the protein as well as increasing protein stability so that E2F-1 accumulates in the cell (Yu *et al.*, 2011). Although regulation of the cell cycle by E2F-1 is complex and not yet fully understood, it is clear that correct regulation of E2F-1 activity is important for normal cellular growth control.

The search for the elusive "switch" that determines whether E2F-1 acts as an oncogene or a tumour suppressor, remains to be discovered. The increased stability of E2F-1 upon DNA damage has led some to suggest that this switch may be governed by a threshold mechanism such that low levels of E2F-1 would trigger E2F-1 oncogenic activity, whereas high levels would cause a switch to apoptotic activity. Alternatively, E2F-1 phosphorylation may result in binding of a specific set of co-activators to E2F-1, targeting it to a distinct gene set under certain conditions (Stevens & La Thangue,

2004). It is evident that more work is needed before we fully understand this fundamental regulator of the cell cycle.

1.10 Regulation of protein activity by PTMs: The Protein Code Hypothesis

The "Histone Code Hypothesis" is often used to explain the phenomenon by which the amino-terminal tails of histones are embellished with a myriad of post translational modifications (PTMs) such as acetylation, methylation, phosphorylation, ribosylation, citrullination, ubiquitination, and SUMOylation (Fernandez-Capetillo *et al.*, 2004; Gardner *et al.*, 2011; Jenuwein & Allis, 2001). These may act in a combinatorial fashion to form binding platforms for the recruitment of effectors that give rise to downstream effects (Filippakopoulos & Knapp, 2012; Sims & Reinberg, 2008), although there is at present some doubt as to the likelihood of many modifications occuring on a single protein moleule simultaneously. It is now known that many non-histone proteins are also subject to extensive PTM and there is a growing amount of evidence for their role in regulating these substrates, which range from mammalian transcription factors such as p53 to viral proteins. This led to the development of the concept of the "Protein Code Hypothesis" to describe the way that PTMs on non-histone proteins regulate their activity (Sims & Reinberg, 2008). The protein code is dynamic in nature with different PTMs continually added and removed, often competing for the same residues yet having opposing effects or mediating further modification by other PTMs at nearby sites. A further layer of complexity arises from variations of specific modifications such as the possibility of mono-, di- and trimethylated forms of a single lysine residue, and the symmetric or asymmetric di-methylation of arginine residues (Bedford & Clarke, 2009; Sims & Reinberg, 2008). The dynamic nature of the protein code is often

demonstrated by p53 which undergoes methylation, acetylation, phosphorylation, ubiquitination, SUMOylation and NEDDylation, and exhibits much interplay between the modifications (Sims & Reinberg, 2008).

As the combination of PTMs on the surface of a protein is unique not only to the protein in question, but also to the specific set of conditions the protein is exposed to at the time, the effector proteins that read them and mediate their downstream ramifications are diverse. Interaction domains usually have a conserved binding pocket for the modified residue, surrounded by a more variable surface that selectively interacts with the flanking amino acids, allowing it to distinguish between different peptide motifs with the same PTM (Seet *et al.*, 2006). A summary of the commonest interaction domains is shown in Table 1.1.

Table 1.1: Summary of the most common reader modules and the PTMs that they recognise

Residue	Modification	Reader module	Full name (where appropriate)
Tyrosine	Phosphorylation	SH2	Src kinase homology domain 2
		PTB	Phosphotyrosine-binding
		C2	Conserved region-2 of protein kinase C
Serine/Threonine	Phosphorylation	14-3-3	
		MH2	MAD-homology-2
		WW	
		BRCT	BRCA1 C-terminal repeat
		SRI	Set2 Rpb1 interacting
		CID	CTD-interaction domains
		KIX	p300/CBP domain for binding to Lys133 phosphorylated CREB
		WD40	
		LRR	Leucine-rich repeat
		Arrestin	
		PBD	Polo-box domain
		FHA	Forkhead-associated domain
Lysine	Methylation	Chromodomain	
		Double chromodomain	
		Chromobarrel	
		Double/tandem tudor	
		Tudor	
		MBT	Malignant brain tumour
		WD40	
		PHD finger	Plant homeodomain
	Acetylation	Bromodomain	
		PHD finger	Plant homeodomain
	Ubiquitination	UIM	Ubiquitin-interacting motif
		UBA	Ubiquitin-associated domain
		CUE	Cue1-homologous domain
		UEV	Ubiquitin E2 enzyme variant
		NZF	Novel zinc finger
		PAZ	Polyubiquitin-associated zinc finger
	SUMOylation	SIM	SUMO-interacting motif
Arginine	Methylation	Tudor	
Asparagine	Glycosylation	Lectin	

(Filippakopoulos & Knapp, 2012; Seet *et al.*, 2006; Taverna *et al.*, 2007; Yang, 2005; Zeng *et al.*, 2010)

Proteins that require multiple PTMs for binding have multiple pockets or interaction domains carefully positioned to accommodate the PTMs on the surface of its target. For others, binding of the effector protein at one PTM may prevent modification by another PTM. The effector proteins themselves can either act as adaptors for the recruitment of other downstream effectors, or in some cases have both a PTM interaction domain and a functional domain, for example a RING (really interesting new gene) finger motif which can cause further modification (such as ubiquitination) to its target (Seet *et al.*, 2006).

Protein regulation by PTM allows very fast responses to cellular stimuli since it does not involve the transcription and accumulation of newly synthesised proteins. For example, global cellular checkpoint networks are activated within seconds of a single localised DNA damage event, resulting from the activation of Chk1 and Chk2 kinases which mediate the phosphorylation of multiple downstream effectors (Kastan & Bartek, 2004).

In the past PTMs and their effects were investigated at the level of single target proteins, but now tools are available to study PTMs at the proteome level. These include mass spectrometry, stable isotope labelling by amino acids in cell culture (SILAC), isobaric tags for relative and absolute quantification (iTRAQ) and absolute quantification (AQUA) (Vertegaal, 2011) (Figure 1.4). Mass spectrometry is used for the identification of proteins by digesting them into smaller peptide fragments and determining their identity based on the mass to charge ratio of these fragments. The technique can be used to identify the presence of a PTM at a specific residue. SILAC is

a type of quantitative mass spectrometry in which two populations of cells (for example control and drug-treated cells) are grown in medium containing either stable heavy or light mono-isotopically labeled amino acids (Kapoor *et al.*, 2012). These are incorporated into cellular proteins which are then pooled together in a defined ratio so that the relative amounts of a specific protein in the two cellular populations can be determined by comparing the ratio of heavy to light peptide peak intensities following mass spectrometry. iTRAQ is used where metabolic labelling is not appropriate since peptides from the different cellular populations are covalently labelled *in vitro* before identification and quantification by mass spectrometry (Kapoor *et al.*, 2012). AQUA involves the addition of a known quantity of labelled pepetide to a protein digest containing the same unlabelled peptide (Sturm *et al.*, 2012). Following mass spectrometry, the peak intensity of lablelled peptide is used as a reference to quantify the unlabelled peptide.

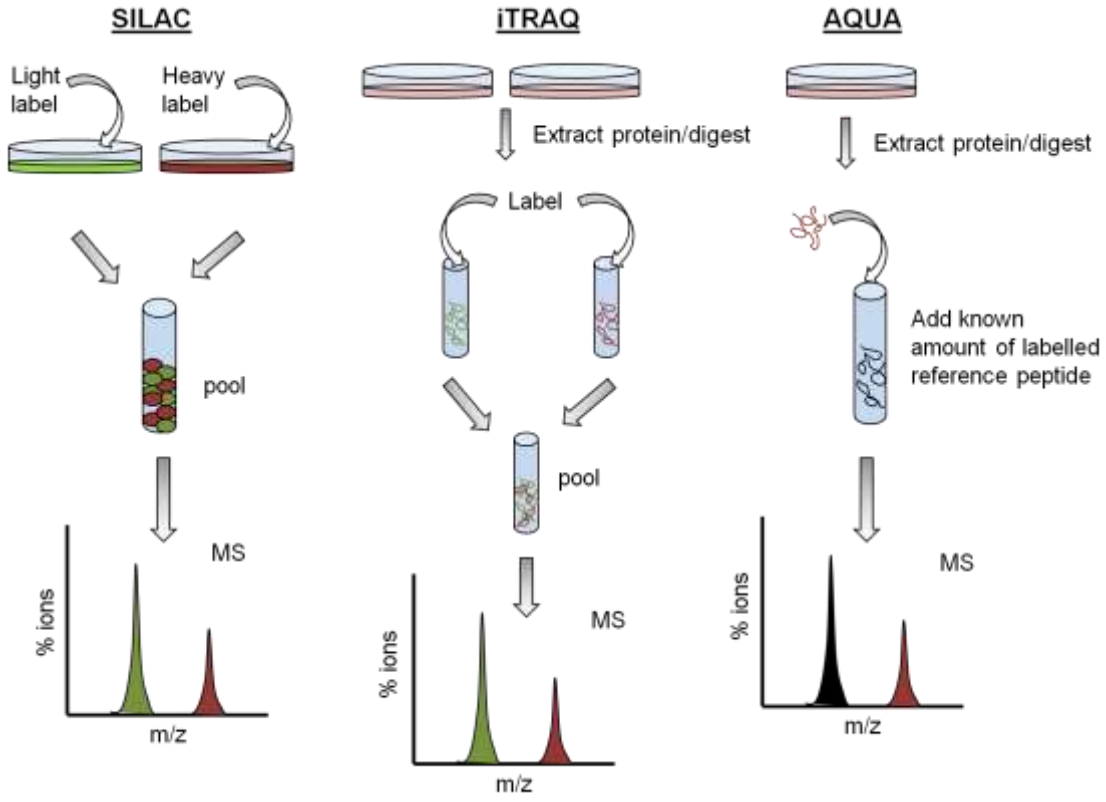


Figure 1.4: Comparison of SILAC, iTRAQ and AQUA methods of protein quantification

For SILAC, cells are metabolically labelled with stable isotopes of different masses before they are harvested, pooled and analysed by mass spectrometry (MS). For iTRAQ, peptides are labelled *in vitro* following extraction and subject to MS analysis. For AQUA analysis, a known amount of a labelled reference peptide is added to the protein digest before MS analysis. m/z, mass to charge ratio.

1.11 Post translational modification of E2F-1

1.11.1 Methylation

E2F-1 is already known to be subject to regulation by multiple PTMs. For example, E2F-1 is known to be methylated at R111 and R113 by PRMT5 both *in vitro* and *in vivo* (Cho *et al.*, 2012). Methylation was found to decrease the stability of E2F-1, resulting from an increase in its ubiquitination. R111 and R113 methylation were additionally found to impede DNA binding and promoter activation by E2F-1. Methylation also hinders the ability of E2F-1 to suppress cell growth and induce apoptosis in a p53

independent manner. The increase in E2F-1 stability upon DNA damage was found to coincide with a decrease in E2F-1 arginine methylation (Cho *et al.*, 2012; Stevens & La Thangue, 2004). In colorectal cancer biopsies, low levels of PRMT5 coincided with high E2F-1 level and a good prognosis. High levels of PRMT5 on the other hand seem to coincide with low levels of E2F-1 and a poor prognosis, presumably resulting from a decrease in the ability of E2F-1 to suppress cell growth (Cho *et al.*, 2012).

E2F-1 has also been reported to be methylated at residue K185 (Kontaki & Talianidis, 2010; Xie *et al.*, 2011). This methylation is also investigated in this report and discussed in more detail in the following chapters.

1.11.2 Ubiquitination

E2F-1 activity is also regulated by ubiquitination. The carboxy-terminal tail of E2F-1 (residues 369-437) is required for its ubiquitination and subsequent proteasomal degradation. There are no lysine residues in the carboxy-terminal tail of E2F-1 *per se*, but it is thought that the region may form a binding platform for a protein that targets amino-terminal lysine residues for ubiquitination. The region of E2F-1 found to be required for its ubiquitination encompasses the Rb-binding domain, and binding of Rb inhibits E2F-1 ubiquitination and turnover (Campanero & Flemington, 1997). The ubiquitination of E2F-1 was also shown to depend on binding of the F-box protein $p45^{SKP2}$, and its E3 ligase partner Cul-1, to E2F-1 (Marti *et al.*, 1999).

E2F-1 was additionally found to be ubiquitinated by the APC/C complex to form K11 linked ubiquitin chains (Budhavarapu *et al.*, 2012) which target E2F-1 for proteasomal degradation. Upon over-expression of the APC/C cofactor Cdh1, the level of E2F-1 protein decreases. Following DNA damage, however, Cdh1 over-expression no longer results in depressed levels of E2F-1, and ubiquitin-K11-linked chains accumulate in the cell. Therefore, the increase in E2F-1 stability observed following DNA damage results from inhibition of the degradation of ubiquitin-K11 linked chains at the proteasome, rather than an increase in E2F-1 ubiquitination (Budhavarapu *et al.*, 2012).

1.11.3 SUMOylation

E2F-1 has also been shown to be modified by the small ubiquitin-like modifier (SUMO) as a result of its interaction with SUMO E3 ligase $p14^{ARF}$ (Rizos *et al.*, 2005). The role of E2F-1 SUMOylation is yet to be determined, but a melanoma associated truncated mutant of $p14^{ARF}$ was found to lack the ability to SUMOylate E2F-1, most likely because its localisation is limited to the cytoplasm.

1.11.4 Phosphorylation

Early E2F-1 studies identified phosphorylation at residues S332 and S337, demonstrating that this phosphorylation occurs in late G_1 where it disrupts the Rb-E2F-1 interaction and enhances promoter binding (Fagan *et al.*, 1994). The authors showed that E2F-1 was phosphorylated by Cdc2 *in vitro*, but acknowledge that, given that this kinase is most active in the G_2 and M-phases of the cell cycle, that other G_1-S-phase kinases are more likely to be responsible for this phosphorylation *in vivo*.

E2F-1 is also known to be phosphorylated at serine 31, within the amino-terminal tail of E2F-1 (Lin *et al.*, 2001). It was found that in response to DNA damage with chemotherapeutic agents, E2F-1 level increased as a result of an increase in protein stability rather than an increase in E2F-1 transcription. Using $ATM^{-/-}$ cell lines, Lin *et al.* demonstrated that the DNA damage responsive ATM/ATR protein kinase was required for the increase in E2F-1 in response to drug treatment, which led to experiments demonstrating that ATM/ATR can directly phosphorylate E2F-1 at S31, and that this phosphorylation event was specific to E2F-1 and not other E2F family members where S31 is not conserved (Lin *et al.*, 2001). It is known that S31 is in a region important for targeted degradation of E2F-1, leading to the discovery that 14-3-3τ binds specifically to phosphorylated S31, inhibiting the ubiquitination and proteasomal degradation of E2F-1, thus stabilising the protein and promoting expression of proapoptotic genes such as *Apaf-1* and *p73* (Wang *et al.*, 2004).

The increase in E2F-1 protein levels upon DNA damage treatment may also in part be attributed to a second E2F-1 phosphorylation event, namely that at residue S364 (Stevens *et al.*, 2003). It was found that S364 phosphorylation was mediated by Chk2, a protein kinase involved in the downstream effects of cell cycle checkpoint regulation. Similar to phosphorylation at S31, S364 phosphorylation was found to stabilise E2F-1. It was also found to increase E2F-1 transcriptional activity and E2F-1 dependent apoptosis, and upon etoposide treatment phospho-S364 was localised to discrete nuclear substructures (Stevens *et al.*, 2003). The authors suggest these nuclear substructures may be related to the activity of phosphorylated E2F-1, similar to the colocalisation of p53 with nuclear proteins such as PML.

E2F-1 is also thought to be phosphorylated at residues S304 and T433, but the literature associated with phosphorylation at these residues is somewhat confusing. An early study showed these phosphorylation events *in vitro*, implicating the CDK7 subunit of transcription factor TFIIH as the kinase responsible. TFIIH purified from extracts and used in an *in vitro* kinase assay resulted in S304 and T433 phosphorylation, and CDK7 inhibition or immunodepletion in these extracts reduced E2F-1 phosphorylation (Vandel & Kouzarides, 1999). The study also showed that phosphorylation decreased the stability of E2F-1, and suggested that phosphorylation occurred in S-phase. Additionally, Rb binding to E2F-1 was found to inhibit phosphorylation of a truncated form of E2F-1 (residues 380-437) *in vitro* (Vandel & Kouzarides, 1999). A later study also identified S403 and T433 phosphorylation *in vitro,* instead implicating glycogen synthase kinase 3 beta (GSK3β) as the kinase responsible (Garcia-Alvarez *et al.*, 2007). A third study showed that S403 and T433 phosphorylation were important for keratinocyte differentiation, implicating p38MAPK as the kinase responsible for phosphorylation at these residues (Ivanova *et al.*, 2009). A fourth more recent study was the first to show S403 phosphorylation *in vivo* (Real *et al.*, 2010). This phosphorylation event was found to be induced by treatment with the DNA intercalating agent, doxorubicin, but the effect was not dependent on the ATM pathway. Surprisingly, it was found that GSK3β or p38MAPK were not the kinases responsible for this phosphorylation event, meaning that there is still no solid *in vivo* evidence identifying the kinase is responsible for S403 phosphorylation (Real *et al.*, 2010). E2F-1 S403 is conserved amongst mammals and some other species and located in the transactivation domain of the protein. Its phosphorylation results in modification of the expression of E2F-1 target genes such as Bcl-2-interacting killer (BiK), X-linked inhibitor of apoptosis protein (XIAP) and p21 in a gene specific manner, with some genes (for

example BiK) showing a decrease in their transcription following E2F-1 phosphorylation and others (for example XIAP and p21) showing an increase in transcription (Real *et al.*, 2010).

1.11.5 Acetylation

In response to the finding that E2F-1 bound to and was synergistically activated by CBP, a transcriptional activator very closely related to the histone acetylase (HAT) p300, Marzio *et al.* showed E2F-1 to be acetylated at K117, K120 and K125 by both HATs (Marzio *et al.*, 2000). These residues are conserved between E2F-1, E2F-2 and E2F-3 which are all acetylated, but E2F-4, E2F-5 and E2F-6, which lack these lysine residues, were not. To show E2F-1 acetylation by p300 and CBP, Marzio *et al.* used immunoprecipitated p300 and CBP to acetylate E2F-1 *in vitro*. Incubation of recombinant E2F-1 and recombinant CBP was shown to have increased binding of E2F-1 to DNA in an electrophoretic mobility shift assay (EMSA), an effect assumed to result from its acetylation by CBP (Marzio *et al.*, 2000). Over-expression of CBP in cells resulted in increased E2F-1 DNA binding in an EMSA and increased transcriptional activity. E2F-1 acetylation by p300 and CBP was never demonstrated in this study in an all-*in vitro* experiment (i.e. using recombinant p300 and CBP) to rule out the effects of other proteins immunoprecipitated with the p300/CBP complex.

Martínez-Balbás *et al* (2000) extended these findings by performing E2F-1 acetylation *in vitro* using recombinant P/CAF, GCN5, CBP and p300 acetylases individually to determine which acetylates E2F-1. It was found that P/CAF, a protein found to be associated with p300/CBP, acetylated E2F-1 very potently, and p300 and CBP were

able to acetylate E2F-1 only very weakly (Martinez-Balbas *et al.*, 2000). P/CAF was found to associate with E2F-1 independently of the interaction between P/CAF and p300/CBP. Using a combination of *in vitro* and *in vivo* experiments, utilising non-acetylatable K117/120/125R triple mutants, acetylation was found to augment DNA binding, stimulate E2F-1 transactivation potential and increase the stability of the protein. Since Rb is known to suppress E2F-1 activity in part by tethering it to the deacetylase HDAC1, the authors investigated whether HDAC1 could act upon E2F-1. It was found that HDAC1 could indeed deacetylate E2F-1 and that this deacetylation was dependent upon the association of E2F-1 with Rb. This suggests that acetylation of E2F-1 is relevant only to the free (non-Rb bound) form of E2F-1 where it is not deacetylated (Martinez-Balbas *et al.*, 2000).

E2F-1 acetylation was found to be important in governing the apoptotic potential of cells, since ectopic expression of acetylation deficient K117/120/125R triple mutant following etoposide treatment induced a significantly lower level of apoptosis compared to wild-type E2F-1 (Pediconi *et al.*, 2003). Additionally, expression of a mutant where K117, K120 and K125 were substituted for glutamine, an acetylation mimic, had higher apoptotic activity under non-damage conditions, an effect not accentuated by etoposide treatment (Pediconi *et al.*, 2003). The observation that the difference between acetylated and unacetylated (K117/120/125R) E2F-1 was only evident upon etoposide treatment suggests that E2F-1 is acetylated in response to DNA damage. Interestingly, E2F-1 acetylation appeared to have different effects on E2F-1 responsive promoters. For example, the K117/120/125Q acetyl-mimic binds and activates the *p73* promoter more efficiently than wild-type E2F-1, but its ability to bind and activate the DHFR promoter was impaired. It was also apparent that the presence of E2F-1 bound to P/CAF at the

p73 promoter is important for transcriptional activation (Pediconi *et al.*, 2003). Acetylation of E2F-1 only occurs under certain types of damage as UV irradiation does not induce its acetylation (Biswas & Johnson, 2012).

E2F-1 acetylation was found to be independent of p53 and Abl as well as the ATM/ATR pathway, and that E2F-1 acetylation, rather than S31 phosphorylation by ATM/ATR, was required for the increased stability of E2F-1 following treatment with doxorubicin (Ianari *et al.*, 2004). This was later explained by the finding that E2F-1 acetylation is promoted by phosphorylation at S31 (although a small amount of acetylation can occur independently of S31 phosphorylation) (Biswas & Johnson, 2012). It was found that acetylation by P/CAF or by p300, although at the same residues, had different roles in the stabilisation of E2F-1 following DNA damage, with doxorubicin treatment causing a dramatic increase in both the amount of P/CAF bound to E2F-1 and the degree at which E2F-1 was stabilised by its HAT activity, but having little effect on the amount of p300 bound to E2F-1 or the amount by which E2F-1 was stabilised by the HAT activity of p300 (Ianari *et al.*, 2004).

Interestingly, acetylation of E2F-1 following DNA damage is accompanied by a concomitant increase in the level of E2F-1 ubiquitination (Galbiati *et al.*, 2005). Over-expression of p300 alone was able to significantly induce E2F-1 ubiquitination in a similar manner to over-expression of the E2F-1 E3 ubiquitin ligase $p45^{SKP2}$ itself. However, the effect did not result from increased activity of $p45^{SKP2}$ *per se*, since other proteins known to be ubiquitinated by this E3 ligase were unaffected by p300 over-expression. This observation points to a potential role for another E3 ligase, although

the authors point out that a role for $p45^{SKP2}$ in the ubiquitination of acetylated E2F-1 cannot be ruled out by their findings. The finding that the K117/120/125R mutant was not ubiquitinated in response to p300 over-expression, suggests that acetylation is a prerequisite for ubiquitination (Galbiati *et al.*, 2005).

The complex nature of E2F-1 regulation by PTM was recently demonstrated in a study in which it was found that there exist two distinct populations of active E2F in response to DNA damage. E2F-1 modified by acetylation at K117/120/125 and phosphorylation at S31 represent a fraction unbound to Rb, whereas E2F-1 phosphorylated at S364 is found in complex with Rb despite hyperphosphorylation of this pocket protein. Interestingly, both populations of E2F-1 are required for effective induction of pro-apoptotic genes such as *p73* (Carnevale *et al.*, 2012).

A summary of known PTMs on E2F-1 is shown in Figure 1.5.

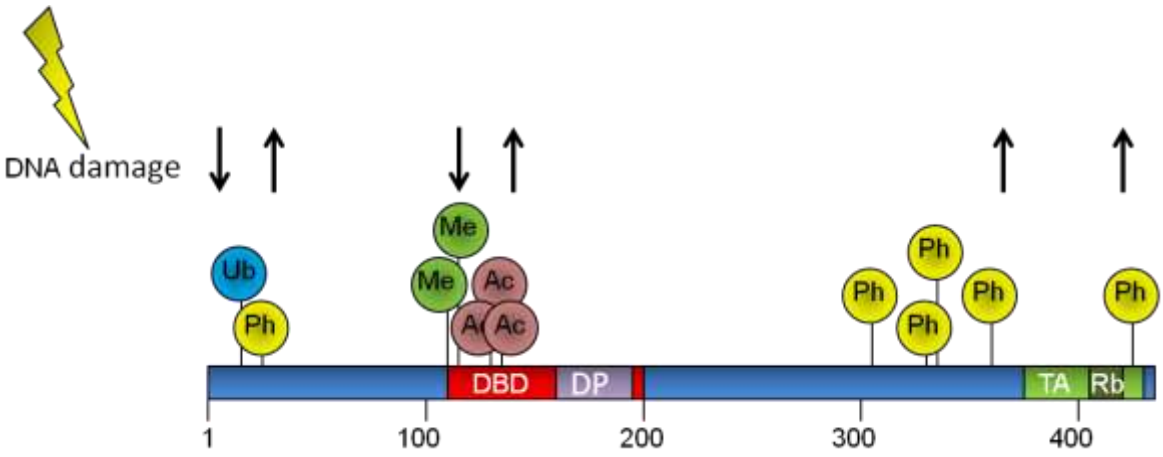


Figure 1.5: Summary of E2F-1 post translational modifications

The positions of known post translational modifications are shown with respect to residue number and various functional domains of E2F-1. Also shown is whether the modification is increased or decreased in response to DNA damage (where known). DBD, DNA binding domain; DP, DP binding (heterodimerisation) domain; TA, transactivation domain; Rb; pocket protein binding domain; Ub, ubiquitination; Me, methylation; Ph, phosphorylation; Ac, acetylation.

1.12 The SET domain superfamily and Set7/9

The first methyltransferase to be discovered was originally identified as one of a group of genes known as *Su(var)* genes which, when mutated, suppressed position effect variegation (PEV) in *Drosophila melanogaster* (Jenuwein, 2006). SUV39H1 was initially believed to be either a phosphatase or a protein that interacts with histones or chromatin and indirectly affects histone tail modifications, owing to its effect of reducing the G_2-dependent accumulation of phosphorylated histone H3. The role of SUV39H1 as a histone methyltransferase was revealed after it was discovered that its evolutionarily conserved SET domain resembled plant methyltransferases. SUV39H1 was found to methylate histone H3K9, inhibiting phosphorylation of H3S10 and resulting the in the recruitment of heterochromatin protein 1 (HP1), a protein involved in the propagation of heterochromatin formation (Jenuwein, 2006). Since these early

discoveries, many other lysine methyltransferases have been identified, most of which contain the SET domain (Dillon *et al.*, 2005).

The SET domain has now been found in all eukaryotic organisms studied and there are approximately 50 predicted SET-domain methyltransferases in the mammalian genome (Dillon *et al.*, 2005; Jenuwein, 2006). The SET domain protein superfamily is known to consist of seven main families, namely SUV39, Set1, Set2, EZ, RIZ, SMYD, SUV4-20 plus a few orphan members which include Set8 (also known as PR-Set7) and Set7/9 (Dillon *et al.*, 2005). All SET domain superfamily members transfer one to three methyl groups from their cofactor, *S*-adenosyl-L-methionine (AdoMet or SAM), to the amino group of a lysine residue on their substrate (Guo & Guo, 2007), leaving behind *S*-adenosyl-L-homocysteine (AdoHcy) as a by-product. Human Set7/9 is best well known for its methylation of histone 3 lysine 4 (H3K4), a modification found predominantly in active chromatin (Nishioka *et al.*, 2002; Pradhan *et al.*, 2009; Wang *et al.*, 2001). H3K4 methylation by Set7/9 is inhibited in the presence of a methylation mark at K9 of the same histone, a heterochromatin mark (Pradhan *et al.*, 2009), yet another example of the interplay between PTMs.

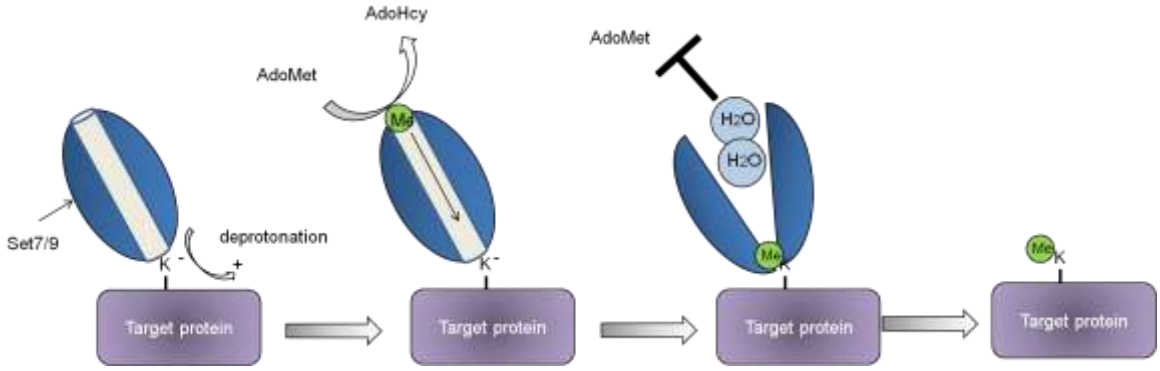
1.13 Mechanism of methylation by Set7/9

The binding sites for SAM and its lysine substrate are situated at opposite sides of the SET domain, and are connected by a pore running through the centre (Dillon *et al.*, 2005) [Figure 1.6(A)]. The lysine binding channel is lined by hydrophobic residues that bind to the lysine via Van der Waals forces. At the base of this channel is a pore connecting it to the AdoMet binding cleft, rimmed with carbonyl oxygens and the

hydroxyl group of an invariant tyrosine residue within the SET domain, which both aid the transfer of the methyl group during catalysis. The methyl group from AdoMet is transferred through the pore and tyrosine residues Y245 and Y305 in the substrate lysine binding cleft hydrogen bond to the ε-amino group of the lysine substrate, positioning it for methyl transfer (Dillon *et al.*, 2005) [Figure 1.6(B)]. The ε-amino group of the lysine substrate is positively charged, so it must be deprotonated to make it nucleophilic since a lone pair of electrons on its ε-amino group is the platform for addition of the methyl group. Y335 has been proposed as the base that is required for this deprotonation (Guo & Guo, 2007). Using molecular modelling, Guo & Guo (2007) propose a mechanism in which this highly conserved tyrosine residue becomes deprotonated by water in the surrounding bulk solvent allowing it to act as a base towards the lysine substrate of Set7/9. However, the high pKA of tyrosine would make this reaction unlikely without the involvement of other nearby basic residues not mentioned in this model and to date this hypothesis is yet to be experimentally proven.

The product specificity of Set7/9 as a monomethyltransferase relies on a combination of steric hindrance by the pore preventing transfer of a second methyl group onto the same lysine, and the integrity of the Y245 and Y305 residues in the substrate binding cleft. Mutation of the Y245 and Y305 residues to phenylalanine results in a change in product specificity of Set7/9 from a monomethyltransferase to di- or trimethyltransferase, respectively (Xiao *et al.*, 2003; Zhang *et al.*, 2003). Additionally, upon binding of methylated lysine within the active site of Set7/9, the binding cleft for SAM (at the other side of the SET domain pore) is opened, which allows water molecules to access the active site, disrupting the catalytic machinery of Set7/9 and inhibiting a di-methylation reaction (Hu *et al.*, 2008).

(A)



(B)

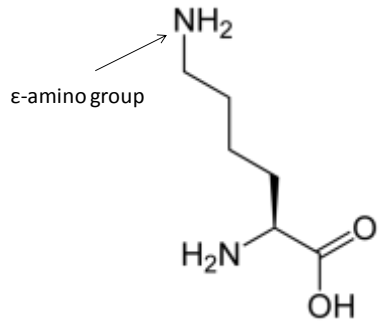


Figure 1.6: Mechanism of monomethylation by Set7/9

(A)The target lysine is first deprotonated to make it nucleophilic in readiness for methyl group addition. The methyl group from cofactor *S*-adenosyl-L-methionine (AdoMet or SAM) is passed through a pore at the centre of Set7/9 to the substrate binding pocket at the opposite end of the enzyme. Methylation causes a conformational change in Set7/9 which prevents a subsequent methylation reaction by allowing water molecules to enter the AdoMet binding pore.

(B) Structure of lysine showing the position of the ε-amino group at which methylation by Set7/9 occurs.

1.14 Non-histone targets for Set7/9

1.14.1 TAF10

In addition to H3K4, Set7/9 is now known to have a number of non-histone targets. The first of these to be discovered was the TATA-box binding protein (TBP) associated factor-10 (TAF10), a component of the general transcription complex TFIID (Kouskouti *et al.*, 2004). TAF10 was found to be methylated by Set7/9 at residue K189.

Interestingly, this methylation event potentiated transcription of two of its targets, ERF1 and ERA1, but had no effect at other TAF10 responsive genes such as *HPRT* and *cyclin E*, suggesting the effects of K189 methylation are gene specific. K189 methylation was also found to have no effect on incorporation of TAF10 into the TFIID complex, but instead enhanced binding to RNA polymerase II (Kouskouti *et al.*, 2004).

1.14.2 p53

The tumour suppressor p53 is also subject to methylation by Set7/9 (Chuikov *et al.*, 2004). p53 was in fact found to be a better substrate for Set7/9 than histone H3, and p53 methylation localised to K372, within the carboxy-terminal regulatory region of the protein. Methylated p53 was found to be restricted to the nucleus where it enhances transcription of p53 target genes such as *p21/WAF/*CIP. p53 is methylated by Set7/9 in response to DNA damage with doxorubicin and this response appeared to be biphasic, with activity peaks at 45 minutes and 4.5 hours post damage (Ivanov *et al.*, 2007). K372 methylation was shown to increase the stability of p53 and Set7/9 activity demonstrated to be critical for induction of p53-dependent apoptosis (Chuikov *et al.*, 2004; Ivanov *et al.*, 2007). In response to DNA damage, methylation of p53 by Set7/9 inhibits methylation of K370 by SMYD2, a modification known to inhibit p53 binding to DNA (Huang *et al.*, 2006). Set7/9 methylation of p53 additionally facilitates subsequent acetylation of the protein at K373 and K382 (Ivanov *et al.*, 2007). Acetylation of these carboxy-terminal located lysines enhances the activity and stability of p53 as a result of direct competition with murine double minute 2 (MDM2)-mediated ubiquitination of the same residues (Brooks & Gu, 2006). It was found that K372 methylation must precede acetylation, as pre-acetylation inhibits methylation (Ivanov *et al.*, 2007).

Using Set7/9$^{-/-}$ and Set7/9$^{+/-}$ mouse models methylation by Set7/9 was shown to be required to drive cells into G_1 arrest following doxorubicin treatment (Kurash *et al.*, 2008). Set7/9 knockout mouse embryonic fibroblast (MEF) cells were also shown to be susceptible to cellular transformation, where addition of either H-ras oncogene or E1A had the effect of inducing transformation (whereas both oncogenes need to be added to transform wild-type MEF cells). Interestingly, it was also found that methyl-K369 (the mouse equivalent of K372) promotes binding of the acetyltransferase Tip60 post-DNA damage, and this may be responsible for the mechanism of methylation-acetylation interplay on p53 (Kurash *et al.*, 2008). It should be noted that the above findings by Kurash *et al*, were recently challenged by two studies showing that Set7/9 activity has no effect on p53-dependent cell-cycle arrest or apoptosis following DNA damage (Campaner *et al.*, 2011; Lehnertz *et al.*, 2011).

Interestingly, Set7/9 additionally regulates p53 activity via a methyl-K372 independent mechanism involving the class III HDAC sirtuin, SIRT1 (Liu *et al.*, 2011). SIRT1 was shown to interact directly with Set7/9 in response to doxorubicin treatment, an interaction found to result in the methylation, by Set7/9, of several lysine residues on the deacetylase. SIRT1 methylation was demonstrated to have no impact on deacetylation of p53, but instead the interaction between Set7/9 and SIRT1 itself could induce the dissociation of SIRT1 from p53, thus enhancing p53 activity. The authors suggest their findings may be explained either by a Set7/9-induced conformational change in SIRT1 that prevents SIRT1 from binding to p53, or direct competition between Set7/9 and p53 for binding to SIRT1 (Liu *et al.*, 2011). However, it is noteworthy that the authors did not directly address the role of K372 methylation and subsequent acetylation of p53 on the SIRT1-p53 interaction.

1.14.3 Oestrogen receptor α

Also amongst the non-histone targets of Set7/9 is oestrogen receptor α (ER), a ligand dependent transcription factor that plays a critical role in normal mammary gland development (Subramanian *et al.*, 2008). Oestrogen binding followed by its interaction with DNA leads to a conformational change in ER which allows binding of multiple cofactors involved in histone modification and subsequent gene expression. Some of these cofactors additionally modify ER itself, in turn regulating ER activity. The hinge region of the protein is particularly susceptible to extensive PTM. Set7/9 was found to methylate ER in its hinge region, but unlike its other PTM mediating enzymes, is not recruited to ER targets themselves, but instead methylates transcription competent-ER away from its promoters. ER was found to be methylated at conserved lysine K302, where methylation stabilises the protein and is required for an efficient oestrogen-induced transcriptional response. K302 is situated amongst a series of residues already known to be subject to PTM and between which there exists known interplay. The authors suggest methylation at K302 may also contribute to this interplay, since residues from K299 through to S305 are known to undergo acetylation (K299, K302 and K303), ubiquitination (K302), and phosphorylation (S305). Mutual exclusivity exists between K303 acetylation and S305 phosphorylation, with ER acetylation (and hence inhibition of S305 phosphorylation) attenuating ER transcriptional activity. The authors suggest that ER K303 acetylation may similarly inhibit K302 methylation leading to its destabilisation (Subramanian *et al.*, 2008).

1.14.4 Retinoblastoma

Retinoblastoma (Rb) is methylated at more than one site by Set7/9 (Carr *et al.*, 2011; Munro *et al.*, 2010). Methylation was first identified at residue K873, at the carboxy

terminus of the protein, where methylation has the effect of facilitating Rb mediated suppression of E2F-1 target gene activity (Munro *et al.*, 2010). The methylation defective K873A mutant was less efficient at driving cells into G_1 arrest and senescence. Methyl-K873 was also found to serve as a binding platform for HP1, a protein best known for its role in gene silencing. HP1 binds trimethylated H3K9 via its chromodomain and recruits the methyltransferase SUV39H1, which binds to HP1 through its chromo-shadow domain. Once bound, SUV39H1 methylates adjacent nucleosomes, in turn recruiting more HP1 and leading to the propagation of heterochromatin and gene silencing (Hediger & Gasser, 2006). Since Rb is known to have a role in transcriptional repression when bound to repressor E2Fs at their promoters, the authors suggest that HP1 bound to methyl-K873 Rb may repress gene activity by a mechanism similar to that seen at H3K9 (Munro *et al.*, 2010).

Set7/9 was later found to additionally methylate K810 of Rb (Carr *et al.*, 2011). This methylation event reduces Rb phosphorylation by CDKs not only at the neighbouring S807 and S811, but at sites across the whole protein. K810 methylation was found to increase under conditions of DNA damage. By inducing hypophosphorylation of Rb, K810 methylation enhances Rb-mediated repression of E2F-1 and therefore decreases activity at E2F-1 responsive promoters (Carr *et al.*, 2011).

1.14.5 Other Set7/9 targets

Other non-histone targets for Set7/9 methylation include NFκB, where methylation at K314 and K315 within its RelA subunit (also known as p65) negatively regulates NFκB activity by promoting proteasomal degradation of promoter associated RelA (Yang *et*

al., 2009). This methylation event is inhibited by acetylation of RelA at K310, as this acetylation interrupts the interaction between RelA and Set7/9 (Yang *et al.*, 2010). The RelA subunit is additionally methylated by Set7/9 at K37 which has the effect of localising NFκB to the nucleus and is required for the expression of a subset of genes in response to tumour necrosis factor (TNF)α stimulation (Ea & Baltimore, 2009).

Set7/9 also methylates DNMT1, where it negatively impacts protein stability (Wang *et al.*, 2009) and the Tat protein of HIV, where methylation positively regulates transactivation of the HIV promoter (Pagans *et al.*, 2010). The acetyltransferase P/CAF is methylated at multiple sites by Set7/9 and methylated P/CAF is localised to the nucleus (Masatsugu & Yamamoto, 2009). The androgen receptor has been reported to be methylated by Set7/9 at K630 and K632 (Gaughan *et al.*, 2011; Ko *et al.*, 2011). Similar to methylation of the oestrogen receptor by Set7/9, this methylation occurs in the hinge region of the protein where it enhances androgen receptor transcriptional activity in multiple cell lines. Set7/9 recruitment to androgen response elements increased with androgen concentration, and corresponded with a decrease in chromatin activation marks (mono- and di-methylated histone H3K4) and an increase in methyl H3K9, a heterochromatin mark (Gaughan *et al.*, 2011; Ko *et al.*, 2011). Set7/9 therefore regulates androgen receptor activity in two ways, by directly modifying the protein and modifying chromatin surrounding androgen response elements. The significance of this modification is demonstrated by the observation of a K630T somatic mutation in prostate cancer (Ko *et al.*, 2011).

1.15 Set7/9 and cancer

SET domain proteins are known to play a role in human cancers (Dillon *et al.*, 2005). For example SMYD3 is known to contribute to the proliferation of hepatocellular and colorectal carcinomas where its upregulation results in activation of oncogenes and genes involved in cell cycle control. The SET domain protein mixed lineage leukaemia 1(MLL1) has been linked to leukaemia and enhancer of zeste homologue 2 (EZH2) (another SET domain protein) to metastatic prostate and breast cancer (Dillon *et al.*, 2005). With its role in regulating the activity of proteins central to cell cycle orchestration such as p53, it seems likely that Set7/9 will also be important in understanding the mechanisms of cancer. Indeed, the COSMIC database currently shows five examples of somatic mutations in Set7/9 in cancers of the central nervous system, large intestine, ovary and upper aerodigestive tract (www.sanger.ac.uk/genetics/CGP/cosmic/). As already mentioned, the Set7/9 target site of the androgen receptor is known to be mutated in prostate cancer (Ko *et al.*, 2011). Additionally, nuclear expression of Set7/9 is up-regulated in epithelial cells and simultaneously down-regulated in stromal cells of prostate cancer tissue compared to benign prostate tissues (Gaughan *et al.*, 2011). Set7/9 was also shown to be pro-proliferative and anti-apoptotic in the prostate cancer cell line, LNCaP (Gaughan *et al.*, 2011).

1.16 NEDDylation Overview

Neural precursor cell-expressed developmentally downregulated protein 8 (NEDD8) is a member of the ubiquitin like family and one of ten known NEDD genes originally identified in a subtractive cloning screen identifying genes enriched in mouse neuronal

precursor cells compared to adult mice (Watson *et al.*, 2011). There are 17 known ubiquitin-like proteins from nine phylogenetically distinct classes (NEDD8, SUMO, ISG15, FUB1, FAT10, Atg8, Atg12, Urm1, and UFM1) all of which attach to substrates in a three step conjugation cascade involving E1 (activating), E2 (conjugating) and E3 (ligating) components, analogous to ubiquitin conjugation (Watson *et al.*, 2006; Watson *et al.*, 2011). There are at least eight known E1 genes in the human genome, 35 active E2 enzymes and over 600 genes for RING-type E3 ligases alone [E3 ligases are divided into RING-type, homology to E6AP carboxyl terminus (HECT)-type and U-type] (Soucy *et al.*, 2010). Despite limited sequence homology between some members of the family, all family members have a characteristic β-grasp ubiquitin fold in their structure (Vertegaal, 2011). Ubiquitin-like proteins are even found in prokaryotes and archaea, known as prokaryotic ubiquitin-like proteins (PUPs) and small archaeal modifier proteins (SAMPs) (Vertegaal, 2011). NEDD8 is 81 residues long and is 60% identical and 80% homologous to ubiquitin (Soucy *et al.*, 2010; Tanaka *et al.*, 2012), the closest in homology of all the ubiquitin-like proteins (Xirodimas *et al.*, 2004). NEDD8 is best known for its conjugation to cullins, proteins that act as scaffolds in E3 ubiquitin ligases, but more recently has also been shown to be conjugated to some non-cullin substrates (discussed later).

NEDD8 is synthesised as a precursor that is processed at a conserved carboxy-terminal glycine by either UCH-L3, which can also process ubiquitin precursors, or the NEDD8 specific protease, NEDP1, removing the five carboxy-terminal amino residues to expose a glycine-glycine motif that is the site of attachment onto its targets (Soucy *et al.*, 2010; Tanaka *et al.*, 2012). MgATP (ATP must be bound to a magnesium ion, Mg^{2+} for it to be active) and NEDD8 bind to the NEDD8 activating enzyme (NAE) heterodimer,

composed of the amyloid precursor protein binding protein 1 (APP-BP1) and ubiquitin-like modifier activating enzyme 3 (UBA3), to form an acyl adenylate intermediate, NEDD8-AMP. This reacts with the active thiol site of the E1 to form NEDD8-NAE thioester, coupled with the release of AMP (Soucy *et al.*, 2010). The composition of NAE as a heterodimer is in contrast to the E1 of the ubiquitin conjugation cascade which consists of a single polypeptide (Xirodimas *et al.*, 2004). Thus the NEDD8 moiety is conjugated via its carboxy-terminal glycine to the cysteine side chain of NAE (Tanaka *et al.*, 2012). A second round of NEDD8 and ATP binding followed by NEDD8-AMP formation results in a ternary complex capable of transferring NEDD8 to the E2 (Soucy *et al.*, 2010). The NEDD8 moiety is then transferred onto one of two known E2 NEDD8 conjugating enzymes, UBC12 or UBE2F by a thioester linkage. UBC12 and UBE2F are each involved in the NEDDylation of different substrates with UBC12 specifically pairing with RING box protein 1 (RBX1) which associates with cullins 1 to 4 in cullin RING ligase (CRL) complexes, whereas UBE2F pairs with RBX2, to mediate cullin 5 NEDDylation (Soucy *et al.*, 2010). Finally, NEDD8 is transferred to the ε-amino group of a lysine residue on its substrate by a substrate specific RING finger domain containing E3 ligase such as RBX1, MDM2, FBXO11, and c-Cbl (Tanaka *et al.*, 2012; Watson *et al.*, 2011) (Figure 1.7). The exception is the E3 DCN1 which does not contain a RING finger domain.

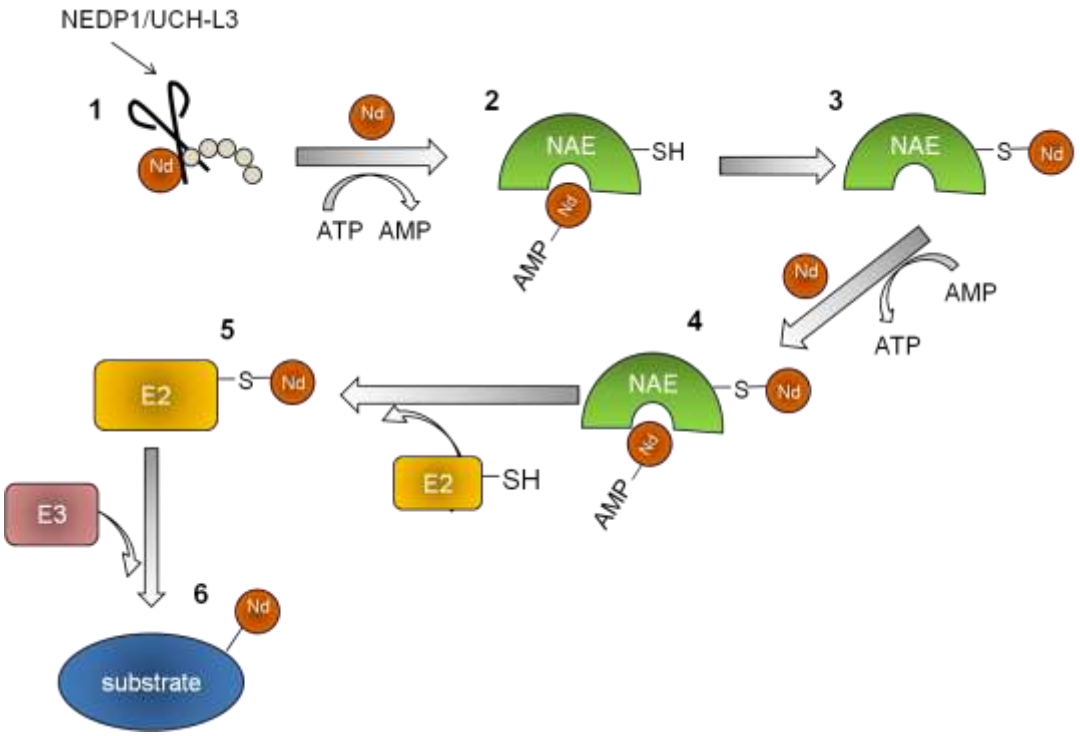


Figure 1.7: The NEDD8 conjugation pathway

1. The NEDD8 protein is processed by NEDP1 or UCH-L3 which cleaves the terminal five amino residues to reveal the glycine-glycine motif required for conjugation onto its substrates.

2. The NEDD8-AMP acyl-adenylate is formed by NEDD8 activating enzyme (NAE) by cleavage of ATP

3. The NEDD8 moiety is then conjugated to a cysteine side chain of the NAE E1.

4-5. A second round of NEDD8-AMP formation produces a NEDD8 capable of being transferred to an E2 enzyme via a thioester linkage.

6. The NEDD8 moiety is conjugated onto its substrate with the aid of a substrate specific E3 ligase.

NEDDylation is reversible as it can be removed from its substrates be the action of the CSN5 subunit of COP9 signalosome (CSN), NEDP1 (also known as DEN1 and SENP8) or USP21 (Mendoza *et al.*, 2003; Tanaka *et al.*, 2012; Watson *et al.*, 2011). *In vivo*, deNEDDylation by CSN is reserved for cullins whereas NEDP1 deNEDDylates other proteins (Soucy *et al.*, 2010). Additionally, NEDDylation is negatively regulated by NUB1 (NEDD8 ultimate buster 1) or inhibited by the binding of CAND1 (cullin-associated and NEDDylation-dissociated 1) to cullins (Tanaka *et al.*, 2012). The balance

between NEDDylation and deNEDDylation may depend on the availability of substrates and adaptor proteins (Soucy *et al.*, 2010).

Ubiquitin is known to form chains and the type of chain formed is crucial in the determination of the functional outcome of this modification (Mocciaro & Rape, 2012). The types of NEDD8 chains formed are only beginning to be discovered. Currently, it is thought that the formation of NEDD8 chains results from the build-up of NEDD8 on the catalytic cysteine of UBC12 prior to transfer onto its substrate (Watson *et al.*, 2011) and it has been shown that K11, K22, K48, and K60 can be linked to form NEDD8 chains *in vivo* (Jones *et al.*, 2008). It is also thought that mixed NEDD8 and ubiquitin may be formed, but as yet this seems to be a rare event with unknown function.

With the similarities between the ubiquitin and NEDD8 moieties as well as many proteins having both NEDD8 and ubiquitin E3 ligase activities, it would seem likely that there is a degree of overlap between their conjugation pathways. However, the specificity of the NEDD8 pathway is determined in several ways. At the E1 stage, the presence of an arginine at position 72 of ubiquitin (which is occupied by an alanine in NEDD8) is repelled by an arginine found in UBA3 of the NEDD8 conjugation pathway. Secondly, a 26 amino acid sequence at the amino terminus of UBC12 docks in a groove exclusive to UBA3 (Watson *et al.*, 2011).

Similar to the ubiquitin conjugation pathway, there are also mechanisms in place to ensure linear progression through the NEDD8 conjugation cascade. For example, when the NAE heterodimer has two NEDD8 moieties loaded onto it, one by a thioester bond

and the other an adenylated NEDD8, the E1 undergoes a conformational change that uncovers a previously hidden UBC12 binding site. Additionally, the NEDD8 E1 and E3 binding sites overlap on UBC12, thus promoting E1-E2-E3 progression (Watson *et al.*, 2011).

The best known substrates for NEDDylation are the cullin family, which is made up of Cul-1, -2, -3, -4A, -4B, -5, and -7 and PARC (Watson *et al.*, 2011). Cullins function as molecular scaffolds for CRLs. CRLs contain a RING finger subunit, either RBX1 or RBX2, which recognises the substrate for ubiquitination and binds either directly or indirectly to the amino terminus of the cullin scaffold. Cullin NEDDylation causes a conformational change that increases binding of the RING finger subunit to the ubiquitin E2 and reduces the gap between the E2 and the substrate recognition component so that the ubiquitin moiety and the substrate are brought closer together. The conformational change additionally promotes ubiquitin chain extension by allowing better access of the E2 to the nascent poly-ubiquitin chain. Cullin NEDDylation also inhibits binding of CAND1, a protein that inhibits CRL activity by preventing cullin binding to its adaptor and substrate recognition components.

The study of NEDDylation is highly relevant to our understanding of cancer as it is essential for efficient control of the cell cycle. Cullin1 containing E3 complexes [known as Skp1-Cul1-F-box (SCF) or Cullin1-CRL (CRL1) complexes] such as SCF^{Skp2}, $SCF^{\beta\text{-}TrCP}$ and SCF^{Fbw7} are amongst the best studied for their role in cancer through their involvement in the degradation of cell cycle regulators such as cyclin D, $p27^{KIP1}$, $p21^{CIP1}$, p73, p130, β-catenin, the NF-κB inhibitor IκBα, cyclin E, c-myc oncoprotein

and Notch (Tanaka *et al.*, 2012). In fact one study predicted SCFs to have over 350 protein substrates. Complexes containing the other cullins are also important in cell cycle control as they control of the levels of proteins involved in cell cycle progression, DNA replication and nucleotide excision repair, spindle assembly, Wnt-β-catenin and JAK-STAT signalling and are known to be amplified in some cancers (Tanaka *et al.*, 2012). To date NEDDylation has been detected on all eight cullins including PARC (Vertegaal, 2011). The discovery that transcription factors central to cell cycle regulation are themselves directly NEDDylated (reviewed below) adds to the large volume of literature supporting a role for NEDD8 in cancer. In support of its potential role as a chemotherapeutic target, it was observed that inactivation of the NEDD8 pathway resulted in multiple rounds of S-phase DNA replication without intervening mitosis in Chinese hamster cells. NEDD8 pathway inactivation also caused hypersensitivity to Ethylnitrosourea (ENU) induced apoptosis in *Caenorhabditis elegans* and a reduced growth phenotype in *Arabidopsis thaliana*. Additionally, inactivation of the NEDD8 pathway resulted in decreased cellular proliferation in oral squamous cell carcinoma cell lines where the level of NEDD8 conjugation was observed to be naturally elevated (Watson *et al.*, 2011).

Many PTMs exert their effects by forming binding platforms for other proteins that generate a downstream effect. NEDDylation is no exception. For example, the DNA methyltransferases DNMT3a and DNMT3b were found to bind to NEDDylated proteins. NEDDylation was found to be essential for DNA methylation since suppression of NAE inhibited DNMT3b-dependent methylation (Shamay *et al.*, 2010).

1.17 Non-cullin substrates of NEDDylation

In addition to cullins, other components of CRLs have been shown to be subject to NEDDylation. These include 12 F-Box proteins (responsible for the substrate specificity of SCF E3 ligases), S-phase kinase-associated proteins (Skp) 1 and 2, several BTB-domain-containing proteins (substrate-specific adaptors in Cul-3-containing E3 ligase complexes) and WD40-repeat-containing proteins (Vertegaal, 2011). In fact, a proteome wide screen utilising single step affinity purification, 1-D gel electrophoresis, and two different tandem mass spectrometers (LTQ MS and QSTAR® XL, a type of QTOF MS) identified 496 NEDD8-modified and associated proteins (Jones *et al.*, 2008). These included not only those associated directly with ubiquitin ligases, but also proteins involved in signal transduction, metabolism, transcription, cell cycle, and apoptosis, DNA repair and replication.

1.17.1 p53/MDM2

The first non-cullin protein to be identified and characterised as a substrate for NEDDylation was the tumour suppressor p53 (Xirodimas *et al.*, 2004). It was found that its negative regulator, MDM2, was the RING finger type E3 ligase responsible for this modification. MDM2 therefore has dual roles, both as a ubiquitin and NEDD8 E3 ligase. However, p53 NEDDylation could occur in the absence of an E3 *in vitro*. MDM2 was found to mediate NEDDylation of itself, in a mechanism requiring the integrity of cysteine 462 (464 in human), a residue known to be required for both the auto-ubiquitination activity of MDM2 and its ubiquitination of p53. NEDDylation of p53 was found to depend on lysines K370, K372, and K373, since triple substitution of these residues abolished NEDDylation, although mutation of individual lysines had no

effect. NEDDylation was found to suppress the transcriptional activity of p53 but also inhibit the suppressive role of MDM2 on p53 activity (Xirodimas *et al.*, 2004). This is analogous to the regulation of p53 by ubiquitination whereby MDM2 controls its own activity by promoting the ubiquitination and proteasomal degradation of p53 and also itself. In line with this, treatment of cells with the proteasome inhibitor MG132 resulted in increased NEDDylation of p53. p53 NEDDylation was additionally found to be regulated by a different mechanism to that of its ubiquitination since they showed different kinetics following damage. p53 ubiquitination and NEDDylation could coexist however, despite the fact that the six lysine residues at the carboxy-terminus of p53 which includes K370, K372 and K373, had previously been implicated in its ubiquitination (Xirodimas *et al.*, 2004). Although the implication is that NEDDylation and ubiquitination occur on different residues at any one time, Jones *et al*. suggest that this finding more likely results from the formation of mixed ubiquitin-NEDD8 chains (Jones *et al.*, 2008).

A later study showed that FBXO11 can additionally act as NEDD8 E3 ligase for p53 (Abida *et al.*, 2007). Interestingly, this protein, which forms part of the SCF^{FBXO11} complex, did not show ubiquitin ligase activity towards p53, yet was able to NEDDylate p53 not only at carboxy terminal lysines, but at two additional sites, K320 and K321, within the nuclear localisation signal of p53. Similar to MDM2, NEDDylation by FBXO11 was shown to inhibit p53 transcriptional activity (Abida *et al.*, 2007).

NEDD8 ultimate buster 1 (NUB1) was identified as an interferon-inducible NEDD8 interacting protein that decreases the levels of both free NEDD8 and NEDDylated

proteins (Liu & Xirodimas, 2010). Ectopic expression of NUB1 caused the expected decrease in p53 NEDDylation, but, intriguingly caused an increase in p53 ubiquitination (and had no effect on SUMOylation). NUB1 enhanced the formation of faster migrating forms of ubiquitinated p53 (i.e. mono-ubiquitinated and multiple mono-ubiquitinated forms) but decreased the formation of slower migrating ubiquitinated p53 (i.e. those conjugated to poly-ubiquitin chains). Under conditions of NEDP1 over-expression, in which p53 NEDDylation is inhibited, the effect of NUB1 on p53 ubiquitination was abolished, suggesting NEDDylation is required for NUB1 mediated p53 ubiquitination. The interaction between NUB1 and p53 was also dependent on NEDDylation, as NEDP1 decreased this interaction. NUB1 was found to stimulate the cytoplasmic localisation of ubiquitinated p53 in a NEDD8-dependent manner and to decrease the transcriptional activity of p53, possibly as a result of its effects on the promotion of p53 ubiquitination (Liu & Xirodimas, 2010). The interplay between NEDDylation and ubiquitination is therefore important in maintaining the fine balance of the activity of transcription factors such as p53.

1.17.2 Ribosomal proteins

In an analysis of the proteome for NEDDylated proteins using a TAP-tag pulldown of NEDD8 associated proteins, 75 proteins were identified with diverse roles including messenger RNA splicing, DNA replication and repair and proteasomal degradation (Xirodimas *et al.*, 2008). Of these 75 proteins, 36 were small and large ribosomal proteins. Further testing of a selection of these ribosomal proteins under denaturing conditions (to rule out the possibility of non-covalent interactions with NEDD8) confirmed NEDDylation of small ribosomal proteins S3, S7 and S20 and large

ribosomal protein L7 but showed that L5 was not directly NEDDylated. This suggested that some of the other candidate proteins for NEDDylation identified in the initial screen may have been indirectly associated with NEDD8. Treatment with the proteasome inhibitor MG132 had the general effect of increasing total NEDDylated substrates, possibly resulting from the presence of ubiquitin in NEDDylated eluates which targets them for proteasomal degradation. However, it was apparent that MG132 treatment increased NEDDylation of some ribosomal proteins but decreased the NEDDylation of others. This suggests ribosomal proteins are differentially regulated by NEDD8. NEDDylation of L11 was also found to cause an increase in its stability (Xirodimas *et al.*, 2008).

NEDDylation of L11 was later found to drive its localisation to the nucleolus (Sundqvist *et al.*, 2009). Nucleolar disruption, for example by actinomycin D, causes relocalisation of ribosomal proteins such as L11 from the nucleolus into the nucleoplasm where it provides the signal for p53 activation by binding and inhibiting its repressor, MDM2. Actinomycin D also causes a rapid decrease in L11 NEDDylation where NEDDylation is proposed to participate in this relocalisation of L11 as well as increasing the susceptibility of L11 to degradation. Like p53, L11 was found to be NEDDylated by MDM2 (Sundqvist *et al.*, 2009).

Interestingly, a recent study showed that out of 36 genes known to be induced upon actinomycin treatment (28 of which are p53-dependent), the induction of 29 genes was dependent on both L11 and NEDD8 (26 out of the 28 p53-dependent genes) (Mahata *et al.*, 2012). Knockdown of either NEDD8 or L11 was found to decrease binding of p300

to p53 and p300 recruitment to p53 responsive promoters upon actinomycin treatment. Acetylation of p53 K382, a PTM known to be induced upon cellular stress, was therefore also decreased by NEDD8 or L11 knockdown. L11 was itself found to be recruited with p53 to the *p21*, *MDM2*, *PUMA*, *BAX* and *PIG3* promoters in a NEDD8 and MDM2 dependent manner (Mahata *et al.*, 2012).

1.17.3 TAp73β

The p53 family member p73 was also found to be NEDDylated by MDM2 (Watson *et al.*, 2006). Alternative promoters and amino-terminal splicing result in the existence of two forms of p73 with opposing functions; a full length version, TAp73, which has pro-apoptotic functions and an amino-terminal truncated version, ΔNp73 which is anti-apoptotic and lacks the MDM2 binding motif. MDM2 was found to NEDDylate only the TAp73 isoform, having the effect of attenuating its transcriptional activity and promoting its cytoplasmic localisation (Watson *et al.*, 2006). As MDM2 is known to promote p73 stability by an unknown mechanism, the authors propose that NEDDylation may stabilise p73, but this hypothesis was not formally tested.

1.17.4 VHL

The von Hippel–Lindau (VHL) protein, responsible for the familial cancer syndrome VHL disease, was also found to be a NEDD8 substrate (Stickle *et al.*, 2004). VHL has two main functions; as the substrate recognition component of Elongin B/C–Cul2–VHL (ECV), an SCF-like E3 ubiquitin ligase that targets hypoxia inducible factor α (HIF1α) for degradation, and a binding protein for fibronectin for the positive regulation of

extracellular matrix assembly. VHL was found to be NEDDylated at residue K159 and this NEDDylation demonstrated to be required for fibronectin binding and therefore extracellular matrix assembly. It was however, found to have no effect on the ability of VHL to regulate HIF1α activity (Stickle *et al.*, 2004). Interestingly, K159 is found to be mutated to a glutamine in some individuals leading to a predisposition to VHL type 2C disease, in which patients experience pheochromocytoma (adrenal gland tumours) without the hemangioblastoma (central nervous system and retina) and renal clear cell carcinomas characteristic of other types of the disease. The authors found that this mutant is non-NEDDylatable and cannot bind fibronectin, but is capable of ubiquitinating HIF1α (Stickle *et al.*, 2004). The mechanism of this function-specific role of VHL NEDDylation was later identified by Russell and Ohh, who showed that NEDDylation prevents association of VHL with Cul2, thereby inhibiting its assembly into the ECV complex. This "freed" VHL, although only a small fraction of the total VHL population, is subsequently deNEDDylated before its association with fibronectin (Russell & Ohh, 2008).

1.17.5 HIF1α

Hypoxia inducible factor α proteins have also been identified as NEDD8 targets (Ryu *et al.*, 2011). These proteins cause the upregulation of various genes during tumour adaptation to hypoxia and angiogenesis. It was found that about one third of HIF-1α was NEDDylated in cells under hypoxic conditions where NEDDylation was found to help increase the stability of the protein. NEDDylation was found to fall between residues 201 and 400 and to occur independently of its negative regulators and known

NEDD8 substrates, VHL and p53. NEDD8 dependent stabilisation of HIF-1α was found to be dependent on the presence of reactive oxygen species (Ryu *et al.*, 2011).

1.17.6 BCA3

Breast cancer associated protein 3 (BCA3) was identified as a NEDD8 substrate in a yeast two-hybrid screen in a human placental cDNA library using NEDP1 as bait (Gao *et al.*, 2006). BCA3 inhibits NFκB transactivation via its binding to p65 protein and the *cyclin D1* promoter. Using a BCA3 mutant in which all 11 lysines had been substituted for arginines in combination with NEDP1 over-expression, it was shown that NEDDylation is required for this inhibitory effect. NFκB signalling protects cells from apoptosis. Its inhibitor BCA3 enhances susceptibility to apoptosis and this is dependent on its NEDDylation. The inhibitory effect of BCA3 on NFκB signalling was also found to depend on recruitment of the HDAC, SIRT1 to promoters. The association between SIRT1 and BCA3 was found to be dependent on NEDDylation of BCA3. Additionally, it was shown that oestrogen could inhibit BCA3 NEDDylation, adding a further layer of complexity to regulation of apoptosis and NFκB signalling by BCA3 (Gao *et al.*, 2006).

1.17.7 IKKγ

Tripartite motif 40 (TRIM 40), a protein highly expressed in gastrointestinal cells, was found to interact with NEDD8 and to promote NEDDylation of IκB kinase subunit, IKKγ (Noguchi *et al.*, 2011). The IKK complex phosphorylates IκB proteins which results in their ubiquitination and subsequent proteasomal degradation. IκB proteins inhibit NFκB activity, so their degradation causes release of NFκB and subsequent

signalling by this transcription factor. IKKγ NEDDylation is unique to this subunit (IKKα and IKKβ were not observed to be NEDDylated) and TRIM40 expression was found to stabilise IκB, thereby inhibiting NFκB signalling and suppressing growth (Noguchi *et al.*, 2011).

1.17.8 EGFR

Epidermal growth factor receptor (EGFR) was found to be NEDDylated by its ubiquitin ligase, c-Cbl (Oved *et al.*, 2006). It was suggested that this NEDDylation occurs at multiple sites since a smear of NEDDylated EGFR upon immunoblot analysis was observed, even after point substitution of K48 of NEDD8 to arginine in an attempt to inhibit chain formation. However, this study was performed prior to that of Jones *et al.* in which NEDD8 was found to form chains through linkages at K11, K22 and K60 in addition to K48 (Jones *et al.*, 2008) and therefore NEDD8 chain formation on EGFR cannot be ruled out. The NEDDylation of EGFR was found to be dependent on the presence of its ligand, EGF, and EGFR NEDDylation was demonstrated to promote ligand induced ubiquitination and downregulation (Oved *et al.*, 2006).

1.17.9 Caspase-7

An RNAi screen in *Drosophila* identified NEDP1 and CSN5 NEDD8 proteases as well as CG1503, a predicted deNEDDylase, as having a role in apoptosis, since their depletion led to decreased apoptosis (Broemer *et al.*, 2010). This suggested that NEDDylation is involved in the suppression of pro-apoptotic proteins leading the authors to identify caspase-7 as a NEDD8 target. It was found that the X-linked

inhibitor of apoptosis, known for its role as a ubiquitin E3 ligase for caspases, was found to act as NEDD8 E3 ligase for caspase-7 and that NEDDylation inhibits the activity of the caspase-7 *Drosophila* homologue, drICE. This explains, at least in part, the observed decrease in apoptosis upon inhibition of NEDD8 proteases in the original RNAi screen (Broemer *et al.*, 2010).

1.18 New methods for identification of NEDD8 substrates

The validity of the studies presented above in identifying NEDD8 substrates has in recent months been thrown into doubt with the publication of several studies showing that the NEDD8 moiety is conjugated to substrates via an "atypical" conjugation pathway under some experimental conditions (Hjerpe *et al.*, 2012a; Hjerpe *et al.*, 2012b; Leidecker *et al.*, 2012). It was demonstrated that free NEDD8 and ubiquitin levels are approximately equal under physiological conditions, but under conditions where the level of free NEDD8 is raised at least 7-fold above that of ubiquitin, NEDDylation occurs via a mechanism that utilises components of the ubiquitin conjugation pathway. This atypical pathway is independent of the NEDD8 conjugation pathway and the array of proteins identified to be NEDDylated is very different under these conditions (Hjerpe *et al.*, 2012a). Perturbations in the NEDD8: ubiquitin ratio can occur when NEDD8 is overexpressed or following treatment with proteasome inhibitors (which results in the accumulation of ubiquitin conjugates and depletion of the free ubiquitin pool), both common experimental techniques in the identification of new substrates for NEDDylation. Changes in the NEDD8:ubiquitin ratio can also occur following heat shock and oxidative stress (Leidecker *et al.*, 2012). It was shown that although cullins and NEDD8 E1 and E2 enzymes were conjugated to NEDD8 under

physiological conditions, other non-cullin substrates were not detected (Hjerpe *et al.*, 2012a). Under conditions of ectopic NEDD8, p53 and caspase-7 were NEDDylated in a manner dependent on the presence of the ubiquitin E1, UBE1, but not on the activity of the NEDD8 E1, since they were still NEDDylated upon treatment with the NAE inhibitor MLN4924. NEDDylation of HIF1α under conditions of ectopic NEDD8 was dependent on both UBE1 and NAE, suggesting that at least some of the observed NEDDylation was via the atypical pathway (Hjerpe *et al.*, 2012b). The authors do not deny the physiological relevance of NEDDylation of non-cullin substrates, but suggest that it may only occur when the ubiquitin pool is depleted, for example when patients are treated with the proteasome inhibitor bortezemib, or in neurodegenerative diseases in which cells accumulate ubiquitin conjugates. They suggest that since the conjugation of ubiquitin to the NEDD8 moiety is inefficient, and that atypical NEDDylation was shown to slow the proteasomal degradation of ubiquitinated substrates, NEDDylation by the atypical pathway may slow the formation of ubiquitin chains, even terminating them before they reach a critical length for targeting to the proteasome, similar to that shown for SUMO1 which terminates SUMO chains (Hjerpe *et al.*, 2012a). This mechanism may exist to preserve the free ubiquitin pool under conditions of ubiquitin-depletion stress.

The above findings have led some to revisit older studies, such as Nagano *et al.* whose data suggest that XIAP does not act as an NEDD8 E3 ligase for caspase-7, although it can mediate NEDDylation of itself (Nagano *et al.*, 2012). Therefore, from now on studies investigating NEDDylation must be structured carefully, including controls to rule out conjugation by the atypical pathway described in these studies.

1.19 Targeting NEDDylation in cancer: MLN4924

Since SCF ubiquitin E3 ligases are known to be dysregulated in a wide range of cancers (Tanaka *et al.*, 2012), and since NEDDylation is important for their activity, NEDDylation is an attractive target for the development of new cancer therapies, especially since defects in NEDDylation itself have been observed in some cancers as well as neurodegenerative diseases (Tanaka *et al.*, 2012; Vertegaal, 2011). For example, the CSN5 subunit of the COP9 signalosome cleaves the isopeptide bond between NEDD8 and its substrate and is known to be overexpressed in breast, liver and pancreatic cancers. An abnormality of the NEDDylation inhibitor, CAND1 was implicated in the observed increased levels of NEDDylated Cul1 in neuroendocrine lung cancer. Over-expression of $p45^{SKP2}$ is also associated with tumorigenesis, and in renal cell carcinoma cells expression of a non-NEDDylatable form of pVHL results in a lack of fibronectin matrix assembly and in tumour formation (Soucy *et al.*, 2010).

An inhibitor of NEDDylation, MLN4924 (Figure 1.8) has recently been developed and is currently in clinical trials for patients with several solid tumour and haematological malignancies such as acute myelogenous leukaemia, where it was found that MLN4924 increases the levels of CRL substrates such as IκBα, having an anti-cancer effect (Soucy *et al.*, 2010). MLN4924 targets NEDDylation at the first step in the conjugation cascade, using NAE catalysis to irreversibly form a covalent adduct with NEDD8 which resembles adenylated NEDD8. This binds the adenylation site of the UBA3 subunit of NAE and, as it cannot be used to form a thioester, blocks the active site deeming NAE inactive (Soucy *et al.*, 2010; Tanaka *et al.*, 2012). As NEDDylation has such an important role in cell cycle control by regulating the activity of E3 ligases as well as transcription factors, there is currently a deluge of interest in MLN4924. In fact the

compound has already been reported to have been tested for its effectiveness in treating Ewing sarcoma (Mackintosh *et al.*, 2012), liver cancer (Luo *et al.*, 2012), breast cancer (Yang *et al.*, 2012), myeloma (McMillin *et al.*, 2012), pancreatic cancer (Wei *et al.*, 2012), lymphoma (Robertson *et al.*, 2011), and head and neck cancer (Zhao *et al.*, 2011). The mechanism or mechanisms by which MLN4924 exerts its anti-cancer effects are not fully understood and remain the subject of much investigation. A proteomics study suggested that as many as 38 proteins are important for the cytotoxicity of MLN4924, having roles in cell cycle, DNA damage repair, and ubiquitin transfer (Liao *et al.*, 2011) illustrating the importance of acquiring a better understanding of NEDDylation.

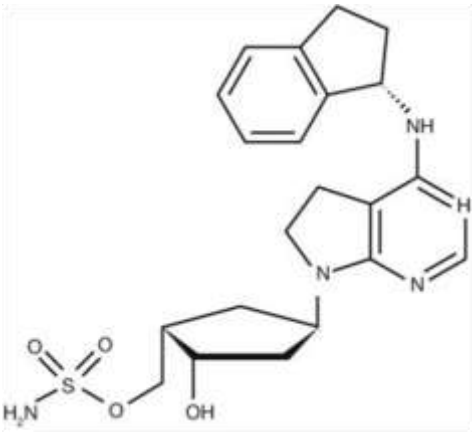


Figure 1.8: Structure of the small molecule inhibitor of NAE, MLN4924
(Soucy *et al.*, 2010)

1.20 Aims and objectives

It is clear that E2F-1 is central to the regulation of the cell cycle whether it is behaving as an oncogene or a tumour suppressor. Since E2F-1 activity is dysregulated in the majority of cancers (Trimarchi & Lees, 2002), an awareness of how E2F-1 is controlled

is critical to our understanding of the disease. It is evident that although there is a wealth of literature associated with E2F-1, our knowledge of its regulation or activity, in particular, what governs its bipolar nature as both a promoter of cell cycle progression and apoptosis, is as yet incomplete. The last decade has seen rapid growth in the amount of literature demonstrating the fine tuning of protein activity by PTM. Proteins subject to regulation by PTM include transcription factors such as p53, p73, NFκB as well as E2F-1 itself, illustrating the importance of the "protein code" in our understanding of the cell cycle (Cho *et al.*, 2012; Chuikov *et al.*, 2004; Sims & Reinberg, 2008; Stevens *et al.*, 2003; Watson *et al.*, 2006; Xirodimas *et al.*, 2004; Yang *et al.*, 2009).

The aim of this study was to identify and characterise novel E2F-1 PTMs, investigating their regulation under various conditions such as DNA damage, their localisation with respect to other known E2F-1 PTMs and their impact on E2F-1 activity. The study began with the identification of E2F-1 as a substrate for methylation by Set7/9, a monomethyltransferase known to regulate other cell cycle regulators such as p53 and Rb (Carr *et al.*, 2011; Chuikov *et al.*, 2004; Munro *et al.*, 2010). Next, E2F-1 was identified as being a substrate for NEDDylation, a modification also known to affect the activity of cell cycle regulators such as p53, p73 and NFκB (Noguchi *et al.*, 2011; Watson *et al.*, 2006; Xirodimas *et al.*, 2004).

Following localisation of E2F-1 methylation and NEDDylation to the DNA binding and heterodimerisation domain, these two modifications were found to exhibit interplay, as methylation promotes subsequent NEDDylation of E2F-1. These new E2F-1 modifications are in a region crucial to its function and are already known to be subject

to PTM by methylation by PRTM5 as well as acetylation. Therefore, there is potential for even further interplay between PTMs in the DNA binding and heterodimerisation domain. With the current deluge of interest in the newly developed small molecule inhibitor of the NEDD8 conjugation pathway, MLN4924, following its success in Phase 1 clinical trials for acute myelogenous leukaemia, the importance of understanding how NEDDylation affects cell cycle regulation has never been greater. The study presented here aims to contribute to our knowledge of this highly important modification.

Chapter 2

Materials and Methods

2.1 Preparation of recombinant proteins

Escherichia coli (E. coli) cells (BL21 strain) were transformed with 500ng pGEX bacterial expression vector, plated onto agar dishes containing ampicillin (100µg/ml) and a single colony amplified, firstly for 8 hours in 5ml Luria Broth (Sigma) containing ampicillin (100µg/ml) at 37ºC, shaking, and this starter culture transferred into a 500ml volume of Luria Broth for amplification at 37ºC, shaking, until the optical density of the culture medium reached OD_{595} ~0.6-0.7. IPTG (VWR International Ltd) was then added to a 1mM final concentration to induce protein expression and the cultures incubated at 20ºC overnight, shaking. Cells were spun down at 4000rpm (Sorvall Evolution centrifuge, SLA3000 rotor) for 30 minutes. The bacterial pellet was resuspended in 20ml buffer [25mM Tris-HCl pH7.5, 0.5mM EDTA, 1mM DTT, 10% sucrose, 5mM benzamidine with protease inhibitors (10µg/ml leupeptin, 10µg/ml aprotinin (both from Sigma), 10µg/ml pepstatin (Roche diagnostics), 1mM AEBSF (Sigma)]. The total volume was made up to 30ml with resuspension buffer and lysozyme and Triton X-100 (Sigma) added to final concentrations of 20µg/ml and 1%, respectively. Samples were left on ice for 10 minutes and then sonicated. Samples were next spun at 13,000rpm for 30 minutes (Sorvall Evolution centrifuge, SLA3000 rotor) and the supernatant mixed with 0.5ml glutathione Sepharose beads (GE Healthcare) pre-washed in resuspension buffer and incubated overnight, rotating at 4ºC.

Glutathione Sepharose beads were then spun down at 1000rpm (Beckman GS-6R centrifuge) and washed 3 times (each for 15 minutes, rotating at 4°C) in wash solution (identical to resuspension buffer minus sucrose). Recombinant proteins were eluted in 1ml 100mM Tris-HCl pH8, 100mM NaCl and 20mM reduced glutathione (Sigma).

Elutions were repeated 3 times and the eluates pooled. Eluates were then subject to buffer exchange by injection into dialysis cassettes (10,000 Molecular weight cut-off, Thermo Scientific) and overnight incubation at 4ºC in dialysis buffer [50mM Tris-HCl pH7.5, 100mM KCl, 20% glycerol, 0.2mM DTT with protease inhibitors (10µg/ml leupeptin, 10µg/ml aprotinin, 10µg/ml pepstatin, AEBSF)].

2.2 In vitro methylation assa***y***

1µg recombinant GST-tagged substrate or calf thymus histones (Roche), 0.5µg recombinant GST-Set7/9(52-366) and 1µCi *S*-adenosyl-[^{3}H-methyl]-methionine (GE Life Sciences) were combined in a 30µl volume of buffer containing 50mM Tris-HCl pH8, 20mM KCl, 5mM DTT and 1mM EDTA and methylation carried out over one hour at 30°C. For a filter assay, the entire reaction was spotted and dried on P81 membrane circles (Whatman). These were washed 3 times for 10 minutes in 200mM $NaHCO_3$ (adjusted to pH 9.2 with $NaHCO_3$), dipped briefly in acetone and dried. Filters were put into scintillation vials, 4ml scintillant mix (Beckman Coulter) added and the uptake of radiolabel measured on a scintillation counter (Beckman LS 6000SE). For autoradiography, the methylation reaction was mixed with 2x SDS loading buffer and separated by SDS-PAGE, transferred onto nitrocellulose, sprayed with EN^{3}HANCE Spray Surface Autoradiography Enhancer (Perkin Elmer) and exposed to light sensitive film (Kodak) at -80°C for 2 weeks. Films were developed using the AGFA CP1000 automatic developer.

2.3 Tissue culture, transfection and siRNA treatment

U2OS, SAOS2 and HeLa cell lines were cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen) and H1299 cells in RPMI, all supplemented with 10% foetal calf serum and penicillin-streptomycin (Invitrogen). Cell lines were cultured at 37°C and 5% CO_2. Transfection of mammalian expression vectors and siRNA was carried out using GeneJuice (Novagen) and Oligofectamine (Invitrogen) transfection reagents, respectively, both according to the manufacturer's guidelines. Cells were harvested 48 hours post plasmid transfection or 72 hours post siRNA transfection.

Table 2.1: siRNA sequences and working concentrations

siRNA	Working Concentration	Sequence	Company
Set7/9	10nm	5'-AGAUAACAUUCGUCAUGGA-3'	Dharmacon (custom)
UBE1 (h)	20nm	Not known	Santa Cruz
UHRF1	50nm	5'-TGTGAAATACTGGCCCGAGAA-3'	Dharmacon, (custom)
Non-targeting control #2	as targeting siRNA	5'-UAAGGCUAUGAAGAGAUACUU-3'	Dharmacon, (custom)

2.4 Generation of NEDD8-inducible cell line

His-NEDD8 plasmid was cloned into a pTre2-hyg vector and transfected into U2OS cells containing the pTet-On plasmid (G418 resistant). After 48 hours transfection, cells were selected and maintained in DMEM containing G418 (100µg/ml) and hygromycin (150µg/ml).

2.5 Plasmids and expression vectors

All expression vectors have been described previously; HA-E2F-1 (Milton *et al.*, 2006), His-NEDD8, His-ubiquitin and NEDP1 (Xirodimas *et al.*, 2004), *Cdc6*-luciferase (Hateboer *et al.*, 1998), *p73*-luciferase, *Apaf-1*-luciferase (Pediconi *et al.*, 2003), *cyclin E*-1uciferase (Botz *et al.*, 1996) pGEX-E2F-1 (Stevens *et al.*, 2003), pGEX-Set7/9 (52-366); (Wilson *et al.*, 2002) UBC12 (C111S) (Wada *et al.*, 2000), UBE2F (C116S) (Huang *et al.*, 2009). Lysine to arginine residue substitution mutants and truncations were derived from their wild-type counterparts as described below. Mammalian expression vectors were prepared by transforming *E. coli* cells (DH5α strain) with 10ng DNA, plating onto agar dishes containing ampicillin (100µg/ml), and amplification of a single colony overnight at 37ºC, shaking, in 200ml Luria Broth containing ampicillin (100µg/ml). DNA was subsequently purified using the Plasmid Maxi Kit or QIAprep® Miniprep Kit (both from Qiagen), according to the manufacturer's instructions.

2.6 Site Directed Mutagenesis

E2F-1 substitution mutants were generated by site directed mutagenesis of 100ng wild-type HA-E2F-1 pcDNA3 construct using the primers described in Table 2.2. For E2F-1 double and triple mutants (K161/164R, K117/120R, K117/120/125R, K182/183/185R), PCR was performed using Pfu Turbo AD DNA polymerase (Stratagene), at an annealing temperature of 55°C and an extension temperature of 65°C. For E2F-1 point mutants (K182R, K183R, K185R), PCR was performed using Platinum Pfx DNA polymerase (Invitrogen) at an annealing temperature of 55°C and an extension time of 68°C.

Following PCR, DNA underwent digestion with Dpn1 (New England Biolabs) to remove template DNA, before transformation of XL10-Gold® ultracompetent cells (Stratagene) with the point substituted E2F-1 plasmid for amplification and plasmid purification.

Table 2.2: Primers used for site directed mutagenesis

Mutant	Primer sequence
K117/120R (forward)	CGCCATCCAGGAAGAGGTGTGAGATCCCCGGGGGAG
K117/120R (reverse)	CTCCCCCGGGGATCTCACACCTCTTCCTGGATGGCG
K161/164R (forward)	GCCGAGGTGCTGAGGGTGCAGAGGCGGCGCATCTAT
K161/164R (reverse)	ATAGATGCGCCGCCTCTGCACCCTCAGCACCTCGGC
K182/183/185R (forward)	CAGCTCATTGCCAGGAGGTCCAGGAACCACATCCAG
K182/183/185R (reverse)	CTGGATGTGGTTCCTGGACCTCCTGGCAATGAGCTG
K182/183R (forward)	ATCCAGCTCATTGCCAGGAGGTCCAAGAACCAC ATC
K182/183R (reverse)	GATGTGGTTCTTGGACCTCCTGGCAATGAGCTGGAT
K182R (forward)	ATCCAGCTCATTGCCAGGAAGTCCAAGAACCAC ATC
K182R (reverse)	GATGTGGTTCTTGGACTTCCTGGCAATGAGCTGGAT
K183R (forward)	ATCCAGCTCATTGCCAAGAGGTCCAAGAACCAC ATC
K183R (reverse)	GATGTGGTTCTTGGACCTCTTGGCAATGAGCTGGAT
K185R (forward)	GGCAAGAAGTCCAGAAACCACATCCAG
K185R (reverse)	CTGGATGTGGTTTCTGGACTTCTTGCC

2.7 Generation of E2F-1 truncated mutants

The region of E2F-1 to be subcloned was generated from 50ng HA-E2F-1 pcDNA3 construct by PCR using Pfu Turbo AD at an annealing temperature of 64°C and an extension time of 72°C. The primers used are described in Table 2.3.

The PCR product was loaded onto a 2% agarose gel containing ethidium bromide (0.003%), alongside DNA ladder (Promega). The band corresponding to the desired PCR product (determined by its size) was cut out and DNA purified using the QIAquick® gel extraction kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 30µl nuclease free water (Qiagen).

Sticky ends were generated by performing a restriction digest of both the PCR-generated insert (using 30µl of total 50µl PCR reaction) and 3µg 3x-Flag vector (Sigma) using XbaI and EcoRI or HindIII and BamHI restriction endonucleases (Promega), as indicated in Table 2.3.

Digests were performed at 37°C for 1 hour. The vector digest was run on a 2% agarose gel with ethidium bromide (0.003%) alongside DNA ladder, an undigested vector control and lambda DNA for an estimation of DNA concentration. The digested vector band was then cut out and purified using the QIAquick® gel extraction kit and the insert digest purified using the QIAquick PCR purification kit (Qiagen).

Ligation of the insert DNA into the 3x-Flag vector was performed using the LigaFast Rapid Ligation System (Promega) according to the manufacturer's instructions, in a total volume of 10µl. 3µl of the ligation was used to transform *E. coli* cells (DH5α strain) and the construct amplified and purified as described in Section 2.6.

Table 2.3: Primers for generation of E2F-1 truncated mutants

Primer	Sequence
1-110 (forward)	ATTAGTAAGCTTATGGCCTTGGC
1-110 (reverse)	AATACTGGATCCTCAGCCCCGAGCTGG
1-145 (forward)	ATTAGTAAGCTTATGGCCTTGGC
1-145 (reverse)	CACGATGCGGCCGCGTGGCTCAGCAGCTCCA
1-194 (forward)	ATTAGTAAGCTTATGGCCTTGGC
1-194 (reverse)	GGATCCTCTAGATCAGTGGCTGCCCAGCCACTGGAT
110-437 (forward)	AGTACTAAGCTTGGCAGAGGCCG
110-437 (reverse)	AGCTTAGCGGGCCGCTCAGAAATCCAGGGGGGT
110-194 (forward)	GCCGCGAATTCAGGCAGAGGCCGCCATCCAGG
110-194 (reverse)	GGATCCTCTAGATCAGTGGCTGCCCAGCCACTGGAT
146-437 (forward)	TATCTAGGATCCTCGGCTGACGGTGTCGT
146-437 (reverse)	AGCTTAGCGGGCCGCTCAGAAATCCAGGGGGGT
194-437 (forward)	ACGTCCAAGCTTCACACCACAGT
194-437 (reverse)	AGCTTAGCGGGCCGCTCAGAAATCCAGGGGGGT
Following PCR, sticky ends were generated using HindII and BamH1 restriction endonucleases in Buffer E for all mutants apart from 110-194, for which XbaI and EcoRI were used.	

2.8 SDS-PAGE and immunoblotting

Samples were mixed with SDS loading dye (final concentrations 125mM β-mercaptoethanol, 50mM Tris-HCl pH6.8, 2% SDS, 7.5% glycerol, 0.1% bromophenol blue) and heat denatured at 100ºC for 5 minutes before separation by SDS-PAGE using the Mini Protean III system (Biorad). Acrylamide gels [resolving: either 8 or 15% acrylamide, 375mM Tris-HCl pH 8.8, 0.1% SDS, 0.15% ammonium persulphate (APS), 0.15% tetramethylethylenediamine (TEMED); stacking gel: 3.2% acrylamide, 125mM Tris-HCl pH 6.8, 0.1% SDS, 0.15% APS, 0.15% TEMED] were run in Laemmli

running buffer (25mM Tris, 192mM glycine, 0.1% SDS) at 180V. ColorPlus Prestained Protein adder (New England Biolabs) was run alongside samples to estimate molecular mass. Electrophoresed samples were transferred onto Protran nitrocellulose membrane (Whatman) in Laemmli transfer buffer (25mM Tris, 192mM glycine) at either 150mA overnight or 400mA for 1 hour.

Membranes were stained with Ponceau S dye (Sigma) to check for even protein transfer and incubated in blocking solution [5% non-fat milk, 0.3% Tween-20, phosphate buffered saline (PBS)] for 15 minutes before overnight incubation at 4ºC with the appropriate antibody (diluted in blocking solution) at the concentration presented in Table 2.4. Membranes were then washed 3 times in 0.3% Tween-20/PBS for 10 minutes per wash before 30 minutes incubation at room temperature with the relevant horseradish peroxidase (HRP)-tagged secondary antibody in blocking solution. Signal intensity was measured using SuperSignal West Pico Substrate (Pierce) and exposing X-ray films (Kodak) to the membranes. Films were developed using the AGFA CP1000 automatic developer.

Table 2.4: List of antibodies and their working dilutions

Antibody	Supplier	Species	Working dilution
HA (11)	Covance	mouse	1/1000
Actin	Sigma	mouse	1/2,000,000
E2F-1 (KH95)	Santa Cruz	mouse	1/1000
p53 (DO-1)	Santa Cruz	mouse	1/1000
NEDP1	Gift from Ron Hay, Dundee, UK	sheep	1/1000
NEDD8	Alexis Biochemicals	rabbit	1/1000
Set7/9	Abnova	mouse	1/1000
methyl K185-E2F-1	Eurogentec (custom synthesised)	rabbit	1/50
Mouse IgG (non-specific)	Jackson Immunoresearch	mouse	As specific antibody
Rabbit IgG (non-specific)	Jackson Immunoresearch	rabbit	As specific antibody
HRP-anti mouse	DAKO	rabbit	1/15,000
HRP-anti rabbit	DAKO	goat	1/15,000

2.9 Methyl-specific antibody characterisation

The E2F-1 K185 methyl-specific antibody was generated by Eurogentec by immunising rabbits with a cys-LIAKKSK[m]HIQW peptide, where K[m] is monomethylated. K185 methyl-specific antibody was purified by first passing serum over a column of Sepharose beads bound to unmethylated E2F-1 peptide to remove non-specific antibodies, and then passing the eluate over a second column of Sepharose beads bound to monomethylated E2F-1 peptide (as above). The methylated peptide column was washed and bound methyl-peptide specific antibody eluted.

In order to test the specificity of the E2F-1 K185-methyl-specific antibody, dot blots and peptide competition assays were used. For dot blots, 25µg synthetic E2F-1 peptide

(either cys-LIAKKSK[m]HIQW or the unmethylated cys-LIAKKSKHIQW) was spotted onto a nitrocellulose membrane and incubated with methyl-K185 E2F-1 antibody in 5% BSA/TBST overnight at 4°C and developed after addition of a HRP-tagged secondary antibody. For peptide competition assays, an *in vitro* methylation reaction was performed as described in Section 2.2 using 1µg E2F-1(146-437) protein and samples subject to SDS-PAGE and immunoblot analysis (Section 2.9). After Ponceau S staining, membranes were cut into strips each consisting of a single lane of the gel. Methyl-K185 E2F-1 antibody in 5% BSA/TBST was mixed with E2F-1 peptides (10µg/ml final concentration) and incubated for 1 hour at 4ºC, rotating. The peptide-antibody solutions were then incubated overnight at 4ºC with the membrane strips and developed after addition of secondary antibody.

2.10 Luciferase reporter assays

U2OS or SAOS2 cells were transfected as indicated and cells harvested after 48 hours, washed once in PBS and lysed in reporter lysis buffer (Promega). 50% lysate was used to measure luciferase counts on a luminometer (MicroLumat Plus LB 96V), 10% to measure β-galactosidase signal (absorbance at 420nm read after addition of buffer to a final concentration of 100mM Na_2PO_4 (pH7.3), 1mM $MgCl_2$, 50mM β-mercaptoethanol and 0.665mg/ml ONPG and incubation at 37°C), and 40% lysate analysed by immunoblot to measure protein levels. All conditions were performed in triplicate, and luciferase values were normalised according to their β-galactosidase reading.

2.11 Immunoprecipitation

U2OS cells were washed once in PBS and then lysed in a buffer consisting of 50mM Tris pH 7.5, 1% Igepal CA-630 (Sigma), 150mM NaCl, 1mM EDTA, 1mM NaF, 1mM Na_3VO_4, with protease inhibitors. Protein concentrations were determined by Bradford assay (Sigma) and equal protein added to each immunoprecipitation. Lysates were incubated overnight at 4°C with 1µg of the appropriate antibody and 30µl pre-washed Protein G agarose beads (Sigma). The beads were subsequently washed 5x with lysis buffer. Following protein elution with SDS loading buffer, analysis by SDS-PAGE and immunoblotting was performed.

2.12 Chromatin immunoprecipitation

U2OS cells were fixed for 10 minutes by the addition of formaldehyde (Sigma) to a 1% final concentration, prior to neutralisation with glycine at a final concentration of 0.125M. Cells were washed and harvested in PBS before lysis with a buffer containing 5mM Tris-HCl pH 8, 85mM KCl, 0.5% and Igepal CA-630. After 20 minutes on ice, cells were spun down and the buffer replaced by a second lysis buffer containing 10mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% Igepal CA-630, 1% deoxycholic acid sodium salt (DOC) and 0.1% SDS and the lysates sonicated 10-12 times at 25% maximum power for 10 second periods. Following a high speed spin, lysates were pre-cleared at 4°C for 3 hours with the appropriate non-specific antibody and 40µl of a 50:50 mix of protein A and protein G agarose beads (pre-blocked in a solution of 1mg/ml BSA, 400µg/ml sonicated salmon sperm and protease inhibitors). After pre-clearing, lysates were incubated overnight at 4°C with 2µg/ml of the appropriate specific antibody. Pre-blocked protein A and protein G agarose beads were added to the

lysates and incubated at 4°C for 2 hours before the beads were washed 4x with Buffer A (see below for buffer recipes), 6x with Buffer B, 6x with Buffer C and twice with 1M TE buffer. Protein/DNA complexes were eluted in a solution of 1% SDS and 0.1M $NaHCO_3$ and the protein and RNA removed from the mix by addition of NaCl (200mM), EDTA (10mM), Tris-HCl pH 6.5 (40mM), proteinase K (40µg/ml) and RNAse A (20µg/ml) (final concentrations). Samples were incubated for 3 hours at 55°C and then overnight at 65°C to allow the optimal temperature for both enzymes to be reached. After DNA purification [using the QIAquick PCR purification kit (Qiagen)], chromatin was analysed by PCR using Taq DNA polymerase (Stratagene) at an annealing temperature of 61°C and an extension time of 72°C. The primers used are described in Table 2.5.

Buffer A: 20mM Tris-HCl pH 8, 150mM NaCl, 2mM EDTA, 0.1% SDS and 1% Triton X-100 (Sigma).

Buffer B: 20mM Tris-HCl pH 8, 500mM NaCl, 2mM EDTA, 0.1% SDS and 1% Triton X-100

Buffer C: 10mM Tris-HCl pH 8, 250mM LiCl, 1mM EDTA, 1% Igepal CA-630 and 1% DOC

Table 2.5: Primer sequences for use in chromatin immunoprecipitation

Target	Direction	Sequence	Position relative to transcription start site
Cdc6	forward	GGCCTCACAGCGACTCTAAGA	-176 to - 156
	reverse	CTCGGACTCACCACAAGC	+ 54 to + 67
Cdc2	forward	GCTTGCGCTCGCACTCAGTTGGCG	-177 to - 154
	reverse	CAGATCCCTGACCTCCAGTCC	+ 199 to +219
Cdc25a	forward	TCTGCTGGGAGTTTTCATTGACCTC	-348 to - 324
	reverse	TTGGCGCCAAACGGAATCCACCAATC	-165 to - 140
Albumin	forward	TACATTGACAAGGTCTTGTGGAG	-166 to - 144
	reverse	TGGGGTTGACAGAAGAGAAAAGC	+ 36 to + 58

2.13 His-purification of NEDD8 substrates under denaturing conditions

H1299 cells were transfected as indicated and treated with MG132 (20µM; Calbiochem) 4 hours prior to harvesting in denaturing lysis buffer (8M Urea pH 8, 0.1M Na_2HPO_4/NaH_2PO_4, 0.01M Tris-HCl). Lysate was passed 6 times through a 23G needle and spun at 13,000rpm for 5 minutes (Eppendorf 5415R). After adjusting lysate volumes for protein concentration (diluting with lysis buffer), 70µl Ni^{2+} NTA-agarose beads (Qiagen) were mixed with lysate and β-mercaptoethanol (Sigma) and Triton X-100 added to a final concentration of 10mM and 0.1%, respectively. Samples were incubated at 4°C overnight, rotating. Beads were then washed twice with denaturing lysis buffer (above) with 10mM β-mercaptoethanol and 0.1% Triton X-100 and 3 times with denaturing lysis buffer adjusted to pH6.3 and with 10mM β-mercaptoethanol and 0.1% Triton X-100. NEDDylated proteins were then eluted with 100µl buffer containing 200mM imidazole, 5% SDS, 150mM Tris-HCl pH 6.8, 30% glycerol,

720mM β-mercaptoethanol and 0.005% bromophenol blue for 30 minutes with rotation at room temperature. 10% total lysate was used for immunoblot analysis of inputs.

2.14 His-purification of endogenous E2F-1 under native conditions

NEDD8-inducible U2OS cells were plated onto 10cm dishes (10 per condition), induced with 1µg/ml doxycycline after 24 hours and treated with MG132 (20µM; Calbiochem) after a further 24 hours. 4 hours after MG132 treatment cells were washed with PBS, lysed, and His-purification of NEDDylated E2F-1 performed using Ni^{2+} NTA-agarose (Qiagen) according to the manufacturer's instructions. For a non-specific binding control, nickel was removed from Ni^{2+} NTA agarose by incubating for 30 minutes with 50mM EDTA and these beads used in the purification.

Chapter 3

E2F-1 Methylation by Set7/9

3.1 Introduction

Since its original discovery as a histone lysine monomethyltransferase (Nishioka *et al.*, 2002; Wang *et al.*, 2001), Set7/9 has additionally been shown to monomethylate lysines on several non-histone proteins, including p53 (Chuikov *et al.*, 2004), Rb (Carr *et al.*, 2011; Munro *et al.*, 2010), ERα, (Subramanian *et al.*, 2008), and DNMT1 (Esteve *et al.*, 2009). In a study by Couture *et al.* (2006) structural analysis of Set7/9 bound to another of its substrates, TAF10, was performed. This was followed by mutational analysis to assess the importance of individual residues within the methylation sites of Set7/9 substrates (Couture *et al.*, 2006). The study revealed that Set7/9 recognises a conserved motif [Figure 3.1(A)(i-ii)] which was used to identify other potential targets for the monomethyltransferase. Amongst the list of candidate substrates identified was the cell cycle regulator E2F-1 [Figure 3.1(A)(iii)] where lysine methylation was predicted to occur within the DNA binding and heterodimerisation domain of the protein [Figure 3.1(B)].

The data presented in this chapter showed that E2F-1 is indeed a Set7/9 substrate and that E2F-1 is methylated by Set7/9 in its DNA binding and heterodimerisation domain. It was found that this methylation event impacted on E2F-1 transcriptional activity.

(A)

(i)	R/K - S/T/A - K- D/K/N/Q	Set7/9 consensus sequence	
(ii)	369-LKSKKGQ-375	p53	*Known Set7/9 substrates*
	186-SKSKDRK-192	TBP associated Factor-10 (TAF10)	
	2-SKSKDDA-8	TBP associated Factor-7 (TAF7)	
	1-ARTKQTA-7	Histone H3	
(iii)	182-KKSKNHI-188	E2F-1	

(B)

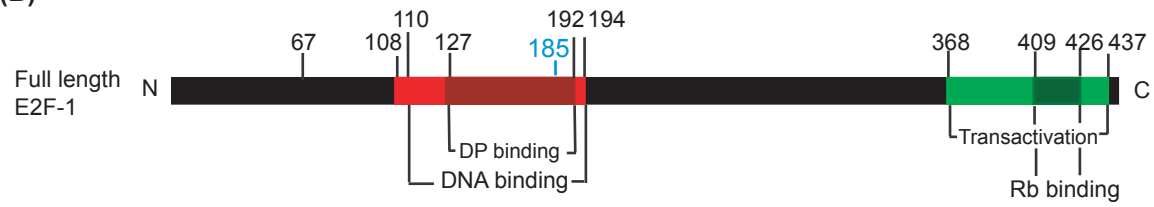


Figure 3.1: Set7/9 consensus in E2F-1
(A)(i) Set7/9 consensus site as described by Couture *et al.* (2006), with the methylated residue shown in red. (ii) Sites of methylation by Set7/9 in previously characterised substrates for the enzyme, the methylated residue is shown in red and numbers indicate the position of the amino acid residues flanking the site. (iii) Proposed Set7/9 target site within the E2F-1 protein, with the potential methylated residue shown in blue. (B) Schematic diagram showing the positions of the major domains of the E2F-1 protein. Amino acid positions are shown above, with the position of the predicted Set7/9 methylation site in blue.

3.2 E2F-1 is methylated by Set7/9 at lysine 185

To test the hypothesis that E2F-1 is a substrate for Set7/9, recombinant E2F-1 and Set7/9 were combined *in vitro* in the presence of radiolabelled Set7/9 cofactor, ^{3}H-SAM, and uptake of radiolabel recorded in a filter assay as a measure of protein methylation [Figure 3.2(A-C)]. Set7/9 is known to target lysine residues (Nishioka *et al.*, 2002), so a series of truncated mutants of E2F-1 were generated in which lysine residues were divided between them so that the region of E2F-1 that is the target for methylation could be identified. It was revealed that full length E2F-1 is methylated by Set7/9 *in vitro* and that this methylation occurrs between residues 146 and 194, since the 146-437 mutant could be methylated but the 1-145 and 194-437 mutants could not [Figure 3.2(A)].

There are five lysine residues between E2F-1 residues 146 and 194. In order to determine which of these is methylated, mutants of the 146-437 E2F-1 truncation were generated in which individual lysine residues within the 146-194 region were substituted for arginine residues and then tested for methylation in the *in vitro* methylation assay. The results suggested that methylation occurs at residue K185, since substitution of this residue significantly reduced the amount of radiolabel taken up by E2F-1 [Figure 3.2(B)].

To confirm that E2F-1 residue K185 is the primary target for methylation by Set7/9 in full length E2F-1, the K185R substitution was generated in the full length E2F-1 construct and tested for methylation in the *in vitro* methylation assay. This confirmed the importance of lysine 185 in the methylation of E2F-1 by Set7/9 as, similar to its

truncated (146-437) counterpart, the K185R mutant showed significantly less methylation than wild-type E2F-1 [Figure 3.2(C)]. It is noteworthy that substitution of residue K185 never completely ablated E2F-1 methylation, suggesting that another Set7/9 methylation site may exist. However, considering the four-fold decrease in radiolabel uptake for both the truncated (146-437) and full length K185R mutants with respect to wild-type E2F-1, it seems likely that the K185 is the major target site for methylation by Set7/9.

These findings were reiterated by autoradiography in which *in vitro* Set7/9 methylation reactions with either wild-type or K185R E2F-1 (full length), Set7/9 and ^{3}H-SAM were performed (as above), subject to SDS-PAGE and exposed to X-ray film [Figure 3.2(D)]. The autoradiograph showed a clear decrease in the amount of radiolabel uptake by (and hence the amount of methylation of) K185R with respect to wild-type E2F-1.

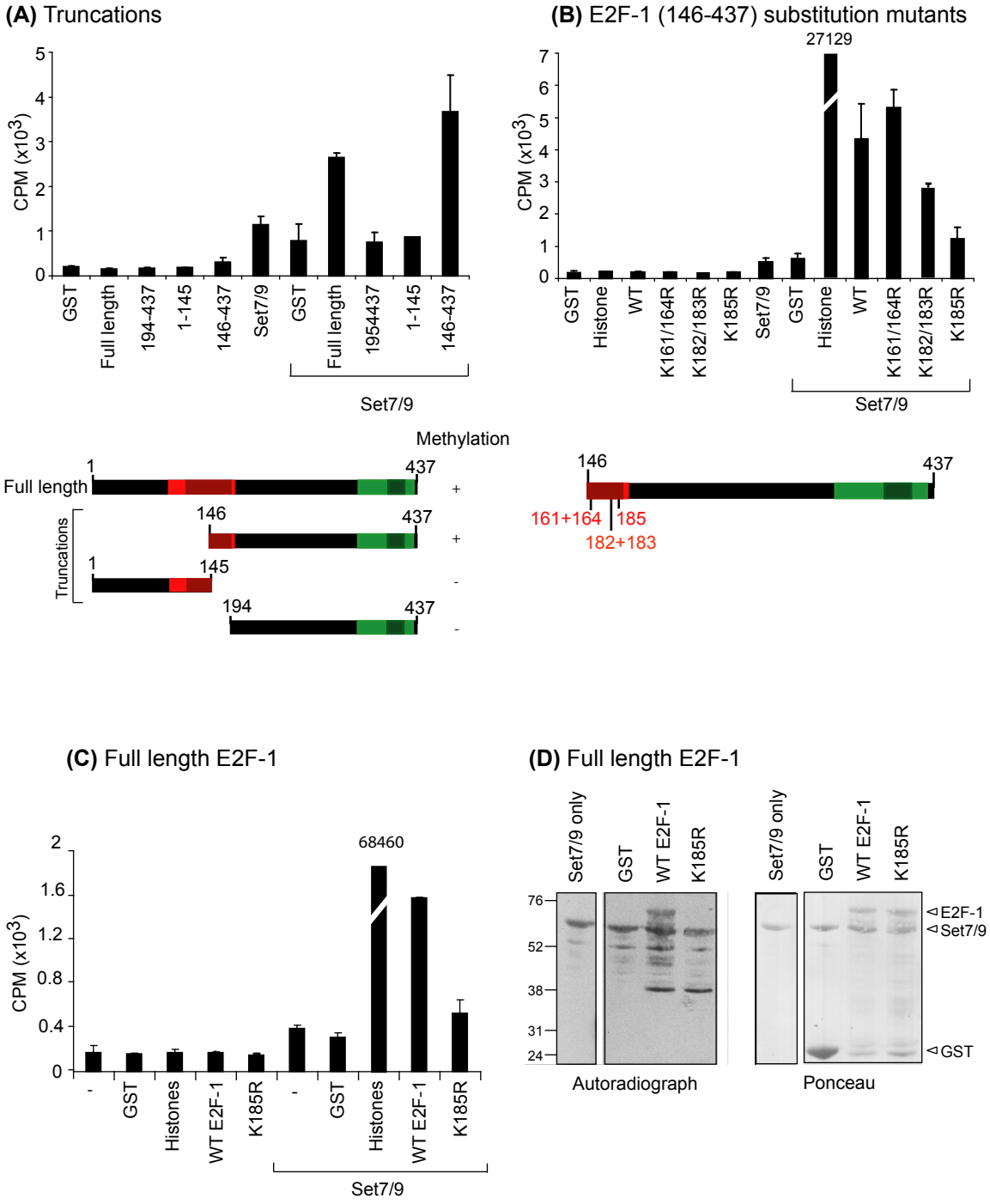


Figure 3.2: E2F-1 is methylated by Set7/9 at lysine 185
(A-C) Recombinant E2F-1, either truncated (A) 146-437 mutant with substitution mutants (B) or full length (C) was incubated with recombinant Set7/9 in the presence of ^{3}H SAM. Substrate methylation was quantified by measuring transfer of radiolabel, detected in a filter assay. A schematic comparing the residues spanned by the truncations relative to full length E2F-1 is shown in (A) and positions of the substitutions (shown in red) within the 146-437 truncated mutant used in (B) is also shown. (D) Autoradiograph of the methylation reaction in (C) showing uptake of radiolabel by recombinant E2F-1. The Ponceau of the membrane is also shown to demonstrate protein loading.

3.3 E2F-1 is methylated at lysine 185 in vivo

To confirm whether E2F-1 is methylated *in vivo*, an antibody was generated to specifically recognise E2F-1 monomethylated at lysine 185. In order to test the specificity of this antibody to its methylated substrate, it was used in a dot blot assay in which synthetic peptides were spotted onto nitrocellulose and incubated with methyl-K185 E2F-1 antibody at various concentrations [Figure 3.3(A)]. The antibody bound only to methylated peptide at all concentrations used, and there was little, if any, binding to the unmethylated peptide. Furthermore, immunoblot analysis of recombinant E2F-1 (146-437) following its methylation *in vitro* by Set7/9 showed specific binding of the antibody to methylated but not unmethylated E2F-1 (tracks 1 and 4). This was further supported by the observation that pre-incubation of the methyl-K185 E2F-1 antibody with synthetic methyl-E2F-1 peptide could compete out the signal generated by methylated recombinant E2F-1 (146-437) (tracks 3 and 6), an effect not observed upon pre-incubation with unmethylated peptide (tracks 2 and 5).

Having confirmed the specificity of the methyl-K185 E2F-1 antibody, it was used in an immunoblot analysis of immunoprecipitated wild-type or K185R E2F-1 from ectopically expressing U2OS cells [Figure 3.3(B)]. It was observed that wild-type E2F-1 was, indeed, methylated at K185 *in vivo* and that this methylation decreased under conditions of DNA damage caused by etoposide treatment.

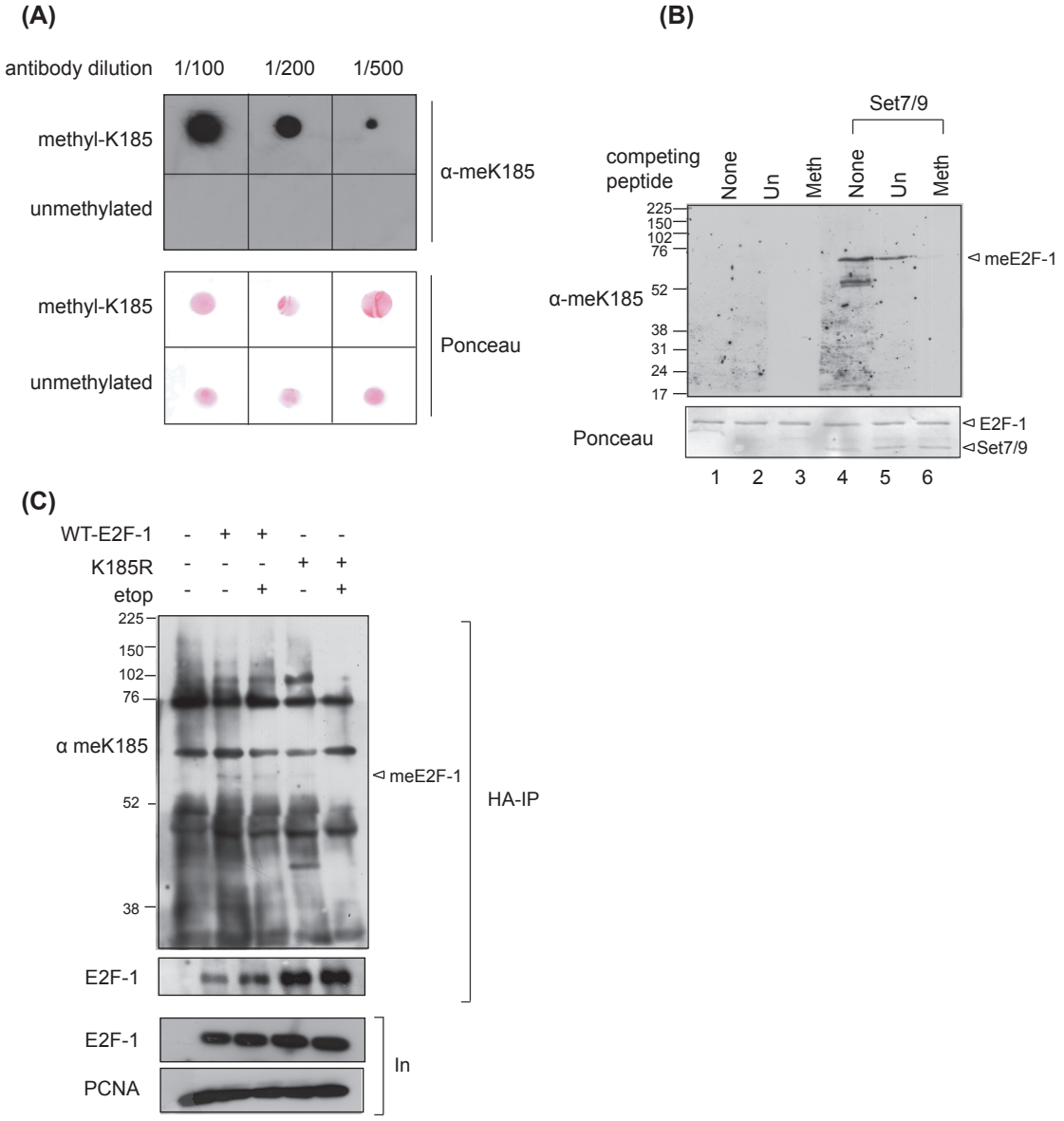


Figure 3.3: E2F-1 is methylated at lysine 185 *in vivo*
(A) 5µg E2F-1 peptide (either methylated at K185 or unmethylated) was spotted onto nitrocellulose and incubated with methyl-K185 E2F-1 antibody at various concentrations to demonstrate the specificity of the antibody. The Ponceau stain of the nitrocellulose is shown as a control for peptide loading (B) 1µg recombinant E2F-1(146-437) and Set7/9 were incubated together *in vitro* in the presence of ^{3}H-labelled SAM and then separated by SDS-PAGE. Methyl-K185 E2F-1 antibody was pre-incubated with either K185-methylated (Meth) or unmethylated (Un) competing E2F-1 peptide (5µg/ml) and then used in immunoblot analysis of the *in vitro* methylation assay. (C) U2OS cells were transfected with HA-E2F-1(1µg) and treated with etoposide (10µM) for 16 hours. E2F-1 was purified by immunoprecipitation and analysed by immunoblotting using the methyl-K185 E2F-1 antibody.

3.4 E2F-1 methylation affects its transcriptional activity

K185 is within the DNA binding domain of E2F-1 and, although it is not itself a direct DNA contact point, it is adjacent to residue N186 and close to K182, which are both DNA backbone contacts (Zheng *et al.*, 1999). The K185 residue itself is conserved throughout the E2F family members (Zheng *et al.*, 1999) suggesting that it is very important for E2F-1 function. Therefore any modifications to it might be expected to have some effect on the efficiency with which E2F-1 binds to its promoters. To investigate whether K185 methylation impacts on E2F-1 transcriptional activity, luciferase reporter assays were performed [Figure 3.4(A-C)]. U2OS cells were transfected with a vector containing the promoter of an E2F-1 responsive gene such as *cyclin E, Cdc6* or *p73* cloned immediately in front of the gene coding for luciferase. Therefore when E2F-1 activates the promoter, the luciferase gene is expressed and E2F activity can be measured indirectly using a luminometer detecting luciferase activity. Wild-type or K185R E2F-1 were ectopically expressed with luciferase reporter construct and β-galactosidase (as a measure of transfection efficiency) and the amount of promoter activity measured. It was evident that for *p73*, *cyclin E*, *Apaf-1* and *Cdc6* promoters, K185 methylation repressed activation by E2F-1, since the methylation defective K185R mutant exhibited greater reporter activity than the wild-type, despite even levels of protein expression [Figure 3.4(A)]. This effect was evident regardless of the amount of E2F-1 DNA transfected [Figure 3.4(A)(iv)], eliminating the possibility that the results observed were influenced by the previously documented "squelching" phenomenon in which the use of highly-expressed eukaryotic expression vectors can sometimes lead to a biased view of transcriptional effects (Lee *et al.*, 1998). However, the difference in transcriptional activation abilities of K185R versus wild-type E2F-1 was reversed at the *E2F-1* promoter, where the K185R mutant exhibited lower promoter

activity, suggesting that methylation at K185 may increase E2F-1 activity at this promoter [Figure 3.4(B)]. These findings suggested that the effect of E2F-1 methylation on promoter activity may be gene specific.

To confirm that the observed differences between wild-type and K185R E2F-1 could be attributed to the activity of Set7/9, both wild-type and K185R E2F-1 were expressed under conditions of Set7/9 siRNA depletion and the effect on the activity at the *Cdc6* promoter observed in a luciferase reporter assay [Figure 3.4(C)]. Despite a surprising overall decrease in promoter activity upon Set7/9 depletion (an effect that may be attributed to a non-specific effect of Set7/9 siRNA treatment since it affects both wild-type and K185R E2F-1 similarly), it was apparent that wild-type and K185R E2F-1 had approximately equal promoter activities under conditions of Set7/9 knockdown. This was in contrast to the significantly increased levels of promoter activity of K185R E2F-1 with respect to wild-type in the presence of Set7/9. This effect provides support for the hypothesis that the differences in promoter activity observed between wild-type and K185R E2F-1 result from E2F-1 methylation by Set7/9.

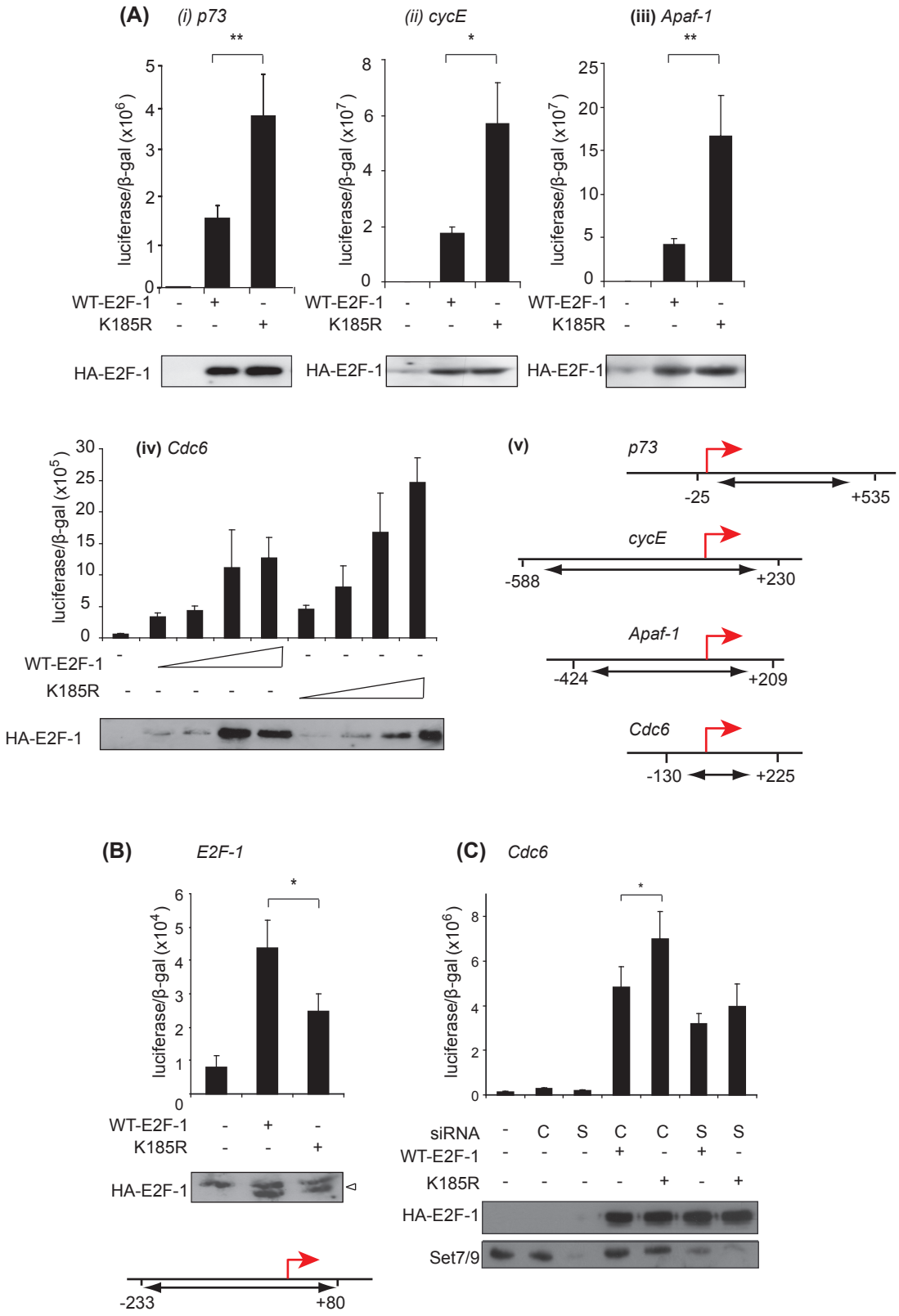


Figure 3.4: E2F-1 methylation affects its transcriptional activity
(A-C) E2F-1 (100ng) was co-expressed in U2OS cells with the indicated luciferase reporter construct (*p73*-, *E2F-1*-, *cyclin E*-, *Apaf-1*- or *Cdc6*-luciferase; 100ng) and β-galactosidase (100ng) and subject to Set7/9 (S) or control (C) siRNA treatment (where indicated). E2F-1 promoter activity was measured as described and input levels analysed by immunoblotting. (** $P < 0.01$, * $P < 0.05$, Student's t-test). Positions of the luciferase reporter constructs with respect to transcription start site (red arrow) are shown in (A)(v) and (B).

Further support for the hypothesis that E2F-1 methylation at K185 regulates E2F-1 activity at its promoters came from chromatin immunoprecipitation (ChIP) analysis in which wild-type or K185R E2F-1 were ectopically expressed in U2OS or SAOS2 cells and E2F-1 binding at various promoter DNA sequences observed [Figure 3.5(A-C)]. In U2OS cells, and consistent with the observations in the reporter assays in Figure 3.4, it was observed that E2F-1 (110-194) bound less efficiently at the *Cdc6* promoter than its methyl-deficient K185R counterpart [Figure 3.5(A)]. However, the effect was reversed at the *Cdc25a* promoter. Full length wild-type E2F-1 similarly showed reduced binding at the *Cdc6* promoter with respect to full length K185R E2F-1, and this effect was also evident at the *Cdc2* promoter. The increased binding of full length K185R E2F-1 at the *Cdc6* promoter was also apparent in SAOS2 cells. Since SAOS2 cells lack functional p53 and Rb (Shreeram *et al.*, 2002), it was concluded that these transcription factors do not influence the effects observed here. These results support the hypothesis that methylation of E2F-1 at K185 regulates its transcriptional activity in a gene specific manner.

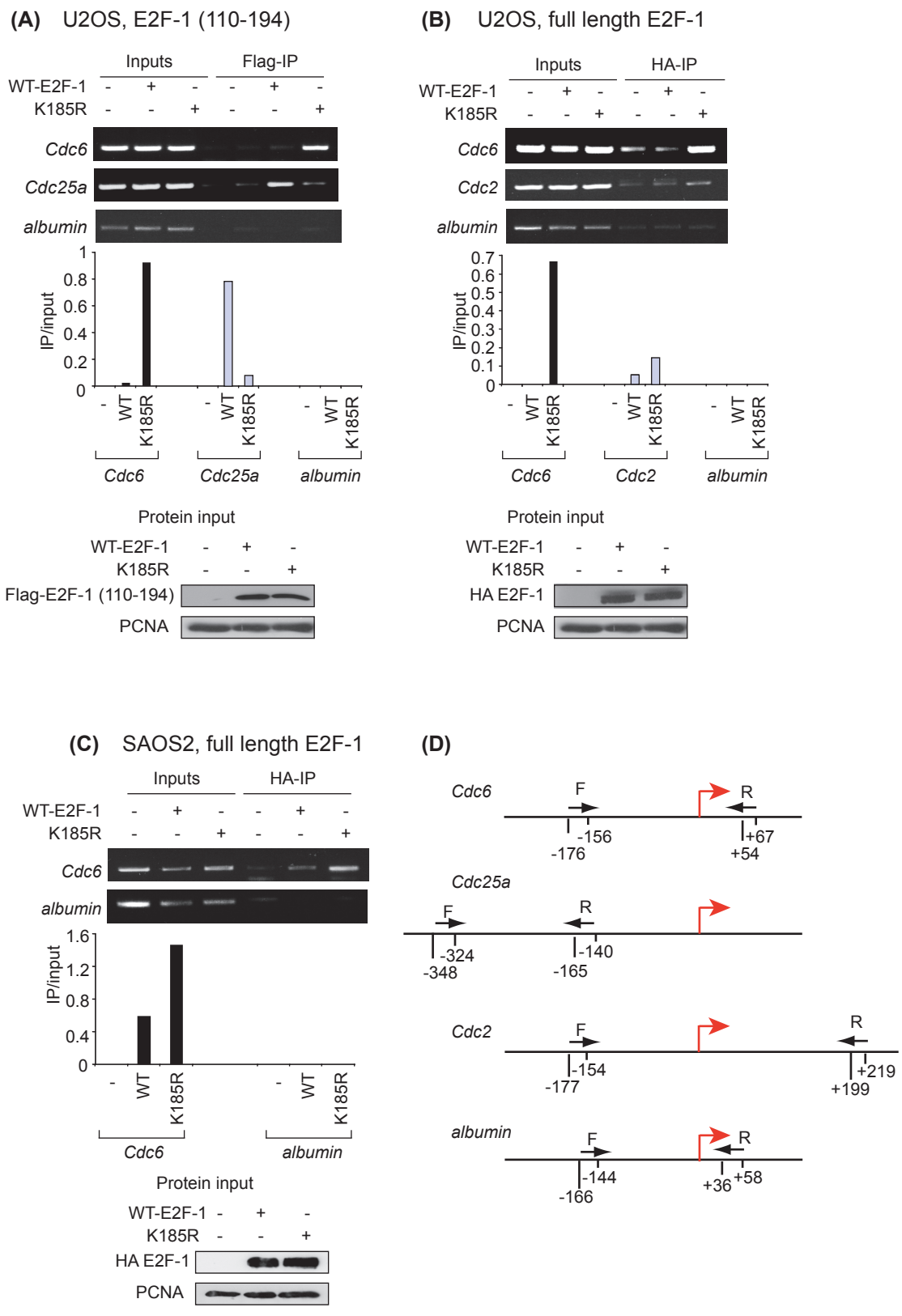


Figure 3.5: E2F-1 methylation affects its DNA binding
U2OS cells (A-B) or SAOS2 cells (C) ectopically expressing 1µg wild-type or K185R E2F-1 (110-194) (A) or full length E2F-1 (B-C) underwent chromatin immunoprecipitation (ChIP) with HA or Flag antibodies and analysis of E2F-1 binding to the indicated promoters performed by PCR. Quantification of agarose gel bands is presented underneath. Protein input levels were analysed by immunoblot. Positions of primers used in ChIP assays (forward, F; reverse, R) with respect to transcription start site (red arrow) are shown in (D).

3.5 Conclusions

The data presented in Chapter 3 found E2F-1 to be a substrate for the monomethyltransferase Set7/9 both *in vitro* and *in vivo* and that E2F-1 is methylated by Set7/9 at residue K185, within the DNA binding and heterodimerisation domain. It was also demonstrated here that methylation of E2F-1 at K185 results in gene specific regulation of E2F-1 activity, with methylation having a negative effect on E2F-1 activity at the *p73*, *cyclin E*, *Apaf-1*, *Cdc6*, and *Cdc2*, promoters, but a positive effect at the *E2F-1* and *Cdc25a* promoters.

The results support the findings by Couture *et al.* (2006) that the consensus site for Set7/9 binding and methylation is R/K - S/T/A - <u>K</u> - D/K/N/Q, where <u>K</u> is the methylated residue, and E2F-1 can now be added to the list of Set7/9 substrates targeted at this motif. By generating an antibody directed against methyl-K185 of E2F-1, methylation was shown to occur in cells. An important experiment to demonstrate that this methylation was Set7/9 dependent would be to observe the disappearance of the methyl-E2F-1 band under conditions of Set7/9 siRNA depletion. However, the methyl-specific antibody was extremely difficult to use as it has high sensitivity and poor selectivity so that immunoblots often had very high background. Therefore it was often challenging to identify specific bands corresponding to methyl-K185 E2F-1 and although the experiment using Set7/9 siRNA depletion was attempted many times, a clear result was never obtained. Despite this shortcoming, the data presented in Chapter 3 strongly support a role for E2F-1 methylation in cell cycle control.

It is evident that the difference in DNA binding between wild-type and K185R E2F-1 at the Cdc6 promoter observed in the ChIP assays [Figure 3.5] is significantly more prominant than the difference in promoter activation at the same promoter demonstrated in reporter assays [Figure 3.4(A)(iv) and (C)]. This discrepancy most likely reflects differences in the assays themselves, for example, reporter assays represent a very artificial system, where E2F-1 binds an artificial plasmid, whereas during ChIP assays E2F-1 binds to native promoters. Since the reporter plasmid was generated in bacteria, perhaps it is modified differently to native DNA, for example by DNA methylation, which may affect E2F-1 binding. Additionally, the reporter plasmid and the ChIP primer sequences encompass different regions of the Cdc6 promoter, perhaps accounting for the differences in binding. The ChIPs presented here are only semi-quantitative, with the quantification performed by comparing intensities of the bands on the agarose gel. Perhaps the differences between the data from ChIP assays and the reporter assays could be minimised by quantifying ChIP assays using real time PCR. Nevertheless, both the reporter assays in Figure 3.4 and the ChIP assays in Figure 3.5 are consistent in that they both demonstrate that methylation of E2F-1 at K185 impacts on its transcriptional activity.

The differential control of E2F-1 target genes by methylation of E2F-1 at K185 is interesting as it suggests that the methylation event is involved in multiple differently-regulated cellular pathways and may have a complex role to play in the bipolar behaviour of E2F-1. It is difficult to conclude how methylation fits into the overall control of E2F-1 cell cycle regulation since the genes activated or repressed in response to methylation do not fit into any discrete categories (for example apoptotic versus cell cycle progression or the number of E2F-1 response elements the promoters contain) and

further experiments are required to determine this. In particular, it would be beneficial to know how E2F-1 methylation impacts on expression of the E2F-1 target genes endogenously by measuring either protein levels in an immunoblot or mRNA levels by RT-PCR. Possible explanations for the similar regulation of apoptotic and tumour suppressive promoters by methylation are discussed in Chapter 7. The positive role of E2F-1 methylation in activity at its own promoter could perhaps be part of a feedback loop to help control the expression of the majority of E2F-1 response genes, which on the whole seem to be negatively regulated by methylation, but further experimental evidence would be required to support this hypothesis.

The observation that E2F-1 methylation by Set7/9 is downregulated under conditions of DNA damage is interesting as previous studies investigating the effect of damage on methylation of Rb and p53 and by Set7/9 have shown that methylation increases upon DNA damage where it promotes apoptosis (Carr *et al.*, 2011; Ivanov *et al.*, 2007). However, since E2F-1 is largely oncogenic and p53 tumour suppressive, and since Rb is a negative regulator of E2F-1 with Set7/9 methylation enhancing its ability to suppress E2F-1 function (Carr *et al.*, 2011; Munro *et al.*, 2010), perhaps this discrepancy is not surprising as it is consistent with a role for Set7/9 in helping govern a cell cycle arrest response to DNA damage.

The position of the K185 E2F-1 methylation site within a region so crucial for its function would suggest that it has a highly important role in controlling E2F-1 activity. The experiments presented here only begin to address this. ChIPseq or microarray analysis of E2F-1 promoter targets, for example, would be a useful tool in which to get

a better overall idea of the types of genes regulated by E2F-1 methylation at K185 and may provide a clearer picture of which pathways are affected by the methylation event. Experiments investigating the growth properties and cell cycle profiles of cells with methylated versus non-methylated E2F-1 would also help explain the role of E2F-1 methylation. Additionally, experiments investigating whether the monomethylation event at K185 promotes further methylation for example by the dimethylase, Suv39H1 would enhance understanding of the mechanism by which methylation by Set7/9 exerts its effects. In a similar vein, mass spectrometry analysis of methylated versus unmethylated E2F-1 could indicate the interplay of K185 methylation with other PTMs.

During the course of this study two independent studies were published, both demonstrating in a similar manner to the work described here that E2F-1 is methylated at K185 by Set7/9 and that this methylation affects E2F-1 transcriptional activity (Kontaki & Talianidis, 2010; Xie *et al.*, 2011). Much of the study by Kontaki and Talianidis (2010) is in agreement with the data presented here as they show that over-expression of Set7/9 resulted in decreased expression of p73 mRNA, reflected by a reduction in the amount of binding of E2F-1 at the *p73* promoter, and reduced cell death, an observation mirrored in this study by the reporter assay in Figure 3.4(A). They also show that the K185R mutant is more stable than wild-type E2F-1 and that methylation of E2F-1 is lost upon DNA damage. These two latter observations are reflected in the present study in Figure 3.3(C) where the level of expression of the K185R mutant is higher than that of the wild-type, despite transfection of an equal amount of DNA. In addition to the results presented in the current study, Kontaki and Talianidis (2010) show that E2F-1 K185 methylation can be reversed by over-expression of the demethylase LSD1. However, a major contradiction between the

Kontaki and Talianidis study and the current study is that the authors reported that their observations are only true in a $p53^{-/-}$ background and when p53 is expressed the effects of Set7/9 over-expression on cell death is reversed. In the current study, most experiments were performed in the p53 positive U2OS cell line and when the p53 null SAOS2 cell line was used [Figure 3.5(C)] the observations were similar to those of U2OS cells. Xie *et al*., similarly found that the effects of the K185 methylation event on E2F-1 transcriptional activity was the same in both p53 positive (U2OS) and p53 null (HCT116 $p53^{-/-}$) cells. However, in contrast to the findings of this study and that of Kontaki and Talianidis, Xie *et al.* (2011) found E2F-1 methylation to enhance its stability and transcriptional activity rather than suppressing them, thereby enhancing E2F-1 mediated cell death upon DNA damage. A comparison of the results presented here with those published in the Kontaki and Talianidis (2010) and Xie *et al*. (2011) studies is discussed in more detail in Chapter 7.

In summary, the data presented in this chapter identify E2F-1 as substrate for monomethylation by Set7/9, an event that impacts on its transcriptional activity, findings now supported by recent studies (Kontaki & Talianidis, 2010; Xie *et al.*, 2011).

Chapter 4

NEDDylation of E2F-1

4.1 Introduction

In addition to methylation, lysine residues are known to modified by several other PTMs including acetylation, ubiquitin and the ubiquitin-like family of PTMs, which includes the small ubiquitin-like modifier (SUMO) and NEDD8 (Herrmann *et al.*, 2007; Sims & Reinberg, 2008). It was therefore decided to investigate whether E2F-1 lysine residues are subject to other novel modifications that may, like methylation and other previously-described lysine-targeted E2F-1 PTMs (Pediconi *et al.*, 2003; Rizos *et al.*, 2005), impact on its activity. The data presented in this chapter investigated whether E2F-1 was subject to modification by the 9kDa protein, NEDD8 (Herrmann *et al.*, 2007)

The best characterised substrates for NEDDylation are cullins, the structural subunits of cullin RING E3 ubiquitin ligases (CRLs) where NEDDylation enhances CRL activity by inducing conformational changes within the complex. In recent years a diverse array of non-cullin substrates for NEDDylation have been identified including the growth factor receptor EGFR, caspase 7, ribosomal proteins and VHL (Broemer *et al.*, 2010; Oved *et al.*, 2006; Stickle *et al.*, 2004; Xirodimas *et al.*, 2008). The effect of NEDDylation on these proteins is diverse including changes in protein stability, protein localisation and recruitment of binding partners (Mahata *et al.*, 2012; Oved *et al.*, 2006; Russell & Ohh, 2008; Ryu *et al.*, 2011; Sundqvist *et al.*, 2009). Amongst the non-cullin NEDD8 substrates identified are transcription factors central to cell cycle regulation such as p53, where NEDDylation by either MDM2 or FBXO11 suppresses its transcriptional activity, and the p53 family member p73, where specific NEDDylation of the proapoptotic isoform, TAp73β, by MDM2 similarly attenuates transcriptional

activity as well as promoting its cytoplasmic localisation (Abida *et al.*, 2007; Watson *et al.*, 2006; Xirodimas *et al.*, 2004).

The data presented in this chapter demonstrated that E2F-1 is indeed a substrate for NEDDylation, and that this NEDDylation, like methylation, occurs within the DNA binding and heterodimerisation domain of the protein.

4.2 E2F-1 is NEDDylated in vivo

To assess whether E2F-1 is a substrate for NEDDylation, E2F-1 was ectopically expressed with His-NEDD8, and covalently modified NEDD8 substrates purified by His-tag purification under denaturing conditions [Figure 4.1(A)]. H1299 cells were used because the experimental conditions for His-NEDD8 purification had previously been optimised in this cell line (Xirodimas *et al.*, 2004). Additionally, H1299 cells are p53 null, meaning that possible effects of p53 NEDDylation could be ruled out. The purified substrates were subject to immunoblot analysis, probing for E2F-1. p53, a known NEDD8 substrate, was used as a positive control. E2F-1 was found to be a NEDD8 substrate as a clear band was observed after His-NEDD8-purification. Further, it could be concluded that the NEDD8-E2F-1 interaction was direct since only covalent modifications are preserved in the denaturing conditions under which the His-NEDD8 purification was performed. For NEDD8-p53, a doublet was evident resulting from polyNEDDylation of the p53 substrate (discussed in Chapter 1 and Xirodimas *et al.*, 2004).

To elucidate whether endogenous E2F-1 is NEDDylated, a stable inducible His-

NEDD8 cell line was developed in U2OS cells. Cells were induced overnight and His-tag purification performed under denaturing [Figure 4.1(B)(i)] or native [Figure 4.1(B)(ii)] conditions using nickel-stripped agarose beads as a negative control. His-NEDD8 substrates were subject to immunoblot analysis and probed for endogenous E2F-1. Using this approach it was evident that endogenous E2F-1 could be NEDDylated directly. The nickel stripped agarose beads were used as a negative control rather than uninduced cells because the His-NEDD8 inducible cell line was found to be slightly leaky (data not shown).

To rule out the possibility that the NEDDylated E2F-1 observed in Figure 4.1(B)(i-ii) was simply an artefact of non-specific binding to nickel itself, U2OS cells were transiently transfected with His-NEDD8 or empty vector and His-tag purification performed using Ni^{2+} conjugated agarose beads throughout [Figure 4.1(B)(iii)]. Despite an increase in the overall level of E2F-1 in the input for cells transfected with His-NEDD8 (perhaps resulting from a general effect of the transfection itself), NEDD8-E2F-1 could be seen after His-tag purification. In contrast, the empty vector control lane was blank. This confirmed that E2F-1 does not non-specifically bind to nickel and that the NEDD8-E2F-1 bands shown in Figure 4.1(B) all represent NEDDylated E2F-1.

For the His-tag purifications performed under denaturing conditions, the immunoblots are presented such that the shift in molecular weight of approximately 10kDa that occurs upon NEDDylation can be observed. The 10kDa shift was evident for all immunoblots in this study in which input protein levels (largely not NEDDylated)

were run alongside the NEDDylated E2F-1. In the interest of space and clear presentation, most blots in the study are presented without showing this mass shift.

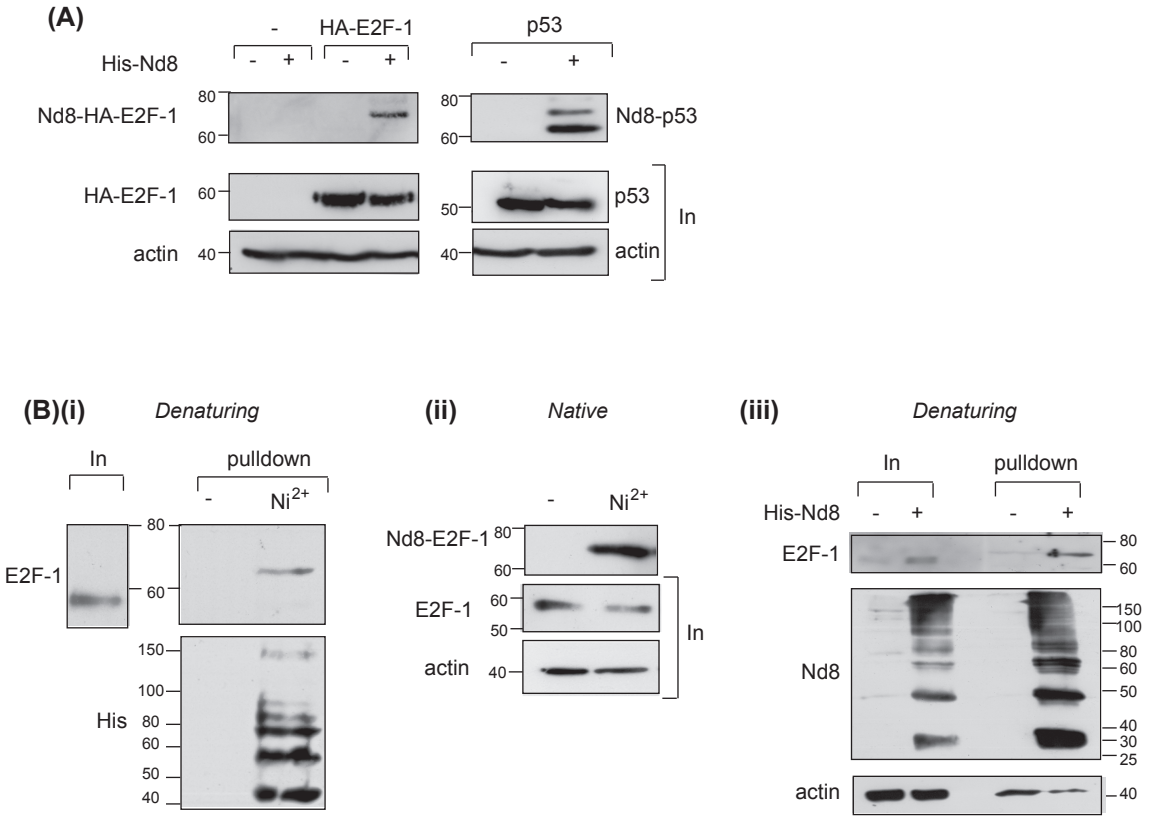


Figure 4.1: E2F-1 is NEDDylated *in vivo*.
(A) HA-E2F-1, empty vector or p53 (1µg) were co-expressed with His-NEDD8 in H1299 cells, NEDD8 substrates purified under denaturing conditions and analysed by immunoblotting with HA or p53 antibody. (B)(i-ii) U2OS cells stably expressing His-NEDD8 were subject to His-tag purification under denaturing (i) or native (ii) conditions using nickel-stripped agarose as a negative control. (iii) U2OS cells were transfected with empty vector or His-NEDD8 (3µg) and subject to His-tag purification under denaturing conditions. NEDD8 substrates were analysed by immunoblotting with E2F-1 antibody.

Further support for E2F-1 NEDDylation came from a double pulldown assay in which H1299 cells ectopically expressing HA-E2F-1 and His-NEDD8 were subject first to His-NEDD8 purification under denaturing conditions to isolate all NEDD8 substrates, and then to immunoprecipitation with HA antibody to purify only NEDDylated E2F-1 [Figure 4.2(A)]. Double purification allowed a greater yield of immunoprecipitated NEDD8-E2F-1 to be obtained, which permitted the visualisation of higher molecular weight species. These higher molecular weight bands may either indicate that E2F-1 is monoNEDDylated at multiple sites across E2F-1, or that a polyNEDD8 or mixed NEDD8-ubiquitin chain is formed on E2F-1, rather than a single NEDD8 moiety being added. Chains such as these have been described previously and are thought to result from a build-up of NEDD8 on UBC12 prior to conjugation (Watson *et al.*, 2011). Although rare, chains consisting of a mixture of NEDD8 and ubiquitin are also known to occur occasionally, but their function is not yet known (Jones *et al.*, 2008).

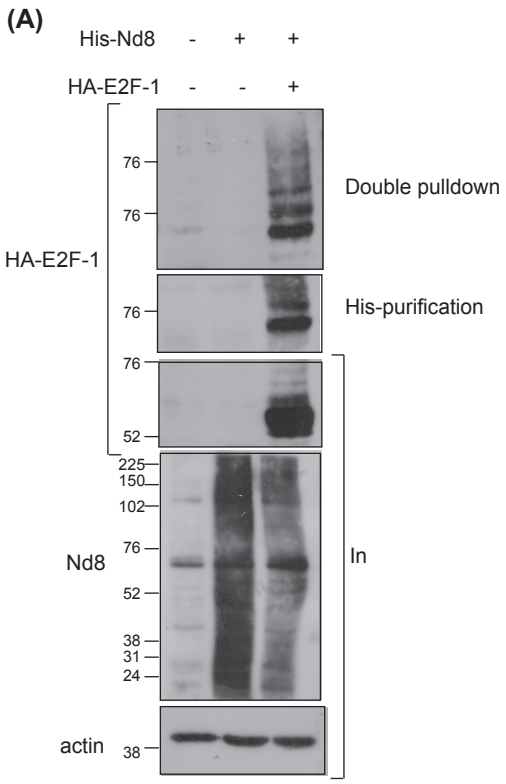


Figure 4.2: E2F-1 is polyNEDDylated
(A) U2OS cells expressing HA-E2F-1 and His-NEDD8 were subject to His-NEDD8 purification under denaturing conditions followed by HA-immunoprecipitation and purified protein analysed by immunoblotting.

4.3 E2F-1 NEDDylation is removed by NEDD8 specific inhibitors

NEDP1 (also known as DEN1 and SENP8) is a NEDD8-specific protease involved in processing the inactive NEDD8 precursor, exposing its diglycine motif so it may be conjugated onto its substrates, but it is also capable of removing NEDD8 from its substrates when overexpressed in cells (Mendoza *et al.*, 2003; Xirodimas *et al.*, 2004). NEDP1 over-expression was therefore used to further confirm the presence of NEDD8-E2F-1, by co-expressing NEDP1 with HA-E2F-1 and His-NEDD8 and performing His-NEDD8 purification under denaturing conditions. In doing so, a NEDP1 concentration-dependent decrease in the intensity of NEDD8-E2F-1 was observed [Figure 4.3(A)].

Further support for E2F-1 NEDDylation came from studying the effect of a dominant negative derivative of the E2 ligase UBC12 (C111S) which contains a single cysteine to serine substitution at the conserved cysteine in the E2 family (Wada *et al.*, 2000). This substitution results in a protein that binds covalently to the processed NEDD8 moiety with an ester bond that is so stable that it prevents subsequent transfer onto a NEDD8 substrate by an E3 ligase. NEDD8 is therefore sequestered by UBC12 (C111S) which diminishes the pool of free NEDD8 for conjugation by wild-type UBC12, thereby inhibiting the NEDD8 conjugation cascade (Wada *et al.*, 2000). Co-expression of UBC12 (C111S) in U2OS cells with HA-E2F-1 and His-NEDD8 followed by His-NEDD8 purification under denaturing conditions revealed a UBC12 (C111S) concentration dependent decrease in E2F-1 NEDDylation [Figure 4.3(B)].

With several recent publications suggesting that NEDDylation can occur via an atypical conjugation pathway in which enzymes of the ubiquitin conjugation cascade mediate

the attachment of NEDD8 when a cell has low levels of free ubiquitin relative to NEDD8 (Hjerpe *et al.*, 2012a; Hjerpe *et al.*, 2012b; Leidecker *et al.*, 2012), it was important to ascertain which conjugation pathway is responsible for the NEDDylation of E2F-1. Under conditions where both E2F-1 and NEDD8 were overexpressed (thereby increasing the level of free NEDD8 with respect to free ubiquitin), E2F-1 NEDDylation in cells could be almost completely ablated by treatment with the inhibitor of the "typical" NEDD8 conjugation cascade MLN4924 [Figure 4.3(C)]. In contrast, there was no change in the level of E2F-1 NEDDylation in this ectopic system upon depletion of UBE1, the E1 of the ubiquitin conjugation pathway [Figure 4.3(D)(i)]. As expected, under these same conditions the level of E2F-1 ubiquitination decreased significantly, thus confirming the inhibition of the ubiquitin conjugation pathway [Figure 4.3(D)(ii)].

These results showed that NEDDylation of E2F-1 can be inhibited by specifically blocking NEDD8 conjugation at either the E1 step using the NAE small molecule inhibitor MLN4924 or the E2 step by overexpressing a dominant negative derivative of UBC12, or by overexpressing the NEDD8 specific protease NEDP1. The experiments presented in Figure 4.3 were all performed under conditions of His-NEDD8 over-expression and proteasome inhibition, when the free NEDD8 to ubiquitin ratio would be expected to be perturbed, and the atypical NEDD8 conjugation pathway potentiated. Therefore, the results strongly support the finding that E2F-1 is NEDDylated and that this NEDDylation occurs via the physiological "typical" NEDD8 conjugation pathway rather than the non-physiological atypical conjugation system.

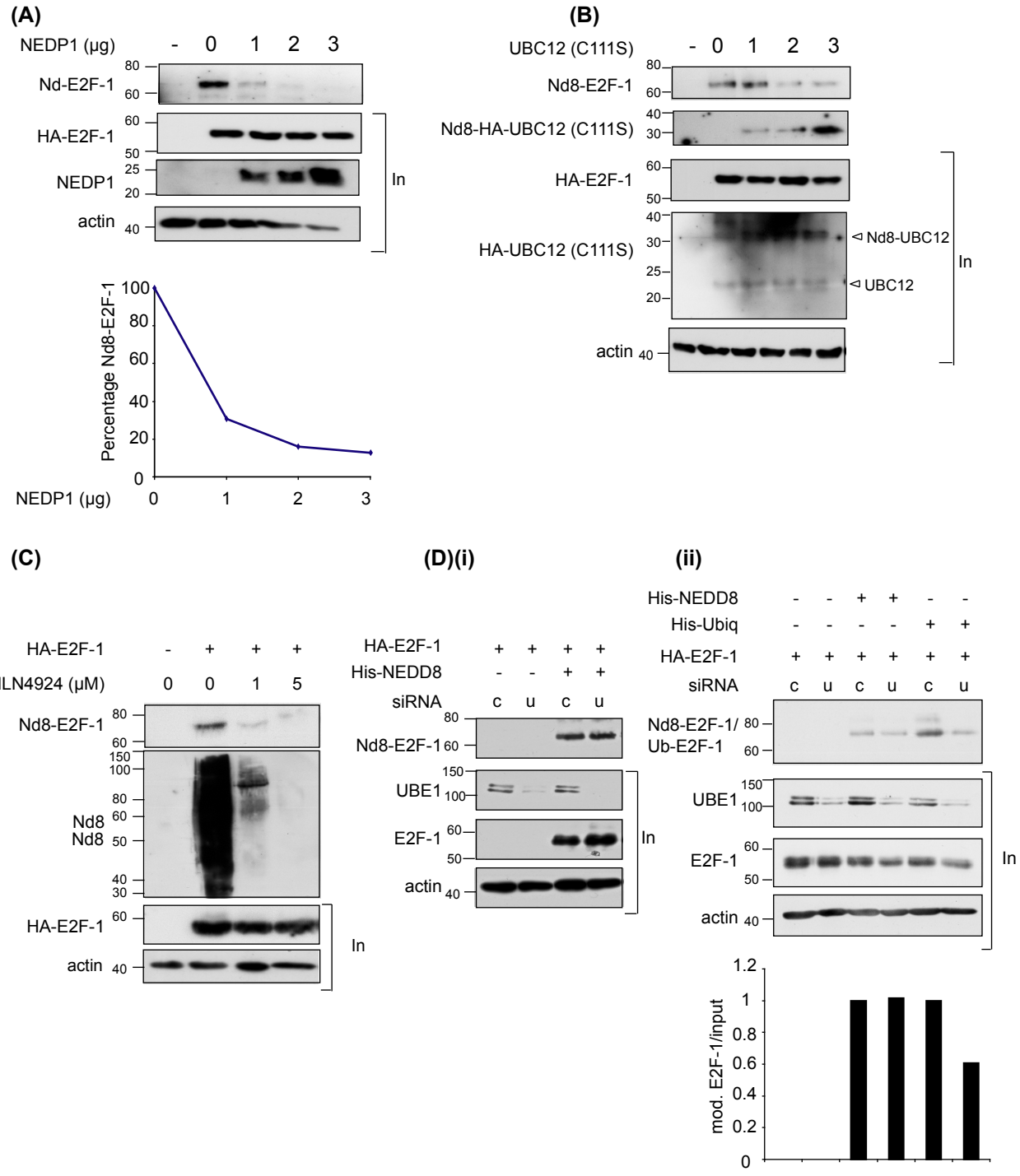


Figure 4.3: E2F-1 NEDDylation is removed by NEDD8 specific inhibitors
(A-B) U2OS cells coexpressing HA-E2F-1, His-NEDD8 and either NEDP1 (A) or UBC12 (C111S) (B) were subject to His-NEDD8 purification under denaturing conditions and NEDD8 substrates analysed by immunblotting with HA antibody. The concentration dependent decrease in NEDD8-E2F-1 with NEDP1 expression is also presented graphically. (C) U2OS cells co-expressing HA-E2F-1 and His-NEDD8 were treated with MLN4924 for 16 hours before they were subject to His-NEDD8 purification followed by immunoblot analysis. (D)(i-ii) U2OS cells co-expressing His-NEDD8 or His-ubiquitin and HA-E2F-1 were treated with either control (c) or UBA1 (u) siRNA, and NEDD8 substrates purified and subject to immunoblot anaylsis

4.4 Localisation of E2F-1 NEDDylation

In order to localise the site at which E2F-1 is NEDDylated, a series of truncations and lysine to arginine substitution mutants of E2F-1 were generated, co-expressed in U2OS cells with His-NEDD8 and the relative level of NEDDylation of these mutants with respect to wild-type E2F-1 observed following His-NEDD8 purification and immunoblot analysis [Figure 4.4(A-B)]. Analysis of NEDDylation of the E2F-1 truncation mutants revealed that NEDDylation occurred within the DNA binding and heterodimerisation domain of the protein (residues 110-194) since mutants not spanning this region (namely the 1-110 and 194-437 truncated mutants) showed evidence of reduced NEDDylation whereas truncations incorporating residues 110-194 [namely, full length (shown in Figure 4.1, Figure 4.2 and Figure 4.3) and the 110-194, 110-437 and 1-194 truncations] were efficiently NEDDylated [Figure 4.4(A)]. These findings were not surprising since NEDDylation occurs on lysine residues (Vertegaal, 2011) and most lysine residues within E2F-1 are situated within the DNA binding and heterodimerisation domain [Figure 4.4(B)(iv)]. Therefore substitution mutants were generated in which individual lysine residues within residues 110-194 were replaced with arginine residues and then assessed for their ability to be NEDDylated [Figure 4.4(B)]. Figure 4.4(B)(i) shows that NEDDylation was decreased for the K182/183/185R mutant with respect to wild-type E2F-1. Further analysis of these three residues in which individual lysines were substituted, revealed that K185R was the substitution responsible for the most significant decrease in NEDDylation [Figure 4.4(B)(ii)], suggesting K185 is important for E2F-1 NEDDylation.

When the array of E2F-1 mutants was expanded to include the K117/120/125R mutant, this too was found to have reduced NEDDylation with respect to wild-type

E2F-1 [Figure 4.4(B)(iii)]. This, when taken together with the observation that the K117/120R double mutant showed no decrease in NEDDylation with respect to the wild-type protein [Figure 4.4(B)(i)], suggests either that K125 is itself important for NEDDylation, or that E2F-1 NEDDylation is dependent on the integrity of all three of the K117, K120, K125 residues.

A noteworthy point to raise is the discrepancy between levels of NEDDylation of the K161/164R mutant with respect to wild-type E2F-1 between Figure 4.4(B)(i) where the level of NEDDylation of the mutant is decreased and Figure 4.4(B)(iii) where it is increased. It was found that there was no consistency in the level of NEDDylation of the K161/164R mutant compared to the wild-type over the course of the study, perhaps resulting from experimental variation, or a more complex level of regulation of NEDDylation at this site.

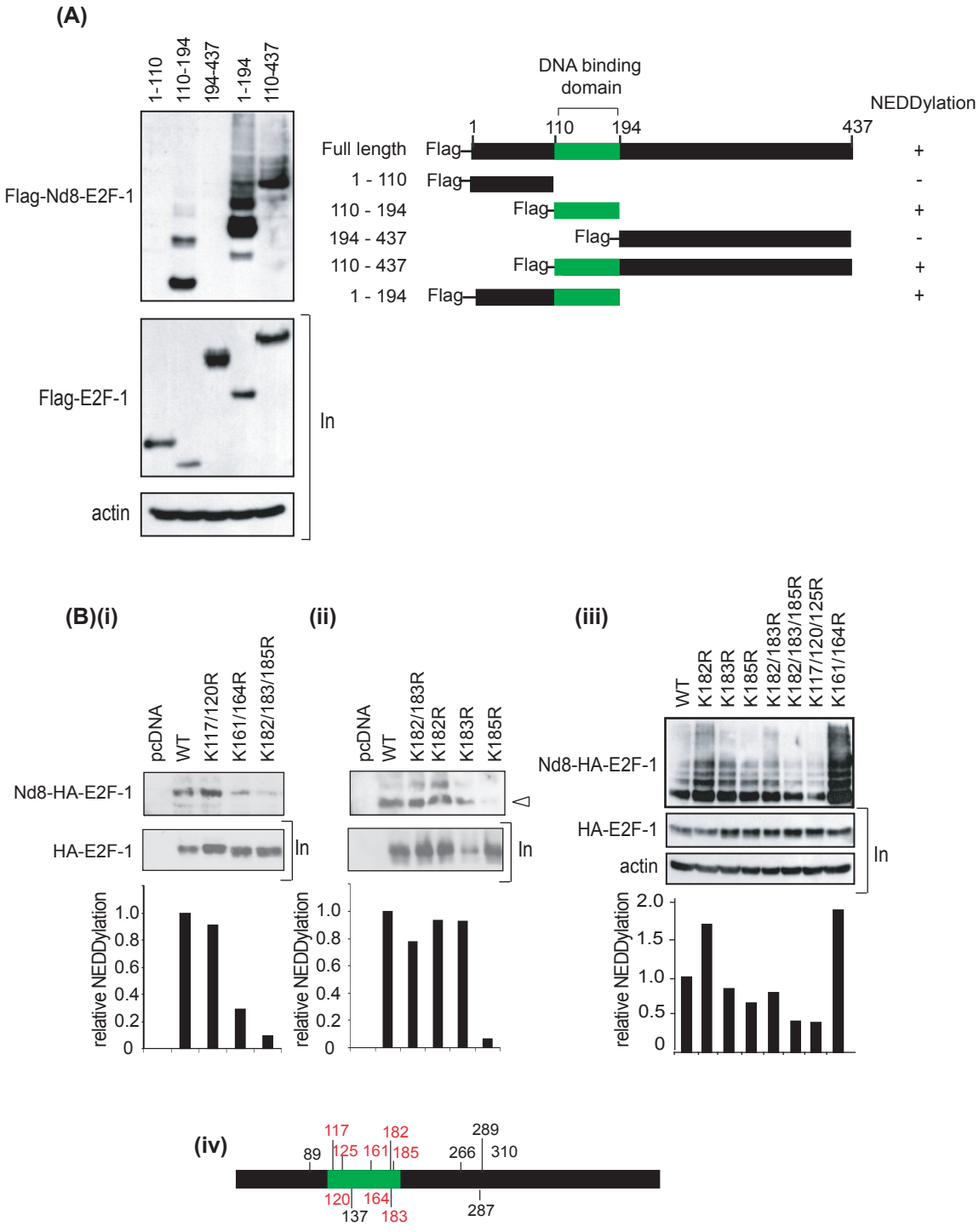


Figure 4.4: Localisation of E2F-1 NEDDylation
E2F-1 wild-type or truncated (A) or substitution (B) mutants were co-expressed with His-NEDD8, NEDD8 substrates purified by His-purification under denaturing conditions and analysed by immunoblotting. A schematic showing truncated mutants and whether they are NEDDylated is shown for (A) and the relative amount of NEDDylation for each mutant (wild-type NEDDylation set at 1) shown for (B). (B)(iv) The positions of all lysine residues in E2F-1 showing residues substituted for arginine in red.

4.5 Conclusions

The data presented in this chapter demonstrated that E2F-1 is NEDDylated *in vivo* and that residues K185 and K125 are important for NEDDylation to occur. E2F-1 may therefore be added to the growing number of non-cullin targets for NEDDylation. NEDDylation of other published targets has been reported to affect protein localisation, stability, transcriptional activity and association with other binding partners (Mahata *et al.*, 2012; Ryu *et al.*, 2011; Stickle *et al.*, 2004; Xirodimas *et al.*, 2004). The residues important for E2F-1 NEDDylation are within the DNA binding and heterodimerisation domain of the protein, which raised the possibility that NEDDylation may regulate E2F-1 transcriptional activity. This and other possibilities are investigated in the following chapter.

Very interestingly, the results from this study showed that E2F-1 residue K185 is important for both methylation by Set7/9 and NEDDylation. This raised the possibility that the two events are interconnected and that there may be a level of interplay between them, similar to that described for other PTM events on both E2F-1 itself (Kontaki & Talianidis, 2010) and other proteins such as Rb (Carr *et al.*, 2011) and p53 (Sims & Reinberg, 2008). This possibility is investigated in Chapter 6.

A limitation of the mutational analysis in Figure 4.4(B) was that technical difficulties prevented the generation of individual point mutants for lysines K117, K120 and K125. The observation that the K117/120R double mutant is as efficiently NEDDylated as wild-type E2F-1 suggests the residue important for NEDDylation is K125, but an alternative interpretation is that all three residues are required for the NEDDylation

event, perhaps due to the way the E3 ligase binds to the NEDD8 substrate. A similar sequence motif, in which three lysine residues are positioned in close proximity, was identified as being important for NEDD8 modification of p53 (Xirodimas *et al.*, 2004). Here, substitution of individual residues had no effect on NEDDylation, but mutation of all three resulted in a significantly reduced NEDD8 signal. There are therefore two possible explanations for the results presented in Figure 4.4(B). For the rest of this study the K185R and K117/120/125R mutants are used as NEDD8-deficient mutants to investigate the effect of NEDDylation on E2F-1 function.

Following the recent publication of studies suggesting that NEDDylation can occur via a non-physiological atypical pathway that involves enzymes of the ubiquitin conjugation pathway under conditions where the free ubiquitin to NEDD8 ratio is perturbed, for example after over-expression of NEDD8 (Hjerpe *et al.*, 2012a; Hjerpe *et al.*, 2012b; Leidecker *et al.*, 2012), it is now important when establishing a new target for NEDDylation to show that this NEDDylation can occur under physiological conditions and that the NEDDylation occurs via the "typical" NEDD8 conjugation cascade. In this chapter, it was shown that NEDDylation of E2F-1 can be ablated by inhibition of the NEDD8 conjugation pathway, but not by inhibition of the ubiquitin conjugation pathway [Figure 4.3(C-D)], thereby demonstrating that, at least for E2F-1, NEDDylation occurs via the physiological "typical" conjugation pathway. As the experiments presented here were all carried out under conditions of His-NEDD8 over-expression, it can be assumed appropriate to use these methodologies in the study of E2F-1 NEDDylation. Therefore, experiments in the following chapters frequently use this approach. Throughout this chapter, cells were treated with the proteasome inhibitor MG132 prior to harvesting since it was found that, without it, the level of NEDD8-E2F-

1 was too low to allow its detection by immunoblotting. Perhaps this is because NEDDylation makes E2F-1 unstable so that its existence is very short lived under physiological conditions. A similar trend was described in the study of p53 NEDDylation (Xirodimas *et al.*, 2004). Hjerpe *et al.* argue that MG132 treatment is one of the ways the free ubiquitin to NEDD8 ratio can be perturbed, since ubiquitin conjugates can no longer be recycled at the proteasome meaning they accumulate in the cell, depleting the free ubiquitin pool (Hjerpe *et al.*, 2012a). However, it was shown here that under conditions of both NEDD8 over-expression and proteasome inhibition by MG132 treatment, E2F-1 NEDDylation can be ablated by inhibition of the NEDD8 conjugation pathway but not the ubiquitin conjugation pathway (Figure 4.3). This suggested that any alterations in the ubiquitin to NEDD8 ratio caused by MG132 treatment do not drive NEDDylation via the atypical pathway and that use of MG132 in the experiments presented in this and the following chapters is justified.

Chapter 5

Impact of NEDDylation on E2F-1 activity

5.1 Introduction

Chapter 4 revealed E2F-1 to be NEDDylated, revealing that residues within the DNA binding and heterodimerisation domain of E2F-1 are important for this NEDDylation to occur. The NEDD8 moiety is a 9kd protein, so if NEDDylation were to occur within this region, it seemed likely that it would have a significant effect on the ability of E2F-1 to act as a transcription factor. Indeed, NEDDylation has previously been shown to affect the transcriptional activity of other important cell cycle regulators such as p53 and TAp73β where NEDDylation was found to attenuate the transactivation abilities of these proteins (Watson *et al.*, 2006; Xirodimas *et al.*, 2004). It therefore seemed likely that NEDDylation may also affect the transcriptional activity of E2F-1. For some NEDD8 substrates NEDDylation affects protein stability. For example NEDDylation of HIF1α and ribosomal protein L11 results in an increase in their stabilities (Ryu *et al.*, 2011; Xirodimas *et al.*, 2008). However NEDDylation destabilises cullins (Wu *et al.*, 2006) and EGFR (Oved *et al.*, 2006). Taken together with the observation that NEDDylation of E2F-1 was difficult to detect unless cells were treated with the proteasome inhibitor MG132 (as discussed in Chapter 4), it seemed likely that E2F-1 NEDDylation may impact on its stability.

Previous studies investigating the role of NEDDylation of non-cullin substrates did not identify the conditions under which NEDDylation occurs. Since E2F-1 has both oncogenic and tumour suppressive activities (Sahin & Sladek, 2010), knowledge of the conditions under which it is NEDDylated, may give a clue as to the role of E2F-1 NEDDylation in the cell, with the possibility that it may help guide E2F-1 towards either its apoptotic or cell cycle promoting functions.

These possibilities were investigated in this chapter where NEDDylation was shown to negatively regulate the activity of E2F-1 at its target promoters. It was also demonstrated that NEDDylation decreased E2F-1 stability, and that under conditions of DNA damage E2F-1 NEDDylation was decreased.

5.2 NEDDylation of E2F-1 negatively regulates its transcriptional activity

The impact of NEDDylation on the transcriptional activity of E2F-1 was investigated by measuring E2F-1 activity in the presence or absence of NEDDylation in luciferase reporter assays. U2OS cells ectopically expressing the NEDD8-specific protease NEDP1 were observed to have increased activity at the *cyclin E, Cdc6* and *p73* promoters with respect to the control [Figure 5.1(A)]. The reverse effect was observed upon ectopic expression of NEDD8 [Figure 5.1(B)]. These observations were recapitulated with the finding that ectopic E2F-1 activity was increased two-fold and four-fold at the *Cdc6* and *p73* promoters, respectively, upon over-expression of NEDP1 [Figure 5.1(C)]. An increase in ectopic E2F-1 promoter activity was also observed upon treatment of both U2OS and SAOS2 cells with the NEDD8 specific inhibitor, MLN4924 [Figure 5.2(A-B)]. When U2OS cells were transfected with either wild-type or the NEDD8 deficient mutants, K185R or K117/120/125R, and activity at the *p73* promoter observed, wild-type E2F-1 was found to have significantly lower promoter activity than the NEDD8-deficient mutants [Figure 5.2(C)]. This provided support for the hypothesis that the effects of the NEDD8 inhibitors observed in Figure 5.1(A-C) and Figure 5.2(A-B) directly resulted from E2F-1 NEDDylation. It could therefore be

concluded that NEDDylation has a negative impact on the transcriptional activity of E2F-1.

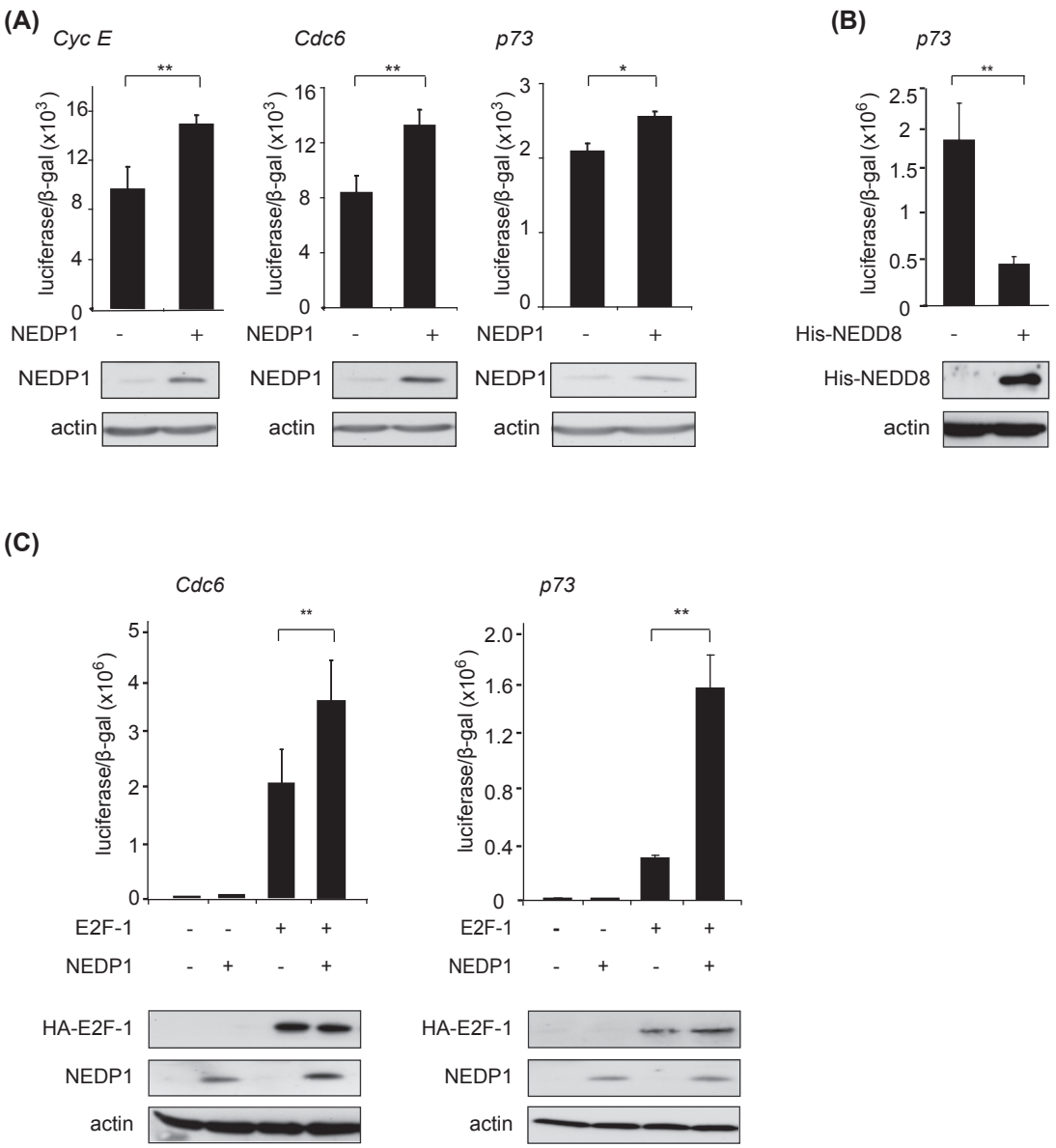


Figure 5.1: NEDDylation of E2F-1 negatively regulates its transcriptional activity
(A-C) HA-E2F-1 (100ng), NEDP1 (200ng) and His-NEDD8 (500ng) (where indicated) were co-expressed in U2OS cells with the indicated luciferase reporter construct (*Cyclin E*- *Cdc6*- or *p73*-luciferase; 100ng) and β-galactosidase (100ng). E2F-1 promoter activity was measured as described and input levels analysed by immunoblotting. (** $P < 0.01$, * $P < 0.05$, Student's t-test) The positions of the reporter constructs with respect to transcription start site are as detailed in Figure 3.4

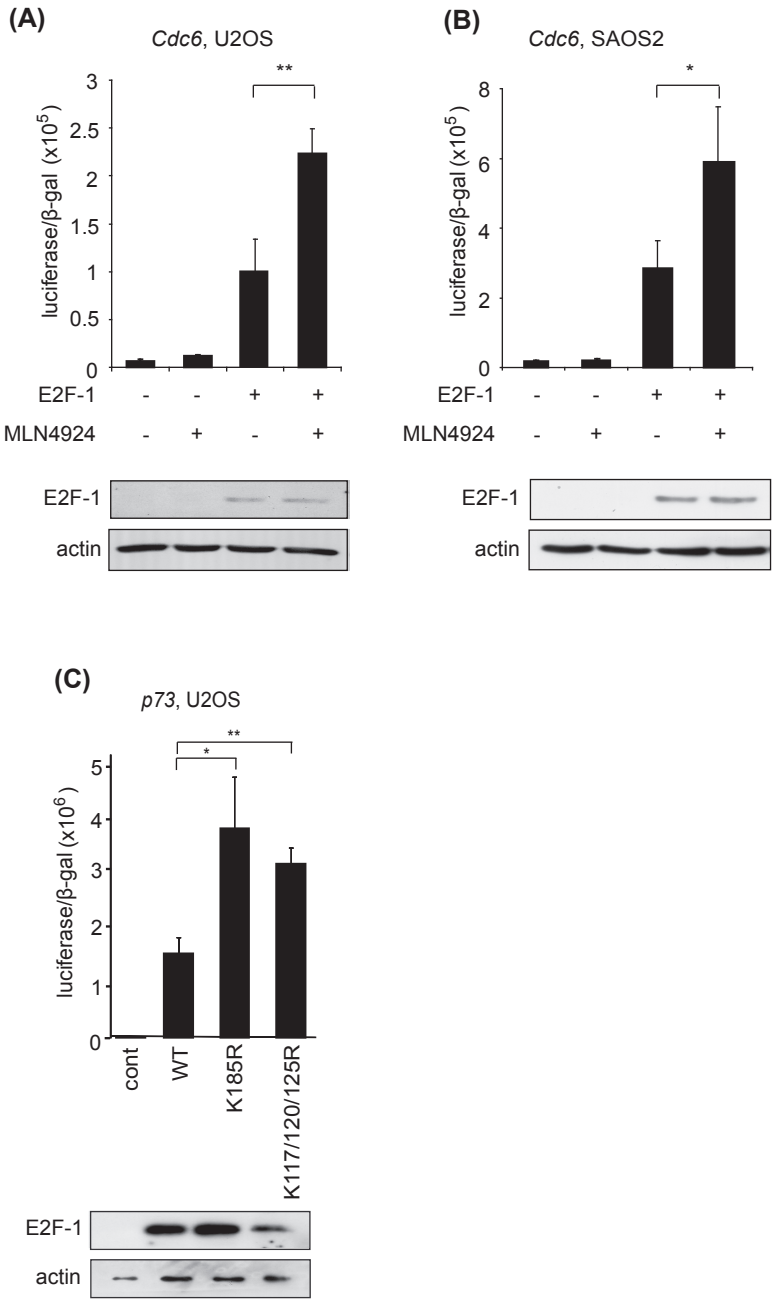


Figure 5.2: NEDDylation of E2F-1 negatively regulates its transcriptional activity
(A-C) E2F-1 (100ng) was co-expressed in U2OS or SAOS2 cells with the indicated luciferase reporter construct (*Cdc6*-luciferase or *p73*-luciferase; 100ng) and β-galactosidase (100ng) and where indicated treated with MLN4924 (1μM) for 16 hours. E2F-1 promoter activity was measured as described and input levels analysed by immunoblotting. (** $P < 0.01$, * $P < 0.05$, Student's t-test)

5.3 NEDDylation of E2F-1 decreases its protein stability

It had been noted that the level of endogenous E2F-1 often increased upon treatment with MLN4924. This effect was apparent for all cell lines tested [Figure 5.3(A)]. This, taken together with the knowledge that NEDDylation had previously been shown to impact on protein stability (Oved *et al.*, 2006; Ryu *et al.*, 2011; Xirodimas *et al.*, 2008), led to the hypothesis that NEDDylation may affect E2F-1 stability. To test this, His-NEDD8 and HA-E2F-1 were co-expressed in U2OS cells and His-tag purification performed under denaturing conditions at various time points after cyclohexamide treatment. NEDD8 substrates were analysed by immunoblotting and probed for HA-E2F-1 [Figure 5.3(B)]. It became apparent that the half-life of purified NEDD8-E2F-1 relative to the total E2F-1 input (assumed to be predominantly un-NEDDylated) was significantly reduced, suggesting that NEDDylation of E2F-1 does indeed decrease its stability. This was recapitulated by the observation that in U2OS cells co-expressing HA-E2F-1 and NEDP1, E2F-1 half-life was longer than that of cells expressing HA-E2F-1 alone [Figure 5.3(C)]. Further support came from the observation that wild-type E2F-1 had a shorter half-life than the NEDD8-deficient mutants, K185R and K117/120/125R [Figure 5.3(C)]. It was also evident from Figure 5.3(C) that the protein level of wild-type E2F-1 was significantly lower than that of the NEDD8-deficient mutants and wild-type E2F-1 when co-expressed with NEDP1, despite transfection of equal amounts of E2F-1 expression vector. Together these results provided strong support for the hypothesis that NEDDylation of E2F-1 decreases its stability.

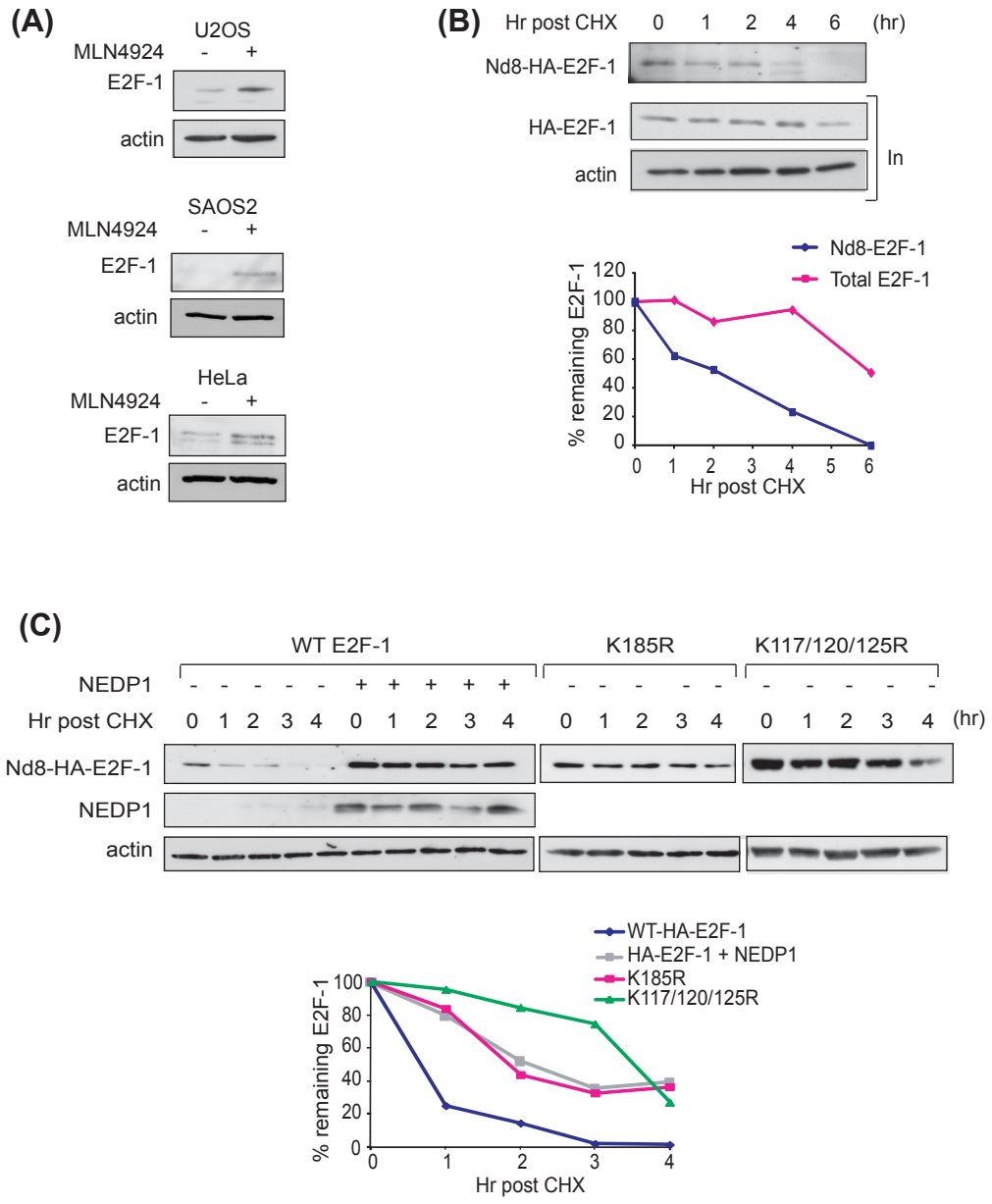


Figure 5.3: NEDDylation of E2F-1 decreases its protein stability
(A) U2OS, SAOS2 and HeLa cells were treated for 16 hours with MLN4924 (1μM), lysed and subject to immunoblot analysis probing for endogenous E2F-1. (B) U2OS cells transfected with expression vectors encoding HA-E2F-1 (1μg) and His-NEDD8 (4μg) were treated with cyclohexamide (100μM) and harvested at time points over 6 hours. NEDD8-E2F-1 was isolated by His-purification under denaturing conditions and the results analysed by immunoblotting, to compare the half-life of NEDDylated E2F-1 with that of total ectopic E2F-1. (C) U2OS cells expressing ectopic NEDP1 (2μg) and HA-E2F-1, K185R or K117/120/125R (1μg) were harvested over 4 hours following cyclohexamide treatment and analysed by immunoblotting. Quantification is shown below. The difference in half-life of WT E2F-1 between (B) and (C) resulted from experimental variation.

5.4 E2F-1 NEDDylation is downregulated upon DNA damage

As E2F-1 is known to be stabilised following DNA damage (Blattner *et al.*, 1999) and NEDDylation destabilises E2F-1, it was possible that E2F-1 NEDDylation was regulated under DNA damage. To investigate this, the effect of etoposide and doxorubicin treatment on the level of E2F-1 NEDDylation was observed and it was found that NEDDylated E2F-1 levels decreased by more than 80% under these conditions [Figure 5.4(A)]. Under conditions of DNA damage, E2F-1 was stabilised but treatment with MLN4924 no longer had the effect of increasing endogenous E2F-1 levels, providing support for the hypothesis that the increase in E2F-1 level under MLN4924 treatment is a direct result of a decrease in its NEDDylation [Figure 5.4(B)].

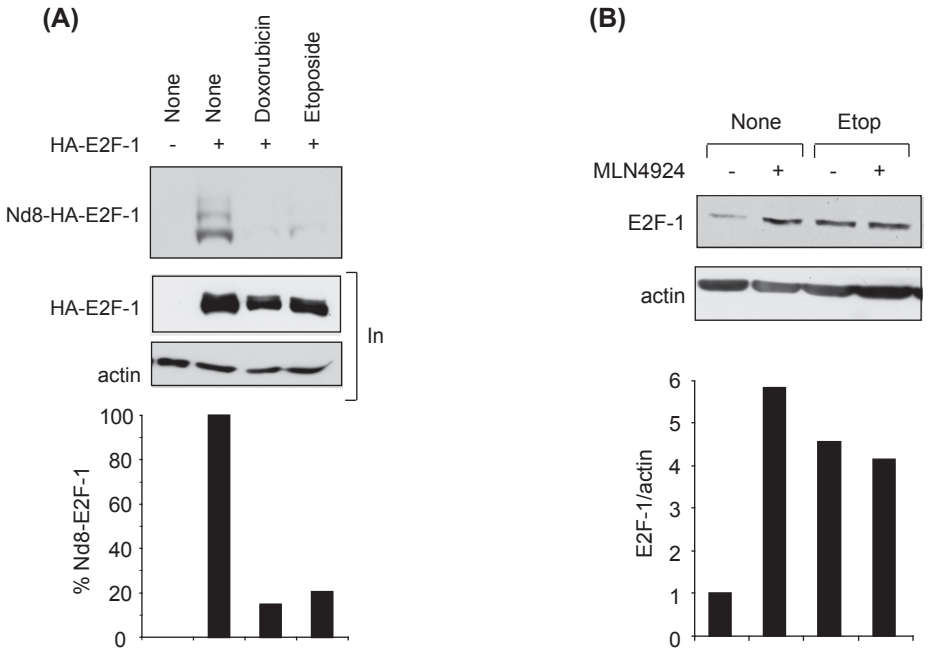


Figure 5.4: NEDDylation of E2F-1 decreases following DNA damage
(A) NEDD8 substrates were purified from U2OS cells ectopically expressing His-NEDD8 and HA-E2F-1 and treated with either etoposide (20μM) or doxorubicin (2μM) for 16 hours and the level of NEDD8-E2F-1 analysed by immunoblotting. (B) U2OS cells were treated with MLN4924 (1μM) and etoposide (20μM) for 16 hours and lysates subject to immunoblotting, probing for endogenous E2F-1.

5.5 Conclusions

The results presented in this chapter demonstrated that NEDDylation of E2F-1 negatively regulates its transcriptional activity and decreases its stability. It was also shown that E2F-1 NEDDylation is downregulated under conditions of DNA damage. These results are provocative since they suggest that E2F-1 activity is mediated via more than one mechanism by its NEDDylation. The absence of NEDDylation under conditions of DNA damage suggests that NEDDylation is specifically involved in the role of E2F-1 as a cell cycle promoter. The clinical relevance of protein NEDDylation has recently been highlighted with the success of the inhibitor of the NEDD8 conjugation pathway, MLN4924, in the treatment of acute myelogenous leukaemia in Phase I clinical trials (Soucy *et al.*, 2010). With the finding that the cell cycle regulator E2F-1 is also subject to NEDDylation, it seems likely that any anti-cancer effects observed following treatment with MLN4924, will at least in part, result from depletion of E2F-1 NEDDylation. Specifically, since it was suggested by the results presented in this chapter that E2F-1 NEDDylation is most active when E2F-1 is acting as an oncogene, inhibiting E2F-1 NEDDylation may be expected to decrease E2F-1 oncogenic potential. Based on data obtained during the current study, MLN4924 treatment would be expected to increase transcription from apoptotic genes such as *p73* and stabilise E2F-1, perhaps increasing its levels to the threshold required to induce its apoptotic activity.

The mechanism by which NEDDylation of E2F-1 exerts its effects is as yet unclear. Chromatin immunoprecipitation (ChIP) showed that expression of NEDP1 increased E2F-1 binding to the *E2F-1* and *Cdc6* promoters (Loftus *et al.*, 2012), suggesting that

NEDDylation downregulates E2F-1 transcriptional activity by preventing its binding to DNA. The large size of the NEDD8 moiety would make it likely that this may result at least in part from steric hindrance of the E2F-1 to access DNA by NEDD8. To investigate this possibility further, it would be interesting to generate a structural model for NEDDylated E2F-1 in complex with DNA. An important consideration that should be addressed is whether E2F-1 NEDDylation impacts on its heterodimerisation with DP-1, as perhaps this forms part of the mechanism of its inhibition of E2F-1 activity. The effects of E2F-1 NEDDylation on its protein stability could potentially stem from the similarity of the NEDD8 moiety to ubiquitin, or due to the formation of NEDD8-ubiquitin mixed chains, which may help target E2F-1 for degradation at the proteasome.

There are some obvious shortcomings in the data presented here, the most significant of which being that, similar to the effect observed for methylation of E2F-1, the reporter assays performed here investigating E2F-1 transcriptional activity showed that NEDDylation has similar effects at both the *Cdc6* and *p73* promoters. Since Cdc6 is concerned with cell cycle progression and p73 is involved in the apoptotic function of E2F-1 it is difficult to envisage a context in which both genes would need to be regulated in the same way. Since it has been shown that E2F-1 NEDDylation is downregulated upon DNA damage where E2F-1 would be expected to activate apoptotic pathways, it would seem logical that NEDDylation predominates when E2F-1 is acting as an oncogene, promoting cell cycle progression. As NEDDylation suppresses E2F-1 activity, perhaps it is involved in suppressing the activation of apoptotic genes by E2F-1. However, the observation here that NEDDylation similarly decreases E2F-1 activity at the *Cdc6* promoter does not fit this hypothesis. Further work is therefore required to uncover the mechanism and the functional consequences of E2F-1

NEDDylation. Possible explanations for the similar regulation of apoptotic and tumour suppressive promoters by NEDDylation are discussed in Chapter 7.

Significantly, the K117/120/125 residues identified here as being important for NEDDylation have also previously been identified as sites of E2F-1 acetylation (Martinez-Balbas *et al.*, 2000). E2F-1 acetylation causes increased protein stability and transcriptional activity, and is upregulated in response to DNA damage (Martinez-Balbas *et al.*, 2000; Pediconi *et al.*, 2003). In other words, acetylation has the opposite effect to NEDDylation. Therefore, it is tempting to speculate that the two modifications are mutually exclusive with acetylation occurring under DNA damage conditions to promote the apoptotic role of E2F-1 and NEDDylation occurring under non-damage conditions to promote the oncogenic role of E2F-1. This is not investigated further in the current study, but is discussed in more detail in Chapter 7.

Since residue K185 is important for both NEDDylation and methylation, some of the effects observed upon E2F-1 NEDDylation may reflect a mechanism involving both modifications, especially as methylation has been shown in the current study as well as by others (Kontaki & Talianidis, 2010) to impact negatively on E2F-1 transcriptional activity as well as its stability, similar to NEDDylation. The potential interplay between E2F-1 methylation by Set7/9 and E2F-1 NEDDylation is explored in the next chapter.

Chapter 6

Interplay between Methylation and NEDDylation on E2F-1

6.1 Introduction

Analogous to the "histone code hypothesis", the "protein code hypothesis" predicts that proteins are embellished at multiple sites with a plethora of PTMs ranging from the relatively small uncharged methyl and acetyl groups to larger charged phospho groups through to very large moieties of the ubiquitin like family (Sims & Reinberg, 2008). PTMs mediate the activity of their substrates in a combinatorial manner by forming binding platforms for cofactors and regulatory proteins. PTMs do not simply represent a permanent modification. Instead, the protein code is in a perpetual state of change involving continuous addition and subtraction of PTMs and interplay between different modifications often targeted to the same or a nearby site (Carr *et al.*, 2011; Ivanov *et al.*, 2007; Sims & Reinberg, 2008).

So far the study had shown that both methylation by Set7/9 and NEDDylation occur on E2F-1 and that K185 is important for both events. This raised the possibility that there exists interplay between these modifications, especially as it seems extremely unlikely that both modifications would be present at the ε-amino group of single lysine residue simultaneously. Furthermore there are many parallels between E2F-1 NEDDylation and its methylation; as both modifications exhibit a similar effect on E2F-1 transcriptional activity (i.e. repression), at least at the *Cdc6* and *p73* promoters, both modifications decrease the stability of E2F-1 and both are downregulated in response to DNA damage. If both methylation and NEDDylation were to occur at the same residue, their roles would be redundant and in direct competition with one another, leading one to question the purpose of the co-evolution of the two pathways. It therefore seemed more likely that methylation and NEDDylation were linked by a more complex mechanism,

with one modification event perhaps being a prerequisite for the conjugation of the other at another site. This would be analogous to the situation observed for the interplay between E2F-1 phosphorylation, acetylation and ubiquitination where phosphorylation at S31 promotes E2F-1 acetylation at K117/120/125 (Biswas & Johnson, 2012) and this acetylation is a prerequisite for its ubiquitination (Galbiati *et al.*, 2005). Methylation at K185 had been confirmed directly by using a methyl-K185 specific antibody, whereas E2F-1 NEDDylation at K185 had only been shown by indirect methods (discussed further in Chapter 7). The possibility therefore existed that methylation is the real modification at K185 and that NEDDylation occurs elsewhere on the protein, but requires the integrity of the K185 residue and, perhaps, its methylation. As the K117/120/125 residues were also shown to be important for NEDDylation, it was possible that these residues were the true NEDDylation sites.

In this chapter, this potential for interplay between methylation and NEDDylation was investigated. It was shown that interplay does indeed exist between these PTMs on E2F-1 and that methylation by Set7/9 is a prerequisite for NEDDylation. Additionally ubiquitin-like protein containing PHD and RING finger domains 1 (UHRF1) was identified as a potential mediator of this methylation-NEDDylation interplay.

6.2 E2F-1 methylation at K185 is required for its NEDDylation

To investigate whether Set7/9 activity impacts on E2F-1 NEDDylation, U2OS cells expressing His-NEDD8 and HA-E2F-1 were subject to Set7/9 depletion by siRNA and the amount of E2F-1 NEDDylation observed by performing a His-tag purification of NEDD8 substrates followed by immunoblot analysis [Figure 6.1(A)]. It was observed

that depletion of Set7/9 resulted in ablation of the NEDD8-E2F-1 signal, suggesting that Set7/9 activity is required for E2F-1 NEDDylation. Although NEDDylation of the K185R mutant also decreased under conditions of Set7/9 depletion, the effect was much smaller when compared to the wild-type. The small decrease observed for this mutant most likely resulted from either non-specific effects of Set7/9 depletion or due to the presence of a second Set7/9 methylation site within E2F-1 (as alluded to in Section 3.2), that potentially also has a minor impact on E2F-1 NEDDylation.

In support of the hypothesis that methylation by Set7/9 is required for E2F-1 NEDDylation, Set7/9 depleted cells had higher levels of promoter activation by E2F-1 than their Set7/9 expressing counterparts, similar to the effect of depleting NEDDylation by NEDP1 over-expression in Set7/9 expressing cells. In cells depleted of Set7/9, NEDP1 over-expression had no significant effect on promoter activity suggesting that methylation is required for E2F-1 to respond to NEDP1 [Figure 6.1(B)]. Similarly, there was no significant change in the transcriptional activity of the methylation-deficient K185R mutant under conditions of NEDP1 over-expression [Figure 6.1(C)]. Therefore it can be concluded that methylation of E2F-1 is required for its efficient NEDDylation.

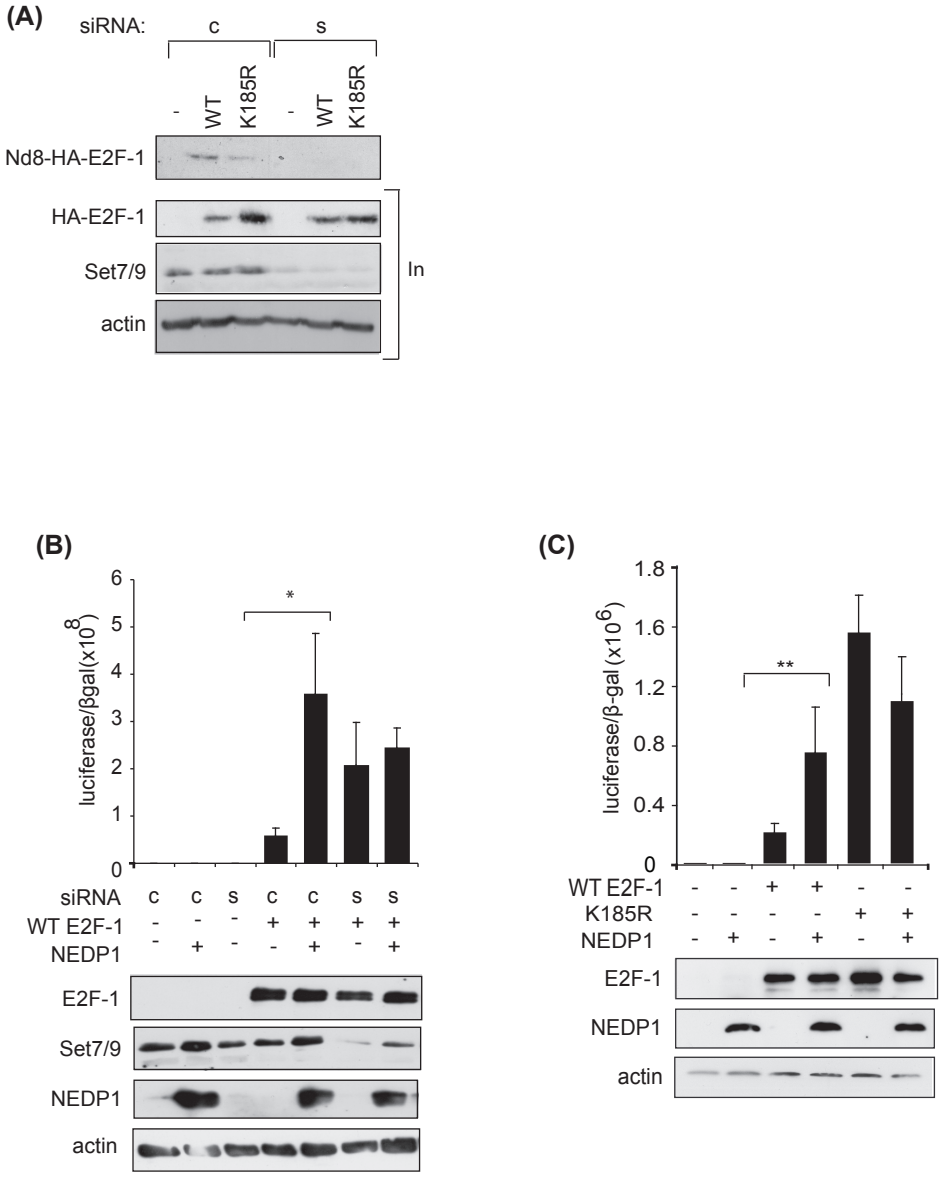


Figure 6.1: E2F-1 methylation at K185 is required for its NEDDylation
(A) NEDD8 substrates were purified from U2OS cells treated with control (c) or Set7/9 (s) siRNA and expressing HA-E2F-1 and His-NEDD8 and analysed by immunoblot. (B) E2F-1 (100ng) and NEDP1 (200ng), as indicated, were co-expressed in U2OS cells with *Cdc6*-luciferase reporter construct (100ng) and β-galactosidase (100ng) following treatment with either Set7/9 (s) or control (c) siRNA (20nM). Promoter activity was measured as described. Immunoblot analysis of protein expression level is shown. (C) Either E2F-1 or K185R (100ng) were co-expressed with NEDP1 (200ng), as indicated, with *Cdc6*-luciferase reporter (100ng) and β-galactosidase (100ng) plasmids and promoter activity measured as described. Immunoblot analysis of the protein expression level is also shown. (*P<0.05, **P<0.01, Student's t-test).

6.3 UHRF1 interacts with E2F-1 at methyl-K185

The finding that methylation of E2F-1 was required for its NEDDylation raised the possibility that another protein such as a cofactor of E2F-1 was mediating these effects. To investigate this, E2F-1 peptides were used in a chromatin-associated domain array (CADOR) (performed in collaboration with Mark T. Bedford, Graduate School of Biomedical Sciences, University of Texas, Houston, Texas, USA) to look for binding partners that specifically associate with E2F-1 methylated at K185. This approach utilises a microscopy slide displaying 174 different recombinant domains from chromatin associated proteins (the full list of domains represented on the CADOR array slide along with the uncropped CADOR array is presented in the Appendix). These peptides span the PTM binding domains of the proteins they represent (such as bromo, chromo, Tudor, PHD, SANT, SWIRM, MBT, CW and PWWP domains) (Kim *et al.*, 2006). Fluorophore-tagged peptides are passed over the chip and interactions detected (Figure 6.2). When E2F-1 peptides were passed over the CADOR chip, UHRF1 and PHF20L1 were identified as binding specifically to methylated, but not unmethylated E2F-1 [Figure 6.3(A)]. More precisely, UHRF1 was found to interact with E2F-1 via its Tudor domain and PHF20L1 via its MBT domain.

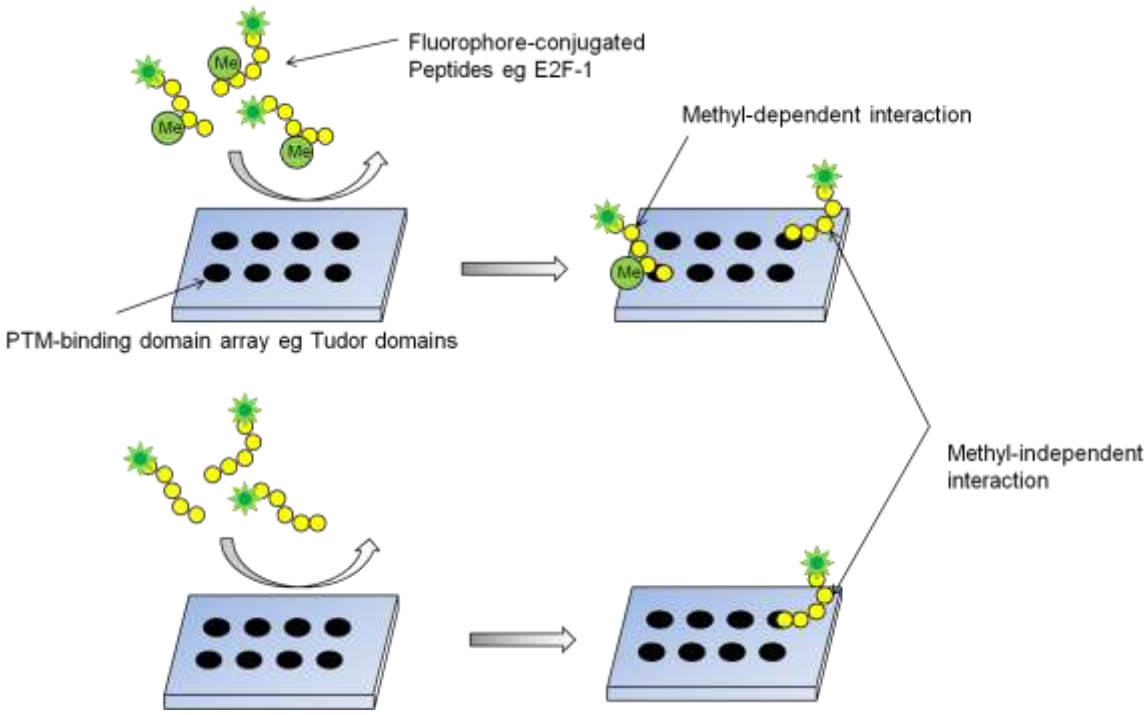


Figure 6.2: CADOR array for detection of methyl-dependent interactions between proteins

Fluorophore-conjugated peptides, either methylated (Me) or unmethylated are passed over a microscope slide displaying an array of PTM binding domains. Those peptides that bind to the slide generate a fluorescent signal in a position corresponding to the PTM-binding domain that it has adhered to.

UHRF1, also referred to as inverted CCAAT box-binding protein of 90kDa (ICBP90) or Nuclear protein 95kDa (Np95), is known to be important for cell cycle regulation owing to its ability to regulate gene expression through epigenetic mechanisms, and is required for cell cycle progression of non-cancerous cells (Tien *et al.*, 2011). It is also involved in conserving epigenetic inheritance (Unoki *et al.*, 2009). It is a multi-domain protein containing an amino-terminal ubiquitin-like domain, a PHD domain, an SRA (SET- and RING finger-associated) domain, a RING finger domain and a Tudor domain (Tien *et al.*, 2011). UHRF1 is extremely interesting since it is known both to bind methylated lysine residues via its PHD and Tudor domains (Karagianni *et al.*, 2008; Nady *et al.*, 2011) and to exhibit ubiquitin E3 ligase activity, attributed to its RING finger domain (Karagianni *et al.*, 2008). As ubiquitin E3 ligases can also function as NEDD8 ligases

(Abida *et al.*, 2007; Broemer *et al.*, 2010; Oved *et al.*, 2006; Watson *et al.*, 2006; Xirodimas *et al.*, 2004), it was possible that UHRF1 could act as mediator for the interplay between methylation and NEDDylation on E2F-1 by binding to methyl-K185 and subsequently promoting NEDDylation at another site. Therefore, UHRF1 was chosen from the CADOR array screen for further investigation.

To confirm that E2F-1 and UHRF1 interact *in vivo*, Flag-tagged UHRF1 was expressed in U2OS cells and immunoprecipitated. Upon immunoblot analysis of immunoprecipitated proteins, endogenous E2F-1 was indeed found to interact with UHRF1 [Figure 6.3(B)]. To confirm that this interaction was specific to E2F-1 methylated at K185, wild-type or K185R E2F-1 were expressed in U2OS cells and immunoprecipitated. Immunoprecipitates were analysed by immunoblotting and UHFR1 found to interact with wild-type but not K185R E2F-1 (although some non-specific binding of UHRF1 to the agarose beads used in the immunoprecipitation was also observed) [Figure 6.3(C)]. This supports the hypothesis that methylation at K185 is required for the UHRF1-E2F-1 interaction.

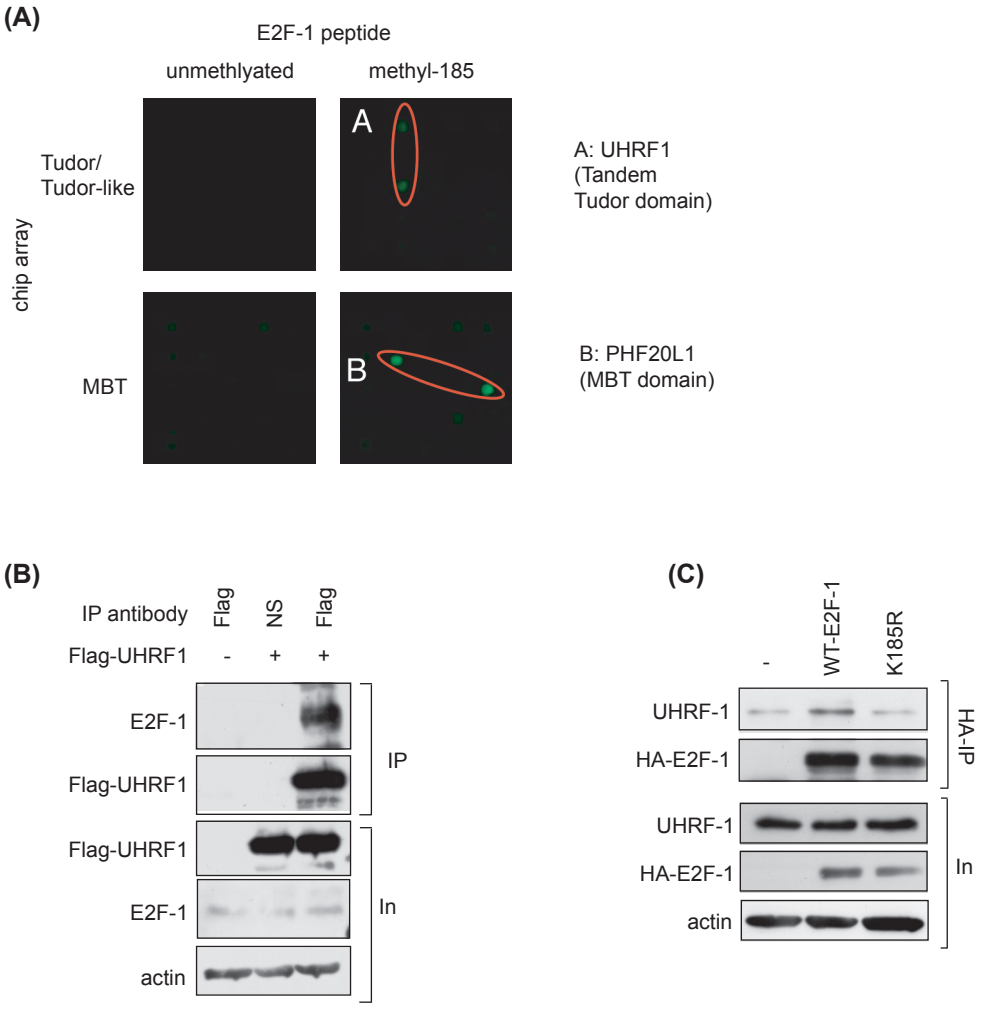


Figure 6.3: UHRF1 interacts with E2F-1 at methyl-K185
(A) Positive hits in a CADOR array to identify peptides that interact specifically with methyl-K185 E2F-1 but not unmethylated E2F-1. Positive hits were detected in the Tudor/Tudor-like domain array and the MBT domiain array. (Courtesy of Mark T. Bedford, University of Texas, USA) (B) U2OS cells transfected with Flag-UHRF1 (1µg) were subject to immunoprecipitation (IP) with either Flag or non-specific (NS) antibody and immunoblot analysis used to detect the presence of endogenous E2F-1 bound to UHRF1. (C) U2OS cells were transfected with wild-type or K185R E2F-1 (1µg) and subject to HA-immunoprecipitation and immunoblot analysis to identify endogenous UHRF1.

6.4 UHRF1 promotes E2F-1 NEDDylation.

To investigate whether UHRF1 enhances E2F-1 NEDDylation, UHRF1 was co-expressed with E2F-1 and His-NEDD8 in U2OS cells and NEDD8 substrates purified and analysed by immunoblotting [Figure 6.4(A)]. It was observed that UHRF1 expression dramatically increased the amount of E2F-1 NEDDylation. It is therefore possible that UHRF1 acts as the NEDD8 E3 ligase for E2F-1. In support of this hypothesis, siRNA depletion of UHRF1 dramatically reduced the level of E2F-1 NEDDylation, despite the relative inefficiency of the UHRF1 knockdown itself (as determined by the UHRF1 input immunoblot) [Figure 6.4(B)].

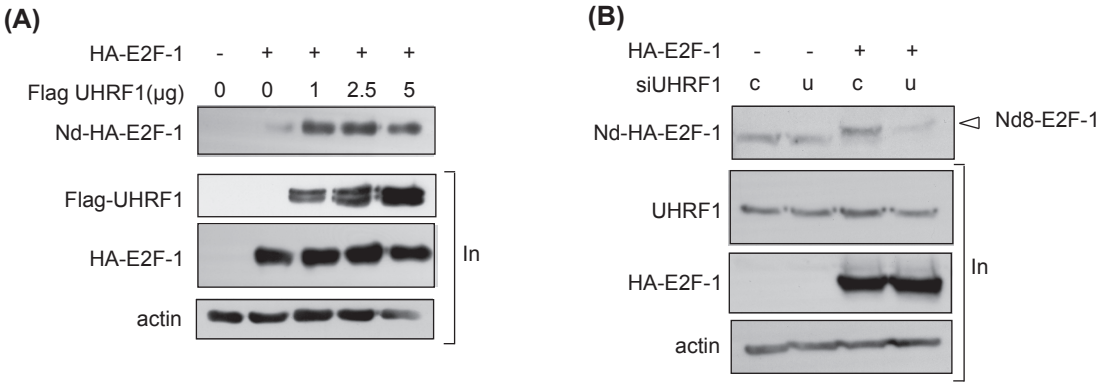


Figure 6.4: UHRF1 enhances E2F-1 NEDDylation
(A) NEDD8 substrates were purified from U2OS cells expressing HA-E2F-1, His-NEDD8 and Flag-UHRF1 and analysed by immunoblotting. (B) NEDD8 substrates were purified from U2OS cells treated with either control (c) or UHRF1 (u) siRNA and expressing HA-E2F-1 and His-NEDD8 and analysed by immunoblotting.

6.5 Conclusions

The results presented in this chapter showed that there exists a level of interplay between E2F-1 methylation by Set7/9 and E2F-1 NEDDylation. These data suggested that methylation by Set7/9 may be required for its subsequent NEDDylation and that the multi-domain protein UHRF1 may mediate this methylation-NEDDylation interplay, perhaps by binding to methyl-K185 E2F-1 via its Tudor domain and promoting E2F-1 NEDDylation at a distant site, perhaps at K117/120/125. The data demonstrate a previously undescribed mechanism of interplay between PTMs, namely that between protein methylation and NEDDylation.

Although provocative, the data presented here only go a short way to demonstrating that UHRF1 mediates methylation-NEDDylation interplay on E2F-1 and more experiments would be required to confirm this hypothesis. For example, mutant UHRF1 harbouring defective Tudor and/or RING finger domains could be used to demonstrate that the catalytic activity of UHRF1 is required for E2F-1 NEDDylation. A Tudor domain mutant would be expected to result in loss of the interaction between E2F-1 and UHRF1 and also lead to loss of E2F-1 NEDDylation, supporting the hypothesis that methylation mediates UHRF1 binding. A RING finger mutant of UHRF1 would be expected to lack the E2F-1 NEDDylation promoting activity of the wild-type protein, supporting its proposed role as NEDD8 E3 ligase for E2F-1, but would be expected to retain the ability to bind methyl-K185 E2F-1. It would also be useful to investigate methylation-NEDDylation interplay *in vitro*, using wild-type and K185R E2F-1 in combination with UHRF1 harbouring RING finger or Tudor domain mutants. These *in vitro* experiments would unequivocally determine whether UHRF1 can enhance E2F-1 NEDDylation

directly (i.e. without involvement of other cofactors present in the cell) as well as demonstrating the dependence of the NEDDylation event on the integrity of the interaction of UHRF1 with methyl K185 E2F-1 via its Tudor domain. Additionally, E2F-1 wild-type and K185R could be methylated *in vitro* prior to the *in vitro* NEDDylation reaction to further establish the importance of K185 methylation on NEDDylation.

With the recent publication of studies suggesting that NEDDylation can occur via an atypical conjugation cascade that utilises enzymes of the ubiquitin pathway (Hjerpe *et al.*, 2012a; Hjerpe *et al.*, 2012b; Leidecker *et al.*, 2012), an experiment demonstrating that UHRF1 can promote E2F-1 NEDDylation when the activity of the ubiquitin conjugation cascade is compromised, for example under conditions of UBE1 siRNA knockdown, will be crucial in determining the physiological relevance of the findings in this chapter. Figure 6.4(A) showed that E2F-1 NEDDylation is enhanced by UHRF1 expression. Here, NEDD8 was overexpressed thereby increasing the free NEDD8 to ubiquitin ratio and UHRF1, a known ubiquitin E3 ligase, was also overexpressed. According to the findings of Hjerpe *et al* (2012a), these two experimental conditions could potentiate NEDDylation by the ubiquitin conjugation cascade (Hjerpe *et al.*, 2012a; Hjerpe *et al.*, 2012b). Therefore, an experiment that unequivocally rules out the atypical conjugation pathway as playing a role in this UHRF1 study will be crucial to understanding whether the observed effects of UHRF1 on E2F-1 NEDDylation are physiologically relevant, or simply an artefact of the experimental technique.

Lack of availability of a modification specific antibody for NEDD8-E2F-1 meant that direct E2F-1 NEDDylation at any particular residue was not established during this study. The conclusions of this study could therefore be supported by use of a technique such as mass spectrometry to identify the site of NEDDylation, confirming the assumption made here that NEDDylation does not occur at K185, but elsewhere on the protein, perhaps at K117/120/125. However, mass spectrometry to identify sites of NEDDylation is extremely challenging since upon tryptic digest (required for most mass spectrometry techniques) a NEDD8 residue is shortened to a di-glycine motif which is undistinguishable from that formed by ubiquitin (Jones *et al.*, 2008) and so methods that exclude the possibility of detecting ubiquitinated E2F-1 need to be employed. The most obvious of these is the over-expression of a tagged version of NEDD8 (for example His-NEDD8 or GST-NEDD8) and performing a purification of NEDDylated substrates prior to mass spectrometric analysis, similar to those performed by Xirodimas *et al.*, 2008 and Jones *et al.*, 2008. However, we now know that over-expression of NEDD8 can result in NEDDylation via a non-physiological atypical pathway (Hjerpe *et al.*, 2012a; Hjerpe *et al.*, 2012b; Leidecker *et al.*, 2012). Therefore this proteomic technique risks the identification of false positives and until new methodologies are developed, we must tentatively use the data obtained by indirect techniques such as those used in this current study.

In summary, the data presented in this chapter suggested an interesting link between methylation and NEDDylation of E2F-1 by the multi-domain protein UHRF1. There is evidently further work required to confirm this connection, but if verified the data reveal a novel regulatory mechanism for E2F-1.

Chapter 7

Discussion

7.1 Overview of findings

The objective of this study was to identify and characterise novel E2F-1 PTMs to glean a better understanding of the role of E2F-1 in governing the cell cycle. The E2Fs are a family of eight transcription factors with diverse roles operating throughout the cell cycle (DeGregori & Johnson, 2006). E2F-1 is known to be important for the decision by a cell to commit to a full round of DNA replication and cell division at the G_1- to S-phase transition, where it has a role in promoting cell cycle progression by activating genes such as *cyclin E* (Sahin & Sladek, 2010). However, E2F-1 also has a role as a tumour suppressor where it drives the apoptotic response to DNA damage in either a p53-dependent or p53-independent manner (Stevens & La Thangue, 2004). With its central role in the orchestration of the cell cycle, E2F-1 activity is extensively regulated, for example by its association with its negative regulator and pocket protein family member, Rb, and its heterodimeric partner DP, without which E2F-1 is unable to bind to DNA and regulate promoter activity (DeGregori & Johnson, 2006; Dimova & Dyson, 2005). The activity of E2F-1 is further fine tuned by the embellishment of the protein with PTMs, and E2F-1 is already known to be phosphorylated, ubiquitinated, acetylated, methylated and SUMOylated in response to various stimuli (Budhavarapu *et al.*, 2012; Cho *et al.*, 2012; Fagan *et al.*, 1994; Lin *et al.*, 2001; Pediconi *et al.*, 2003; Rizos *et al.*, 2005; Stevens *et al.*, 2003). However, despite a large wealth of literature associated with E2F-1, its regulation is still not fully understood. In particular, it is not understood what governs the switch changing E2F-1 function from oncogene to tumour suppressor under conditions of DNA damage, an aspect of E2F-1 crucial to our understanding of the protein and our elucidation of how it is dysregulated in cancer.

In this study, it was demonstrated that E2F-1 is regulated by two additional PTMs, methylation at K185 by Set7/9 and NEDDylation. These modifications were both downregulated in response to DNA damage, conditions under which E2F-1 governs an apoptotic response (Stevens & La Thangue, 2004).

Both K185 methylation and NEDDylation have the effect of diminishing the transcriptional activity of E2F-1, although some gene specific effects may exist, at least regarding K185 methylation. Methylation and NEDDylation of E2F-1 also have the effect of reducing the stability of E2F-1. The similarities between the effects of methylation and NEDDylation on E2F-1 activity led to experiments demonstrating that a level of interplay exists between E2F-1 K185 methylation and NEDDylation in which methylation is required for subsequent NEDDylation of E2F-1. These data illustrated a novel E2F-1 regulatory mechanism and the first example of interplay between methylation and NEDDylation.

7.2 Methylation of E2F-1 at K185

During this study, recombinant wild-type and substitution mutants of E2F-1 were subject to methylation *in vitro* and residue K185 identified as a target for Set7/9 monomethyltransferase (Figure 3.2). The rationale for these experiments was to follow up the prediction made by Couture *et al.* (2006) that E2F-1 was a Set7/9 target as it had the Set7/9 consensus in its sequence. Following identification of E2F-1 K185 as a Set7/9 target *in vitro*, methylation at this site was confirmed *in vivo* using an antibody generated specifically against the methyl-K185 residue in E2F-1 (Figure 3.3). Using this antibody it was additionally shown that K185 methylation decreases upon DNA

damage, suggesting that this methylation event has a role in promoting expression of cell cycle progression genes and/or inhibiting expression of apoptotic genes in the absence of DNA damage.

The identification of E2F-1 K185 as a Set7/9 substrate provides support for the Set7/9 consensus site proposed by Couture *et al*. (2006). However, since this consensus was established, more recent studies have updated and expanded this sequence to account for the finding that many more recently identified Set7/9 substrates do not conform to it (Dhayalan *et al.*, 2011). Rather than simply comparing a handful of known Set7/9 targets (as was the method used in the Couture study), Dhayalan *et al.* took a different approach, preparing an array of histone H3 tail (1-21) peptides by exchanging each residue with all 20 other natural amino acids to identify an optimal target sequence for Set7/9. This lead to the identification of an updated consensus sequence (Figure 7.1).

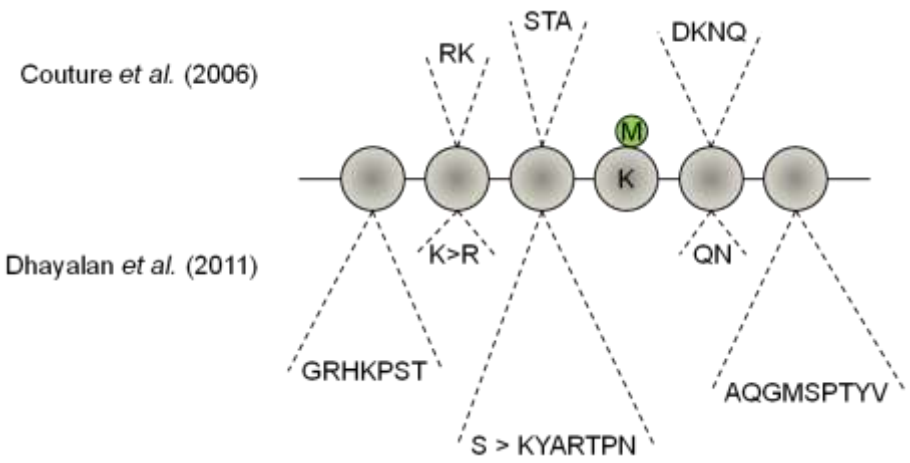


Figure 7.1 Set7/9 consensus sequence

Consensus sequences for Set7/9, as determined by Couture *et al.* (2006) (top) and Dhayalan *et al* (2011) (bottom). The position of each residue is represented by a grey circle with the possible residues at each position shown by their single letter codes. ">" indicates where there is a preference for one residue over another, for example K>R, lysine preferred over arginine. The position of the methylated lysine is indicated. M, methyl group.

Although the new consensus incorporates the sequence predicted by Couture *et al.* (2006), a bioinformatics study using this new sequence found that some previously unidentified targets of Set7/9 are methylated more efficiently than known Set7/9 targets such as DNMT1, ER, Tat and even histone H3, which is methylated at the Couture *et al*. consensus. It was also found that MINT, one of the most efficiently methylated proteins, could in fact be dimethylated, raising the possibility that Set7/9 has different product specificity depending on the level of similarity of the consensus site to the optimal (Dhayalan *et al.*, 2011).

Therefore the candidate substrates proposed by Couture *et al.* (2006), including E2F-1, may only represent weak targets (i.e. their consensus sequences are not as close to the optimal as other substrates and therefore at the peptide level are less efficiently methylated than other proteins whose consensus sequences are closer to the optimal).

However, this does not diminish the significance of E2F-1 methylation since, as Dhayalan *et al.* (2011) point out, less efficient methylation at the peptide level may be balanced by a strong molecular interaction between Set7/9 and its substrate when considered at a whole protein level, which may push the reaction kinetics in favour of methylation. Additionally, weak methylation may allow the potential for fine tuning of E2F-1 so that it may readily be demethylated, for example by LSD1, as already described (Kontaki & Talianidis, 2010; Xie *et al.*, 2011). A more strongly methylated substrate may be more difficult to demethylate as the balance between methylation by Set7/9 and demethylation by another enzyme such as LSD1, may be in favour of methylation.

Functional studies presented in Figure 3.4 and Figure 3.5 suggested a role for methylation in the regulation of E2F-1 transcriptional activity, where luciferase reporter assays revealed that the K185R non-methylatable mutant had greater promoter activity and promoter binding than wild-type E2F-1 at most of the promoters studied. These observations were supported by the publication of two similar studies which also demonstrated E2F-1 methylation at K185 by Set7/9, similarly showing a role for this methylation event in the regulation of E2F-1 transcriptional activity (Kontaki & Talianidis, 2010; Xie *et al.*, 2011). In agreement with the findings presented here, the Kontaki and Talianidis (2010) study showed that methylation at K185 resulted in decreased *p73* promoter occupancy and activation, leading to decreased E2F-1 mediated apoptosis. They also demonstrated that methylation decreases the stability of E2F-1, as was evident in the current study [Figure 3.3(C)]. However, in contrast to the findings in the current study, Kontaki and Talianidis showed that their findings were only relevant in a $p53^{-/-}$ background, since when ectopic p53 was introduced, cell death was instead

observed to be induced rather than inhibited by Set7/9 over-expression. This is in agreement with other studies such as those by Chuikov *et al*., (2004) demonstrating that in a p53 positive background apoptosis is induced by Set7/9 activity resulting from methylation of p53, which enhances its transcriptional activity. It also supports studies showing that methylation of Rb by Set7/9 enhances Rb mediated repression of E2F-1, driving cells into G_1 arrest or senescence under conditions of DNA damage (Carr *et al.*, 2011; Munro *et al.*, 2010).

The discrepancy between the two studies most likely arose due to differences in experimental approach. For example, Kontaki and Talianidis (2010) demonstrate that cell death is reduced by Set7/9 over-expression in p53 null cells but increased when p53 is reintroduced by ectopic expression. They do not unpick the specific effects of Set7/9 over-expression on E2F-1 regulation by methylation *per se*, for example by investigating the stability or transcriptional activity of E2F-1 following Set7/9 over-expression under both p53 positive and negative backgrounds. Neither do the authors make use of the K185R methyl-deficient mutant in the experiments comparing p53 positive and negative backgrounds. Therefore it cannot be concluded that the effects of Set7/9 over-expression on the cell cycle are mediated by E2F-1 methylation under both sets of conditions. Since it is known that p53 is also methylated by Set7/9 (Chuikov *et al.*, 2004), it is possible that the effects of p53 methylation may override any effects of E2F-1 methylation under the experimental conditions used by Kontaki and Talianidis, particularly as p53 was ectopically expressed. p53 may also be a stronger target for methylation by Set7/9. With these points in mind, it seems likely that, as observed both in the current study and by Xie *et al*. (2011), methylation has similar effects on E2F-1 activity in both p53 positive and negative backgrounds.

Conversely, Xie *et al.* (2011) observed E2F-1 transcriptional activity to increase rather than decrease upon ectopic Set7/9 expression at the BIM promoter in a luciferase assay. In the current study, the effects of K185 methylation were demonstrated to be gene specific. Since BIM promoter activity was not tested in the current study, it may be that E2F-1 activity at the BIM promoter does indeed increase after methylation, similar to its effects at the E2F-1 promoter shown in Figure 3.4(B). However, this does not explain why Xie *et al.* observed increased p73 mRNA levels upon Set7/9 over-expression, which contrasts the findings in both the current study and those by Kontaki and Talianidis (2010) showing that E2F-1 activity at the *p73* promoter decreases following E2F-1 methylation. The primary difference between the experimental approach used in the current study and the Xie *et al*. study is that in the current study the effect of methylation on E2F-1 transcriptional activity was observed by comparing wild-type E2F-1 to the K185R mutant. In contrast Xie *et al.* used Set7/9 over-expression to observe the effect of methylation. Perhaps, as discussed previously, the effects of Set7/9 over-expression on another cell cycle regulating protein such as p53 may override any effect on E2F-1 activity, and therefore may explain the discrepancy between the findings. This may also explain why Kontaki and Talianidis observed decreased p73 mRNA levels following Set7/9 over-expression in $p53^{-/-}$ cells whereas Xie *et al*. saw the reverse in p53 positive cells.

It is logical that Set7/9 methylation would have opposing effects on tumour suppressor proteins such as Rb and p53, than on E2F-1, which has a significant role to play in promoting cell cycle progression in unstressed cells (DeGregori & Johnson, 2006; Trimarchi & Lees, 2002). The fact that E2F-1 methylation by Set7/9 is downregulated under DNA damage [Figure 3.3(C)], whereas that of tumour suppressors such as Rb is

upregulated (Carr *et al.*, 2011), provides support for this opposing role of Set7/9 methylation for oncogenes and tumour suppressors. It therefore seems likely that under conditions of DNA damage, methylation of p53 and Rb predominate, resulting in increased p53 transcriptional activity, enhanced suppression of E2F-1 by Rb and apoptosis induction. Methylation of E2F-1, however, occurs mainly under non-damage conditions where transcription from its apoptotic promoters is suppressed and the protein is destabilised. This destabilisation may bring overall levels of E2F-1 to below a threshold that triggers the switch from tumour suppressor to growth promoting behaviour of E2F-1, as suggested previously (Stevens & La Thangue, 2004). LSD1 has been shown to remove the K185 methyl mark from E2F-1 (Kontaki & Talianidis, 2010; Xie *et al.*, 2011) and may therefore govern its demethylation following DNA damage, and hence the switch of E2F-1 function from growth promoter to tumour suppressor.

The functional effects of E2F-1 methylation by Set7/9 described in this report are consistent with those demonstrated for other known Set7/9 targets, since methylation has previously been shown to impact on the transcriptional activity and stability of its targets. For example, methylation of the NFκB RelA subunit and DNMT1 were found to negatively regulate their stability (Wang *et al.*, 2009; Yang *et al.*, 2009). In contrast, methylation by Set7/9 was shown to enhance the stability and transcriptional activity of the ERα (Subramanian *et al.*, 2008) and enhance the transcriptional activity of the Tat protein of HIV (Pagans *et al.*, 2010). Methylation by Set7/9 also enhances the transcriptional activity of p53 and the TFIID complex (Chuikov *et al.*, 2004; Kouskouti *et al.*, 2004). Regulation of TFIID by methylation occurred in a gene specific manner similar to that revealed in this study for E2F-1 methylation (Figure 3.4). Methylation by Set7/9 therefore clearly has an important role to play in regulating the activity of

transcription factors either by mediating protein stability or by promoting or inhibiting the binding of cofactors. However, it seems that methylation can have either a positive or a negative effect on transcription factor activity depending on the protein concerned and the context in which it occurs.

For some proteins such as p53 and PCAF, methylation affects their localisation (Masatsugu & Yamamoto, 2009; Xirodimas *et al.*, 2004). There were no observed changes in localisation for methylated versus unmethylated E2F-1 (Loftus *et al.*, 2012). The effect of methylation on the localisation of some proteins most likely reflects binding of cofactors of these proteins to the methyl mark.

To help understand the relationship between methylation of E2F-1 and Rb by Set7/9 it would be useful to determine whether E2F-1 is methylated in its free (unbound) or Rb-complexed state. This could be achieved by performing immunoprecipitations to purify E2F-1 bound to Rb or E2F-1 alone (perhaps under conditions of Rb siRNA depletion) and comparing K185 methylation levels using the methyl-specific antibody characterised in Figure 3.3. Since methylation has a negative impact on E2F-1 transcriptional activity, it may be expected that E2F-1 is methylated when bound to Rb, its negative regulator, but alternatively methylation may be an Rb-independent means of regulating E2F-1.

7.3 E2F-1 NEDDylation

In this study, E2F-1 was shown to be NEDDylated using a combination of NEDD8 purifications performed under both native and denaturing conditions and utilising both endogenous and ectopically expressed E2F-1 (Chapter 4). Despite contradictory publications suggesting that the methodologies used in this study (in which the ratio of NEDD8 to ubiquitin was frequently increased by over-expression of NEDD8 and by the use of the proteasome inhibitor MG132) would lead to NEDDylation via a non-physiological atypical conjugation pathway involving the enzymes of the ubiquitin system (Hjerpe *et al.*, 2012a; Hjerpe *et al.*, 2012b), it was demonstrated that NEDDylation of E2F-1 does indeed occur via the physiological NEDD8 conjugation system (Figure 4.3). This was achieved by specifically inhibiting either the NEDD8 or the ubiquitin conjugation pathways at the E1 stage using the small molecule inhibitor of NAE, MLN4924, or UBE1 siRNA, respectively [Figure 4.1(C); Figure 4.3(C-D)]. Additionally, NEDDylation was inhibited more generally by overexpressing the NEDD8 protease NEDP1 and the dominant negative inhibitor of NEDDylation UBC12 (C111S). The latter remove the NEDD8 moiety from its substrates and deplete the free NEDD8 pool, respectively (Mendoza *et al.*, 2003; Wada *et al.*, 2000) [Figure 4.3(A-B)].

In contrast to the findings presented here, Hjerpe *et al.* performed very similar experiments to those presented in Figure 4.3(C-D), showing that previously published NEDD8 substrates p53 and caspase-7 were NEDDylated in a UBE1 dependent fashion and that MLN4924 treatment had no effect on NEDDylation of these substrates (Hjerpe *et al.*, 2012b). Therefore, it seems that E2F-1 NEDDylation is an even more unique event than first thought, as the number of non-cullin substrates NEDDylated under physiological conditions is now fewer than when the current E2F-1 study began. One

noteworthy difference between E2F-1 NEDDylation and that of many other substrates, including p53, caspase-7, and EGFR, is that for the latter substrates, the E3 responsible for their ubiquitination is also responsible for their NEDDylation (Broemer *et al.*, 2010; Oved *et al.*, 2006; Xirodimas *et al.*, 2004). Preliminary experiments performed during this study showed that the ubiquitin ligases for E2F-1, $p45^{SKP2}$ and MDM2 were unable to NEDDylate E2F-1 (data not shown) and later in the study UHRF1 was identified as a possible E3 for E2F-1 NEDDylation (Figure 6.4). Perhaps this possession of discrete ubiquitin and NEDD8 E3 ligases determines whether a substrate is targeted by the typical or atypical NEDD8 conjugation pathways. It is noteworthy however, that UHRF1 has not yet been tested for its ubiquitin ligase activity towards E2F-1. If NEDDylation really is such a rare event, its potential as a successful target for cancer therapy is increased, as the risk of unwanted side effects is reduced. For example, if E2F-1 activity can be regulated without influencing other transcription factors such as p53, the therapeutic benefit of such a compound could be enhanced.

7.4 E2F-1 NEDDylation functional studies

NEDDylation was found to negatively regulate E2F-1 transcriptional activity in luciferase reporter assays (Figure 5.1 and Figure 5.2) and to decrease E2F-1 stability in cells treated with cyclohexamide (Figure 5.3). NEDDylation of E2F-1 was also found to be downregulated upon DNA damage (Figure 5.4). These findings are similar to those observed for other NEDD8 targets such as p53 and TAp73, for which NEDDylation similarly decreases transcriptional activity (Watson *et al.*, 2006; Xirodimas *et al.*, 2004). Stability changes are also commonly associated with NEDDylation, for example, HIF-1α, MDM2 and IκB, where NEDDylation enhances protein stability (Noguchi *et al.*,

2011; Ryu *et al.*, 2011; Watson *et al.*, 2010), and L11 and EGFR where it decreases stability (Oved *et al.*, 2006; Sundqvist *et al.*, 2009). The data presented in this study are also consistent with previous findings showing that DNA damage induces expression of NEDP1, which results in a decrease in NEDDylation of substrates such as MDM2. Under these conditions of deNEDDylation MDM2 becomes destabilised, thereby releasing p53 from repression and inducing apoptosis (Watson *et al.*, 2010). Perhaps the decrease in E2F-1 NEDDylation upon DNA damage observed in this study is in part mediated by an increase in NEDP1 expression. This observation additionally provides support for a role for E2F-1 NEDDylation in promoting the oncogenic rather than the tumour suppressive properties of E2F-1 function, perhaps by negatively regulating E2F-1 apoptotic activity.

The findings in this study are also consistent with a more recent study showing that an increase in NEDDylation (induced by siRNA knockdown of NEDD8 proteases such as NEDP1) resulted in decreased apoptosis, mediated in part through the inhibition of caspase-7 (Broemer *et al.*, 2010). Perhaps NEDDylation has a general role in apoptosis inhibition, and E2F-1 NEDDylation plays its part by switching E2F activity from tumour suppressor to cell cycle promoter.

Similar to methylation, NEDDylation is also commonly associated with changes in the localisation of a protein, for example L11 is driven to the nucleolus following its NEDDylation (Sundqvist *et al.*, 2009) and TAp73 NEDDylation promotes its cytoplasmic localisation (Watson *et al.*, 2006). However, no differences were observed in the localisation of the NEDD8 deficient mutants of E2F-1 (K185R and

K117/120/125R) with respect to wild-type E2F-1 (Loftus *et al.*, 2012). It is likely that the changes in localisation of L11 and TAp73 are not caused by their NEDDylation *per se*, but rather by binding of cofactors specific for these proteins to the NEDD8 moiety. This is interesting since NEDP1 is localised exclusively to the cytoplasm, where NEDDylation is also assumed to occur (Sundqvist *et al.*, 2009). It must therefore be assumed that E2F-1 moves from the nucleus into the cytoplasm to be NEDDylated in a transient manner or that NEDDylation occurs immediately after translation and prior to nuclear entry so that the level in the cytoplasm at any time is too small to observe in immunofluorescence experiments. It would be interesting to investigate this further.

It is as yet unknown how E2F-1 NEDDylation decreases its stability. For some proteins, such as EGFR, NEDDylation promotes subsequent ubiquitination of the protein which in turn results in the targeting of the substrate to the proteasome for degradation (Oved *et al.*, 2006). During this study, the generation of high molecular weight species was evident when NEDD8-E2F-1 was isolated from cells (Figure 4.2). These may either be indicative of the NEDDylation of E2F-1 at multiple sites across the protein, or the generation of poly-NEDD8 chains as described by Jones *et al.*, (2008) (Jones *et al.*, 2008). These chains are known to occasionally be mixed with ubiquitin but as yet this seems to be a rare event with unknown function (Watson *et al.*, 2011). With the similarities between NEDD8 and ubiquitin [60% identical and 80% homologous (Soucy *et al.*, 2010)], it seems likely that NEDD8 or mixed chains could be mistaken for ubiquitin chains by the proteasomal degradation machinery, especially for those chains linked via K48 conjugation. It is also possible that NEDDylation provides a platform for the recruitment of binding partners that somehow affect E2F-1 stability. Kontaki and Talianidis (2010) demonstrated that E2F-1 methylation enhanced ubiquitination. Since

the data presented here show that methylation promotes subsequent NEDDylation, it seems likely that the decreased stability observed here for either methylated or NEDDylated E2F-1 stems from this effect on its ubiquitination, and subsequent proteasomal degradation.

For the luciferase assays presented in this study, E2F-1 level was kept constant by adjusting the amount of E2F-1 expression vector transfected. The results presented in Figure 5.1 and Figure 5.2 therefore demonstrate that NEDDylation of E2F-1 impedes its transcriptional activity independently of its effects on E2F-1 stability. The mechanism for this could be that NEDDylation either sterically inhibits the access of E2F-1 to DNA directly, or that it prevents the assembly of a transcription factor complex, for example by inhibiting the association between E2F-1 and a cofactor. The former hypothesis is supported by the finding that NEDP1 over-expression enhances binding of E2F-1 to the *E2F-1* and *Cdc6* promoters and that the NEDD8 deficient K185R mutant binds to these promoters more efficiently than wild-type E2F-1 (Loftus *et al.*, 2012). Alternatively the NEDD8 moiety may somehow promote association with its negative regulator, Rb.

Very recently, the results presented in this study were supported by publication of an analogous study which similarly demonstrated E2F-1 NEDDylation (Aoki *et al.*, 2012). Using overexpression and knockdown of NEDP1, NEDDylation was shown to inhibit E2F-1 transcriptional activity at the *p73* promoter yet have no effect at the E2F-2 promoter. NEDP1 expression was found to enhance the interaction between E2F-1 and its cofactor, MCPH1, a protein known to affect E2F-1 activity at a subset of its promoters. In keeping with the data presented in this current study, Aoki *et al*. (2012)

demonstrate that E2F-1 NEDDylation is reduced in response to DNA damage, and that this effect is dependent on the expression of NEDP1. Interestingly, NEDP1 expression was found to decrease NEDDylation of E2F-2, E2F-3, E2F-4 and DP1. E2F-1 was observed to specifically inhibit the apoptotic activity of E2F-1 without affecting its role in cell cycle progression.

7.5 Interplay between methylation and NEDDylation on E2F-1

A mutational analysis revealed that residues K117, K120 and K125 (in a combinatorial fashion) and K185 were important for E2F-1 NEDDylation (Figure 4.4). Having already demonstrated that E2F-1 K185 is the site of methylation by Set7/9, the possibility was raised that interplay may exist between methylation and NEDDylation on E2F-1. This possibility was made even more likely following the observation of so many parallels between the functional effects of E2F-1 NEDDylation and methylation. For example, both modifications negatively regulate the transcriptional activity of E2F-1, both decrease its stability and both are downregulated in response to DNA damage. Since both methylation by Set7/9 and NEDDylation occur on the ε-amino acid of a lysine residue, it seemed improbable that both methylation and NEDDylation would occur simultaneously, at least on the same residue. It was possible that the modifications were mutually exclusive at the K185 residue, but since both modifications have the same effect on E2F-1 activity, this hypothesis assumed the two modifications to be redundant. It was more likely that one modification would promote modification by the other at a distant site. In this study, E2F-1 methylation at K185 had been confirmed using an antibody specifically directed against the methyl-K185 residue. However, no such direct methods of measuring NEDDylation at K185 itself had been used in this study. Figure 4.4 showed K185 only to be important for NEDDylation of E2F-1 by observing a decrease in overall E2F-1 NEDDylation for the K185R substitution mutant with respect

to wild-type E2F-1. This was supported by the observations presented in Chapter 5 showing that the effects of substituting the K185 residue were similar to inhibiting NEDDylation, without showing that these effects were through K185 NEDDylation directly.

It therefore seemed likely that methylation was the true modification at K185 and that this methylation event could promote NEDDylation at another site. Therefore experiments were conducted assessing the effect of Set7/9 knockdown on E2F-1 NEDDylation and it was found that loss of Set7/9 activity inhibited E2F-1 NEDDylation [Figure 6.1(A-B)]. Interplay between PTMs has been previously observed on E2F-1 where phosphorylation at S31 promotes E2F-1 acetylation at K117/120/125 (Biswas & Johnson, 2012) and this acetylation is a prerequisite for ubiquitination (Galbiati *et al.*, 2005).

The observation of interplay between E2F-1 methylation and NEDDylation led to experiments attempting to reveal a protein or proteins that may mediate this interplay. As methylation is required for NEDDylation and the site of methylation was known to be K185, a CADOR array screen was performed to identify a protein that specifically binds to the methly-K185 site on E2F-1 and had the potential to mediate subsequent NEDDylation of E2F-1. The CADOR array identified the multi-domain protein, UHRF1 as a methyl-K185 specific interacting protein of E2F-1 [Figure 6.2(A)]. UHRF1 was found to interact with E2F-1 via its tandem Tudor domain. UHRF1 is also known to have ubiquitin E3 ligase activity, attributed to its RING finger domain (Karagianni *et al.*, 2008; Nady *et al.*, 2011; Unoki *et al.*, 2009). Since ubiquitin E3 ligases have often

been observed to act as NEDD8 E3 ligases (Broemer *et al.*, 2010; Oved *et al.*, 2006; Xirodimas *et al.*, 2004), the possibility was raised that UHRF1 may directly promote NEDDylation of methylated E2F-1. Over-expression of UHRF1 was subsequently found to enhance NEDDylation of E2F-1 whilst UHRF1 siRNA treatment produced the reverse effect (Figure 6.4). This suggests that UHRF1 can indeed act as NEDD8 E3 ligase for E2F-1 (Figure 7.2).

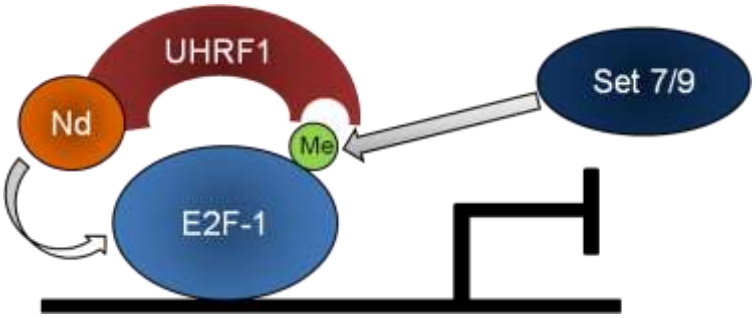


Figure 7.2: Model for the interplay between E2F-1 methylation and NEDDylation

The findings of this study suggest that E2F-1 is methylated by Set7/9 and that this leads to recruitment of UHRF1 which results in subsequent NEDDylation of E2F-1.

The finding that UHRF1 mediates the interplay between E2F-1 methylation and NEDDylation is very interesting since UHRF1 has never before been implicated in the direct control of a transcription factor. UHRF1 is a multi-domain protein that contains an amino terminal ubiquitin-like domain, overlapped partially by a leucine zipper domain, followed by two zinc finger domains (a PHD finger and a RING finger) (Arima *et al.*, 2004). It also has two Rb-binding sites in the PHD and RING finger domains. UHRF1 is known to provide the link between DNA methylation and histone modifications by binding to hemi-methylated DNA via its SET and Ring Associated (SRA) domain, a domain unique to the UHRF family, as well as binding trimethylated histone H3K9 via its tandem Tudor domain (Unoki *et al.*, 2009). It forms a complex

localised to the methylated promoters of tumour suppressor genes with HDAC1, Tip60, G9a, and DNMT, many of which are implicated in carcinogenesis (Unoki *et al.*, 2009). UHRF1 is best known for its role in maintaining DNA methylation and hence transcriptional repression from mother to daughter cells following cell division (Bronner *et al.*, 2010). UHRF1 expression peaks at late G_1 (similar to E2F-1) and at G_2/M-phase of the cell cycle in normal cells but is itself upregulated in various cancers, (Alhosin *et al.*, 2011; Arima *et al.*, 2004). It has also been proposed as a biomarker for the diagnosis of cervical and pancreatic cancer, amongst others (Alhosin *et al.*, 2011).

UHRF1 E3 ligase activity can ubiquitinate all histones *in vitro*, although histone H3 is its preferred substrate *in vivo*. This E3 ligase activity has been shown to be required for tumour cell progression (Unoki *et al.*, 2009). The data presented here could imply that this results not only from histone ubiquitination, but also perhaps due to the ability of UHRF1 to NEDDylate E2F-1 and promote its oncogenic activities. Additionally, E2F-1 is known to be an upstream regulator of UHRF1, since it binds to the *UHRF1* promoter and promotes its transcription (Unoki *et al.*, 2004), providing an interesting negative feedback loop in which UHRF1 inhibits E2F-1 activity by causing its NEDDylation.

UHRF1 has also been reported to be downregulated upon DNA damage by the action of p53 and p21 (Arima *et al.*, 2004), a finding consistent with the study presented here in which E2F-1 NEDDylation, for which UHRF1 is proposed to be E3 ligase, is similarly downregulated under DNA damage (Figure 5.4). Additionally, UHRF1 downregulates Rb activity and promotes S-phase entry in two ways; by binding to the Rb promoter and also to the protein itself when Rb is in its hypophosphorylated form. The latter

mechanism most likely results from the ubiquitin E3 ligase activity of UHRF1 promoting the proteasomal degradation of Rb (Jeanblanc *et al.*, 2005). This ability of UHRF1 to promote the transition from G_1- to S-phase by regulating of Rb, the negative regulator of E2F-1, is consistent with a role for UHRF1 in mediating the oncogenic properties of E2F-1 activity by promoting its NEDDylation.

Examples of proteins that, like UHRF1, act as adaptors mediating the interplay between PTMs have previously been reported at non-histone proteins. For example, during the response of a cell to double stranded DNA breaks, the mediator of DNA damage checkpoint 1 (MDC1) is phosphorylated by ATM and recruits the RING-finger ubiquitin E3 ligase RNF8, which in cooperation with RNF168, ubiquitinates histones to mediate changes in chromatin structure around the DNA lesion (Giunta *et al.*, 2010). Another example is the acetyltransferase Tip60, which binds to methyl K372 of p53, possibly by its chromodomain, resulting in subsequent acetylation of p53 (Kurash *et al.*, 2008).

7.6 Interplay with other post translational modifications

The K117/120/125 residues identified as being likely targets for E2F-1 NEDDylation are also targets for acetylation (Martinez-Balbas *et al.*, 2000; Marzio *et al.*, 2000). E2F-1 acetylation was found to augment DNA binding, stimulate transactivation potential and increase the stability of E2F-1 (Martinez-Balbas *et al.*, 2000). Additionally, E2F-1 is acetylated in response to DNA damage where it enhances apoptosis by binding to and activating promoters such as *p73* (Pediconi *et al.*, 2003). This transcriptional effect is gene specific (Pediconi *et al.*, 2003). It is evident that acetylation has exactly the

opposite effect on E2F-1 as NEDDylation. It therefore seems likely that there is interplay between NEDDylation and acetylation and that perhaps the two events are mutually exclusive, with acetylation replacing NEDDylation on lysine residues following DNA damage. Acetylation stabilises E2F-1 protein levels and promotes apoptosis, whereas NEDDylation regulates E2F-1 cell cycle control in the absence of a damage stimulus (Figure 7.3). In support of this hypothesis, methylation of E2F-1 at K185, now known to promote its NEDDylation, has been shown to inhibit its acetylation and vice versa (Kontaki & Talianidis, 2010). Additionally, E2F-1 K185 methylation was demonstrated to inhibit its phosphorylation (Kontaki & Talianidis, 2010), an event known to be associated with the DNA damage response where it promotes subsequent acetylation of E2F-1 (Biswas & Johnson, 2012). Further experiments comparing levels of E2F-1 NEDDylation, methylation, phosphorylation and acetylation with time after damage will be helpful to determine whether the hypothesis is true since upon damage E2F-1 NEDDylation and methylation would be expected to decrease with similar kinetics, whereas acetylation and phosphorylation would be expected to increase.

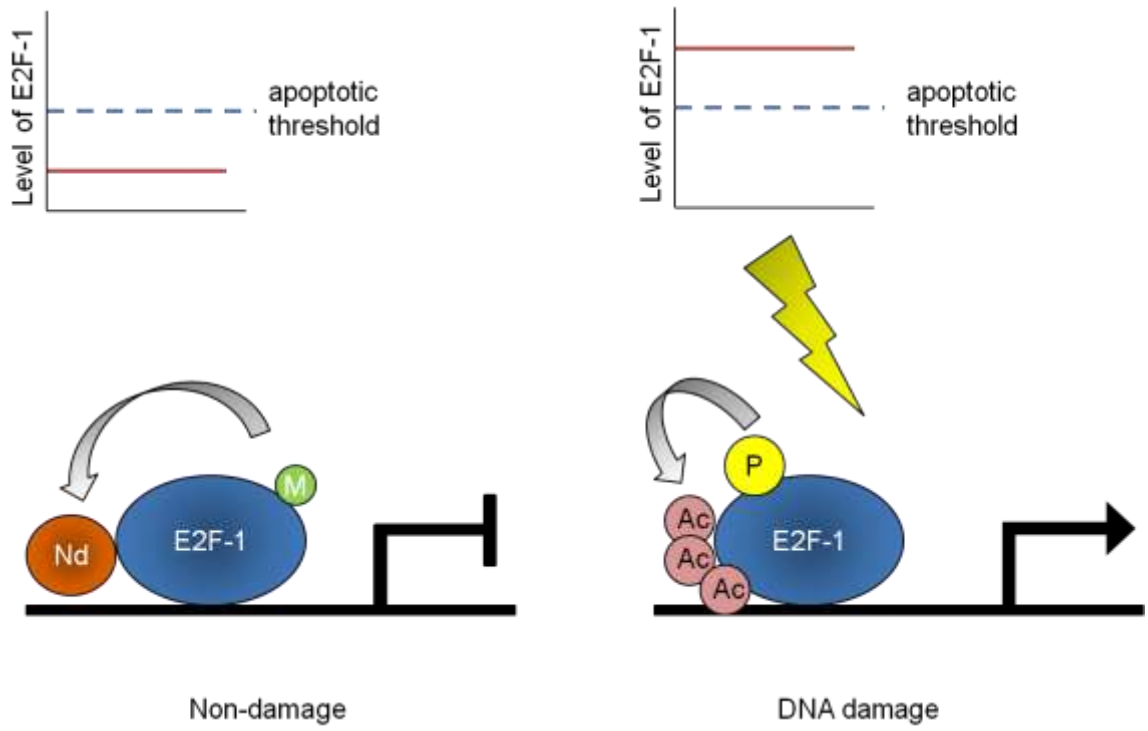


Figure 7.3: Model for the interplay between methylation, NEDDylation, phosphorylation and acetylation on E2F-1.

In unstressed cells, E2F-1 is methylated which promotes its subsequent NEDDylation, potentially within residues K117/120/125. Under these conditions E2F-1 levels are relatively low and presumed to be below the threshold for apoptosis induction. Following DNA damage, E2F-1 methylation and NEDDylation are downregulated and E2F-1 is phosphorylated by damage responsive kinases ATM/ATR. This phosphorylation event promotes acetylation of E2F-1 at K117/120/125 leading to increased stability and activation of E2F-1 responsive genes such as *p73*. Under DNA damage E2F-1 is stabilised, possibly increasing its levels to above the threshold for apoptosis induction.

The reason why the K117/120/125R triple mutant used in the current study behaves in a manner that reflects its deficiency in NEDDylation [i.e. increased transcriptional activity and stability with respect to wild-type E2F-1, Figure 5.2(C) and Figure 5.3(C)] rather than its deficiency in acetylation is unclear. The most likely explanation is that all of the experiments in this study using the triple mutant were under non-damage conditions, when NEDDylation is at its maximum levels and acetylation presumed to be at its lowest levels. Perhaps if the functional experiments had been repeated under DNA

damage conditions where acetylation is presumed to predominate, the reverse effect would have been evident.

7.7 Parallels with p53

During the course of this study, it became evident there were many similarities between the regulation of E2F-1 and p53 by PTM along with some very important differences (summarised in Table 7.1). Firstly both p53 and E2F-1 are targets for methylation by Set7/9 (Chuikov *et al.*, 2004). Methylation of E2F-1 is within the DNA binding and heterodimerisation domain of the protein whereas p53 methylation occurs in the carboxy terminus regulatory region. Modification of both proteins appears to have opposite effects; whereas methylation of E2F-1 diminishes its transcriptional activity and decreases its stability, p53 methylation enhances transcriptional activity and increases stability (Chuikov *et al.*, 2004). Additionally, p53 methylation is induced under DNA damage where it promotes p53 dependent apoptosis (Ivanov *et al.*, 2007), whereas E2F-1 methylation is downregulated under DNA damage and reduces cell death (Kontaki & Talianidis, 2010).

The opposite effects of Set7/9-mediated methylation on p53 and E2F-1 most likely result from the binding of different cofactors to the methyl mark. For p53, the methyl-mark reader is Tip60, which binds methylated p53 and subsequently acetylates the protein (Kurash *et al.*, 2008). The data presented in this study suggest the protein that binds to the methyl mark and propagates the methylation signal is the multi-domain protein, UHRF1.

Similar to E2F-1, p53 is also subject to NEDDylation where it has a similar effect of decreasing transcriptional activity of the protein (Xirodimas *et al.*, 2004). The site found to be NEDDylated was a KSKK motif found in the carboxy-terminal regulatory domain at residues 370-373, similar to the KKSK motif at 182-185 in the DNA binding and heterodimerisation domain of E2F-1 identified to be important for E2F-1 NEDDylation. Significantly, the methylation site for Set7/9 methylation in p53 (K372) falls within the three residues identified as being required for its NEDDylation raising the very strong possibility that there is interplay between methylation and NEDDylation similar to that observed in this study for E2F-1. The p53 NEDDylation study, similar to the current study, used mutagenesis to determine the residues important for NEDDylation rather than a direct approach to identifying the NEDDylation sites. Therefore the possibility cannot be ruled out that a very similar mechanism exists for p53 where residues 370-373 are required for NEDDylation since methylation at K372 by Set7/9 is required for binding of a cofactor analogous to UHRF1 to NEDDylate p53 at an undetermined distant site.

The interplay between p53 methylation, acetylation and phosphorylation has been extensively studied. For example, Set7/9 methylation of p53 facilitates subsequent acetylation of the protein at K373 and K382 (Ivanov *et al.*, 2007). Acetylation of these carboxy-terminal located lysines enhances the activity and stability of p53 as a result of direct competition with MDM2 mediated ubiquitination of the same residues (Brooks & Gu, 2006). It was found that K372 methylation must precede acetylation, as pre-acetylation inhibits methylation (Ivanov *et al.*, 2007). Here, a similar mechanism for the regulation of E2F-1 activity by methylation is proposed (but having the opposite end effect to the methylation of p53) in which Set7/9-mediated methylation promotes its

subsequent NEDDylation, resulting in destabilisation and transcriptional repression of E2F-1 under non-damage conditions. As discussed, NEDDylation perhaps competes with P/CAF which, in response to DNA damage, acetylates E2F-1 at the same residues, K117/120/125, where it has the opposite effect, i.e. stabilisation and transcriptional activation (Martinez-Balbas *et al.*, 2000).

In response to DNA damage, methylation of p53 by Set7/9 inhibits a nearby methylation event, namely methylation of K370 by SMYD2, a modification known to inhibit p53 binding to DNA (Huang *et al.*, 2006). As there are so many parallels between E2F-1 and p53 regulation by PTMs, perhaps a similar mechanism exists for Set7/9 methylation induced inhibition of methylation at nearby site. Indeed, Kontaki and Talianidis (2011) showed that E2F-1 methylation inhibits E2F-1 acetylation and phosphorylation and enhances ubiquitination suggesting that K185 methylation shows much interplay with other PTMs.

Table 7.1: Comparison of the similarities and differences between methylation and NEDDylation of E2F-1 and p53

E2F-1	p53
Target for methylation by Set7/9	Target for methylation by Set7/9
Methylation decreases transcriptional activity and decreases stability	Methylation increases transcriptional activity and increases stability
Methyl mark reader: UHRF1	Methyl mark reader: Tip60
Target for NEDDylation	Target for NEDDylation
NEDDylation decreases transcriptional activity	NEDDylation decreases transcriptional activity
NEDDylation occurs at KKSK motif	NEDDylation occurs at KSKK motif
Site identified to be important for methylation and NEDDylation overlap	Site identified to be important for methylation and NEDDylation overlap
Methylation promotes NEDDylation and ubiquitination. Methylation inhibits acetylation and phosphorylation	Methylation promotes acetylation which in turn inhibits ubiquitination

7.8 Limitations and future work

The purpose of this study was to identify and characterise novel E2F-1 PTMs with the aim of gleaning a better understanding of the role of E2F-1 as both an oncogene and a tumour suppressor. Firstly, the study established that E2F-1 is methylated at K185 by Set7/9 in undamaged cells and that this methylation impacts on E2F-1 transcriptional activity in a gene specific manner and also decreases protein stability. It was then demonstrated that E2F-1 is also NEDDylated and that K185 as well as K117/120/125 are important for this NEDDylation. NEDDylation was found to have similar effects on E2F-1 activity as methylation which led to experiments demonstrating the interplay between methylation and NEDDylation. It was established that methylation is required for NEDDylation and that UHRF1 may be both the methyl mark reader and the NEDD8 E3 ligase that mediates this interplay. Since UHRF1 binds methyl-K185, it is presumed

that the actual site of NEDDylation is K117/120/125 since it is unlikely both modifications would occur at the same residue simultaneously.

There are evidently some limitations in the data, the most significant being the lack of any direct evidence for the site of E2F-1 NEDDylation. Despite multiple attempts at mass spectrometric analysis to confirm K117/120/125 as the methylation site, direct evidence for NEDDylation at K117/120/125 was never obtained. Secondly, the effect of E2F-1 methylation on its transcriptional activity was observed to be gene specific. It was hoped that assessing the activity of E2F-1 at an array of promoters in reporter and ChIP assays would illuminate differential regulation of cell cycle progression associated genes and apoptotic genes. However, the majority of genes tested were downregulated in response to E2F-1 methylation which included both apoptotic genes such as *p73* and *Apaf-1* and those involved in cell cycle progression such as *Cdc6*. Therefore it is difficult to imagine where E2F-1 methylation fits in with the E2F-1 "switch" between oncogene and tumour suppressor, but as discussed, methylation may play a wider role in E2F-1 control via its interplay with other PTMs. With the data presented here, it can be assumed that K185 methylation plays a role in promoting E2F-1 mediated cell cycle progression since methylation is at its highest level in undamaged cells where it promotes NEDDylation, inhibiting E2F-1 activity and destabilising the protein, perhaps by inhibiting its acetylation at K117/120/125. It will be interesting to determine whether the gene specific effects are similar for E2F-1 NEDDylation. This possibility has already been alluded to by Aoki *et al.* (2012).

Another important experiment missing from this study is an immunoprecipitation demonstrating the impact of E2F-1 methylation and NEDDylation on DP-1 binding. This information will be essential when trying to elucidate a mechanism for how these modifications regulate E2F-1 transcriptional activity. K185 is in the heterodimerisation domain of E2F-1 and so could potentially influence DP-1 binding directly. Although K117, K120 and K125 are not in the heterodimerisation domain itself, they are in close proximity and the large size of the NEDD8 moiety would make steric hindrance between NEDDylated E2F-1 and DP-1 very likely. Alternatively, NEDDylation at K117/120/125 may have its effect through inhibiting binding to the DNA itself as these residues are within the DNA binding domain, although they are not themselves direct DNA base or backbone contacts (Zheng *et al.*, 1999). As already discussed, ChIP experiments have illustrated that NEDDylation does indeed inhibit E2F-1 DNA binding ability (Loftus *et al.*, 2012). Structural modelling of methylated and NEDDylated E2F-1 bound to DNA would provide a useful tool for illuminating the mechanism by which these PTMs abrogate E2F-1 transcriptional activity. As E2F-1 residue K185 is conserved amongst E2F family members (Zheng *et al.*, 1999), it would be interesting to investigate whether other E2Fs are methylated by Set7/9 and whether they are also subsequently NEDDylated.

Another major limitation of the data presented here is the largely ectopic nature of most of the experiments. For the E2F-1 methylation study, this mainly resulted from the already discussed limitations of the methyl-specific antibody so that ectopic expression of the K185R mutant was required as a control for unmethylated E2F-1. For the NEDDylation study, expression of His-NEDD8 was required in most experiments so that His-purification could be performed to isolate NEDDylated E2F-1 from non-

NEDDyated E2F-1. The reason for this was again lack of a direct tool such as a NEDD8 site specific antibody, or a NEDD8 antibody that was appropriate for use in immunoprecipitations. The strength of the study could therefore be greatly improved by performing experiments under more physiological conditions. In the first instance, these would involve experiments showing that E2F-1 can be methylated and NEDDylated in an unperturbed cellular system, for example by immunoprecipitating endogenous E2F-1 using an antibody targeted against endogenous NEDD8. The MLN4924 compound provides a useful tool for investigating the effects of inhibiting NEDDylation without ectopic expression of any proteins, and if used in conjunction with a tool such as mass spectrometry for directly identifying NEDDylated endogenous E2F-1, could yield some interesting data. For example SILAC could be used to compare the levels of E2F-1 target genes before and after treatment with MLN4924 to investigate the effects of NEDDylation in a more physiological system. Further experiments could investigate the role of E2F-1 methylation and NEDDylation in a whole organism such as a mouse. For example, immunohistochemistry could be used to determine the cell types or organs that exhibit most or least E2F-1 methylation and NEDDylation so that we can gain a better understanding of the function of these modifications. Additionally, tumour biopsies could be probed with the methyl-K185 E2F-1 antibody to identify whether some tumours show more methylation of E2F-1 than others. If MLN4924 becomes more widely used in the clinic to treat cancer patients, it would be interesting to investigate whether the level of methylated E2F-1 in a tumour corresponds with the responsiveness of a patient to MLN4924 treatment. Based upon the findings of the current study, it would be predicted that higher levels of methylated E2F-1 would lead to a more significant response to MLN4924 treatment, resulting from increased E2F-1

NEDDylation. If this were found to be true, K185-methylated E2F-1 could be used as a biomarker for response to this chemotherapeutic agent.

Further experiments are also required to fully elucidate the mechanism by which E2F-1 methylation and NEDDylation exert their effects. For example, as yet we do not know how methylation or NEDDylation inhibit E2F-1 transcriptional abilities. Although we know that both modifications impede binding of E2F-1 to DNA (Figure 3.5; Loftus *et al.*, 2012), we do not know how this occurs, for example whether it is by steric hindrance or whether these modifications result in binding or inhibition of binding to a cofactor.

7.9 Clinical relevance and conclusion

The success of the small molecule inhibitor of the NEDD8 conjugation pathway, MLN4924, in early stage clinical trials, especially for the treatment of acute myelogenous leukaemia (Soucy *et al.*, 2010), clearly demonstrates the clinical relevance of NEDDylation in the treatment of cancer. As E2F-1 is central to the orchestration of the cell cycle, the ability to fine tune its activity using a highly specific small molecule inhibitor is a powerful tool. Although the success of MLN4924 has so far been attributed to its effects on regulating cullin NEDDylation and hence CRL activity, the study presented here suggests that E2F-1 NEDDylation may also play a role in governing therapeutic response. Specifically, since it was suggested by the results presented in Figure 5.4 that E2F-1 NEDDylation is most active when E2F-1 is acting as an oncogene, inhibiting E2F-1 NEDDylation may be expected to decrease E2F-1 oncogenic potential. Based on data obtained during the current study, MLN4924

treatment would be expected to increase transcription from apoptotic genes such as *p73* and stabilise E2F-1, perhaps increasing its levels to above the threshold required to induce its apoptotic activity.

If, as the data presented here suggest, methylation and NEDDylation are linked, at least on E2F-1, by a single protein (i.e. UHRF1), there is potential for an even more specific therapeutic agent to be developed in which E2F-1 activity is altered by disrupting the interplay between methylation and NEDDylation by targeting UHRF1. This could help avoid likely non-specific side effects brought about by whole-cell NEDDylation inhibition.

In conclusion, the data presented here demonstrate a novel mechanism of interplay between PTMs on E2F-1. NEDDylation had not previously been described on E2F-1 and methylation had never been reported to exhibit interplay with NEDDylation until now. Both modifications are evidently important in the regulation of E2F-1 activity and hence the orchestration of the cell cycle by this transcription factor. The findings are highly relevant to our understanding of cell cycle regulation, which is required for our appreciation of how it goes wrong during tumorigenesis. The data presented here will additionally be important as we elucidate the mechanism of action of MLN4924 and also suggest that methyl-K185 E2F-1 could be used as a biomarker for response to this drug. All of these factors will be crucial as we progress towards a stratified medicine approach to cancer therapy.

Chapter 8

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Appendix

EMBO reports

scientific report

NEDDylation regulates E2F-1-dependent transcription

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The ubiquitin-like molecule NEDD8 modifies cullin-RING ubiquitin E3 ligases. NEDD8 has been shown to have a few additional substrates, but the extent to which this modification targets non-cullins and the functional significance of such modifications remain unclear. Here, we demonstrate that the cell-cycle-regulating transcription factor E2F-1 is a substrate for NEDD8 post-translational modification. NEDDylation results in decreased E2F-1 stability, lower transcriptional activity and slower cell growth. The lysine residues in E2F-1 targeted for NEDDylation can also be methylated, pointing to a possible interplay between these modifications. These results identify a new mode of E2F-1 regulation and highlight the emerging role of NEDD8 in regulating transcription factor stability and function.
Keywords: cell cycle; E2F-1; methylation; NEDD8
EMBO *reports* advance online publication 27 July 2012; doi:10.1038/embor.2012.113

INTRODUCTION

It is now clear that the biological complexity of an organism is not simply a product of the number of genes it possesses. The 'histone code hypothesis', in which gene expression is mediated by the combination of post-translational modifications (PTMs) embellishing the surrounding histones, is now widely accepted. With the discovery that non-histone proteins are similarly subjected to PTM, it now seems possible that analogous 'protein codes' exist to mediate the activity of proteins such as transcription factors [1]. An example is the regulation of p53, which can be modified by methylation, acetylation, phosphorylation, ubiquinitination, SUMOylation and NEDDylation [2]. Interplay exists between these modifications so that some only exist in the absence or presence of others. The combination of modifications existing at any time can dictate which binding partners are recruited and thus whether activity is up- or downregulated.

Neural precursor cell-expressed developmentally down-regulated 8 (NEDD8), also known as Related to Ubiquitin 1 in yeast, is a member of the ubiquitin-like family of PTMs, which also includes the small ubiquitin-like modifier (SUMO). It is a 9 kDa protein that shares 80% sequence homology with ubiquitin [3]. NEDD8 is conjugated to its substrates in a three-step pathway analogous to that of ubiquitin, involving NEDD8 activation by an E1 (the APPBP-1-UBA3 heterodimer, also known as NEDD8-activating enzyme), transfer onto the catalytic cysteine of an E2 (either UBC12 or UBE2F) and then covalent attachment to the substrate with the aid of a substrate-specific E3 ligase [4]. The predominant and best-characterized NEDD8 substrates are the cullins, which are structural subunits of cullin-RING E3 ubiquitin ligases (CRLs). Cullin NEDDylation induces conformational changes within the CRL that enhance its binding to the E2 and decrease the distance between the E2 and the substrate recognition component, thereby increasing the proximity of ubiquitin (carried by the E2) to its target. Ubiquitin chain extension is also enhanced by cullin NEDDylation as it makes the nascent ubiquitin chain more accessible to the E2. Cullin NEDDylation further enhances CRL activity by hindering binding of the CRL inhibitor CAND1 [4]. More recently, a small number of non-cullin targets for NEDD8 have been identified [5–7]. Among this list of substrates are transcription factors such as p53 [8] and its family member TAp73β [9]. In both of these examples, NEDDylation impedes transcriptional activity.

The clinical relevance of NEDDylation has in recent years been demonstrated by the use of MLN4924, a small molecule inhibitor that targets NEDD8-activating enzyme by forming an adduct with NEDD8, which blocks the active site of the E1 [10]. Early laboratory studies showed the drug to have great potential as an antitumour agent, and clinical trials are at present underway to evaluate the role of MLN4924 in the treatment of haematological and solid tumour malignancies [11].

We reasoned that other cell-cycle regulators might be NEDDylated and focussed our attention on the E2F-1 transcription factor, which has a central role in mediating the cell cycle, in particular during the G1- to S-phase transition. It operates in a somewhat bipolar manner, exhibiting both oncogenic and tumour suppressor activities depending on the biological context [12,13]. Already E2F-1 is known to be methylated [14], phosphorylated [15], acetylated and ubiquitinated [16]. These properties of E2F-1 suggest that its activity might be subject to intricate mechanisms of control.

Here, we report that E2F-1 is regulated by NEDDylation. NEDDylation influences E2F-1 stability as well as transcriptional and growth control. Moreover, we provide evidence for interplay between NEDDylation and methylation. Our results thus hint towards a level of complexity that is consistent with an E2F-1 protein code.

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Fig 1 | E2F-1 is NEDDylated. (**A**) HA-E2F-1(1 μg), p53 or empty vector were transfected with His-NEDD8 (4 μg) into H1299 cells and NEDD8 substrates isolated by His purification under denaturing conditions and subject to immunoblot analysis to show that E2F-1 is a substrate for NEDDylation. $n = 2$. (**B**) NEDD8 substrates were isolated from U2OS cells stably expressing His-NEDD8 using His purification under native conditions and analysed by immunoblotting. NTA-Ni^{2+} agarose beads in which the Ni^{2+} group was removed by EDTA were used as a control for nonspecific binding. $n = 2$ (**C**) HA-E2F-1(1 μg) was transfected with His-NEDD8 (4 μg) into U2OS cells and cells treated with the indicated concentration of MLN4924 for 16 h. NEDD8 substrates were isolated by His purification under denaturing conditions and subject to immunoblot analysis. $n = 2$ (**D**) U2OS, SAOS2 and HeLa cells were treated for 16 h with MLN4924 (1 μM), lysed and subject to immunoblot analysis probing for endogenous E2F-1. $n = 4$ (**E**) NEDD8 substrates were isolated from H1299 cells co-expressing HA-E2F-1 (1 μg) and the NEDD8-specific protease NEDP1 (0.5, 1, 2 and 5 μg), using His purification under denaturing conditions and NEDD8-E2F-1 detected using immunoblot analysis (i). The decreasing level of NEDD8-E2F-1 with increasing NEDP1 was quantified and is shown graphically (ii). $n = 5$ (**F**) Immunoblot analysis of His-purified NEDD8 substrates from cells expressing the indicated E2F-1 mutants (1 μg) and His-NEDD8 (4 μg). The immunoblot (ii) is shown alongside a schematic (i) showing the mutants with respect to full-length E2F-1. $n = 2$ (**G**) Immunoblot analysis of His-purified NEDD8 substrates from cells expressing the indicated E2F-1 mutants (1 μg) and His-NEDD8 (4 μg). The immunoblot (ii) is shown together with a schematic (i) indicating the positions of the lysine residues, with red numbers representing lysine residues that were substituted for arginine. The level of NEDDylation of the mutants relative to wild-type is shown graphically. $n = 4$.

RESULTS

NEDDylation of E2F-1

We first expressed His-NEDD8 and E2F-1 in H1299 cells and used His purification followed by immunoblot analysis to identify E2F-1 as a substrate for NEDDylation, using a known NEDD8 substrate, p53, as a positive control [8] (Fig 1A). We then purified NEDD8 substrates from a stable His-NEDD8-inducible U2OS cell line under both native (Fig 1B) and denaturing (supplementary Fig S1A online) conditions and probed for endogenous E2F-1, which also indicated that E2F-1 is NEDDylated. When MLN4924 was used to treat U2OS cells ectopically expressing His-NEDD8 and E2F-1, E2F-1 NEDDylation was observed to decrease in a dose-dependent fashion (Fig 1C). This confirms that the observed NEDDylation on E2F-1 occurs via the classical NEDD8 pathway rather than an 'atypical' pathway in which components of the ubiquitin conjugation pathway can mediate NEDDylation [17,18]. Further support for E2F-1 NEDDylation via the typical conjugation pathway came from the observation that siRNA depletion of UBE1 had no effect on the level of E2F-1 NEDDylation (supplementary Fig S1Bi online). Moreover, a decrease in E2F-1 ubiquitination was observed in cells depleted of UBE1 (supplementary Fig S1Bii online). Furthermore, treating cells with MLN4924 caused an increase in endogenous E2F-1 levels in U2OS, SAOS2 and HeLa cells (Fig 1D), a result consistent with the NEDDylation of endogenous E2F-1.

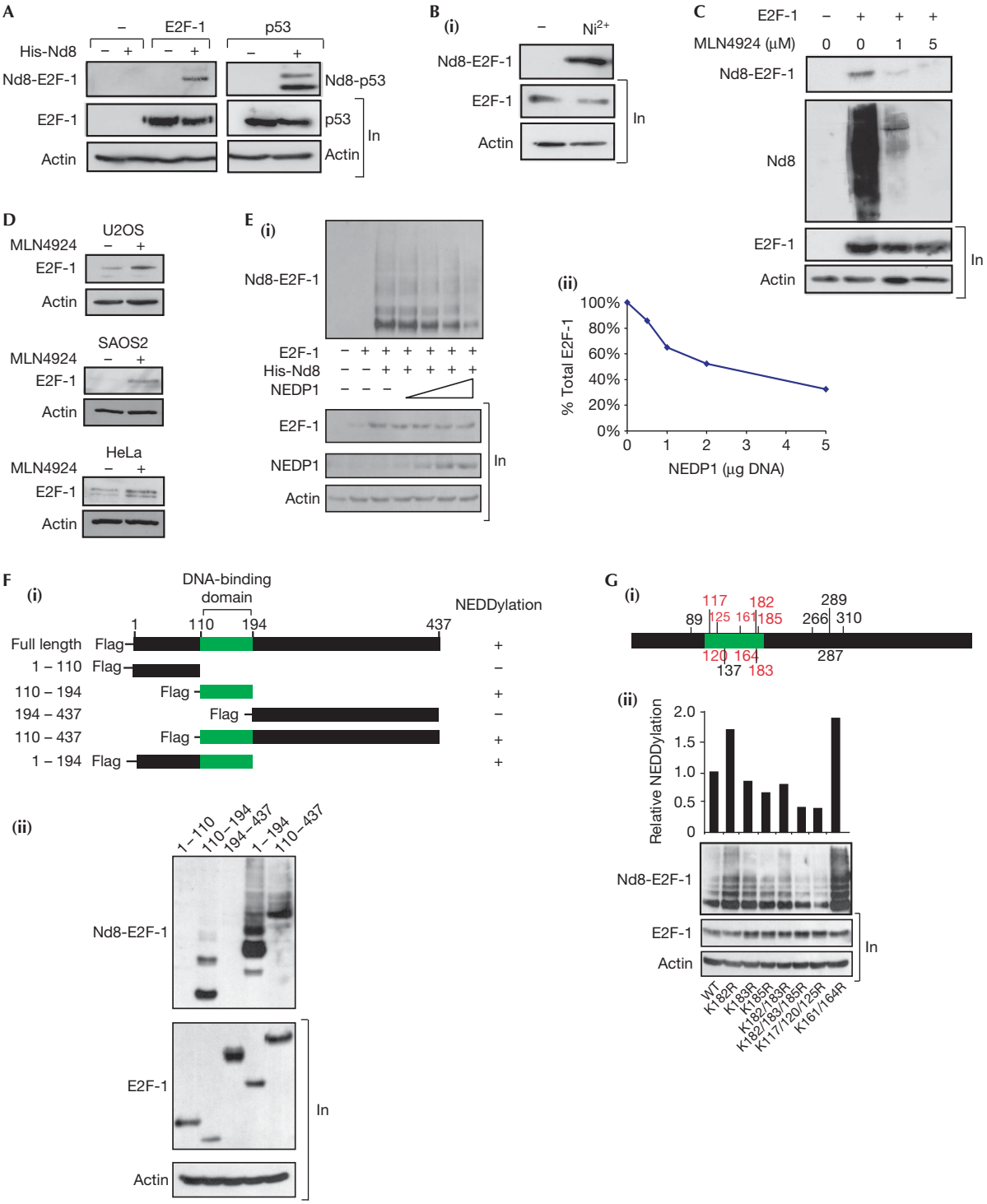
NEDP1 (also known as DEN1 and SENP8) is a NEDD8-specific protease involved in processing the inactive NEDD8 precursor, exposing its diglycine motif so it might be conjugated onto its substrates, but it is also capable of removing NEDD8 from its substrates [19]. By co-expressing NEDP1 with E2F-1 and His-NEDD8, we observed a NEDP1 concentration-dependent decrease in the intensity of NEDD8-E2F-1 (Fig 1E). Further support for E2F-1 NEDDylation came from studying the effect of dominant-negative derivatives of the E2 ligases, UBE2F (C116S) [20] and UBC12 (C111S) [21] (supplementary Fig S1C online). A decrease in E2F-1 NEDDylation on expression of UBE2F (C116S) was observed, in a fashion dependent on the concentration of the E2 derivative.

NEDDylation occurs on lysine residues [22]. To identify residues targeted by NEDDylation, we generated a panel of mutant derivatives (truncations and substitution mutants) and assessed their ability to be NEDDylated (Fig 1F,G). We found E2F-1 to be NEDDylated in its DNA-binding domain as the truncated mutants incorporating only residues 1–110 or 194–437 (namely mutants that did not include the DNA-binding domain) showed no evidence of NEDDylation (Fig 1F). Using substitution mutants in this region (residues 110–194), we identified lysine residues 182, 183, 185, 117, 120 and 125 to be required for efficient NEDDylation of E2F-1 (Fig 1G; supplementary Fig S1D online). The integrity of residue K185 appeared to be particularly important, as did residues K117, K120 and K125, as NEDDylation was decreased in mutants carrying substitutions in these residues. The K117/120R double mutant showed no decrease in NEDDylation with respect to wild-type E2F-1 (supplementary Fig S1D online) suggesting that either residue K125 is important for NEDDylation, or that, similar to the NEDD8 site on p53 [8], all three lysines need to be mutated to observe a decrease in NEDDylation. We therefore used K117/120/125R and K185R as NEDDylation-deficient mutants for further analysis.

NEDDylation affects transcriptional activity

We investigated whether NEDDylation impacted on the transcriptional activity of E2F-1 by co-expressing E2F-1 with luciferase reporter constructs and thereafter assessing the effect of NEDP1 and MLN4924 on promoter activity. An increase of promoter activity on NEDP1 expression or MLN4924 treatment was apparent (Fig 2A–D), suggesting that E2F-1 NEDDylation downregulates transcriptional activity. As the effect was recapitulated in the SAOS2 cell line, which has defective Rb and p53 (Fig 2D), we could rule out the possibility that the observed effects of MLN4924 treatment were mediated by NEDDylation of p53 or by the interaction of Rb with E2F-1, although we cannot, as yet, rule out any influence by other E2F-1-binding partners such as DP-1 and p300. The hypothesis that E2F-1 NEDDylation downregulates its transcriptional activity was further supported by the observation that the K185R and K117/120/125R mutant derivatives had higher levels of reporter activity than the wild-type (Fig 2E). This effect did not arise from any perturbations in intracellular localization resulting from the residue substitution, as both E2F-1 mutants had a nuclear location (supplementary Fig S1E online). Further, when His-NEDD8 was ectopically expressed, a reduction in p73 promoter activity was observed (Fig 2F). The increased transcriptional activity that occurred on the expression of NEDP1 reflected enhanced E2F-1 DNA-binding activity, detected by chromatin

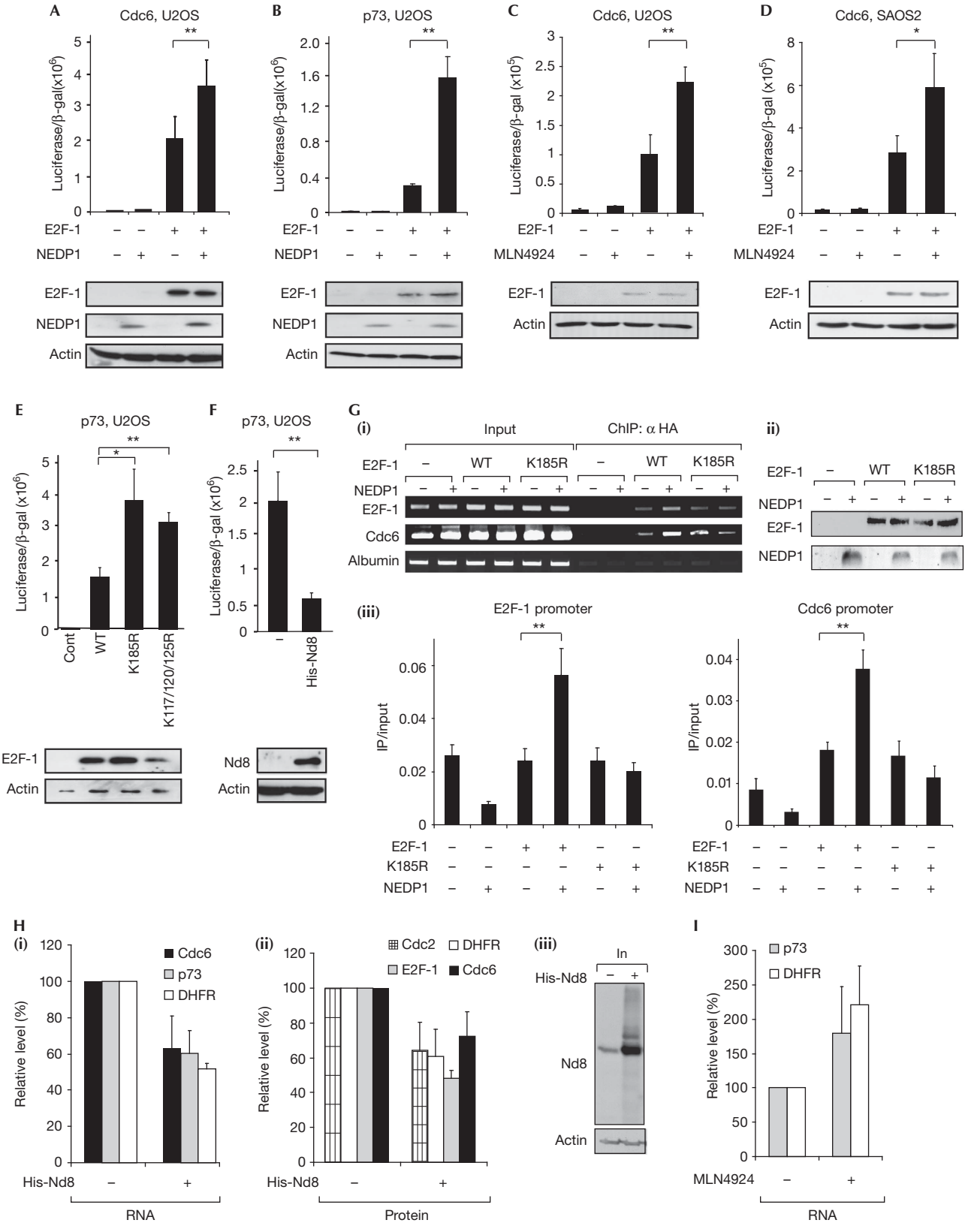
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immunoprecipitation (ChIP), and was not apparent with the K185R mutant, for which NEDP1 failed to augment DNA-binding activity (Fig 2G).

We used a His-NEDD8-inducible stable cell line to investigate whether NEDDylation influenced RNA and protein levels of E2F-responsive genes. We observed a decrease of dihydrofolate reductase (DHFR), Cdc6 and p73 RNA, and E2F-1, Cdc6, Cdc2 and E2F-1 proteins on induction of His-NEDD8 expression (Fig 2H), providing further support that NEDDylation downregulates E2F-1 activity. These results were recapitulated by treating cells with MLN4924, which caused an increase in expression of E2F-1 target genes (Fig 2I), which might at least in part result from the

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◀ **Fig 2** | E2F-1 NEDDylation influences its transcriptional activation. (**A–F**) E2F-1 (100 ng), NEDP1 (200 ng) and His-NEDD8 (500 ng) (where indicated) were co-expressed in U2OS (**A–C; E,F**) or SAOS2 (**D**) cells with the indicated luciferase reporter construct (Cdc6-luciferase or p73-luciferase; 100 ng) and β-gal (100 ng) and where indicated treated with MLN4924 (1 μM) for 16 h. E2F-1 promoter activity was measured as described and input levels analysed by immunoblotting. For **E**, quantification and normalization of E2F-1 input level (normalized to actin) showed the ratio of WT:K185R:K117/120/125R to be 1:1.1:0.7. (**$P<0.01$, *$P<0.05$, Student's *t*-test) $n=4$. (**G**) ChIP from U2OS cells expressing either wild-type E2F-1 or K185R (1 μg) and NEDP1 (1 μg) as indicated (i). An immunoblot of ectopic protein levels is also shown (ii). When quantified, NEDP1 input level fluctuates by 6% between wild-type and K185R treatments. (iii) Quantification of E2F-1 binding to Cdc6 and E2F-1 promoters by real-time PCR. (**$P<0.01$, Student's *t*-test) $n=4$. (**H**) RNA was purified from stable inducible His-NEDD8-expressing U2OS cells and RT–PCR performed to measure the level of the indicated RNA species (i). The decrease in DHFR and p73 levels are significant to a 1% confidence level and that of Cdc6 is significant to a 5% confidence level (Student's *t*-test). Protein levels of the indicated E2F target genes in these cells are shown in (ii). The decrease in Cdc2, DHFR and E2F-1 levels is significant to a 1% confidence level and that of Cdc6 significant to a 5% confidence level (Student's *t*-test). Protein inputs for NEDD8 are shown in (iii). $n=4$. (**I**) RNA was purified from U2OS cells and RT–PCR performed to measure the level of the indicated RNA species following treatment with MLN4924 (1 μM) for 16 h. The observed increases in DHFR and p73 RNA were significant to a 5% confidence level. $n=3$. In all panels, error bars represent s.e.'s. ChIP, chromatin immunoprecipitation; RT–PCR, reverse transcriptase PCR; WT, wild-type.

increased E2F-1 levels observed on MLN4924 treatment (Fig 1D). Thus, NEDDylation influences the transcriptional and DNA-binding properties of E2F-1.

NEDDylation regulates stability and growth control

NEDDylation has previously been shown to regulate the stability of its substrates [5,6]. To investigate this possibility for E2F-1, we compared the level of His-tag-purified NEDDylated E2F-1 with the level of total ectopic E2F-1 at several time points following cyclohexamide treatment and observed that E2F-1 is destabilized on NEDDylation (Fig 3A). This effect was also apparent with NEDP1, because E2F-1 in NEDP1-expressing cells had an extended half-life (Fig 3B). It is also consistent with this effect that the K185R and K117/120/125R mutants had an increased half-life (Fig 3B).

As E2F-1 is known to be stabilised under DNA damage [23], we investigated the effect of etoposide and doxorubicin treatment on E2F-1 NEDDylation and found NEDDylated E2F-1 levels to decrease by more than 80% under these conditions (Fig 3C). Further, treatment with MLN4924 no longer had the effect of increasing endogenous E2F-1 levels upon DNA damage (Fig 3D). These results suggest that E2F-1 NEDDylation is regulated during the DNA damage response.

As E2F-1 is important for growth control, we reasoned that NEDDylation could affect cell growth. We therefore expressed wild-type E2F-1 and its mutants, and thereafter assessed their activity in a cell proliferation assay (Fig 3E). Very interestingly, U2OS cells expressing the NEDDylation-deficient K185R and K117/120/125R mutants proliferated faster than those expressing wild-type E2F-1, suggesting that NEDDylation impacts on the growth-regulating properties of E2F-1.

Interplay with E2F-1 NEDDylation

Our results indicate that residue K185 is important for efficient NEDDylation of E2F-1. We found the identification of K185 to be provocative because the same residue is methylated by Set7/9 (Fig 4A–C) [14]. We surmised, therefore, that there might be a level of interplay between methylation and NEDDylation of E2F-1. To explore this idea, we established conditions for measuring Set7/9-dependent methylation of K185, which was apparent both *in vitro* and in cells (Fig 4A–C). Under conditions of K185 methylation in cells, we depleted Set7/9 and thereafter monitored the level of E2F-1 NEDDylation. Strikingly, depletion of Set7/9 reduced the level of wild-type E2F-1 NEDDylation, with a lesser effect evident on the K185R mutant (Fig 4D). At the transcriptional level, in Set7/9 depleted cells E2F-1 failed to respond to NEDP1 (Fig 4E). Furthermore, K185R exhibited enhanced transcriptional activity and, when compared with wild-type E2F-1, was less responsive to NEDP1 (Fig 4F). These results suggest a regulatory role for methylation of E2F-1 that is mediated in part through an influence on site-specific NEDDylation.

DISCUSSION

We have demonstrated that E2F-1 is NEDDylated, that NEDDylation negatively regulates E2F-1 activity and that there exists a level of crosstalk between NEDDylation and methylation. Our results are somewhat analogous to those seen for p53, where NEDDylation requires a tetrapeptide motif consisting of three lysines and a serine residue (similar to the E2F-1 NEDDylation site), and results in attenuation of its transcriptional activity [8]. Residue K372 of p53 is both required for NEDDylation and is a target for Set7/9 [24], thus raising the interesting possibility that protein regulation by NEDDylation and methylation by Set7/9 are closely linked events. In addition, BCA3 NEDDylation has been shown to affect transcription, with BCA3 NEDDylation resulting in suppression of NFκB transcription via its ability to bind the p65 and cyclin D1 promoter [25]. Perhaps, therefore, NEDDylation has a more general role in gene control, reflecting a combination of its direct regulation of transcription factors and enhanced ubiquitination from its role in CRL activity.

There are also other reported examples of NEDDylation influencing protein stability [5,6]. A noteworthy example is the observation of MDM2 stabilization by NEDDylation [26]. DNA damage was found to increase NEDP1 levels which reversed MDM2 stabilization, causing a rise in p53 level and apoptosis. This is in support of our observations that NEDP1 reduces E2F-1 NEDDylation and that E2F-1 NEDDylation decreases under conditions of DNA damage. In addition, as we have observed many parallels with E2F-1 and p53 NEDDylation, it is plausible that NEDDylation provides a link between the E2F-1 and p53 cell-cycle regulatory pathways. The interesting observation that the NEDDylation and Set7/9 methylation sites on both E2F-1 and p53 overlap raises the possibility that a wider interplay between

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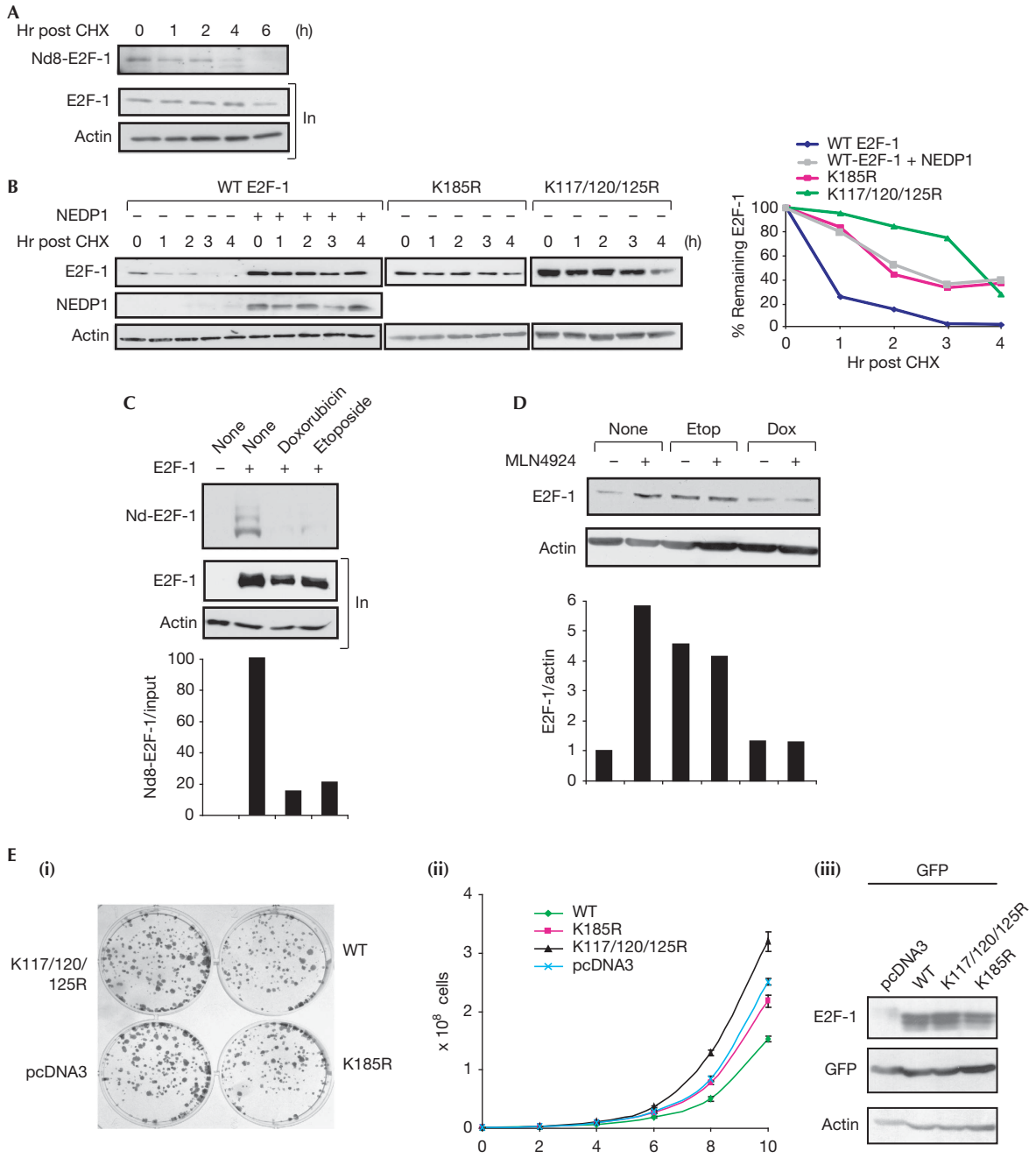


Fig 3 | E2F-1 NEDDylation results in its destabilisation. (**A**) U2OS cells transfected with expression vectors encoding HA-E2F-1 (1 μg) and His-NEDD8 (4 μg) were treated with cyclohexamide (100 μM) and harvested at time points over 6 h. NEDD8-E2F-1 was isolated by His purification under denaturing conditions and the results analysed by immunoblotting, to compare the half-life of NEDDylated E2F-1 with total ectopic E2F-1. Quantification showed that NEDD8-E2F-1 had a half-life that was 32% shorter than that of total E2F-1. $n = 2$. (**B**) U2OS cells expressing ectopic NEDP1 (2 μg) and HA-E2F-1, K185R or K117/120/125R (1 μg) were harvested over 4 h following cyclohexamide treatment and analysed by immunoblotting. Quantification is shown alongside. $n = 2$. (**C**) NEDD8 substrates were purified from U2OS cells ectopically expressing His-NEDD8 and HA-E2F-1, and treated with either etoposide (20 μM) or doxorubicin (2 μM) for 16 h, and the level of NEDD8-E2F-1 analysed by immunoblotting. $n = 3$. (**D**) U2OS cells were treated with MLN4924 (1 μM), etoposide (20 μM) or doxorubicin (2 μM) for 16 h and lysates subject to immunoblotting, probing for endogenous E2F-1. (**E**) U2OS cells were transfected with either wild-type, K185R or K117/120/125R E2F-1 (1 μg), and cell colonies stained with crystal violet after 10 days (i). Quantification showed colony numbers to be as follows: pcDNA3 171; wild-type 120; K185R 148 and K117/120/125R 225. The rate of proliferation of transfected cells was measured by counting cells every 2 days for 10 days after staining with trypan blue and is presented graphically in (ii). s.e.'s are representative of three independent repeats. Protein levels at the end of the 10-day period are shown in (iii). $n = 3$. GFP, green fluorescent protein; WT, wild-type.

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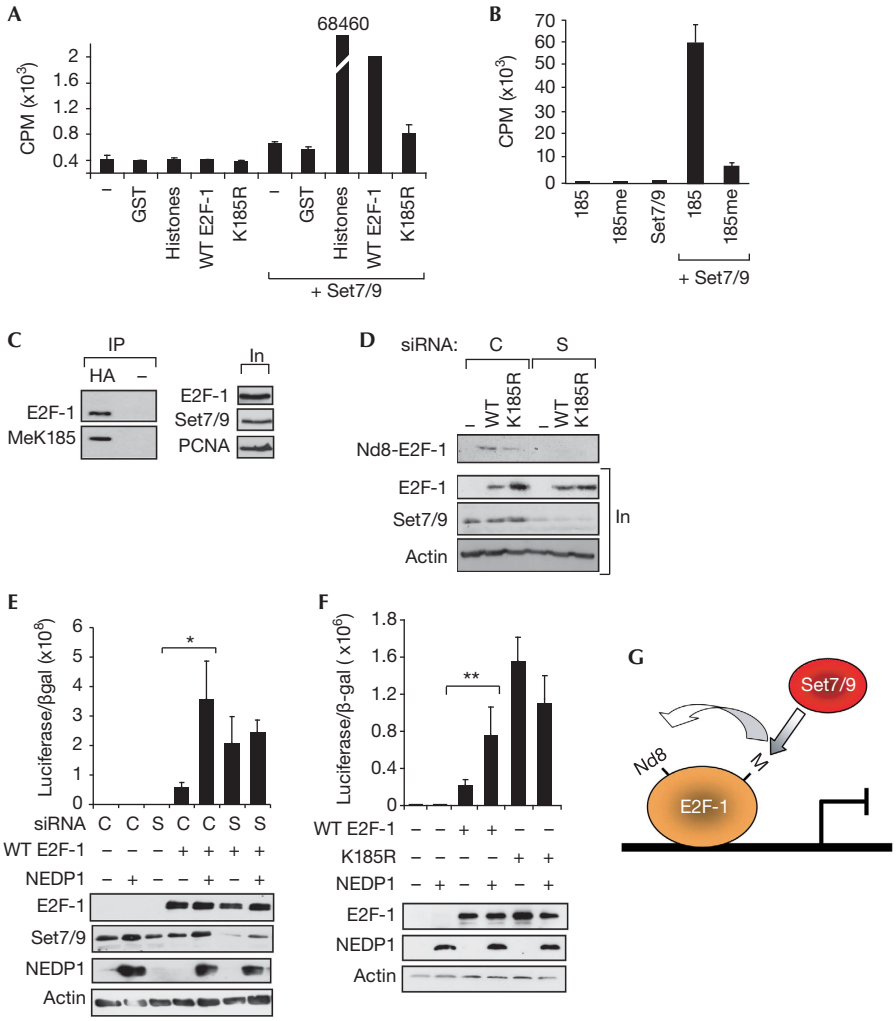


Fig 4 | E2F-1 methylation at K185 may be required for its NEDDylation. (**A**,**B**) Recombinant E2F-1 (1 μg) (**A**) or peptides (5 μg), either methylated (185me) or unmethylated (185) (**B**), were incubated with Set7/9 (0.5 μg) in the presence of ^{3}H-labelled SAM. Substrate methylation was quantified by measuring the transfer of radiolabel, which was detected in a filter assay. $n = 5$. In all panels, error bars represent s.e.'s. (**C**) HA-E2F-1 (4 μg) was immunoprecipitated from U2OS cells with either anti-HA or control (−) antibody, and analysed by immunoblotting with either anti-E2F-1 or a site-specific methyl-K185 antibody. Input (In) levels are also shown. $n = 2$. (**D**) NEDD8 substrates were purified under denaturing conditions from H1299 cells treated with Set7/9 (S) or control (C) siRNA (20 nM) under ectopic HA-E2F-1 (1 μg) and His-NEDD8 (4 μg) expression. The results were analysed by immunoblotting. $n = 3$. (**E**) E2F-1 (100 ng) and NEDP1 (200 ng), as indicated, were co-expressed in U2OS cells with Cdc6-luciferase reporter construct (100 ng) and β-gal (100 ng) following treatment with either Set7/9 (S) or control (C) siRNA (20 nM). Promoter activity was measured as described. Immunoblot analysis of protein expression level is shown. (*$P < 0.05$, Student's t-test) $n = 5$. (**F**) Either E2F-1 or K185R (100 ng) were co-expressed with NEDP1 (200 ng), as indicated, with Cdc6-luciferase reporter (100 ng) and β-gal (100 ng) plasmids and promoter activity measured as described. Immunoblot analysis of the protein expression level is also shown. (**$P < 0.01$, Student's t-test) $n = 5$. In panels **E** and **F**, error bars represent s.e.'s. (**G**) Proposed model of the interplay between methylation and NEDDylation. It is suggested, based on the results presented, that Set7/9 methylation of E2F-1 influences subsequent NEDDylation of E2F-1, which then impacts on the biochemical and functional properties of E2F-1.
IP, immunoprecipitation; SAM, *S*-adenosyl-L-methionine; siRNA, short interfering RNA; WT, wild-type.

transcription factor regulation by these modifications exists, and this should be investigated further.

METHODS

His purification under denaturing conditions. Cells were treated with MG132 (20 μM) (Calbiochem) 4 h before harvesting and lysates prepared and His-NEDD8 substrates purified using Ni^{2+}-NTA agarose beads (Qiagen) according to the manufacturer's instructions but with several modifications as outlined in the supplementary information online.

Luciferase reporter assays. H1299 cells were plated onto 6 cm dishes and transfected as indicated, harvested after 48 h and lysed in 100 μl Reporter Lysis Buffer (Promega). Fifty percent of lysate was used to measure luciferase, 10% to measure β-gal

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(absorbance at 420 nm read after addition of buffer to a final concentration of 100 mM Na_2PO_4 (pH7.3), 1 mM $MgCl_2$, 50 mM β-mercaptoethanol and 0.665 mg/ml ONPG and incubation at 37 °C) and 40% lysate analysed by immunoblot to measure protein levels. Luciferase values were normalized according to the β-gal reading.

Chromatin immunoprecipitation. Chromatin was prepared as described previously [27]. The recovered DNA was analysed by real-time PCR as described [28] on the MX3005P QPCR system (Stratagene) using Brilliant II SYBR Green QPCR Master Mix (Stratagene) according to the manufacturer's instructions. Primer sequences are available on request.

Reverse transcriptase PCR. RNA was isolated from cells with the use of Trizol (Invitrogen). Five microgram of total RNA was used to generate complementary DNA using an oligo$(dT)_{20}$ primer and Superscript III reverse-transcriptase enzyme (Invitrogen). Two microlitre of this reaction was subsequently used as a template for PCR using gene-specific primers (sequences available on request) and Paq 5000 DNA polymerase (Agilent Technologies).

***In vitro* methylation assay.** *In vitro* methylation assays were carried out using bacterially expressed recombinant GST-E2F-1 or GST-K185R E2F-1 as described previously [29].

Further Methods can be found in supplementary information online.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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Author contributions: S.J.L., G.L., S.M.C. and S.M. undertook all experimental work, S.J.L. and N.B.L.T. wrote the manuscript and N.B.L.T. directed the project.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Supplementary Information

SI Figure 1

(A) NEDD8 substrates were isolated from U2OS cells stably expressing His-NEDD8 using His-purification under denaturing conditions and analysed by immunoblotting. NTA-Ni^{2+} agarose beads in which the Ni^{2+} group was removed by EDTA were used as a control for non-specific binding. The positions of molecular weight markers (in kDa) are indicated to demonstrate the ~10kDa shift observed for NEDDylated E2F-1 with respect to its unmodified form; n=4

(B (i-ii)) NEDD8 or ubiquitin substrates were isolated by His-purification from U2OS cells co-expressing E2F-1 (1µg) and, where indicated, either His-NEDD8 or His-Ubiquitin (3µg) under conditions of either control (C) or UBE1 (U) siRNA depletion (50nM). His-purification was performed under denaturing conditions and NEDD8-E2F-1 or ubiquitin-E2F-1 detected by immunoblotting. The level of modified (mod.) E2F-1 normalised to its respective level under conditions of normal UBE1 levels and with resepct to E2F-1 input level was quantified and is shown graphically; n=6

(C) NEDD8 substrates were isolated from H1299 cells co-expressing E2F-1 (1µg) and His-NEDD8 (3µg) and one of two E2 mutants (0.5µg, 1µg and 2µg) which act as dominant negative regulators of NEDDylation [1, 2], namely UBE2F(C116S) or UBC12(C111S). His-purification was performed under denaturing conditions and NEDD8-E2F-1 detected by immunoblotting (i). The decreasing level of NEDD8-E2F-1 with increasing UBE2F or UBC12 was quantified and is shown graphically (ii). n=3

(D) Immunoblot analysis of His-purified NEDD8 substrates from cells expressing the indicated E2F-1 mutants (1µg) and His-NEDD8 (4µg). Quantification showed the ratio of wild-type: K117/120R: K161/164R: K183/183/185R to be 1: 1.1: 5.1:0.67.

(E) Cells were subject to immunofluorescence analysis in U2OS cells expressing wild-type, K185R or K117/120/125R E2F-1 (250ng).

(F) Protein inputs for the growth assay in Fig 3E(ii) shown at 2 day time points throughout the duration of the experiment.

Additional materials and methods

Cell culture and transfection: H1299 cells were cultured in RPMI supplemented with 5% fetal calf serum (Invitrogen) and U2OS, SAOS2 and HeLa cells cultured in Dulbecco modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum and penicillin-streptomycin (Invitrogen). All cell lines were cultured at 37°C and 5% CO_2. Transfections for ectopic protein expression and siRNA were carried out using GeneJuice (Novagen) and Oligofectamine (Invitrogen), respectively, both according to the manufacturer's instructions. Cells were harvested 48 hours post plasmid transfection or 72 hours post siRNA transfection.

Generation of NEDD8-inducible cell line: His-NEDD8 plasmid was cloned into a pTre2-hyg vector and transfected into U2OS cells containing the pTet-On plasmid (G418 resistant). After 48 hours transfection, cells were selected and maintained in DMEM containing G418 (100µg/ml) and hygromycin (150µg/ml).

Plasmids and expression vectors: All expression vectors have been described previously; UBE2F (C116S) [1], UBC12 (C111S) [2], HA-E2F-1 [3], Cdc6-luciferase [4], p73-luciferase [5], pGEX-E2F-1 [6], pGEX-Set7/9 (52-366); [7]. His-NEDD8, His-ubiquitin and NEDP1 vectors were a kind gift from Ron Hay, Dundee, UK. Lysine to arginine substitution mutants and truncations were derived from their wild-type counterparts using standard molecular techniques.

Antibodies: HA (11) (Covance); Actin (Sigma); E2F-1 (KH95), p53 (DO-1), Cdc6, DHFR, Cdc2 (Santa Cruz), NEDP1 (Gift from Ron Hay Dundee, UK), Set7/9

(Abnova), UBE1 (Abcam). The methyl-specific K185-E2F-1 antibody was generated by Eurogentec.

siRNA: UBE1 siRNA (h) (Santa Cruz); Set7/9 siRNA (Dharmacon custom synthesis); non-targeting control #2 (Dharmacon).

His-purification of NEDD8 substrates under denaturing conditions: H1299 cells were and transfected as indicated and treated with MG132 (20μM; Calbiochem) 4 hours prior to harvesting in denaturing lysis buffer (8M Urea pH 8.0, 0.1M Na_2HPO_4/NaH_2PO_4, 0.01M Tris-HCl). Lysate was passed 6 times through a 23G needle and spun at 13,000 rpm for 5 minutes. After adjusting lysate volumes for protein concentration (diluting with lysis buffer), 70μl Ni^{2+} NTA-agarose beads (Qiagen) were mixed with lysate and β-mercaptoethanol (Sigma) and Triton X-100 (Sigma) added to a final concentration of 10mM and 0.1%, respectively. Samples were incubated at 4°C overnight, rotating. Beads were then washed twice with denaturing lysis buffer (above) with 10mM β-mercaptoethanol and 0.1% Triton X-100 and 3 times with denaturing lysis buffer adjusted to pH6.3 and with 10mM β-mercaptoethanol and 0.1% Triton X-100. NEDDylated proteins were then eluted for 30 minutes rotating at room temperature in 100μl buffer containing 200mM imidazole, 5% SDS (Sigma), 150mM Tris-HCl pH6.8, 30% glycerol, 720mM β-mercaptoethanol and 0.005% bromophenol blue. 10% total lysate was used for immunoblot analysis of inputs.

For purification of endogenous E2F-1 by His-purification under denaturing conditions, His-NEDD8 inducible U2OS cells were harvested in PBS, spun down by

centrifugation and lysed in 4ml denaturing lysis buffer with Benzonase (125U/ml, Novagen). Lysate was divided equally into two and His-purification performed as above. For a non-specific binding control, nickel was removed from Ni^{2+} NTA-agarose by incubating for 30 minutes with 50mM EDTA and these beads used in the purification

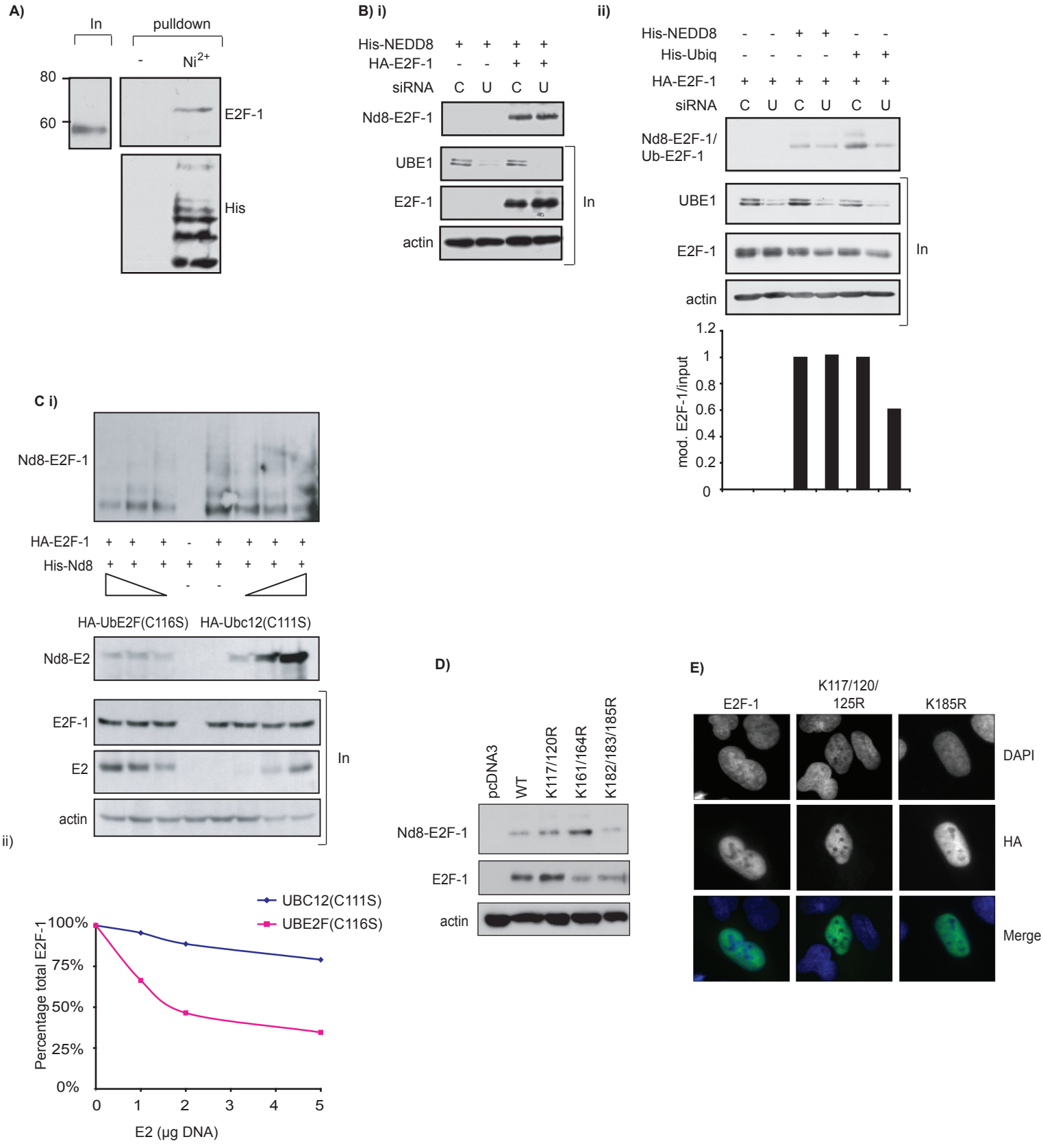
His-purification of endogenous E2F-1 under native conditions: His-NEDD8 inducible cells were plated onto 10cm dishes (10 per condition), induced with 1µg/ml doxycycline after 24 hours and treated with MG132 (20µM; Calbiochem) after a further 24 hours. 4 hours after MG132 treatment, cells were washed with phosphate buffered saline (PBS) and cells lysed and His-purification of NEDDylated E2F-1 performed using Ni^{2+} NTA-agarose (Qiagen) according to the manufacturer's instructions. For a non-specific binding control, nickel was removed from Ni^{2+} NTA-agarose by incubating for 30 minutes with 50mM EDTA and these beads used in the purification

Immunoprecipitation: U2OS cells were washed in PBS and then lysed in a buffer consisting of 50mM Tris pH 7.5, 1% Igepal CA-630 (Sigma), 150mM NaCl, 1mM EDTA, 1mM NaF, 1mM Na_3VO_4, with protease inhibitors. Lysates were incubated overnight at 4°C with 10µg HA-agarose (Sigma). Beads were washed 5x with lysis buffer as described above but with 0.1% Igepal CA-630 (Sigma). Following protein elution with 4x SDS loading buffer, analysis was by SDS PAGE and immunoblotting.

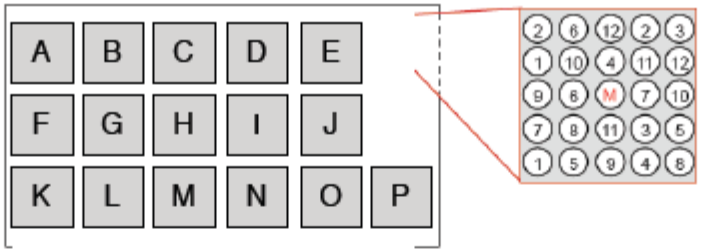
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Supplementary Information Figure 1

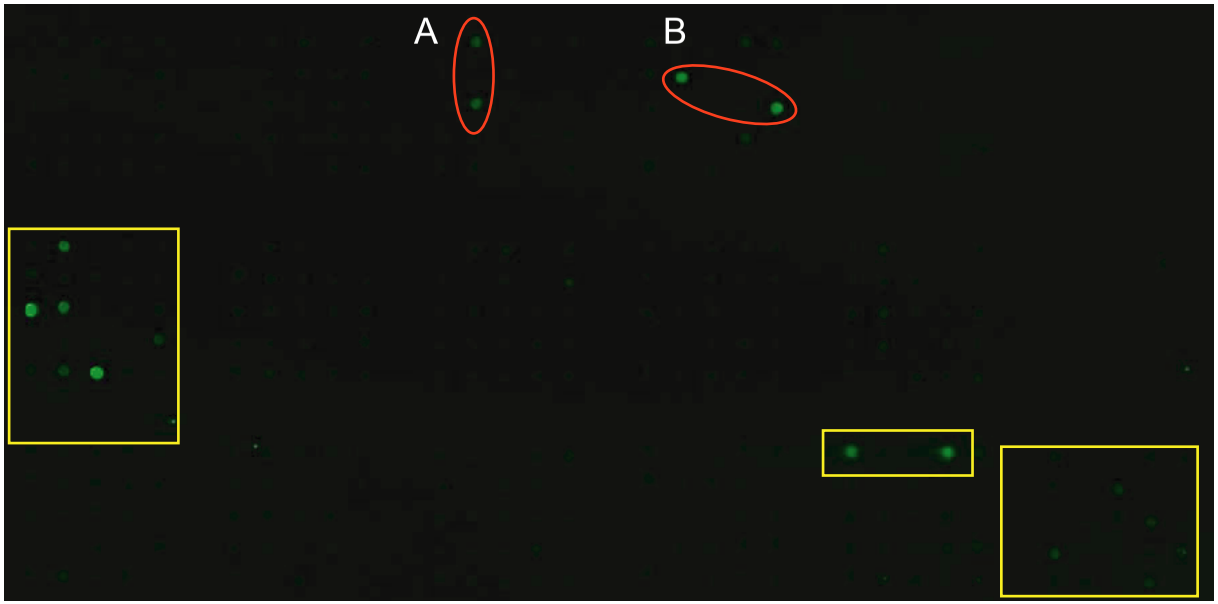


List of recombinant peptide domains used in the CADOR array



TUDOR A1 TDRD2(NP_006853) A2 TDRD3(Q9H7E2) A3 TDRD4-1(Q9NUY9) A4 TDRD4-2(Q9NUY9) A5 TDRD4-3(Q9NUY9) A6 TDRD7-1(NP_055105) A7 TDRD7-3(NP_055105) A8 EBNA-2Co-A(NP_056205) A9 Ret-bp1(AAB28543) A10 M96(AAH10013) A11 53BP1(1-2)(NP_005648) A12 53BP1(1)(NP_005648)	**TUDOR +** B1 2C(NP_055876) B2 RBP1 like-2 (NP_112739) B3 SMN (NP_075012) B4 CG1-72(NP_057102) B5 PHF20(NP_057520) B6 PHF20 MBT+TDR(NP_057520) B7 Pombe 1(CAA22823) B8 JMJD2A-2(NP_055478) B9 JMJD2A1-2(NP_055478) B10 LBR TDR(NP_919424) B11 LBR211(NP_919424) B12 SPF30(c)(O75940)	**TUDOR / Tudor-Like** C1 Lin9 TDR(b)(AAH65302) C2 JMJN 2B WT(f)(NP_055830) C3 ARI4A (e)(NM_002892) C4 PHF19(NP_001009936) C5 SND1 (e)(NM_014390) C6 UHRF1 Tudor-like(a) C7 C420 Tudor-Like(p) C8 C460 Tudor-Like(p)	**MBT** D1 SFMBT F.L(j) D2 SFMBt 4xMBT(j) D3 L(3)MBT(NP_056293) D4 L(3)MBT1-3(NP_056293) D5 SCMH1(AAH21252) D6 SCML1(NP_057413) D7 SCML2(AAH64617) D8 LML2(Q969R5) D9 PHF20 MBT(NP_057520) D10 CG1-72-MBT(NP_057102)	**His-MBTs / WD40** E1 SFMBT1(a) E2 SFMBT2(a) E3 L3MBTL1(a) E4 L3MBTL2(a) E5 L3MBTL3(a) E6 SCMH1(a) E7 WD40 -WDR5(d)(NP_543124) E8 WD40 -WDR9(NM_018963) E9 WD40 -RbAb46(d)(BT007309) E10 WD40 -RbAb48(d)(X74262) E11 WD40 -HIRA(CR456503) E12 WD40 -Mep50(d)(AF478464)	
PhD + F1 BPTF(P+B)(BAA89208) F2 ING2(e)(AAH50003) F3 PHF2(NP_005383) F4 PHF8(CAI42860) F5 DATF1(CAI96708) F6 Rag2(NM_000536) F7 PCCX1(NP_055406) F8 P300(P+B)(NM_001429) F9 PHF20 PHD(NP_057520) F10 PHD PHF3 F11 PHD PHF5 F12 PHD CHD5 (1-2)	**PhD** G1 Dnmt3a-His/GST(g) G2 Dnmt3b-His/GST(g) G3 DnMT3L N-term-His/GST(g) G4 Trim24 Brd-PhD(l) G5 ING3(e) (NP_061944) G6 ING4(e) (NP_001121054) G7 ING5(e) (NP_115705) G8 PHD _TIF1A(e) (O15164) G9 TRI66(e) (O15016) G10 BRPF1(e) (P55201) G11 MLL4(e) (Q9UMN6) G12 MTF2(e) (Q9Y483)	**PhD +** H1 JMJD2APhD+2Tudor(NP_055478) H2 JMJCPhD(NP_055478) H3 M96Tudor+PhD(AAH10013) H4 MYST4PhD+PhD(AAH48199) H5 NSD1 PhD+PWWP(Q96L73) H6 WHSC1 PhD+PWWP(NP_579877) H7 BS69PhD+ BRD(AAH12586) H8 ATRX H9 RAL1 H10 BAZ1b/WSTF H11 CBP H12 TAF3 (k)	**BROMO** I1 GCN5(Q92830) I2 TAF1- D1(NP_620278) I3 TAF1- D2(NP_620278) I4 P/CAF (S71788) I5 SNF2 beta(S45252) I6 BAF180 1-2(NP_060635) I7 BAF180-3(NP_060635) I8 BAF180 3-4(NP_060635) I9 BAF180 5-6(NP_060635) I10 KAP-1(AAB37341) I11 P300(NP_004371) I12 WDR9 2(Q9NSI6)	**BROMO /SANT / TSN** J1 Bromo-BAZ(NP_075381) J2 Bromo-BRDT 1(AAH62700) J3 SANT-MPP11-like(XP_379909) J4 SANT-N-CoR2-2(Q9Y618) J5 SANT-RERE(AAH62342) J6 SANT-ADA2(NP_001479) J7 SANT-Zuotin Rel.(XP_166590) J8 TSN-p100(o) (NP_055205) J9 TSN-p100 m5(o) (NP_055205) J10 TSN-p100 m6(o) (NP_055205)	
CHROMO K1 TIP60(h)(AAB18236) K2 CHD2(h)(AAB87382) K3 CHD4(h)(AAH38596) K4 MPP8(h) (NP_059990) K5 SMARCC2(h)(AAH26222) K6 MRG15(h)(AAD29872) K7 RBBP1(h)(AAD41239) K8 PC2(h)(AAB60718) K9 PC3(h)(AAG09180) K10 CHD5(h)(AAK56405) K11 CHD7 (1-2)(AB037837) K12 CBX6/NPCD(BC012111)	**CHROMO** L1 MI-2(h)(CAA60384) L2 HP1alpha(h)(P45973) L3 HP1gamma(h)(NP_057671) L4 Msl3-like(h)(AAD38499) L5 SUV39H1(h)(AAB92224) L6 CBX1/HP1beta(h)(AAD21972) L7 HP1 beta(h)(P23197) L8 CDY1(h)(AAD22735) L9 CHD1(e) (NP_001261) L10 CBX4/PC2(e) (NM_003655) L11 CBX7/RP4(e) (NM_175709) L12 CBX5/HP1alpha(e)NM_012117)	**CHROMO / BRK /MRG** M1 CBX3(e) (NM_016587) M2 CBX2(e) (NM_005189) M3 CDYL2(e) (NM_152342) M4 CBX8(e) (NM_020649) M5 BRK_SMCA2 (e)(NM_003070) M6 BRK_SMCA4(e) (NM_003072) M7 BRK_CHD6(e) (NM_032221) M8 BRK_CHD7 (e)(NM_017780) M9 BRK_Q6DTK9 (e)(NM_025134) M10 MRG_MS3L1(e) (NM_079629) M11 MRG_MO4L1(e) (NM_206839)	**PWWP** N1 BRPF1(AAH53851) N2 DNMT3B(Q9UBC3) N3 HDGF (P51858) N4 HRP-3(BAA90477) N5 MSH6(P52701) N6 NSD1(Q96L73) N7 WHSC1-1(NP_579877) N8 PSIP1(e) (NM_033222 N9 BRD1(e) (NM_014577 N10 ZCPW1 (e)(AL136735 N11 MBD6 (e)(NM_018328	**PWWP / CW / SWIRM** O1 PWWP_PKCB1(e) (NM_183047 O2 PWWP_HDGR3(e) (NM_016073) O3 PWWP_DNM3A (e) (NM_175629) O4 CW3(AAH02725) O5 CW5(BAA09485) O6 CW6(XP_087384) O7 SWIRM_ KIAA1915(BAB67808) O8 SWIRM_KIAA0601(CAB72299) O9 SWIRM_ADA2(NP_001479)	**ANK** Q1 ANK-BARD1 (NP_000456) Q2 ANK-GLP(m) (AAM09024.1) Q3 ANK-Notch (NP_060087) Q4 ANK-IKB alpha F.L. (e) **Other** Q5 TULP1 Q6 MeCP2(l) Q7 PHD_ ZFP-1 His/GST(n)

Uncropped CADOR Array



biotin-PEG-IQLIAKKSK[Me]NHIQWLGS



biotin-PEG-IQLIAKKSKNHIQWLGS

A UHRF-1

B PHF20L1

Non-specific binding

Methyl-K185 specific interaction