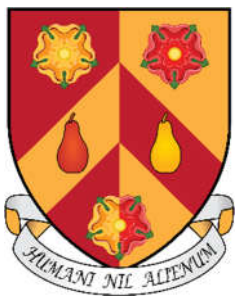
EXPLORING THE EFFECTS OF SYMMETRIC ARGININE METHYLATION READERS ON E2F1-DEPENDENT TRANSCRIPTIONAL OUTPUT *IN VITRO*

Thesis submitted for the MSc by Research in Oncology
University of Oxford

By

Arjelle Decasa Agupitan
Wolfson College

Hilary 2017





To my mother and father,
without whom, none of this would be possible.

Declaration

All of the work in this thesis was conducted solely by myself. No part of this thesis has been accepted, or is currently being submitted for any other degree or diploma in this university or elsewhere. My work and research was conducted under the supervision of Professor Nicholas La Thangue, Department of Oncology, University of Oxford.

Arjelle Decasa Agupitan

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Exploring the effects of symmetric arginine methylation readers on E2F1-dependent transcriptional output *in vitro*

Arjelle Decasa Agupitan, Wolfson College

Submitted for the degree of MSc by Research in Oncology

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Abstract

E2F1 plays contrasting biological roles in both cellular proliferation and apoptosis. Recent studies have demonstrated that residue-specific arginine dimethylation marks deposited by PRMT1 and PRMT5 mediate the apoptotic and proliferative activity of E2F1 respectively. Two readers of the proliferative symmetric arginine dimethylation mark deposited by PRMT5 on E2F1 have since been identified: TSN and TDRD1. In this study, qRT-PCR analysis was used to validate the deregulation of candidate genes in response to TSN or TDRD1 knockdown in MCF7, SKBR3 and DuCaP cells. Transient siRNA co-depletion of readers along with E2F1 confirmed that the deregulation observed was achieved in an E2F1-mediated manner in MCF7 and SKBR3 cellular backgrounds. Furthermore, ChIP analysis revealed that the levels of E2F1 binding to target promoters were affected by reader knockdown. Moreover, E2F1-dependent and -independent recruitment of TSN was confirmed on the promoters of target genes in MCF7 cells. In addition to direct transcriptional effects, analysis of the splice variant expression of several target genes revealed that E2F1 could potentially play a role in TSN-associated splicing activity. The results obtained from this study implicate TSN and TDRD1 in mediating the proliferative activity of E2F1 through proposed regulatory roles in the transcription and alternative splicing of E2F1 targets. This study thus refines our current understanding of how symmetric arginine dimethylation mediates E2F1 proliferative activity, and forms the foundation for further research to strengthen and establish the mechanistic bridge between TSN, TDRD1 and the E2F1 symmetric arginine dimethylation mark.

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Abbreviations

% v/v	volume/volume percent
°C	degrees Celsius
ΔΔCT	relative fold change according to threshold cycle
µg	Microgram
µL	Microliter
µM	Micromolar
53BP1	p53 binding protein 1
aa	amino acid
Ac	Acetylation
ADAR	adenosine deaminase acting on RNA
ADMA	asymmetric dimethylarginine
AEG1	astrocyte elevated gene 1
AGE	agarose gel electrophoresis
Ago	Argonaute
AKAP	a-kinase anchor protein
APAF-1	apoptotic protease activating factor 1
ARF	alternate reading frame
Arg	Arginine
ATM	ataxia-telangiectasia mutated serine/threonine kinase
ATR	Ataxia telangiectasia and Rad3 related serine/threonine kinase
BCL-2	B-cell lymphoma 2
BCLX	BCL-like 2 protein
BLAST	Basic Local Alignment Search Tool
Blimp1	PR domain zinc finger protein 1
bp	base pairs
B-Raf	v-Raf murine sarcoma viral oncogene homologue B
BRCA	breast cancer susceptibility gene
BRG1	brahma-related gene-1
BRM	Brahma gene
BSA	bovine serum albumin
CADOR	chromatin-associated domain array
CARM1	coactivator-associated arginine methyltransferase 1, PRMT1
Cas9	CRISPR-associated protein-9
CASP8AP2	caspase 8 associated protein 2
Cat No	catalogue number
CBP	CREB-binding protein
CDC6	cell division cycle 6
CDK	cyclin-dependent kinase
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CHK2	checkpoint kinase 2
Chromo	chromatin-binding
CKI	cyclin dependent kinase inhibitor
CO_2	carbon dioxide
co-IP	co-immunoprecipitation
COPR4	COPF repressor

CRISPR	clustered regularly interspaced short palindromic repeats
CT	threshold cycle
CTD	carboxy terminal domain
C-terminus	carboxyl terminus
CXCL16	chemokine (C-X-C motif) ligand 16
DBD	DNA binding domain
DEXD	DEAD-like helicase domain
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	deoxynucleotide
double E	double enhancer box motif
DP	dimerization partner
DTT	dithiothreitol
DuCaP	dura mater Cancer of the Prostate
E2F	early 2 factor
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EGF4	epidermal growth factor 4
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ENCODE	The Encyclopaedia of DNA Elements
ERG	ETS-related gene
ETS	E26 transformation-specific
eTud	extended Tudor domain
F	forward primer
FBS	foetal bovine serum
FBXO11	F-Box Protein 11
FLAG	FLAG octapeptide
G_1	gap phase 1
G_2	gap phase 2
G3BP	Ras-GAP SH3 binding protein
GAR	glycine and arginine-rich motif
GFP	green fluorescent protein
G_o	quiescence
H3	histone 3
H3K27me3	histone 3 lysine 27 trimethylation
H4	histone 4
HAT	histone acetyltransferase
HDAC	histone deacetylase
HELIC	helicase super family
HER2	human epidermal growth factor receptor 2
hnRNP	heterogeneous nuclear ribonucleoprotein
HRP	horseradish peroxidase
IDT	Integrated DNA Technologies
IER2	immediate early response factor 2
IgG	immunoglobulin G

IP	immunoprecipitation
KH	heterogeneous nuclear ribonucleoprotein K homology domain
KLF2	Kruppel-Like Factor 2
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue
LSD1	lysine-specific histone demethylase 1A
Lys	lysine
M	molar
MB	marked box
MBT	malignant brain tumour
MCF7	Michigan Cancer Foundation-7 cell line
MCM	mini-chromosome maintenance protein complex
MDC1	mediator of DNA damage checkpoint 1
Mdm2	mouse double minute 2 homologue/ E3 ubiquitin-protein ligase
Me	methylation
mg	milligram
miRNA	microRNA
MIWI	murine homologue of PIWI
mL	millilitre
mm	millimetre
MMA	monomethylarginine
M-MLV	Moloney Murine Leukaemia Virus
M-phase	mitotic phase
Mre11	meiotic recombination 11
mRNA	messenger RNA
mTOR	mechanistic target of rapamycin
Myb	myeloblastosis virus oncogene cellular homologue
MYC	myelocytomatosis oncogene cellular homologue
MYND	myeloid, Nervy, and DEAF-1
n	number of biological replicates
n/a	not applicable
NCBI	National Centre for Biotechnology Information
Nd	NEDDylation
NEDD8	neural precursor cell expressed, developmentally down-regulated 8
NF-κb	nuclear factor κ B
NLS	nuclear localisation signal
nM	nanomolar
NTD	amino terminal domain
N-terminus	amino terminus
OB-fold	oligonucleotide/oligosaccharide binding fold
P	phosphorylation
P/CAF	P300/CBP-associated factor
p107	retinoblastoma-like 1 protein
p130	retinoblastoma-like 2 protein
p38	P38 mitogen-activated protein kinase
p53	tumour suppressor protein 53
p73	tumour suppressor protein 73
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PBST	Tween-20 in phosphate buffered saline
PCNA	proliferative cell nuclear antigen
PCR	polymerase chain reaction
Pfam	protein family
PGM	proline-glycine-methionine motif
pH	potential of hydrogen scale
piRNA	PIWI interacting RNA
PIWI	P-element induced wimpy testis protein
PIWIL	PIWI-like
POLD1	polymerase delta 1
Polβ	polymerase beta
PPAR	peroxisome proliferator-activated receptor
pRB	retinoblastoma protein
PRMT	protein arginine methyltransferase
Prp8	human UT-220 kDa protein
PSMC3	proteasome 26S subunit ATPase3
PTEN	phosphatase and tensin homologue
PUMA	p53 upregulated modulator of apoptosis
PVDF	P polyvinylidene difluoride
PWWP	proline-tryptophan-tryptophan-proline motif
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcription PCR
R	restriction point
R	reverse primer
RAD54L	RAD54-like protein
RING	Really interesting new gene
RIP	RNA immunoprecipitation
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNA-Seq	RNA sequencing
RRM	RNA recognition motif
S	synthesis phase
SAM	S-adenosyl-L-methionine, AdoMet
SCAF1	SR-related CTD-associated factor 1
SDMA	symmetric dimethylarginine
SDS	sodium dodecyl sulphate
Ser	serine
SET	Su(var)3-9, Enhancer-of-zeste and Trithorax
SH3	SRC Homology 3 Domain
shRNA	short hairpin RNA
siRNA	small interfering RNA
SKBR3	Memorial Sloan–Kettering Cancer Centre Breast Cancer cell line 3
SKP2	S-phase kinase associated protein2/ E3 ubiquitin protein ligase
SMART	simple modular architecture research tool
SMN	survival motor neuron
SNAIL	zinc finger protein SNAI1
SND1	staphylococcal nuclease and Tudor domain containing protein 1

SN-like	staphylococcal nuclease-like domain
snRNP	small nuclear ribonucleoprotein
SPF30	splicing factor 30
SRC	proto-oncogene tyrosine-protein kinase
SRC3	steroid receptor coactivator-3
STAT	signal transducer and activator of transcription
SYNGR4	Synaptogyrin 4
TAD	transactivation domain
TBP1	TAT-binding protein 1
TCF19	Transcription Factor 19
TDRD	Tudor domain-containing protein
TE	Tris-EDTA
TFIID	transcription factor II D
TGFβ1	transforming growth factor beta 1
THW	threonine-histidine-tryptophan motif
TOPBP1	topoisomerase (DNA) II binding protein 1
Tris	tris(hydroxymethyl)aminomethane
TUD	Tudor domain
U2OS	U-2 human osteosarcoma cell line
UBA	ubiquitin-associated domain
UV	ultraviolet radiation
UV-Vis	ultraviolet-visible
VCaP	vertebral metastatic lesion cancer of the prostate
w/v	weight/volume percent
WDR34	WD repeat domain 34
xG	gravity
ZBED8	zinc finger BED-type containing 8
ZnF	zinc finger domain
ZNF367	zinc Finger Protein 367
ZNF524	zinc Finger Protein 524

Chapter 1 : Introduction

1.1 An Overview of Cancer Biology

Cancer is a complex multi-factorial disease that is caused by aberrances in the genome which confer cells with various growth advantages (Cooper 1992; Bertram 2000; Schneider 2001; F.Macdonald; et al. 2005). These abnormal cells subsequently obstruct normal body functions and lead to chronic illness or death. Over one hundred types of cancer exist across a variety of genetic and environmental backgrounds globally, and an encompassing treatment for the diversity of cancer has remained elusive. The biological complexity in cancer has thus made it one of the most challenging health problems in the world. It remains one of the leading causes of morbidity and mortality with approximately 14.1 million new cases reported in 2012, and further accounting for 8 million deaths worldwide in the same year (International Agency for Research on Cancer, 2014).

Advancements in modern genetics and molecular biology has propelled the knowledge of cancer biology to greater heights. Cancer, essentially a disease of the genome, has also started to become understood in the wider contexts of epigenetics, the immune system, and metabolism. A logical framework to better address the underlying complexity of cancer has since been formulated using the concept of sequentially acquired hallmark capabilities in cells which predispose them to malignant growth, widely known as the 'hallmarks of cancer' (Hanahan et al. 2011) (Figure 1.1). Mutations which canonically occur in two classes of genes, oncogenes and tumour suppressor genes, facilitate the acquisition of these capabilities in cancer. Mutations in tumour suppressor genes, which normally function to prevent aberrant cell proliferation, often lead to the loss or interference of their function in cancer. On the other hand, mutations in proto-oncogenes, which usually serve to promote controlled cell growth and division, convert them to oncogenes which stimulate uncontrolled cell proliferation. Furthermore, these mutations either occur through genetic inheritance, through random errors in DNA

synthesis and replication, or through exposure to various carcinogenic stimuli (Hanahan et al. 2011). Affected cells, as a result, are able to transcend natural homeostasis through alterations in their internal architecture and metabolism, gene expression profiles, cell signalling, and external microenvironments. Ultimately, cancer cells acquire the ability to migrate from their primary site to invade other tissues and organs via vascular or lymphatic structures, seeding new sites for tumour initiation. These collective mutations thus allow a cell to undergo malignant transformation over time (Leber & Efferth 2009).

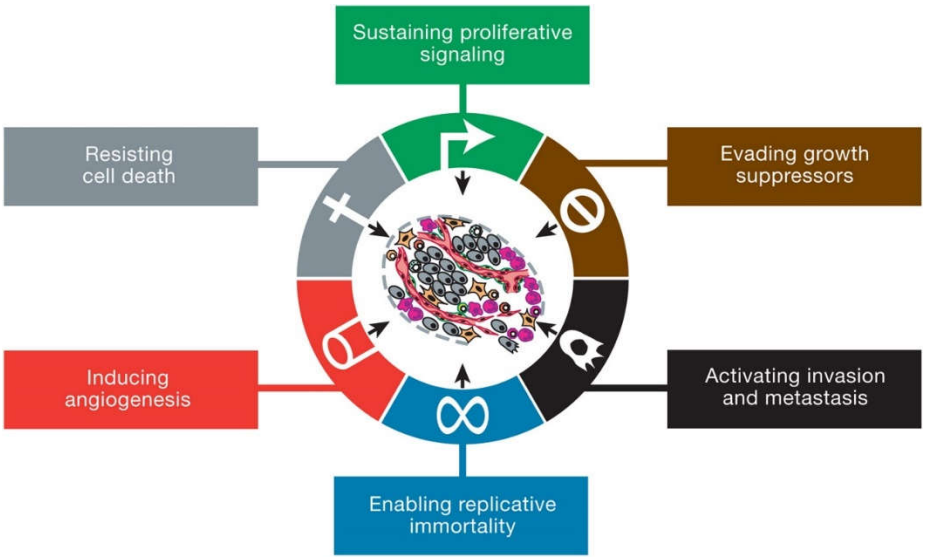


Figure 1.1 The Hallmarks of Cancer.
The Hallmarks of Cancer highlight sequentially acquired capabilities that allow cancer to sustain proliferative signalling, evade common growth suppressive regulation, resist apoptosis and other forms of cell death, enable replicative immortality, induce angiogenesis, and activate invasion and metastasis (Used with permission from Hanahan et al., 2011).

A better understanding of the underlying mechanisms and aberrant pathways in cancer has supplemented usual surgical procedures like whole tumour removal in cancer treatment (Hanahan et al. 2011) (Figure 1.2). Advancements in radiotherapy, hormonal therapy, immunotherapy, and chemotherapy have greatly been aided by the identification of new drug targets, diagnostic and prognostic biomarkers, and early detection methods (Sudhakar 2009).

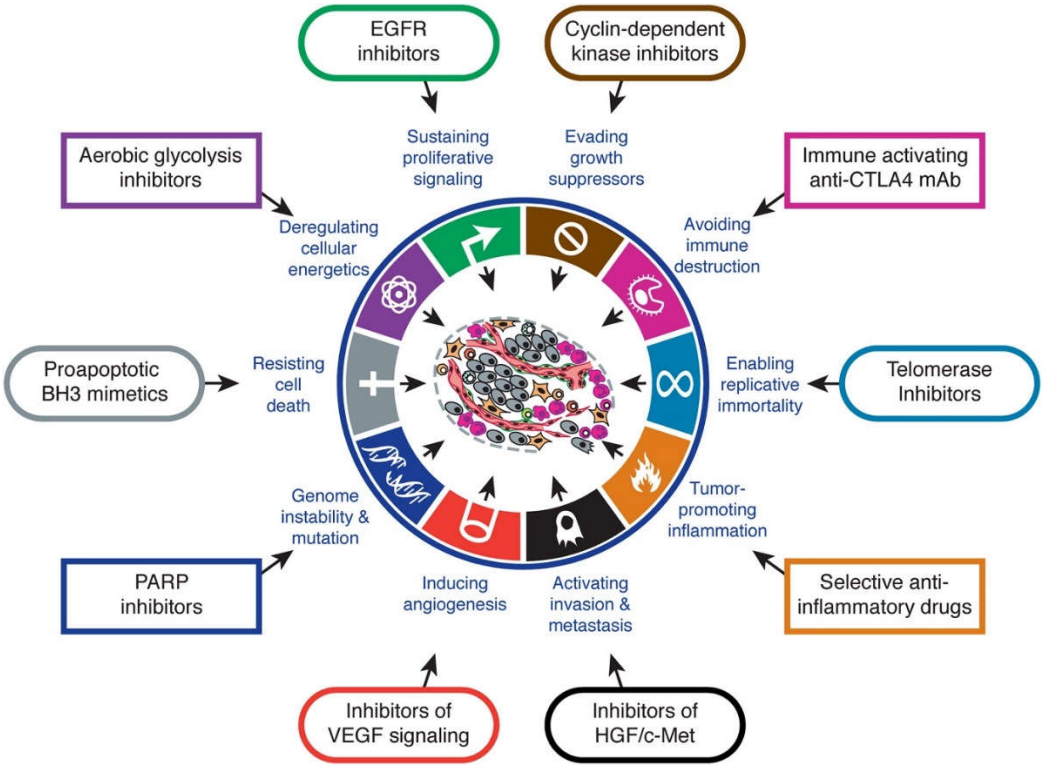


Figure 1.2 Targeted therapies against the different Hallmarks of Cancer.
Targeted approaches the therapy have been enabled by newly acquired understanding of cancer. Drugs that interfere with different hallmark capabilities are listed above (Used with permission from Hanahan et al., 2011).

Extensive research on the mechanisms of cancer has identified a number of commonly defective pathways. Two particular pathways that are completely non-functional, or compromised to some degree in most cancer, are the RB-E2F pathway and the p53 pathway. The RB-E2F pathway is responsible for regulating and ensuring proper progression though the cell cycle (J W Harbour & Dean 2000; Nevins 2001; Indovina et al. 2015), while the p53 pathway, in addition to physiological roles in female fertility and reproduction, embryogenesis, energy metabolism and antioxidant defence, is responsible for the induction of anti-proliferative effects like apoptosis under non-ideal or stress conditions (Lane 1992; Choi & Donehower 1999; Sherr & McCormick 2002; Molchadsky et al. 2010; Feng et al. 2011; Hu et al. 2011; Olovnikov et al. 2009). These frequently aberrant pathways thus highlight the importance of the cell cycle and its corresponding regulatory checkpoints in cancer.

This chapter will discuss the cell cycle in the context of cancer biology, highlighting the role of the RB-E2F pathway in cell cycle maintenance and regulation, and discussing emerging concepts which shed light on the complexity involved in the functions of E2F1. Forming the background of the study, this chapter will also cover the role of arginine methylation in the dual roles of E2F1 as both an oncogene and tumour suppressor. Additionally, this chapter will briefly cover the readers and writers which mediate the biological outcome of this post-translational modification. Finally, this chapter will include a brief project overview, providing the rationale for the proposed study and insight into its relevance and future application.

1.2 The Cell Cycle – An Overview

The cell cycle is a complex regulated process that directs the cell through a specific sequence of events, eventually leading to cell division. This involves two consecutive processes: the replication of DNA, and the segregation of the replicated genetic material into two separate cells. Between two mitotic (M) phases of the cell cycle is an interlude called the interphase, which can be divided in to G_1, S, and G_2 phases. The S phase is characterized by the synthesis of DNA, while the G_1 and G_2 phases represent gaps which allow the cell to grow and prepare for DNA synthesis and mitosis respectively. Cells are able to exit the cell cycle before G_1 and enter a resting phase called G_o (Schafer 1998; Humphrey & Pearce 2010; Williams & Stoeber 2012).

In the G_1 phase of the cell cycle, the point at which the cell is fully committed to replicating is called the restriction point (R). This all or nothing signal ensures that proper requirements have been fulfilled to ensure seamless DNA replication and cell division. Other checkpoints or controls also exist to ensure the orderly sequence of phases in the cell cycle and to detect defects during the course of DNA synthesis, chromosome segregation, and cell division. For example, checkpoints positioned before commitment

to DNA replication in the S phase or before mitosis (G_2-M checkpoint) are activated in response to DNA damage, inducing cell cycle arrest and allowing DNA repair mechanisms to be set in place. If the extent of damage is sensed to be irreparable, induction of apoptosis can occur, preventing the further spread of damaged DNA (Kastan & Bartek 2004).

The cell cycle and its checkpoints form the foundation for maintaining a balance between cell proliferation and death that is required for proper tissue homeostasis. Cancer results from the loss of this balance, favouring uncontrolled cell proliferation and loss of apoptotic regulation (Evan & Vousden 2001; Vermeulen et al. 2003; Kastan & Bartek 2004; Malumbres & Barbacid 2009).

1.2.1 Cyclins and Cyclin-Dependent Kinases

The complex regulation of the coordinated transition from one cell cycle phase to another is mediated by numerous regulatory proteins. The key regulators of the process are cyclin-dependent kinases (CDKs), which become sequentially activated when complexed with different temporally regulated cyclins (Schafer 1998; Sanchez & Dynlacht 2005; Malumbres & Barbacid 2009; Lim & Kaldis 2013) (Figure 1.3).

Different CDKs are active at distinct phases of the cell cycle: CDK4, CDK6, and CDK2 are active during G_1; CDK2 during S; and CDK1 during M. The levels of cyclins vary throughout the cell cycle, unlike CDKs which are stably expressed throughout the cell cycle. Cyclins D1, D2 and D3 bind to CDK4 and CDK6 and are required for G_1 entry. Cyclin E, which binds to CDK2, begins to be expressed in G_1 and is active throughout S. Cyclin A, which binds to both CDK2 and CDK1 begins to be expressed at S phase and is required for entry into M. Cyclin B complexes with CDK1 which regulates proteins required for the completion of M. The active complexes go on to induce downstream processes by phosphorylating cell cycle proteins. The levels and biological activities of

these CDK-cyclin complexes are in turn regulated by various other proteins and pathways to maintain tissue homeostasis and ensure proper cell turnover. (Schafer 1998; Vermeulen et al. 2003).

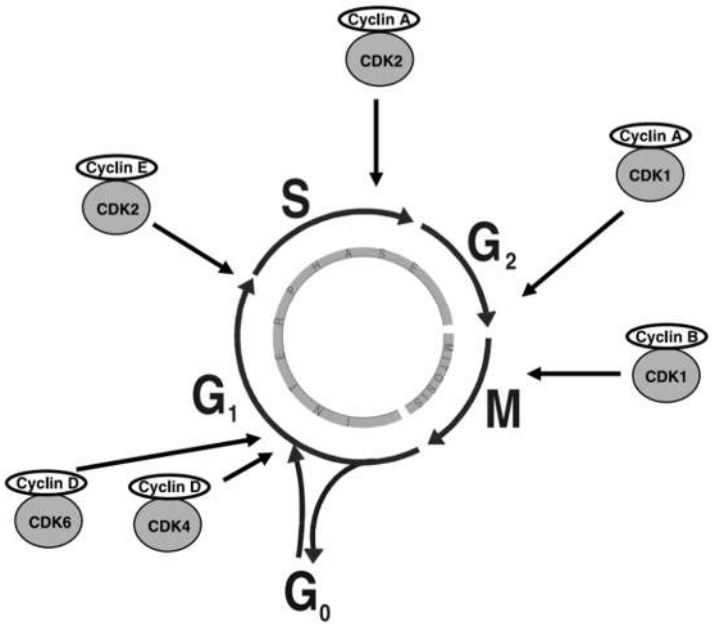


Figure 1.3 The Cell Cycle.
The stages of the cell cycle are regulated by the actions of different cyclin and CDK complexes, which control downstream targets required for each respective phase (Used with permission from Vermeulen et al., 2003).

1.2.2 The Cell Cycle and Cancer

Normal tissue homeostasis involves a delicate balance between cell death and proliferation. This homeostasis is maintained through a number of apoptotic stimuli and strict control of cell cycle progression to prevent the proliferation of cells which have sustained genetic lesions. In cancer, fundamental changes in the genetic control of cell division result in the deregulation of these pathways (Sherr 1996). In fact, the ability of cancer to sustain chronic proliferative signalling is widely considered its most fundamental trait (Hanahan et al. 2011).

Sustained mitogenic signalling is the driving force behind cell cycle deregulation. The overproduction of growth factor ligands which bind cell-surface receptors can

stimulate uncontrolled entry into the cell cycle and autocrine proliferative stimulation. Similarly, overexpression of the cognate receptors which convey the external stimuli to intracellular signalling pathways results in similar deregulation. (Cheng et al. 2008). Downstream of ligand-receptor interaction, the constitutive activation of pathways can sustain the cell and promote growth factor independence, as in the case of mutations in B-Raf, EGFR, KRAS, and phosphoinositide 3-kinase. Negative feedback mechanisms also prevent proper checkpoint properties in mitogenic signalling, as with mTOR kinase, and PTEN (Hanahan et al. 2011).

In the absence of deregulated mitogenic signalling, unrestrained cell proliferation can result from overexpression of CDKs through genetic amplification (Sheppard & McArthur 2013), or through mutations which result in the loss of CDK inhibitor (CKI) binding (Ortega et al. 2002; Vermeulen et al. 2003; Musgrove et al. 2011). Aberrant expression of cyclins resulting from translocations juxtaposing cyclin genes to active promoters or through defects in turnover rates have also been reported in various cancers (Keyomarsi & Pardee 1993; Diehl 2002; Sutherland & Musgrove 2004). Furthermore, aside from direct genetic defects on CDKs and cyclins themselves, mutations and deregulation of CDK-activating enzymes and CKIs also contribute to oncogenic effects (Sherr 1996; Vermeulen et al. 2003; Kastan & Bartek 2004; Malumbres & Barbacid 2009).

Arguably the most common cell cycle defects occur in the G_1 CDK substrate pRB. The absence of functional pRB due to genetic deletion, truncation, or viral inactivation is associated with unrestrained cell cycle progression. Almost all human cancers display abnormalities in some component of the pRB pathway (J W Harbour & Dean 2000; Sherr & McCormick 2002).

In addition to pRB, mutations in checkpoint proteins which normally result in cell cycle arrest, further leading to either DNA damage repair or apoptosis, are also frequently observed in cancer. The tumour suppressor, p53, is widely considered the most mutated gene in human cancer. Mutations can lead to conformational changes which inactivate p53 and prevent its induction of cell cycle arrest and apoptosis. Indirect inactivation of p53 through its negative regulator, Mdm2, is also widely observed (Lane 1992; Sherr & McCormick 2002; Polager & Ginsberg 2009). Together, defects in the RB-E2F pathway and the p53 pathway are considered the most commonly observed aberrations in cancer.

1.3. The RB-E2F Pathway

The Retinoblastoma and Early 2 Factor (RB-E2F) pathway is critical for the proper maintenance and control of cell proliferation, differentiation and survival. This is evidenced by its inactivation in a variety of human cancers (J W Harbour & Dean 2000; Nevins 2001)

The Retinoblastoma (RB) gene, initially of interest due to the frequency of its mutation in retinoblastoma (Lee et al. 1987), was the first tumour suppressor identified. RB codes of the retinoblastoma protein (pRB). Subsequent experiments have revealed RB to be a target for direct tumorigenic mutations, and indirect deregulation in a number of different cancers, and a key player in cell cycle control and regulation (Sherr & McCormick 2002). Though pRB possesses multiple binding partners, the role of pRB in G_1 phase arrest primarily involves its interaction with the E2F family of transcription factors, which is dependent on its phosphorylation status as regulated by Cyclin D-CDK complexes (Chellappan et al. 1991; Nevins et al. 1997). The RB-E2F complex is transcriptionally repressive, preventing the activation of cell cycle genes required for cell proliferation. Hyperphosphorylated forms of RB are functionally inactive and are

predominant in proliferating cells, whereas the hypophosphorylated state promotes pRB binding in quiescent or differentiating cells (J W Harbour & Dean 2000).

In G_1 E2F is able to direct the expression of its transcriptional targets, cyclin E and CDK2, required for progression into the S phase. The cyclin E-CDK2 complex further phosphorylates pRB, inactivating it and locking the cell into proliferation (J W Harbour & Dean 2000).

The RB-E2F pathway is also linked to the induction of cell cycle arrest and apoptosis. The E2F target $p14^{ARF}$ inhibits the ubiquitin ligase Mdm2 which is responsible for targeted p53 degradation. This allows p53 accumulation and facilitates p53-dependent apoptosis. A number of p53-independent pathways to apoptosis have also been identified, specifically enabled by E2F1. In the absence of functional RB-E2F, p53 is protective against tumorigenesis through induction of apoptosis. Lesions in both the RB-E2F and p53 pathway additively contribute to sustained proliferation (Nevins 2001; Sherr & McCormick 2002; Dick & Rubin 2013; Indovina et al. 2015).

1.4. The E2F Family of Transcription Factors

E2F refers to the collective term for transcription factors designated E2-promoter binding factors, originally identified as cellular factors capable of binding the adenoviral E2 gene promoter. The members of the E2F family of transcription factors are largely thought to be concerted regulators of the cell cycle (Nevins 2001; Heuvel 2005). Known as downstream effectors of the pRB tumour suppressor, they are responsible for controlling cell cycle progression at the G_1 to S phase transition. Microarray and chromatin immunoprecipitation (ChIP) assays have also revealed binding to genes unrelated to the canonical role of E2Fs in the cell cycle. E2Fs are thus also thought to regulate aspects of DNA replication, mitosis, apoptosis, DNA damage checkpoints, DNA repair, differentiation, and autophagy via E2F-mediated transactivation and repression of

downstream gene expression (Ginsberg 2002; Cam & Dynlacht 2003; Bracken et al. 2004; DeGregori & Johnson 2006).

The E2F family is made up of eight genes which encode a number of different proteins. E2Fs 1 to 5 have the ability to bind and interact with the RB family of proteins (pRB, p107, and p130), also referred to as pocket proteins for their observed binding to the groove between the conserved A and B transactivation regions of these E2Fs. The transcriptional activity of E2Fs 1-6 is mediated by their DNA binding and dimerization partner (DP) binding domains which enable heterodimerization with DP1 and DP2. This heterodimerization is in turn inhibited by the binding of pocket proteins. E2F7 and E2F8, on the other hand perform DNA binding independent of DP proteins. (Figure 1.4) (Rowland & Bernards 2006).

When bound to pocket proteins, E2Fs form transcriptional repressor complexes via facilitated interaction with histone deacetylases (HDACs), chromatin remodelling proteins, and histone methyltransferases. This binding also masks the transcriptional activation domains at the carboxy terminus of E2Fs 1 to 5, as well as blocks heterodimerization with DPs. Pocket protein binding to E2Fs thus effectively prevents recruitment of the basal transcription factor TFIID and prevents binding of cis-acting proteins required for target gene activation. In turn, the release of E2Fs from pocket protein binding, in addition to enabling DP heterodimerization, also enables interaction with histone acetyltransferases (HATs) like p300, CBP, Tip60 and P/CAF (J. William Harbour & Dean 2000; Cam & Dynlacht 2003; Rowland & Bernards 2006; DeGregori & Johnson 2006).

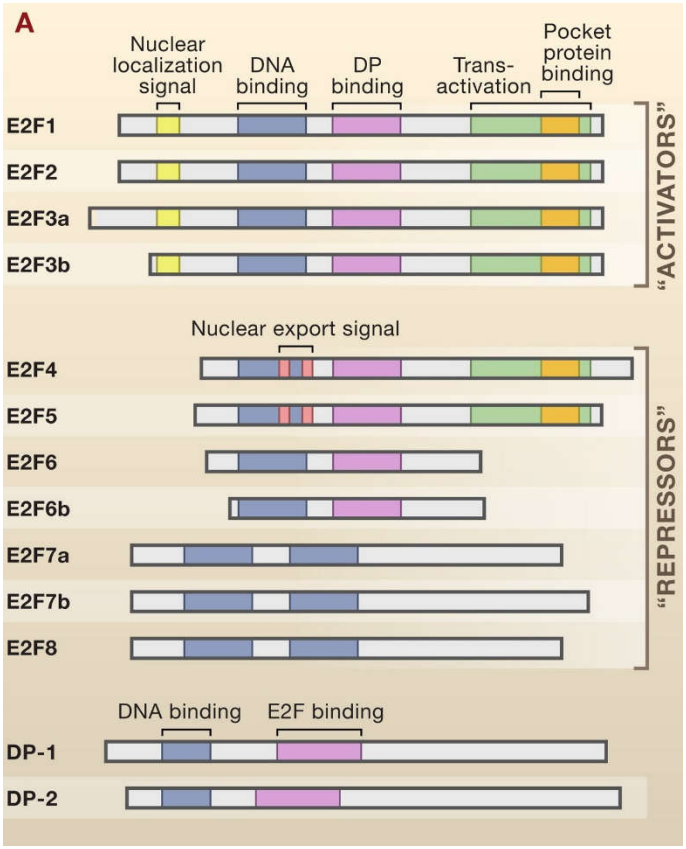


Figure 1.4 The E2F family of transcription factors.
In mammalian cells, at least eight different E2F transcription factors exist, some of which have multiple isoforms. The different structural domains of each E2F allow them to be loosely classified into activators or repressors based on their activated functional activity. Some E2Fs require heterodimerization with DP1 or DP2 to carry out their biological function (Used with permission from Rowland and Bernards, 2006).

The E2F family can be loosely classified into activators or repressors of transcription. While E2Fs 1 to 5 contain both activation and repression domains, E2F4 and E2F5 contain nuclear export signals which mediate their export from the nucleus when not bound to pocket proteins, making them transcriptionally inactive. However, when released from pocket protein binding, the nuclear localization signals found on E2Fs 1 to 3 enable them to carry out their transactivational activity. Thus, E2Fs 1 to 3 are generally considered activator E2Fs, while E2F4 and E2F5 are considered repressor E2Fs. E2Fs 6 to 8 do not contain transactivation domains, and are considered repressors (Rowland & Bernards 2006; Johnson & DeGregori 2006).

Despite opposing effects on transcription, members of the E2F family share overlapping subsets of target genes, and their functions are controlled by a number of

shared upstream regulators to maintain tight and correct progression of cells through the cell cycle (Rowland & Bernards 2006). Most E2Fs demonstrate extensive functional redundancy, but also maintain certain degrees of target specificity, tissue-dependent expression, and activity. As such, the loose classification of E2Fs into activators or repressors of transcription is an oversimplification based solely on molecular structure. It is through the formation of different complexes that the biological function of an E2F is carried out, allowing some E2Fs to function both as activators and repressors (DeGregori & Johnson 2006).

Though much progress has been made to understand the role of the E2F family of transcription factors in cell proliferation, increased complexity of the functions of the E2F family has since become apparent, due to the multiplicity of E2F target genes that both positively and negatively regulate the cell cycle, as well as due to observed activity outside of the G_1/S transition (J. William Harbour & Dean 2000; Cam & Dynlacht 2003; Bracken et al. 2004; Johnson & DeGregori 2006). The additional role of E2F in conjunction with RB in apoptosis also raises questions as to the levels of control which determine the biological outcomes of E2F activity (J W Harbour & Dean 2000). Understanding the complexities of this pathway may be key to improved targeted therapy against cancer.

1.4.1 Emerging complexity in E2F function

As mentioned previously, the classification of E2Fs as activators or repressors of transcription is an over-simplistic framework for understanding the diverse roles that these E2Fs play. The functional framework represented by the activation model of E2F function paints its role as a direct activator of transcription as the primary mediator of its cell cycle effects. However, experiments conducted on mouse embryonic fibroblasts which lack one or more members of the E2F family, with the exception of E2F3-null

cells, show little impairment of cell proliferation (Attwooll et al. 2004). It has been shown that proliferation can occur without the three known activator E2Fs (Wenzel et al. 2011). A model which proposes these E2Fs as anti-repressors, that is, occupants of E2F binding sites on promoters to prevent repressive E2F binding has since then been proposed (Rowland & Bernards 2006).

ChIP assays have shed light on the promoter-specific and context-dependent recruitment of E2Fs, revealing that repressor E2Fs play a greater role in G_0 and early G_1 target promoter occupancy, and that activator E2Fs either activate or repress transcription depending on the presence of various co-factors. Additionally, these roles do not necessarily occur in a cell-cycle dependent manner (DeGregori & Johnson 2006).

1.4.2 The Complex Roles of E2F1

The complexity in E2F functions can be demonstrated by taking a closer look at the varying roles of E2F1 in particular. As a promoter of cellular proliferation, E2F1 acts a transactivator of various cell cycle genes (Bracken et al. 2004). E2F1 can alter epigenetics by facilitating genetic activation through interaction and recruitment of HAT subunits, resulting in the hyperacetylation of histones H3 and H4 (Taubert et al. 2004). E2F1 also mediates gene activation dependent on histone methylation (Tyagi et al. 2007).

Though the role of E2F1 in proliferation is largely redundant to that of E2F2 and E2F3, a unique feature of E2F1 is its ability to induce DNA damage-induced checkpoint control and apoptosis in addition to its canonical proliferative function (Huang et al. 1997; Lin et al. 2001). Illustrating this functional dichotomy, overexpression of E2F1 in the absence of functional p53 in mouse models promotes tumorigenesis (Pierce et al. 1998; Pierce et al. 1999; Laurie et al. 2006). However, $E2F1^{-/-}$ mice also show spontaneous tissue-specific tumour formation (Yamasaki et al. 1996; Yamasaki et al. 1998). E2f1 has been implicated in the oncogenic transformation of cells in culture when overexpressed

(Johnson et al. 1994; Xu et al. 1995; Johnson & DeGregori 2006), yet supporting the additional role of E2F1 as a tumour suppressor, somatic mutations of DP1 which disable E2F1-DP1 heterodimer transcriptional activity decreases DNA damage-induced apoptosis induction (Munro et al. 2014). The immensely diverse functional complexity that E2F1 displays gives insight to the intricate regulatory cross-talk which establishes the balance between cell proliferation and apoptosis.

1.4.2.1 E2F1 Regulates a Variety of Transcriptional Targets

The multifaceted roles of E2F1 are mediated by the plethora of E2F1 target genes. Target genes may contain canonical E2F consensus binding sites (5'-TTTSSCGS-3' or 5'-GGCGCAAA-3' where S is C or G) or variations of the consensus sequence. However E2F1 has also been found to bind to target genes in the absence of a consensus sequence (Bieda et al. 2006). Several E2F1 targets are directly required for cell cycle progression and DNA synthesis. Microarray-based analysis of E2F1 binding targets has also identified genes related to DNA damage response, metabolism, apoptosis, angiogenesis and cellular membrane components (Bracken et al., 2004; Roworth et al., 2015). Cyclin E, E2Fs 1 to 3, and MYC are also direct E2F1 targets, promoting a positive feedback loop for E2F1 activation during the G_1/S transition (Araki et al. 2003). Other canonical targets of E2F1 are CDC6, which stimulates DNA replication; MCM2-7, proteins that form the pre-initiation complex required for DNA replication; cyclins A2, B1 and E which all contribute to cell cycle progression; BRCA1, involved in double-strand break repair; and p73, a transcription factor which targets pro-apoptotic and DNA repair genes. These examples highlight the profound diversity of E2F1 target genes (Roworth et al., 2015).

1.4.2.2 E2F1 and the DNA Damage Response

A number of experiments demonstrate the counter-intuitive function of E2F1 as a tumour suppressor (Phillips & Vousden 2001). In several instances, ectopic overexpression of E2F1 has been demonstrated to cause apoptosis in cultured cells (Wu & Levine 1994; Qin et al. 1994; Kowalik et al. 1995; Adams & Kaelin 1996). Mice that are E2F1-deficient experience defects in thymocyte apoptosis, leading to an excess of mature T-cells (Field et al. 1996). Furthermore, the aberrant apoptosis observed in mice carrying homozygous RB mutations is suppressed when E2F1 is suppressed or inactive (Pan et al. 1998; Tsai et al. 1998).This interesting feature is shared with E2F2 and E2F3 in a few systems, but is by and large considered and E2F1-specific effect (DeGregori et al. 1997; DeGregori & Johnson 2006; Kowalik et al. 1998; Vigo et al. 1999).

The observed DNA damage response and apoptotic effects of E2F1 can occur in p53-dependent (Wu & Levine 1994; Sherr & McCormick 2002; Polager & Ginsberg 2009) and p53-independent manners (Holmberg et al. 1998; Phillips et al. 1997). In response to DNA damage, E2F1 and p53 may cooperate to bring about DNA repair mechanisms or apoptotic induction. E2F1 influences the expression and activity of ATM, CHK2, and p38, known phosphorylators of p53. E2f1 activity thus induces the stabilization of p53 to through its increased phosphorylation state (Lin et al. 2001; Rogoff et al. 2002; Berkovich & Ginsberg 2003; Powers et al. 2004; Rogoff et al. 2004; Hershko et al. 2006). Additionally, ectopic expression of E2F1 results in direct transactivation of the $p14^{ARF}$ tumour suppressor gene which codes for the ARF protein. The regulation of ARF by E2F1 is accomplished through a non-consensus binding site and its induction is only achieved through deregulated E2F1 activity as in the case of DNA damage. ARF prevents the E3 ubiquitin ligase Mdm2 from targeting p53 for ubiquitination and thus leads to p53 stabilisation (Bates et al. 1998; Robertson & Jones 1998; Sherr et al. 1999).

E2F1 has also been found to bind to p53 when p53 is phosphorylated at Ser315, increasing p53 nuclear retention and improving p53 binding in response to DNA Damage (Hsieh et al. 2002; Fogal et al. 2005). E2F1 also affects p53 both positively and negatively through the regulation of various genes involved in p53 regulatory pathways (Zhang & Wang 2006; Kitagawa et al. 2008; Mantovani et al. 2007).

Independent of p53, E2F1 transactivates a variety of pro-apoptotic genes such as caspases, APAF1, NOXA and PUMA (Nahle et al. 2002; Moroni et al. 2001; Hershko & Ginsberg 2004). E2F1 also displays the capability to negatively regulate anti-apoptotic members of the BCL-2 family (Eischen et al. 2001). The transactivation of $p14^{ARF}$ by E2F1 also facilitates p53-independent anti-proliferative activities (Lowe & Sherr 2003). Most notably, E2F1 directly activates the expression of p73, a p53 homologue. The consequent p73 activation results in activation of apoptotic target genes (Stiewe & Pützer 2000; Irwin et al. 2000).

Under DNA damage E2F1 can also block apoptotic signalling to enable DNA repair. The association of TOPBP1 relocalizes E2F1 and likely prevents the transcription of canonical pro-apoptotic targets like $p14^{ARF}$ and p73. This interaction also recruits BRG1/BRM components of the SWI/SNF chromatin remodelling complex to target genes, repressing apoptotic signalling to allow for DNA repair pathways. Furthermore, acetylation of E2F1 promotes interaction with TOPBP1 as opposed to pRB, forming a complex that is localized to the BRCA1 repair complex at sites of DNA damage (Liu et al. 2003; Liu et al. 2004). E2F1 also possesses a number of target genes responsible for regulating DNA repair (Ren et al. 2002; Polager et al. 2002; Black et al. 2005).

1.4.2.3 Post-translational modifications on E2F1

The different biological capabilities that E2F1 demonstrates are mediated in part by a variety of post-translational modifications which lead to its differential affinity with interaction partners, changes in stability, and target specificity.

The induction of ATM/ATR and CHK2 activity in response to DNA damage quickly results in E2F1 phosphorylation at Ser31 and Ser364 respectively (Stevens et al. 2003) (Figure 1.5). This modification enhances pRB affinity for E2F1 and consequent transcription of pro-apoptotic genes (Carnevale et al. 2012; Bertin-Ciftci et al. 2013). E2F1 is also acetylated by various acetyltransferases on Lys117, 120 and 125, close to its DNA binding domain, increasing its stability, particularly to the p73 promoter (Taubert et al. 2004; Galbiati et al. 2005; Ozaki et al. 2009). These largely stabilising activities are thought to be predominantly pro-apoptotic. Perhaps as a regulatory mechanism, ubiquitination levels of E2F1 increases under DNA damage conditions. This ubiquitination has been found to be dependent on the acetylation of E2F1 (Budhavarapu et al. 2012). Ubiquitination of E2F1 decreases its half-life through proteasome-dependent degradation, and blockage of ubiquitination by pRB binding or displacement of the E3 ubiquitin ligase SKP2 by Mdm2 stabilises it (Campanero & Flemington 1997; Zhang et al. 2005; Budhavarapu et al. 2012).

Post-translational modification of E2F1 residues can also reduce its pro-apoptotic activity. E2F1 is methylated at Lys185 by SET9/SETD7, decreasing apoptotic activity by hindering acetylation and phosphorylation of E2F1 at distal sites (Xie et al. 2011). As such, methylation is considered a destabilising modification and demethylation of E2F1 by LSD1 in response to damage stabilises it (Kontaki & Talianidis 2010). Methylation at Lys185 also promotes NEDDylation of E2F1, targeting it for degradation in the absence of DNA damage (Loftus et al. 2012).

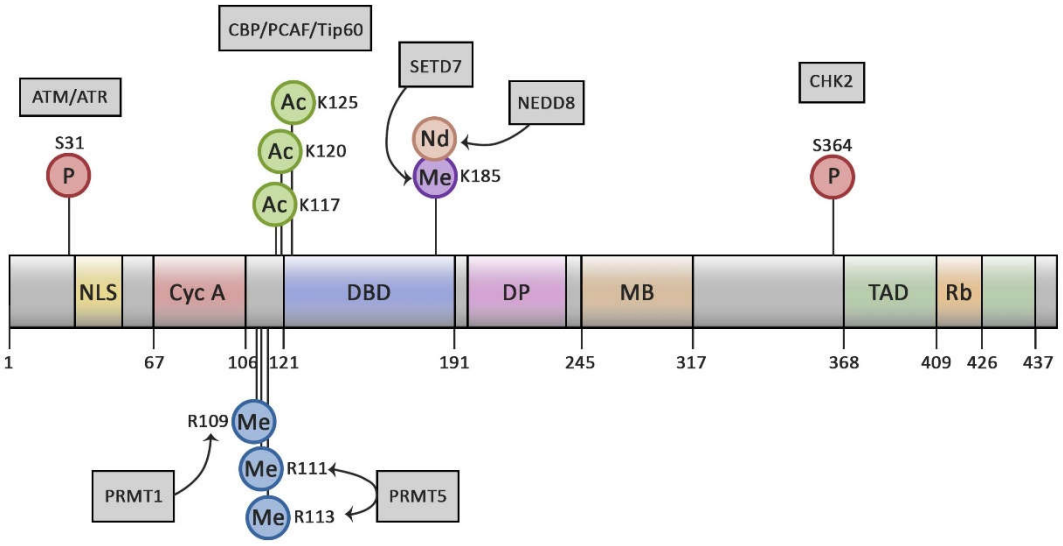


Figure 1.5 Post-translational modifications on E2F1.
A number enzymes are responsible for the post-translational modifications on E2F1 which influence its stability and ultimate functional activity. The respective residues, modifications and enzymes responsible are outlined. The the main functional domains of E2F1 that the modifications occur around are delineated. (NLS: nuclear localisation signal, Cyc A: Cyclin A binding domain, DBD: DNA binding domain, DP: DP binding domain, MB: marked box, TAD: transactivation domain, RB: pRB binding domain, P: phosphorylation, Me: methylation, Ac: acetylation, Nd: NEDDylation; R: Arginine; S: Serine; K: Lysine)

Most recently, E2F1 has been found undergo arginine methylation by protein arginine methyltransferases (PRMTs), the effects of which will be discussed further below (Cho et al. 2012; Zheng et al. 2013).

The multitude of post-translational modifications on E2F1 adds yet another layer of complexity to the regulation of its opposing biological functions. Further study of the readers and effectors of these modifications may be the key to understanding the multifaceted functions of E2F1.

1.4.2.4 E2F1 – Apoptosis versus Proliferation

Aside from the post-translational modifications discussed above, a number of biological mechanisms have been shown to tip the scales between the dichotomous roles of E2F1 in apoptosis and proliferation.

The E2F1-ARF-p53 relationship, for example, constitutes two negative feedback loops. ARF is able to stabilise and activate p53, but is in turn repressed by p53 through the restriction of the ARF promoter from transactivation (Kontaki & Talianidis 2010). At the same time, ARF interacts with E2F1 *in vivo*, inhibiting its transcriptional activity and targeting it for degradation in a p53 dependent manner (Bertin-Ciftci et al. 2013; Galbiati et al. 2005). This facilitates a largely context dependent model for determining the biological outcome of E2F1. A deficiency in p53 or ARF can thus tip the scales toward E2F1 acting in a tumorigenic manner stabilised, rather than promoting apoptosis (Johnson & DeGregori 2006).

Aside from a number of cellular and biological contexts outlined (Johnson & DeGregori 2006), little is known about the molecular mechanism which dictates the biological activity of E2F1. Recently, it has been shown that E2F1 undergoes residue-specific methylation on a small arginine-rich motif displaying considerable sequence homology with a similar motif in p53 (Jansson et al. 2008; Cho et al. 2012). This motif is competitively methylated by different PRMTs. Specifically, it is dimethylated asymmetrically by PRMT1 on Arg109 and symmetrically dimethylated by PRMT5 on Arg111 and Arg113. Furthermore, these modifications occur in a mutually exclusive manner and target E2F1 to opposing functional outcomes. Asymmetric dimethylation accumulates under conditions of DNA damage, promoting E2F1-dependent apoptosis, whereas symmetric dimethylation favours E2F1 in cell cycle progression. The asymmetric dimethylation mark further hinders the interaction between E2F1 and cyclin A, stabilising E2F1 and enhancing p53-associated activity; whereas symmetric methylation enhances cyclin A binding, hindering E2F1-DP interaction and promoting proper progression through the cell cycle after DNA replication (Xu et al. 1994; Cho et al. 2012; Zheng et al. 2013). As such, arginine methylation is emerging as a previously

overlooked post-translational modification on E2F1 and provides a functional model which explains its two opposing outcomes (Figure 1.6).

1.5 Arginine methylation

Arginine methylation is a widely occurring post-translational modification and has been found in both nuclear and cytoplasmic proteins. It has become apparent that methylation status, like phosphorylation, is a significant controlling element in protein function. Methylation often occurs on the side chains of a few amino acid residues as well as at the N- and C- termini of proteins, which in turn generate distinct sets of chemical reactions and regulate a variety of regulatory pathways (Smith & Denu 2009).

While lysine methylation is common, arginine side chain methylation is thought to be the most quantitatively extensive methylation reaction in mammalian cells (Gary & Clarke 1998; Boisvert, Chénard, et al. 2005). Arginine methylation plays significant roles in diverse cellular processes such as DNA damage response, signalling pathways, transcriptional regulation, and RNA metabolism (Bedford & Richard 2005; Auclair & Richard 2013). Interestingly, aberrant arginine methylation can be observed in various diseases, as in cardiovascular disease, tumours in cancer, viral infection and autoimmune disease (Bedford & Richard 2005; Wang et al. 2009). While its occurrence in various proteins is very common, compared to other post-translational modifications such as acetylation and phosphorylation, very little is known about their biological function and the enzymes responsible. The study of this area of post-translational modification holds much potential for the discovery of disease biomarkers as well as therapeutic targets for drug discovery (Copeland et al. 2009).

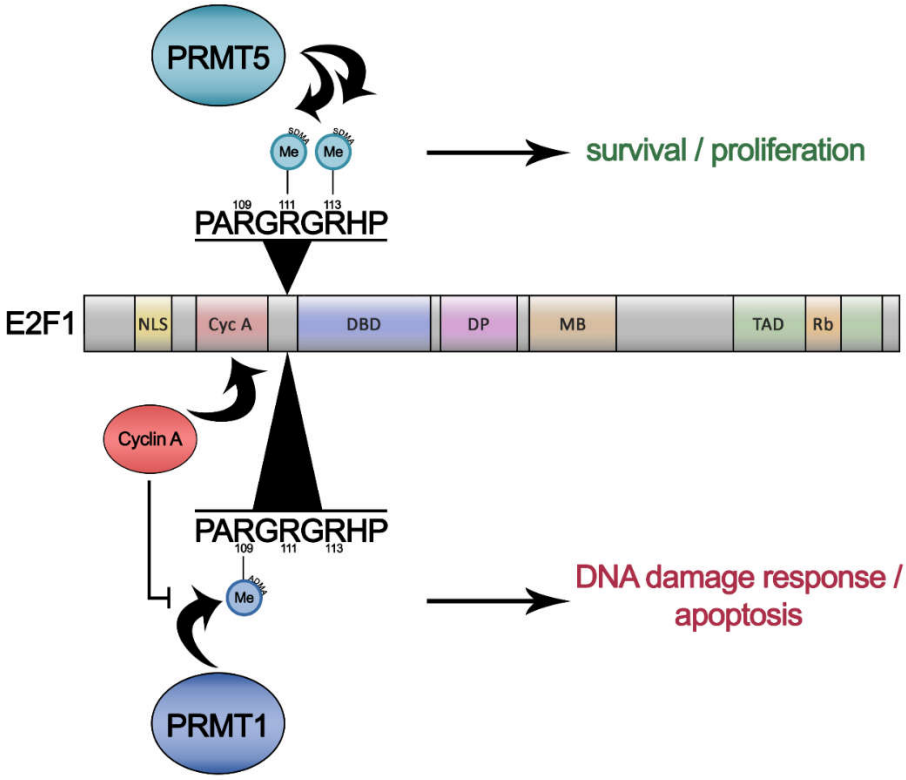


Figure 1.6 Model: arginine dimethylation in E2F1 acts as a switch between its biological activities. PRMT1 asymmetrically dimethylates Arg109, preventing the dimethylation of Arg111 and 113 and thus triggering DNA damage response and apoptosis activity of E2F1. Cyclin A binding inhibits PRMT1 methylation and promotes symmetrical dimethylation of Arg111 and 113 by PRMT5. The presence of symmetric dimethylation marks further impedes asymmetric dimethylation by PRMT1 and favours proliferative activity of E2F1. NLS: nuclear localisation signal, Cyc A: Cyclin A binding domain, DBD: DNA binding domain, DP: DP binding domain, MB: marked box, TAD: transactivation domain, RB: pRB binding domain; ; P: proline; A: alanine R: arginine; G: glycine; H: histidine; ADMA: asymmetric dimethylation; SDMA: symmetric dimethylation (adapted from Zheng et al., 2013)

Arginine is considered the amino acid with the highest basicity. The guanidino group of arginine contains five potential hydrogen-bond donors with positions that favour interactions with existing biological hydrogen bond acceptors. This unique property facilitates intricate protein-nucleic acid complexes and each addition of a methyl group to an arginine residue both changes its conformation as well as removes a potential hydrogen bond donor, altering these protein-nucleic acid interactions (Jones et al. 1999; Luscombe et al. 2001). Arginine methylation is mediated by PRMTs which catalyse the transfer of a methyl group from the universal methyl donor, S-adenosyl-L-methionine (SAM; also known as AdoMet) to the guanidino nitrogen of arginine, resulting in methylarginine and an S-adenosylhomocysteine by-product (Katz et al. 2003).

In mammalian cells, three main forms of methylated arginine have been identified: monomethylarginine (MMA), asymmetric dimethylarginine (ADMA); and symmetric dimethylarginine (SDMA) (Figure 1.7).

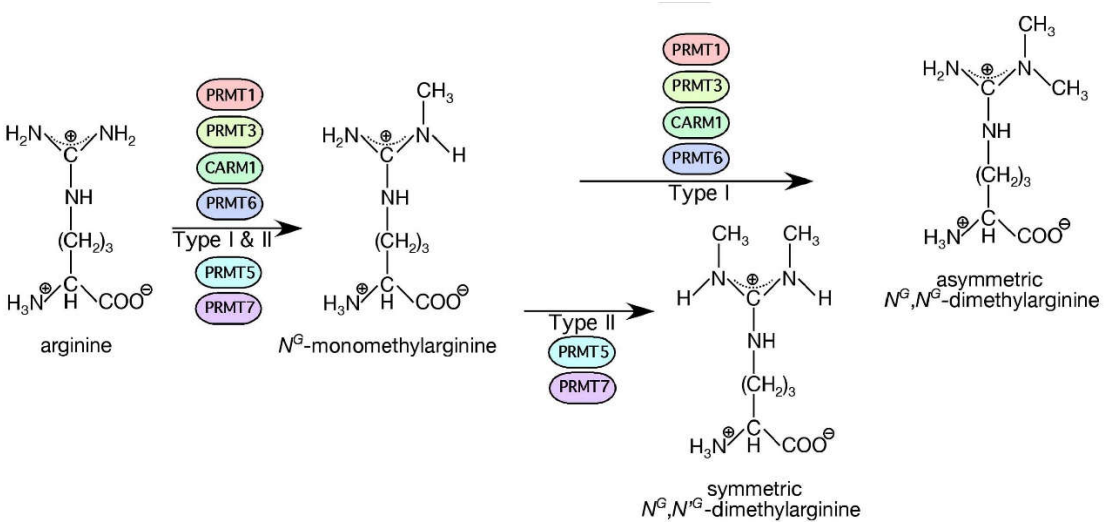


Figure 1.7 Types of arginine methylation.
Three main forms of methylated arginine have been identified in mammalian cells: monomethylarginine (MMA), asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA). The formation of the various methylarginine derivatives is facilitated by the catalytic activity of various protein arginine methyl transferases (Used with permission from Bedford and Richard, 2005).

ADMA occurs when two methyl groups are transferred to one terminal nitrogen atom of the arginine guanidino group and is considered the more prevalent dimethylarginine (Tang et al. 2000). The SDMA derivative is obtained when one methyl group is transferred to each of the terminal guanidino nitrogen of arginine and occurs at 20% the level of ADMA. MMA is derived from the single addition of a methyl group to one terminal nitrogen of arginine and constitutes 50% of the levels of ADMA in mammalian cells (Bedford & Richard 2005). Arginine methylation often occurs in areas flanked by multiple glycine residues though exceptions to the rule are common in various biological systems. These motifs are termed glycine and arginine-rich (GAR) motifs (Najbauer et al. 1993; Gary & Clarke 1998). In proteins, arginine thus constitutes a potential for a variety of conformational changes enabled by its methylation status.

1.5.1 Protein arginine methyltransferases (PRMTs)

PRMTs fall under Class I methyltransferases, which are generally comprised of a common seven-stranded β-sheet structure. Class II methyltransferases are SET lysine methyltransferases, whereas Class III are defined as membrane associated methyltransferases. PRMTs, in addition to the characteristic β-sheet structure of Class I methyltransferases, harbour "double E" and "THW" sequence motifs which are particular to the PRMT subset (Katz et al. 2003).

While the exact number of genes which encode for the various catalytic PRMT subunits is under investigation, ten sequence related PRMTs have been identified to date, of which eight have demonstrated known catalytic activity (Bedford 2007) (Figure 1.8). Notably, the FBXO11 protein, which does not possess the canonical "double E" and "THW" motifs, has been found to display potential arginine methyltransferase activity (Cook et al. 2006; Krause et al. 2007).

PRMTs can be divided in to three distinct types based on their catalytic activity (Figure 1.7). Types I, II and III are all capable of methylating terminal guanidino nitrogen atoms. While Type III PRMTs are able to generate MMA on substrates, the reaction terminates and does not proceed to the formation of a dimethylated derivative. Such activity is displayed exclusively by PRMT7. Type I PRMTs further catalyse the formation of ADMA from the MMA intermediate. PRMTs 1, 3, 4 (also known as CARM1), 6 and 8 all display Type I activity. Type II PRMTs (PRMT5, PRMT7, and FBXO11) produce SDMA from the MMA intermediate. PRMT2 and PRMT9, though structurally related to other PRMTs, have not demonstrated arginine methylation activity (Gary & Clarke 1998; Bedford, Mark T., Clarke 2009). Capability to catalyse the production of ADMA and SDMA has been found to be mutually exclusive and to date, no enzyme has been found to form both dimethyl derivatives.

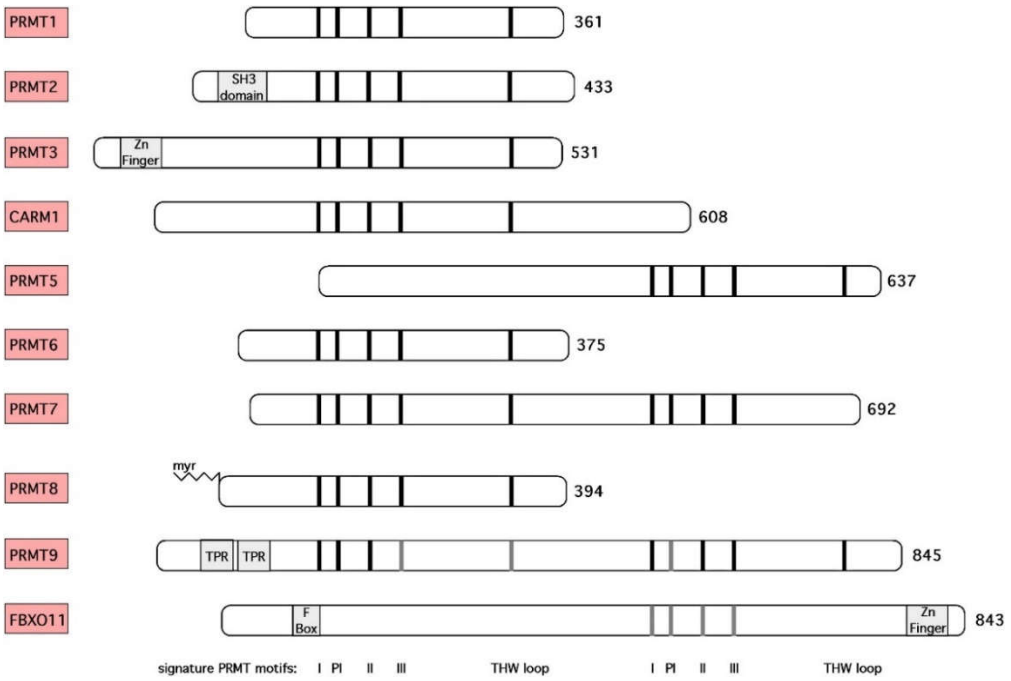


Figure 1.8 The protein arginine methyltransferase (PRMT) family.
Ten proteins demonstrate arginine methyltransferase activity, nine of which are structurally related and fall under the canonical PRMT subset consisting the signature PRMT motifs (in black). FBXO11, a structurally unrelated PRMT is aligned to identify putative signature motifs which may facilitate substrate recognition (in grey) (Used with permission from (Bedford, Mark T., Clarke 2009).

1.5.1.1 PRMT1 – The prototypical Type I PRMT

PRMT1 is the predominant type 1 enzyme and possesses broad target specificity, generally recognizing GAR motif-containing substrates (Gary & Clarke 1998; Lee & Bedford 2002). PRMT1 is broadly expressed in multiple cell types and localizes to both the cytoplasm and the nucleus; unlike PRMT3 which is located exclusively in the cytosol and PRMT8 which is membrane associated and only expressed in the brain (Bedford, Mark T., Clarke 2009). Not surprisingly, substrates of PRMT1 are extensive and are located in both cytosolic and nuclear cellular compartments (Herrmann et al. 2004; Herrmann et al. 2005).

PRMT1 substrate binding is mediated by its interaction with other proteins. While the three-dimensional structure of PRMT1 suggests it is active even as a homodimer, PRMT1 is associated with a number of different proteins in the cell suggesting oligomerization which facilitates it broad target specificity (Gary & Clarke 1998; Zhang

& Cheng 2003; Hung et al. 2007). Three different peptide-binding channels have been identified. This suggests that substrates of PRMT1 make contact with the active site at different angles (Zhang & Cheng 2003). Unlike PRMT1, PRMT4/CARM1 and PRMT6 catalyse the methylation of only a few distinct substrates and do not target general GAR motifs (Lee & Bedford 2002).

PRMT1 targets a number of hnRNP molecules, facilitating the shuttling of these proteins between the nucleus and cytoplasm (Herrmann et al. 2004). Histone H4 is also a target of PRMT1 and is methylated at Arg3, adding a layer of modification to the histone code as a transcriptional activation mark (Wang et al. 2001).

PRMT1 acts as a transcriptional co-activator recruited to promoters by various transcription factors (Bedford & Richard 2005). Though PRMT1 is dispensable in some cultured cells, it is considered to be a central regulator of protein function *in vivo* as PRMT1-knockout mice die shortly after implantation (Pawlak et al. 2000).

1.5.1.2 PRMT5 – The Major Type II PRMT

PRMT5, like PRMT1, exhibits broad expression and localizes to both the cytoplasm and the nucleus (Bedford & Richard 2005). It likely carries out its function through its interaction with multiple binding factors, though the identification of specific binding interaction has been confounded by the finding that αFLAG M2 agarose enriches for PRMT5 activity (Nishioka & Reinberg 2003).

PRMT5 demonstrates transcriptional corepressor functions through its binding with COPR5 that results in preferential H3R8 repressive methylation over H4R3 activating methylation (Lacroix et al. 2008). PRMT5 also displays coactivator activity by complexing with hSWI/SNF ATP-dependent chromatin remodelling proteins BRG and BRM, which facilitates H3R8 methylation (Pal et al. 2007; Dacwag et al. 2007; Stopa et

al. 2015). PRMT5 has also been found to associate with regulators of transcriptional elongation (Liu et al. 2007).

In the cytoplasm, PRMT5 contributes to snRNP biogenesis by methylating a number of Sm proteins in conjunction with PRMT7, another type II PRMT (Neuenkirchen et al. 2008; Gonsalvez et al. 2007).

1.5.2 Biological Roles of Arginine Methylation

Aside from the roles of PRMT1 and PRMT5 outlined above, PRMTs are able to methylate a variety of different proteins and thus regulate their function in different biological pathways.

PRMTs function as transcriptional co-activators by regulating post-translational modifications on transcription factors, other coactivators and histones (Bedford, Mark T., Clarke 2009). Histone acetyltransferases p300, CBP and SRC3 are all arginine methylation substrates (Bedford & Richard 2005). Although PRMTs were initially identified as nuclear receptor coactivators, their modification of a number of different transcription factors like p53, NF-κb, PPARγ and E2F1 has extended their coactivator function (Yadav et al. 2008; Zhao et al. 2008; Cho et al. 2012). In these contexts, PRMT1 and CARM1 are considered the general transcriptional coactivators which facilitate the coactivation of a large number of transcription factors.

PRMT repressor activity was first demonstrated by the synergy between PRMT5 activity and E2F1 in cyclin E1 corepression (Fabbrizio et al. 2002). PRMT5 has since been considered the general transcriptional repressor that regulates and interacts with several transcription factors and repressor complexes, most notably BRG1 and hBRM, Blimp1 and Snail to influence H3R8 and H43R methylation status (Pal et al. 2004; Ancelin et al. 2006; Hou et al. 2008).

The biological role for arginine methylation in the regulation of nucleocytoplasmic shuttling is well established. Sixty percent pf the ADMA found in the nucleus occurs on RNA binding proteins which are shuttled between the nucleus and cytoplasm like snRNPs and hnRNPs rich in GAR and proline-glycine-methionine (PGM) motifs (Bedford & Richard 2005). While PRMT1 is responsible for targeting majority of the GAR rich proteins, CARM1 is able to methylate arginines flanked by PGM motifs (Najbauer et al. 1993; Cheng et al. 2007). In certain cases, hypomethylated proteins have been found to accumulate in the nucleus and others in the cytoplasm, indicating the impairment of shuttling when arginine methylation is repressed (Shen et al. 1998; Aoki et al. 2002; Iacovides et al. 2007).

In DNA repair, arginine methylation has been found to regulate the 3' to 5' exonuclease activity of Mre11 on double-stranded DNA (Boisvert, Hendzel, et al. 2005; Boisvert, Déry, et al. 2005). Mre11 is a major component of mammalian homologous recombination repair. 53BP1, another protein involved in double strand break repair is also methylated by PRMT1 *in vivo*, though its regulatory role has yet to be elucidated (Boisvert, Rhie, et al. 2005). DNA polymerase β (Polβ), involved in base excision repair, associates with PRMT6 and PRMT1 through its lyase domain. These interactions facilitate distinct, non-redundant roles in regulating the function of Polβ. PRMT6 methylation on Arg38 and 152 augments polymerase activity while PRMT1 methylation on Arg137 blocks the Polβ-PCNA interaction (El-Andaloussi et al. 2006; El-Andaloussi et al. 2007).

PRMT8, the only membrane associated PRMT harbours an additional role in signal transduction, presumably through its proline-rich domain which can serve as a signalling hub with SH3 domain-containing proteins (Lee et al. 2005; Sayegh et al. 2007). PRMT1 has also been found to bind to a variety of receptors and in certain contexts of

insulin treatment localizes to membrane fractions, implying its potential role in insulin signalling and glucose metabolism (Bedford & Richard 2005; Iwasaki & Yada 2007). PRMT1 has also been reported to play a role in non-genomic oestrogen signalling both in the cytoplasm and the nucleus, generating distinct complexes involved in transduction and epigenetic modulation (Le Romancer et al. 2008).

1.5.3. Arginine Methylation and Splicing

The roles of arginine methyltransferases in splicing have been studied extensively. This role comes as no surprise, as a majority of PRMT substrates are also associated with RNA, implicating them heavily not only in RNA splicing but in RNA metabolism, transport, translation and turnover (Pahlich et al. 2006). Indeed, arginine methylation was demonstrated to facilitate *in vitro* splicing reactions, as pan-SDMA-specific antibodies could block splicing activity in this setting (Boisvert et al. 2002).

The SDMA and ADMA marks have been demonstrated to play roles in the regulation of *in vitro* and *in vivo* splicing. PRMT4/CARM1 in particular has been clearly identified as a regulator of alternative splicing. Additionally, CARM1 methylates a variety of splicing factors (Boisvert et al. 2002; Ohkura et al. 2005; Cheng et al. 2007).

Arginine methylation also plays an important role in the maturation of U-snRNPs from core Sm proteins (SmB/B', SmD1, SmD2, SmD3, SmE, SmF, and SmG) and snRNA (U1, U2, U4, or U5). SDMA facilitates the binding of the Tudor domain of SMN to methylated Sm and Sm-Like proteins. PRMT5 facilitates the specific arginine dimethylation in these proteins (Brahms et al. 2000; Yu & C. 2011).

Furthermore, Sam68, a protein involved in post-transcriptional regulation and localisation of RNA-protein complexes, is a direct target of PRMT1. The resulting arginine methylation compromises its ability to interact with nucleic acids (Rho et al. 2007; Yu & C. 2011; Sánchez-Jiménez & Sánchez-Margalet 2013). In contrast, the

recognition of SDMA on Sam68 and other Sm proteins by the Tudor domain of TSN, facilitates productive spliceosome assembly (Yang et al. 2007; Hossain et al. 2010; Gao et al. 2012; Cappellari et al. 2014).

Splicing regulators SFRS9/SRp30c, which play a role in early spliceosome assembly and formation of U4/U6/U5 tri-snRNP complex, are both targets for PRMT1-mediated ADMA and MMA, which influences their sub-nuclear localisation (Bressan et al. 2009).

Coming back full-circle, alternative splicing is able to dictate isoform expression of arginine methyltransferases, affecting distinct activities, substrate specificity, and subcellular localisation. This forms and intricate regulatory loop with arginine methylation at the core of splicing-associated activity (Goulet et al. 2007).

1.5.4. Arginine Methylation in Cancer

The extensive biological functions and ubiquitous expression of PRMTs (excluding PRMT8) demonstrate their importance in the maintenance of a variety of cellular pathways and processes. It would not be surprising then if aberrant PRMT activity played role in oncogenic transformation.

Prostate and breast cancers are often hormone dependent. The involvement of PRMTs as coactivators of hormone dependent nuclear receptors make them likely candidates for deregulation in these types of cancer. PRMT1 and CARM1 inhibitors can suppress oestrogen and androgen receptor mediated transcriptional activation (Cheng et al. 2004). CARM1 correlates with androgen independence in human prostate carcinoma and in CARM1-null mouse embryos, oestrogen receptor-mediated transactivation is attenuated (Yadav et al. 2003; Hong et al. 2004). In the same vein, PRMT1 mRNA levels are higher in a panel of breast cancer cell lines compared to control and is overexpressed or aberrantly spliced in breast prostate, lung, colon and bladder cancer and leukaemia

(Goulet et al. 2007; Seligson et al. 2005; Le Romancer et al. 2008; Le Romancer et al. 2010; Cheung et al. 2007; Shia et al. 2012; Yoshimatsu et al. 2011; Guendel et al. 2010; Mathioudaki et al. 2008; Papadokostopoulou et al. 2009; Mathioudaki et al. 2011).

Overexpression of PRMT5 has also been found to promote anchorage-independent growth (Pal et al. 2004). The interaction of PRMT5 with SNAIL also highlights the role of PRMT5 in cancer (Hou et al. 2008). SNAIL is a transcription factor which represses the expression of E-cadherin, and thus promotes epithelial-mesenchymal transition in cancer. When PRMT5 is knocked down, levels of E-cadherin are elevated, strengthening the role of PRMT5 as a critical SNAIL corepressor (Pal et al. 2004). The corepressive activity of PRMT5 may thus contribute to the inhibition of tumour suppressor gene transactivation. Consistent with the notion that PRMT5 is an oncoprotein candidate, PRMT5 levels are elevated in lymphoma, gastric cancer, and leukaemia cells and have been demonstrated to suppress the RB family of tumour suppressors (Kim et al. 2005; Pal et al. 2007). Additionally PRMT5 enzymatic activity is increased in colorectal and lung cancer (Wang et al. 2008; Wei et al. 2012).

The involvement of PRMTs in DNA damage may also provide a precedent for genomic instability in the presence of arginine methylation deregulation. The recent description of arginine methylation in p53 and E2F1 also implicates PRMTs in two of the most deregulated pathways in cancer (Jansson et al. 2008; Cho et al. 2012).

While much progress has been made in the front of the role of arginine methylation in cancer, arginine methylation effector molecules need to be identified to fully characterize the functional mechanisms behind this post-translational modification. The ability of methylarginine sites to serve as binding motifs for members of the Tudor protein family has become a highlight in these efforts. Tudor proteins may thus constitute

the functional bridge between arginine methylation and its biological outcome (Seet et al. 2006).

1.6 Tudor Domains – Readers of Arginine Methylation

To date, Tudor domains remain the only major group of protein molecules capable of binding methylated arginines. Its name is derived from the original Tudor (TUD) gene discovered in *Drosophila melanogaster* (Boswell & Mahowald 1985). The resulting Tudor protein contains 11 repeats of a domain, which was subsequently found in many other proteins displaying functions in RNA metabolism (Ponting 1997b; Callebaut & Mornon 2010). Further study has demonstrated that Tudor domain proteins regulate not only RNA metabolism, but histone modification, germ cell development, transposon silencing, chromatin remodelling and DNA damage response as well (Chen et al. 2011; Siomi et al. 2010; Thomson & Lasko 2005; Lasko 2010).

The Tudor domain is a conserved motif of approximately sixty amino acids that is characterized by a bent anti-parallel β-sheet comprised of five β-strands formed into a barrel-like fold with an aromatic binding pocket (Sprangers et al. 2003). This pocket allows Tudor domain proteins to bind to methylated arginine and lysine residues and act as molecular adaptors for the recruitment of various macromolecular complexes (Kim et al. 2006; Chen et al. 2011). Tudor domains are part of the 'royal' domain superfamily and are structurally related to chromo, MBT, PWWP and plant Agenet domains (Maurer-Stroh et al. 2003). Approximately thirty proteins containing Tudor domains are encoded in the mammalian genome. Based on their ability to bind methylarginine or methyllysine, these proteins are divided into two groups. A large number of Tudor domains identified have been found to be primarily expressed in the germ line where their activity is centred on interaction with arginine methylated PIWI proteins and the coordination of PIWI-

interacting RNA (piRNA). The piRNAs act as guides to silence gene expression in animal gonads functioning in gametogenesis (Siomi et al. 2010).

While some Tudor domains possess methyllysine-binding capabilities (Kim et al. 2006), a subset of Tudor domains specifically and exclusively recognises methylarginine. These include the domains found in survival motor neuron (SMN) protein, splicing factor 30 (SPF30) and the Tudor domains of the Tudor domain-containing (TDRD) protein family.

1.6.1 The mammalian TDRD protein family

The TDRD proteins, comprised of TDRD1-TDRD12 are a functionally related group of methylarginine binding proteins (Figure 1.9). Known to participate primarily in small RNA silencing pathways, many TDRD proteins contain RNA-related domains such as the K homology (KH) domain, the RNA helicase domain or nuclease domains in addition to their multiple tandem Tudor domain repeats (Jin et al. 2009).

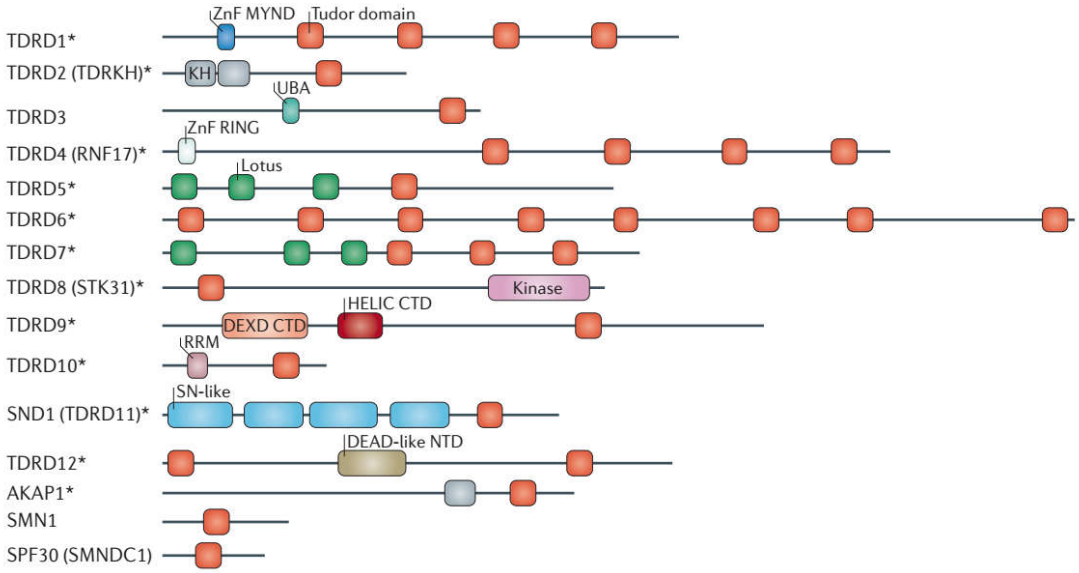


Figure 1.9 Domain architecture of methylarginine binding Tudor domain containing proteins. Domain structure predictions according to SMART (simple modular architecture research tool) and Pfam (protein families) databases. Asterisks indicate proteins which contain eTuds; AKAP1, A-kinase anchor protein 1; interactive domain (also known as BRIGHT); CTD, carboxy-terminal domain; DEXD, DEAD-like helicase domain; HELIC, helicase superfamily; KH, K homology domain; NTD, amino-terminal domain; RRM, RNA recognition motif; SMN1, survival motor neuron 1; SND1, SN-like domain containing protein 1; SN-like, staphylococcal nuclease-like domain; SPF30, splicing factor 30; UBA, ubiquitin-associated domain; ZnF MYND, MYND-type zinc-finger domain; ZnF RING, RING-type zinc-finger domain. (Used with permission from Chen et al., 2011)

In many TDRD proteins, the canonical 60-amino acid core of the Tudor domain is flanked by additional conserved structural elements of around 180 residues similar to the extended domains of the original *D. melanogaster* TUD. These elements are thus termed extended Tudor domains (eTuds) (Jin et al. 2009). The additional residues which make up the extended region of an eTud constitute an α-helix and two β-strands amino-terminal to the canonical core domain and several strands and helices carboxy-terminal to the core (H. Liu et al. 2010).

1.6.2 Structural insight into Tudor recognition of methylarginine

Structural insight into the binding of Tudor domains to their methylarginine targets has been shed by reports on co-crystal and solution structures. These reports aid our understanding of methylarginine recognition by Tudor domains through the analysis of the interaction between eTuds and SDMA modifications on PIWI proteins (H. Liu et al. 2010; K. Liu et al. 2010), the recognition of ADMA and SDMA by the Tudor domains of SMN and SP30 (Tripsianes et al. 2011), as well as the binding of TDRD3 to ADMA (Sikorsky et al. 2012).

This new information outlines the structural determinants which enable general arginine dimethylation recognition, or specific SDMA or ADMA recognition. In the case of TDRD11 (also known as SND1 or p100-TSN) and TUD, the canonical eTud structure allows preferred interaction with SDMA because of hydrogen bonds formed between SDMA and the asparagine within the binding pocket (H. Liu et al. 2010; K. Liu et al. 2010). SMN and SPF30 lack this hydrogen bond and use an alternative strategy for dimethylarginine recognition through tryptophan, a fused-ring heterocyclic amino acid, at the corresponding position within the binding pocket. Such a substitution facilitates altered stacking interaction between the aromatic cavity and ligand. This allows for their comparable affinity for both SDMA and ADMA marks (Tripsianes et al. 2011). In

TDRD3, the same position is occupied by a tyrosine, driving a combination of cation-π, CH-π and stacking interactions with ADMA specifically. This interaction is additively stabilised by the N-H···O hydrogen bond between the ADMA amino group and the hydroxyl group of tyrosine (Sikorsky et al. 2012). Such studies extend our understanding of the coordination of Tudor domain methylarginine recognition.

1.6.3. TDRD proteins – readers of E2F1 arginine methylation

Discussed briefly in a previous subchapter (1.4.2.4), E2F1 has been found to contain arginine residues which are differentially methylated by PRMT1 and PRMT5, generating ADMA on Arg109 and SDMA on Arg111/113 respectively (Cho et al. 2012; Zheng et al. 2013). The mutual exclusivity of these marks, as well as their opposing biological outcomes, constitute a working model explaining the molecular switch between of the roles of E2F1 in proliferation and apoptosis. The description of TDRD proteins which recognise specific arginine methylation ligands supports the possibility that different biological readers mediate the biological effects associated with symmetric or asymmetric dimethylation on E2F1 providing a potential molecular switch between the opposing functions of E2F1.

While readers for E2F1 ADMA have yet to be identified, two readers of the symmetric dimethylation mark have since been identified: Tudor domain proteins SND1/TSN (Zheng et al. 2013) and TDRD1 (Moehlenbrink et al., unpublished). The need for two readers of the same methylation mark alludes to possible branched differences in the pathways utilized which lead to the observed biological outcome of the E2F1 SDMA mark or protective functional redundancy. The reported biological activity of each TDRD protein supports them as candidate effectors of this post-translational modification.

1.6.3.1 SND1/TSN

Tudor-staphylococcal nuclease (TSN), also known as the staphylococcal nuclease and Tudor domain containing 1 (SND1) is an evolutionarily conserved protein in eukaryotes (except *Saccharomyces cerevisiae*) suggesting important physiological functions. Through its various domains and the interactions that they mediate, TSN is localized both to the nucleus as well as the cytoplasm where TSN functions as a transcriptional co-activator, a component of the RNA-induced silencing complex as well as a regulator of mRNA editing, splicing, and stability, potentially allowing it to play various roles in carcinogenesis (Jariwal et al. 2015; Gutierrez-Beltran et al. 2016a).

Human TSN is a 910-amino acid containing protein and is comprised of tandem repeats of four staphylococcal nuclease (SN)-like domains and a fifth Tudor-partial SN-domain fusion (Callebaut & Mornon 1997; Ponting 1997a; K. Liu et al. 2010) (Figure 1.10).

The SN domains of TSN are part of the oligonucleotide-/oligosaccharide-binding fold (OB-fold) superfamily, a family of binding proteins which is remarkably conserved throughout the course of evolution (Theobald et al. 2003). Each SN domain consists of an OB-fold, a characteristic five-strand β-barrel, flanked by three α- helices. These domains enable nucleic acid binding and processing, echoing the primary function of the original bacterial enzyme.

In mice TSN is ubiquitously expressed, apart from in muscle tissues. Overall, TSN is overexpressed in precursor cells or cells that are rapidly proliferating. In contrast, it is downregulated in terminally differentiated cells (Fashe et al. 2013).

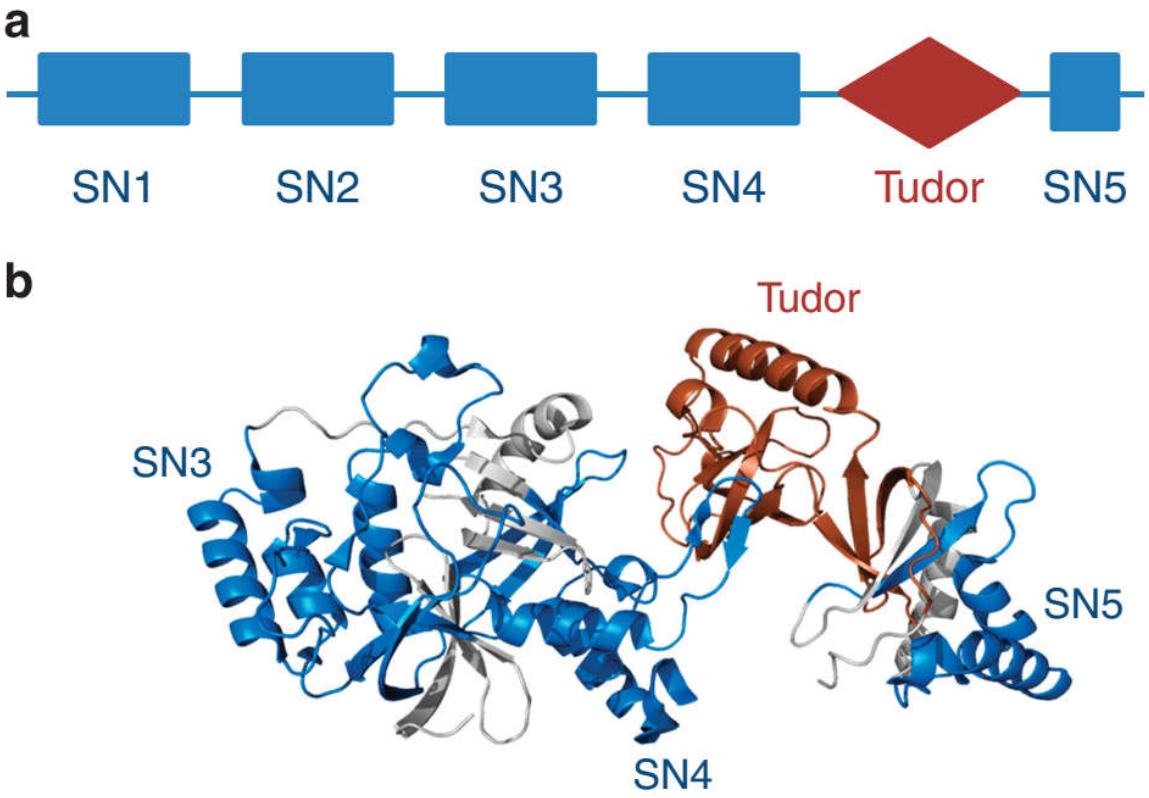


Figure 1.10 Structure of TSN.
(a) The domain structure of TSN consists of four SN domains and a fifth Tudor-SN fusion domain. (b) The ribbon model of truncated human TSN as described by Li et al., 2008. The colours correspond to the domain architecture in (a). SN, staphylococcal nuclease-like. (Used with permission from Gutierrez-Beltran et al., 2016)

1.6.3.1.1 Biological Functions of TSN

The singular SN domains of TSN have been found to interact with astrocyte elevated gene-1 (AEG-1) and Argonaute 1 and 2 (Ago1/2) components of the RNA-induced silencing complex (RISC) (Kim et al. 2005; Gao et al. 2014). The SN domains also enable the degradation of hyper-edited double-stranded RNA and miRNA precursors which are produced by adenosine deaminases acting on RNA (ADARs). This process involves TSN in stress granule formation through its interaction with ADAR1, RNA-binding Ras-GAP SH3 binding protein (G3BP) and other stress granule core components (Scadden 2005; Gao et al. 2010; Weissbach & Scadden 2012; Gao et al. 2014). TSN is also able to bind several components of the basal transcription machinery and plays a

large role in transcriptional co-activation (Tong et al. 1995; Leverson et al. 1998; Paukku et al. 2003; Välineva et al. 2005; Välineva et al. 2006; Low et al. 2006; Duan et al. 2014).

The Tudor domain of TSN structurally constitutes an eTud domain capable of binding methylated arginines and lysine (K. Liu et al. 2010). In such interactions, TSN is able to act as a molecular scaffold or adaptor facilitating the assembly of large protein complexes and the interaction of methylated arginines to effector molecules with specific catalytic activities (Friberg et al. 2009; K. Liu et al. 2010; Liu et al. 2011; Pek et al. 2012). The Tudor domain also mediates the interaction of TSN with core components of RNA splicing, including the human U5-220 kDa protein (Prp8), SmB, SmD1/D3 and the splicing factor Src-associated in mitosis of 68kDa (SAM68) (Yang et al. 2007; Hossain et al. 2010; Gao et al. 2012; Cappellari et al. 2014). Recently, TSN has also been demonstrated to mediate E2F1 binding to target RNA species, potentially influencing the alternative splicing of these genes (Roworth, unpublished). TSN is also implicated in the biogenesis of noncoding RNAs through its interaction with specific members of the Argonaute family PIWIL1/Miwi during development (K. Liu et al. 2010; Ku et al. 2016).

1.6.3.1.2 TSN and cancer

The extensive roles that TSN plays in RNA metabolism make it a candidate for deregulation in various cancers. Recent findings demonstrate that TSN is in fact overexpressed in various cancers and is associated with poor prognosis. TSN overexpression promotes uncontrolled proliferation and accomplishes this through transcriptional co-activation of various oncogenes as well as through the increased degradation of tumour suppressor mRNAs (Tsuchiya et al. 2007; Tsuchiya & Nakagama 2010; Yoo et al. 2011; Santhekadur et al. 2012; Jariwal et al. 2015). TSN also contributes to angiogenesis through the activation of the NF-κB and subsequent induction of oncomiR-221, angiogenin, and CXCL16 (Santhekadur et al. 2012). Additionally, TSN

increases epithelial-mesenchymal transition in breast cancer through the TGFβ1 signalling pathway (Yu et al. 2015).

In tumour-initiating cells, TSN regulates oncogene-induction and expansion through its interaction with AEG1, supporting cell survival even under oncogenic/stress conditions. Disruption of either AEG1 or TSN abrogates tumorigenic potential *in vitro* and *in vivo* (Wang et al. 2012). The simultaneous overexpression of c-Myb and TSN also plays a role in breast cancer progression (Quintana et al. 2011). TSN overexpression is also able to suppress the activity of pro-apoptotic genes leading to chemoresistance in non-small cell lung adenocarcinoma (Zagryazhskaya et al. 2015). Overall TSN has presented itself as a good prognostic marker for various types of cancer, as well as a potential drug target for treatment (Jariwal et al. 2015).

1.6.3.2 TDRD1

Compared to TSN, there is comparatively less known about Tudor-domain containing protein 1 (TDRD1). TDRD1 is a multi-domain protein containing an N-terminal myeloid translocation protein zinc finger domain (ZnF MYND) followed by four tandem eTud domains(Figure 1.9) (Chuma et al. 2003).

In mice, TDRD1 expression tightly corresponds with Piwi proteins during mouse spermatogenesis and functions as a molecular scaffold for mouse PIWI proteins (Mathioudakis et al. 2012a). In embryonic germ cells, TDRD1 interacts with MILI and MIWI2 implicating it in secondary piRNA biogenesis (Vagin et al. 2006; Reuter et al. 2009). It is believed that this interaction is facilitated by eTud recognition of a variety of symmetric arginine methylation marks on Piwi and Piwi-associated proteins whose affinity is determined by various residues flanking the SDMA mark. TDRD1 thus contributes as a molecular scaffold in these contexts and functions in transposon silencing in the mouse germline (Reuter et al. 2009; Mathioudakis et al. 2012a). Interestingly

TDRD1, normally a germline-specific gene that is repressed in somatic tissues, is epigenetically activated in ERG-rearrangement positive prostate cancer and is a prognostic marker for high-risk prostate cancer. Additionally, it is expressed in some cancer cell lines (Kacprzyk et al. 2013; Boormans et al. 2013; Litovkin et al. 2014; Xiao et al. 2016). Despite its activation, it has not been found to contribute to functional piRNA pathways with human orthologues of piRNA-associated proteins or influence retrotransposon activity in these cells.

1.7 Summary of the study: The role of TSN and TDRD1 in E2F1-dependent activity

Reports of differential arginine dimethylation on E2F1 draw focus to potential readers which can recognise and thus mediate their associated proliferative or pro-apoptotic activity (Cho et al. 2012; Zheng et al. 2013). Recently, TSN and TDRD1 have been identified as readers of the E2F1 SDMA mark (Zheng et al. 2013; Moehlenbrink et al., unpublished). Additionally TSN has also been implicated as a transcriptional co-activator facilitating the G_1/S transition in conjunction with E2F1 (Su et al. 2015).

In order to better understand the role of these two readers in determining the functional outcome of E2F1 in the cell cycle, this study aimed to assess the effects of reader depletion on E2F1-dependent transcription and target gene expression on the mRNA level. This was achieved through qRT-PCR validation of the deregulation of candidate target genes obtained from preliminary RNA Sequencing (RNA-Seq) analysis of TSN and TDRD1 knockdown cells (data provided by Chan, unpublished). Further co-depletion experiments demonstrated that these effects were mediated by E2F1.

In order to clarify the mechanism by which this deregulation was achieved, chromatin immunoprecipitation (ChIP) experiments were conducted, illustrating the consequent effects of reader depletion on E2F1 recruitment and vice versa, strengthening the mechanistic bridge between the two arginine methylation readers and E2F1-

dependent transcription. This allowed for the conceptualisation of mechanistic models for TSN- and TDRD1- dependent transcriptional regulation of E2F1 target genes.

Additionally, the previously discussed role of TSN in splicing, along with reports of TSN-mediated E2F1 binding to target RNA species, provided reasonable incentive to explore the possibility of E2F1-mediated splicing events in E2F1 target genes. Reader-dependent differences in splice variant expression of various target genes was thus explored, revealing a cooperative role for E2F1 and TSN in differential splicing which could also contribute to the opposing biological outcomes of E2F1.

Overall, the results of this study provide new insight into one side of the arginine methylation-mediated molecular switch in the opposing functions of E2F1. In this regard, TDRD1 and TSN were demonstrated to play functionally redundant roles in the regulation of the E2F1- target genes but could potentially do so by employing different regulatory strategies.

This newly acquired knowledge supplements our understanding of what mediates the proliferative and potentially oncogenic roles of E2F1 in cancer. This opens doors towards identifying novel approaches to target the specific role of E2F1 in oncogenesis, while leaving its tumour suppressive roles intact. As such, the E2F1 symmetric dimethylation mark, and the readers which mediate its recognition may be used as potential targets in the treatment of cancer.

Chapter 2 : Materials and Methods

2.1 Cell Lines

2.1.1 Cell Line Maintenance

MCF7 and DuCaP cells were cultured and maintained in Dulbecco's modified Eagle's medium (Sigma Cat No. D6429) with 5% (v/v) penicillin and streptomycin (Sigma), and supplemented with 10% heat inactivated foetal bovine serum (FBS) (Labtech, Cat No. FCS-SA/500, Lot No. 40507-CO5). SKBR3 cells were cultured and maintained in McCoy's 5a Modified Medium (Lonza Biowhittaker) with 5% (v/v) penicillin and streptomycin (Sigma), and supplemented with 10% FBS unless otherwise stated. Cells were grown in a humidified incubator at 37°C and 5% carbon dioxide (CO_2).

2.1.2 Stable shRNA Knockdown Cell Lines

MCF7, SKBR3 and DuCaP cells exhibiting stable knockdown of TSN or TDRD1 were previously created by Cheryl Chan via transduction of short hairpin RNA (shRNA) lentiviral particles targeting either gene according to the manufacturer's protocol (Table 2.1). These cells were maintained under constant selection using aforementioned media supplemented with 2 μg/ml puromycin (Insight Biotechnology).

Table 2.1 Product details of shRNA lentiviral particles used in transduction

Target Gene	shRNA Lentiviral Particles
TSN	sc-45514-V (Santa Cruz Biotechnology)
TDRD1	sc-90747-V (Santa Cruz Biotechnology)
Control shRNA	sc-108080 (Santa Cruz Biotechnology)

2.2 Oligonucleotide-based Transient siRNA Knockdown

Short interfering RNA (siRNA) sequences were designed previously by members of the La Thangue Lab (Table 2.2) and were synthesized by Dharmacon. The siRNA duplexes were diluted in universal siRNA buffer (20 mM KCl, 6 mM HEPES-KOH pH 7.5, 0.2 mM $MgCl_2$; Dharmacon) as instructed in product data sheets. Cells were transfected with 12.5-50 nM siRNA using Oligofectamine reagent (Invitrogen) according to manufacturer's instructions with slight modifications.

Table 2.2 Sequences of oligonucleotides used for siRNA transient knockdown

Target Gene	Oligonucleotide Sequence
GFP	5'-AGCUGACCCUGAAGUUCUU-3'
E2F1	5'-AACUCCUCGCAGAUCGUCAUCUU-3'
TSN	5'-AAGGAGCGAUCUGCUAGCUACdTdT-3'
TDRD1	5'-GAUGAGACUUUGUCCCAUAUU-3'

Briefly, 600,000-800,000 cells were plated onto a 100 mm plate in 5 mL complete media and allowed to attach by incubation at 37°C and 5% CO_2 for a minimum of 3 hours. Per 100 mm plate to be treated, 12.5-50 nM siRNA was diluted in 900 µL Opti-MEM™ Reduced Serum Media (Gibco™). Separately, 4 µL Oligofectamine was added to 96 µL Opti-MEM™ and mixed gently before incubating for 5-10 minutes at room temperature. The diluted siRNA was then combined with the diluted Oligofectamine to a total volume of 1 mL, mixed gently, and incubated for 15-20 minutes at room temperature. During incubation, growth medium was removed from previously plated cells and cells were washed with media without FBS before ultimately substituting media with 5 mL FBS-free media. After incubation, the complexed Oligofectamine-siRNA solution was added to cells before incubating at 37°C and 5% CO_2 overnight. The next day, FBS was added to a concentration of 10% and allowed to incubate for another 48 hours before further downstream assessment. GFP was used as a non-targeting siRNA and was transfected at an equal amount as a control. Cells were incubated for a total minimum of 72 hours prior to harvest or further drug treatment.

2.3 Antibodies and Western Blot

2.3.1 Protein Extraction

Cells were harvested by trypsinization or scraping and washed in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4). Cells were pelleted by centrifugation at 200xG for 5 minutes and lysed in modified lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630, 1 mM EDTA, 1

mM NaF, 1X protease inhibitor cocktail [Aprotinin from bovine lung, Pepstatin A, Leupeptin Hemisulfate 1 μg/mL]) for 20 minutes on ice. Cell debris was removed and lysate was clarified through centrifugation for 10 minutes at 16,000xG, 4°C. Clarified lysate was transferred to a new tube. Protein concentration was determined using Bradford reagent (Sigma) and absorbance was read at 595 nm using a Tecan Sunrise absorbance microplate reader.

2.3.2 Western Blot

Equal amounts of protein (20-30 μg) were used for each round of Western Blot analysis. Lysates were run on 6-12% SDS PAGE gels and transferred onto Immobilon®-P polyvinylidene difluoride (PVDF, Merck-Millipore) or Protran nitrocellulose (Whatman) membranes. Membranes were then blocked with 5% (w/v) skimmed milk in PBS for a minimum of one hour. Subsequently, membranes were probed with primary antibodies (Table 2.3) diluted in 5% (w/v) skimmed milk in PBS for one hour at room temperature or overnight at 4°C. Membranes were then washed thrice with PBST (0.1% Tween-20 in PBS) for five minutes before being probed with Horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 2.3) diluted 1:10,000 in 5% (w/v) skimmed milk in PBS for 45 minutes to an hour. Membranes were washed thrice more with PBST for five minutes and signals were detected by incubation with enhanced chemiluminescence (ECL) substrate (Bio-Rad) for 1-2 minutes and allowed to X-ray develop.

Table 2.3 Antibody descriptions and usage information for Western Blot

Antibody	Species and Description	Supplier	Cat. No.	Concentration (Dilution)
β-actin	mouse monoclonal IgG_{2a}	Sigma-Aldrich	A2228	2 mg/mL (1:10000)
E2F1 KH95	mouse monoclonal IgG_{2a}	Santa Cruz Biotechnology	SC-251	200 µg/mL (1:500)
E2F1 C20	rabbit polyclonal IgG	Santa Cruz Biotechnology	SC-193	100 µg/mL (1:1000)
TSN	rabbit polyclonal IgG	Bethyl Laboratories	A302-883A	200 µg/mL (1:3000)
TDRD1	rabbit polyclonal IgG	Atlas Antibodies	HPA037729	300 µg/mL (1:1000)
PRMT5	rabbit polyclonal IgG	Merck Millipore	07-405	200 µg/mL (1:1000)
p53 DO-1	mouse monoclonal IgG_{2a}	Santa Cruz Biotechnology	SC-126	200 µg/mL (1:1000)
Cleaved PARP (Asp214)	rabbit polyclonal IgG	Cell Signaling Technology	9541	100 µg/mL (1:1000)
α Mouse IgG	rabbit polyclonal IgG HRP-conjugated	Merck Millipore	AP160P	800 µg/mL (1:10000)
α Rabbit IgG	goat polyclonal IgG HRP-conjugated	Merck Millipore	AP187P	800 µg/mL (1:10000)

2.4 Quantitative Reverse-transcription Polymerase Chain Reaction

2.4.1 RNA Extraction and Quantification

Cells were harvested via trypsinization and washed twice with PBS before total RNA was extracted using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. The extracted RNA was then stored at -80°C until further needed.

RNA concentration and purity was quantified using a NanoDrop 2000 UV-Vis Spectrophotometer.

2.4.2 First Strand cDNA Synthesis

First strand cDNA was generated from 5 µg total RNA using M-MLV Reverse Transcriptase (Promega) according to the manufacturer's instructions. Per microgram RNA, 0.5µg oligo-dT primer was added to RNA in a sterile microcentrifuge tube and diluted with sterile nuclease free water to a total of 15 µL. Samples were heated to 70°C for five minutes then cooled immediately only ice for 2-3 minutes. Samples were

centrifuged briefly to collect solution to the bottom of the tube before adding 5 µL 5X M-MLV Reaction Buffer (Promega, 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15mM $MgCl_2$, 50mM DTT), 1.5 µL 10 mM dNTP mix (Promega), 0.625 µl (25U) Recombinant RNasin® Ribonuclease Inhibitor (Promega, 40 U/µl), 1 µL (200U) M-MLV RT (Promega, 200 U/µL), and sterile nuclease-free water to a final volume of 25µl per microgram RNA. Samples were mixed gently by flicking before incubation at 37°C for one hour. First-strand cDNA samples were stored at -20°C until further use.

2.4.3 Target gene amplification (Second strand cDNA synthesis)

2.4.3.1 Primer Design

Primers were designed specifically for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) purposes using reference mRNA sequences of each gene target as listed on National Centre for Biotechnology Information (NCBI) and transcript exon and intron sequences listed on the Ensembl database (release 88).

The Integrated DNA Technologies (IDT) PrimerQuest online tool (https://www.idtdna.com/Primerquest/Home/Index) was used to determine optimal oligonucleotide primer sequences limiting product sizes to between 70 and 250 base pairs and optimum annealing temperatures to 60°C. Secondary structure and dimer formation between primer pairs and was assessed using the Premier Biosoft NetPrimer analysis tool (http://www.premierbiosoft.com/NetPrimer/AnalyzePrimer.jsp). Finally, specificity was confirmed using the NCBI Primer Basic Local Alignment Search Tool (Primer-BLAST, https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All oligonucleotides were ordered from Sigma Life Science. A number of primer sequences were obtained from previously published material. For a full list of qRT-PCR primers and details refer to Table 2.4.

Table 2.4 Oligonucleotide sequence of primers used for qRT-PCR

Gene (RefSeq)	Sequence		Product Size	Origin/Reference
β-actin (NM_001101.3)	F	AGAGCTACGAGCTGCCTGAC	184	(Zhou et al. 2014)
	R	AGCACTGTGTTGGCGTACAG		
E2F1 (NM_005225.2)	F	AGCAAGAACCGCTGTTGTCCC	155	La Thangue Lab
	R	GGGTGGGGAAAGGCTGATGA		
TSN (NM_014390.3)	F	GCTGAACTCAGGCGATTACA	181	La Thangue Lab
	R	CACCGTCACATTGACCTTCT		
TDRD1 (NM_198795.1)	F	ACCACCCTATAGACCAAGAAT	137	La Thangue Lab
	R	TTTCAATGTTTCCATAGTCTGCA		
KLF2 (Total) (NM_016270.3)	F	CGTCCGCGTCCTTTCTC	80	Designed
	R	GGCTGGCGAAAGTGGAG		
MDC1 (Total) (NM_014641.2)	F	AATGCCTGACTGCTCTGTG	104	Designed
	R	CACAGTCTCGGAGGATAGGT		
SKP2 (Total) (NM_005983.3)	F	ATGAGCTGCTCTTGGGAATC	86	Designed
	R	TAGGCGATACCACCTCTTACA		
TCF19 (NM_001077511.1)	F	CCACAGCAGCCAAGGTACTTTG	135	Designed
	R	ATGAAGTAGAACTCCGAGGGGCT		
CDC6 (NM_001254.3)	F	GAGATGTTCGCAAAGCACTGGATG	126	Designed
	R	TGGGAATCAGAGGCTCAGAAGGTG		
ZNF367 (NM_153695.3)	F	AGGATGGAATCCGACGTGGTAG	161	Designed
	R	CCTCTCACCTGTATGAGTCCTTTTG		
POLD1 (NM_001256849.1)	F	CTGTGGAACTGTGCTCCCGAG	212	Designed
	R	GTCCGTGTCCACCATGAACC		
PSMC3 (NM_002804.4)	F	CAGACGTACTTCCTTCCTGTGATTG	90	Designed
	R	CAGATAGGAGTCTTTGTTCACACCC		
SCAF1 (NM_021228.2)	F	GCGAAAGTGAGGACATGCTGG	144	Designed
	R	TAGAGGAGTTCGACTGTGGAGAAAC		
SYNGR4 (NM_012451.3)	F	GGACTTCATCCTGGCTGTTCTC	215	Designed
	R	TGTAAGGGACTGGAGCATCATTTC		
RAD54L (NM_003579.3)	F	AGAGAGGGAGTGAAATTCCTGTGG	298	Designed
	R	CTTCCAGCTTTTGGTCTATTTCATC		
IER2 (NM_004907.2)	F	GTGTCAGTGGAAAGGCATAAGGC	123	Designed
	R	CCGCACGAAAGGTAAACAAAGC		
ZNF524 (NM_153219.3)	F	GCGACATGCAAATGAGCTGG	130	Designed
	R	GAGGAGATAAGGCCAGAGGTTTCTC		
WDR34 (NM_052844.3)	F	CGAGCAGCAGCAGATGGTGTC	173	Designed
	R	AGGCACACACGAAGGACTTAAGC		
CASP8AP2 (NM_001137667.1)	F	AATGGCAACTGGTGAAACAAACTC	105	Designed
	R	AGAGGACTGATGGCTTCTGGAATAG		
ZBED8 (NM_001303251.1)	F	TAAGCAAAATTCTGTGGCATCTCTG	99	Designed
	R	ACCAGTAGCGAACATAGTCATCATC		
KLF2_001	F	CCAGCCTTCGGTCTCTTC	125	Designed
	R	TTTGGCTTGGCCTCCAG		

KLF2_002	F	AGGAGGTGAGAAGCCCTAC	122	Designed
	R	ACAGATGGCACTGGAATGG		
MDC1_001	F	GTCCTCAAGGATCACACAAAGA	99	Designed
	R	ATCTGCTTCCACACTGTCATC		
MDC1_007	F	CAGGAGGCATCAGAAAGGTAAG	109	Designed
	R	AAAGGTGGCTGAGTTCCAAG		
MDC1_008	F	GGAAGGAAGAGGTGAGGAGA	98	Designed
	R	GTCACGACATCCTGAGATTGAG		
SKP2_001	F	TCCAGCTCAACTACCTCCA	84	Designed
	R	CAGCTCTTGTCACTAATAGTAAAGTTTC		
SKP2_002	F	GCATTTAGTGTGGTATGCCATTT	101	Designed
	R	AGCATATGAAGTAGATGGGTCAAA		
SKP2_009	F	GAGCTGCTCTTGGGAATCTTT	102	Designed
	R	AGTAGAGAGATGCTAGGCGATAC		
SKP2_202	F	GAAACGGCTGAAGAGCAAAG	94	Designed
	R	GAGACTCATCAGACCTGGAAAG		

2.4.3.2 Polymerase Chain Reaction

Primers to be used for qRT-PCR were first tested by target gene amplification using normal polymerase chain reaction (PCR) using the generated cDNA from subchapter 2.4.2 as a template. Briefly, a master mix consisting of 2 µL of cDNA template, 2 µL of 10X Paq5000 DNA polymerase buffer (Agilent), 2 µL of 5 M Betaine (Sigma-Aldrich), 0.5 µL of 10 µM dNTP mix (Promega), 1 µL each of 10 µM forward and reverse primer solutions, and 0.2 µL of 5U/µL Paq500 DNA Polymerase filled to 20 µL with sterile nuclease-free water per reaction was prepared. PCR was performed using a Bio-Rad T100 Thermal Cycler using the following thermal cycling conditions: one initial denaturation cycle for 5 minutes at 95°C; 30 amplification cycles consisting of a 1-minute denaturation step at 95°C, a 1-minute primer annealing step at 60°C, and a 30-second extension step at 72°C; and an indefinite hold at 4°C. PCR products were visualized on a 2% agarose gel containing 0.5 µg/mL ethidium bromide.

2.4.3.3 Quantitative Reverse-Transcription Polymerase Chain Reaction

In order to measure the relative expression of RNA species from samples collected, SYBR Green-based qRT-PCR was conducted using cDNA obtained in subchapter 2.4.2 as template. cDNA was diluted 1:5 in sterile nuclease-free PCR-grade water prior to use. qRT-PCR reactions were prepared by mixing 10 µL- of 2X Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent), 1 µL of 10 µM forward and reverse primer solution, and 7 µL sterile nuclease-free PCR-grade water for a total of 18 µL per reaction in a sterile microcentrifuge tube. SYBR green mix was then distributed into an Mx3005P 96-well non-skirted PCR plate (Agilent). In triplicates, 2 µL of diluted cDNA template was added to each well for a total of 20 µL per reaction. Plates were then covered with optical strip caps and centrifuged briefly to collect mixture at the bottom. Reactions were then amplified in an Agilent Mx3005P qPCR machine using a PCR setup consisting of a 3-minute pre-melt segment at 95°C; a two-step amplification segment of 40-55 cycles of 20 seconds at 95°C and 20 seconds at 60°C; and a standard 55°C-95°C dissociation segment. Amplification data was collected and analysed using MxPro-Mx3005P (Agilent) software.

2.5 Chromatin Immunoprecipitation

2.5.1 Bead Preparation

Beforehand, 700 µL of Protein A/G Sepharose Fast Flow beads (GE Healthcare) in 30% ethanol was transferred to a 2-mL tube and resuspended in 700 µL of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.1 mM EDTA, 20 mM Tris-HCl pH 8.0, 167 mM NaCl) by inversion. Beads were pelleted at 500xG, 4°C for 2 minutes and washed with 700 µL ChIP dilution buffer three times before resuspension in 700 µL 10 mg/mL filtered bovine serum albumin (BSA) in ChIP dilution buffer. After resuspension, 20 µL of 10 mg/mL salmon sperm DNA (Invitrogen) was added. Beads were then allowed to

mix by rotation at 4°C for at least one hour or until required. Before use, beads were washed twice with ChIP dilution buffer and finally resuspended in 700 µL ChIP dilution buffer.

2.5.2 Crosslinking, Harvest, and Lysis

Per ChIP reaction, three 100 mm plates at 70-85% confluence were used. In a tissue culture hood, formaldehyde was added directly to media to a final concentration of 1%, mixed well and left at room temperature for 10 minutes. Media was then neutralized by adding 1 M glycine solution to a final concentration of 0.125 M and left to incubate at room temperature for 10 minutes. Neutralized media was then aspirated off the plates and cells were washed two times with sterile PBS. Cells were then harvested by scraping in 1 mL PBS per plate and then transferred into a falcon tube. Cells were pelleted at 200xG for 5 minutes at 4°C and resuspended by pipetting in 0.5 mL nuclear lysis buffer (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 M EGTA, 1X protease inhibitor cocktail [Aprotinin from bovine lung, Pepstatin A, Leupeptin Hemisulfate 1 µg/mL]) per 100 mm dish harvested. Suspension was then allowed to mix on rotating wheel for 10 minutes at room temperature. Following mixing, cells were pelleted at 200xG for 5 minutes at 4°C and resuspended in 0.5 mL shearing buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 M EGTA, 0.1% (w/v) Na-deoxycholate, 0.5% Na-Lauroylsarcosine, 1X protease inhibitor cocktail) per 100 mm dish harvested. The resulting suspension was then sonicated using a Branson Digital Sonifier for 10 rounds of 25% intensity pulse on for 10 seconds, and off for 20 seconds, while being kept on ice to generate chromatin fragments of 300-800 base pair lengths. Following sonication, molecular biology grade Triton X-100 (Sigma-Aldrich) was added to a final concentration of 1% (v/v) and vortexed to mix. The lysate was then clarified by centrifugation at 16,000xG, 4°C for 2 minutes. Clarified lysate was transferred to a new

tube, leaving debris. From the clarified lysate, 5% volume was set aside in a sterile tube to serve as the input sample and stored at -20°C until the elution step (2.6.5).

2.5.3 Pre-clearing of Chromatin

Chromatin obtained was then pre-cleared with 2 µg non-specific antibody (anti-rabbit (I5006)/anti-mouse (I5381)/anti-goat (I5256) IgG, Sigma) and 40 µL precleared beads (2.5.1), then left to mix by rotation for 1-3 hours at 4°C. After clearing, beads were spun down at 510xG, 4°C for 2 minutes, transferring cleared chromatin to a new sterile nuclease-free tube and discarding beads.

2.5.4 Immunoprecipitation

Fragments of interest were immunoprecipitated from pre-cleared chromatin (2.5.3) by adding 2 µg of specific antibody to each experimental sample and 2 µg of non-specific antibody to IgG control sample. Samples were mixed by rotation overnight at 4°C.

The next day, 40 µL of precleared beads (2.5.1) was added and allowed to mix further for 2-3 hours. Following antibody binding, beads were washed two times with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), four times with LiCl Buffer (0.25 M LiCl, 1% IGEPAL CA-630, 1% deoxycholate, 1 mM EDTA, 20 mM Tris-HCl pH 8.0), and twice more with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). For each wash, beads were pelleted by centrifugation at 700xG, 4°C for 2 minutes and wash buffer was decanted gently to avoid loss of beads. On the last TE wash, beads were resuspended gently by inversion and the resulting suspension was transferred to a new tube before pelleting beads and discarding supernatant.

2.5.5 Elution and Reverse Crosslinking

Chromatin was eluted by resuspending beads in 60 µL freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) by vortex. The resulting suspension was incubated at 65°C for 15 minutes while being vortexed every 3 minutes. Beads were pelleted by centrifugation at 700xG for 2 minutes at room temperature and eluted sample was transferred to a new sterile nuclease-free tube. The elution step was then repeated with the leftover beads a second time and eluted samples pooled for a total of 120 µL of eluted sample. Beads were then discarded. For the input sample (2.5.2), elution buffer was added to a total volume of 120 µL.

Reverse crosslinking was performed on all samples by adding 6 µL of 5M NaCl to eluted sample. Samples were reverse crosslinked overnight at 65°C.

The next day, 10 µL of 10mg/mL Proteinase K (Promega) was added to each sample and incubated at 55°C for 1-2 hours.

2.5.6 DNA Purification

DNA fragments were purified using the Qiagen QIAquick Purification kit according manufacturer's instructions. Final eluted DNA was then stored at -20°C until further use.

2.5.7. Target Gene Amplification

Primers were designed either to encompass putative E2F1 consensus binding sites (5'-TTTSSCGS-3' or 5'-GGCGCAAA-3' where S is C or G) on target gene promoters or were designed within E2F1 ChIP peaks as called using the available MCF-7 E2F1 Track on the University of California Santa Cruz (UCSC) Genome Browser produced by the Farnham lab (ENCODE Project Consortium 2012; Rosenbloom et al. 2015) (Table 2.5). Target protein recruitment to genes of interest was measured through qRT-PCR, as described in 2.4.3.3 using immunoprecipitated chromatin as template.

Table 2.5 Oligonucleotide sequence of primers used for ChIP analysis

Gene (RefSeq)	Sequence		Product Size	Origin/Reference
Albumin	F	TGGGGTTGACAGAAGAGAAAAGC	224	(Zalmas et al. 2008)
	R	TACATTGACAAGGTCTTGTGGAG		
KLF2	F	CCATCCGGGACGCGTTT	77	Designed
	R	GCCCGCCCAAGCCTTATAG		
MDC1	F	GACACCACCTATTTGAACTCTAAGG	125	Designed
	R	GACTGCGTGTGCCGAAA		
SKP2	F	CTACTGTCACCACTCAGCAG	90	Designed
	R	GAGAGCAGGCGGAAAGAG		
CDC6	F	GGCCTCACAGCGACTCTAAGA	132	La Thangue lab
	R	CTCGGACTCACCACAAGC		
E2F1	F	TAAAGCCAATAGGAACCGC	166	La Thangue lab
	R	AGGGCTCGATCCCGCTCCG		

2.6 Cell Line Characterisation

2.6.1 Flow Cytometry

One-hundred thousand cells were seeded into each well of a 6-well tissue culture treated plate in 3 mL of respective media in triplicates with an extra well corresponding to unstained control. After 48 hours, media of each well was collected and pooled with harvested cells per replicate. Cells were washed with PBS twice before dropwise resuspension in 1 mL 70% ethanol in PBS while vortexing. Cells were allowed to fix overnight at 4°C.

The next day, cells were pelleted and washed with 1 mL PBS twice before treatment with 100 µL 0.2 mg/mL RNase A (Thermo Scientific™) and 450 µL 10 µg/mL propidium iodide (Sigma) in PBS for at least twenty minutes at 4°C in the dark.

After staining asynchronous cell cycle profiles of each replicate and unstained control were measured and collected from 20,000 events using a BD Accuri™ C6 Cytometer and CFlow Plus software.

2.6.2 Growth Curve Analysis

Twenty-thousand cells were seeded in 3 mL of appropriate media into 6-mm plates in replicates of eight. Every 24 hours, cells from one plate were harvested and counted using a haemocytometer. Cell numbers were plotted against time to generate a growth curve for each cell line.

Chapter 3 : Transcriptional effects of TSN and TDRD1 on E2F1 target genes

3.1 Introduction

The different arginine dimethylation marks deposited by PRMT1 and PRMT5 on E2F1 have been found to be mediators of its functions in apoptosis and cell cycle progression respectively (Cho et al. 2012; Zheng et al. 2013), demonstrating a working model for a mechanistic switch between two opposing biological outcomes (Figure 1.6).

Previously, TSN was identified as a candidate reader of the E2F1 SDMA mark in a chromatin-associated domain (CADOR) array conducted in collaboration with the Bedford laboratory at the University of Texas (Zheng et al. 2013). Following various *in vitro* cell-based approaches, the functional connection between E2F1 and TSN was strengthened. It was demonstrated that TSN mediated the decrease in E2F1 half-life which abrogated its pro-apoptotic effects. Additionally, these effects were dependent on the presence of PRMT5, and consequently the SDMA mark on E2F1. Not surprisingly, depletion of TSN resulted in increased apoptosis in an E2F1 dependent manner. TSN has also been detected on the promoters of E2F1, Cyclin E, CDC6 and DHFR, supported by decreased recruitment in the absence of PRMT5 (Zheng et al. 2013).

More recently however, TDRD1 was demonstrated to recognize the same symmetric dimethylation mark in a fluorescence polarisation assay conducted in collaboration with the Structural Genomics Consortium and University of Toronto (Moehlenbrink., unpublished). Interestingly, the structure of the second TDRD1 Tudor domain is strikingly similar to that of the eTud domain of TSN which recognizes the SDMA mark on E2F1 and echoes the structure of its interaction (Mathioudakis et al. 2012a). Furthermore, TDRD1 and E2F1 have been shown to interact endogenously in cells, dependent on the presence of the SDMA mark on E2F1 (Moehlenbrink, unpublished).

Notably, both TSN and TDRD1 have been shown to be upregulated in cancer (Jariwal et al. 2015; Xiao et al. 2016). This raises the question of whether their expression profiles in cancer are functionally connected to their role of recognizing the E2F1 SDMA mark associated with increased cell proliferation, and whether this functional connection is mediated through downstream E2F1-dependent changes in target transcription.

Additionally, the need for two readers of the same methylation mark may allude to possible branched differences in the pathways utilised, or mutually exclusive regulation of different genes, leading to the overall observed biological outcome of E2F1 SDMA mark. Alternatively, the presence of two readers may serve as protective functional redundancy and an additional oncogenic mechanism if either of the two is overexpressed.

While the biological functions of TSN in RNA biology are extensive (Gutierrez-Beltran et al. 2016a), the functions of TDRD1 in human biology in somatic cells are generally unknown. TSN is ubiquitously expressed in a variety of somatic cells, while TDRD1 expression is limited. Its overexpression in certain cancers also hints that its expression may be hormone-dependent.

An initial screen conducted in the La Thangue laboratory revealed that TDRD1 is only expressed in certain breast and prostate cancer cell lines, including, but not limited to MCF7, SKBR3, VCaP and DuCaP cells, prompting their use in this study. The MCF7 cell line is derived from pleural effusions of invasive breast ductal adenocarcinoma and is a member of the Luminal A class which is oestrogen receptor-positive, progesterone receptor-positive and HER2-negative (Holliday & Speirs 2011; Lee et al. 2015). SKBR3s, much like MCF7s, are also derived from breast adenocarcinoma, but represent an oestrogen receptor-negative, progesterone receptor-negative and HER2-positive population of breast cancer (Holliday & Speirs 2011). DuCaP cells, on the other hand are

derived from metastatic prostate carcinoma obtained from brain dura matter. DuCaPs are androgen-receptor positive and are androgen responsive (Lee et al. 2001).

This chapter outlines the experimental foundation which implicates TSN and TDRD1 in E2F1-mediated transcriptional regulation in these cell lines, and supplementary experiments which demonstrate possible mechanisms by which this is achieved.

3.2 Cell Line Characterisation

PRMT5 depletion has been shown to correspond to decreased growth rate in U2OS cells and increased apoptosis in U2OS, HCT116 and SAOS2 compared to control after 96 hours (Cho et al. 2012). On the other hand, PRMT1 depletion resulted in an increased growth rate compared, and decreased apoptosis compared to control in SAOS2 cells (Zheng et al. 2013). It was thus speculated that because TSN and TDRD1 are readers of the SDMA mark deposited by PRMT5, TSN or TDRD1-knockdown cells would display similar cellular characteristics to that of PRMT5-depleted cells. Stable TSN and TDRD1 knockdown MCF7, SKBR3, and DuCaP cells used in this study, however, showed little difference in cell cycle profile and sub-G_1 levels compared to control (Figure 3.1b, c). A small decrease in growth rate, however was observed in MCF7 TDRD1-knockdown cells compared to control (3.1a.).

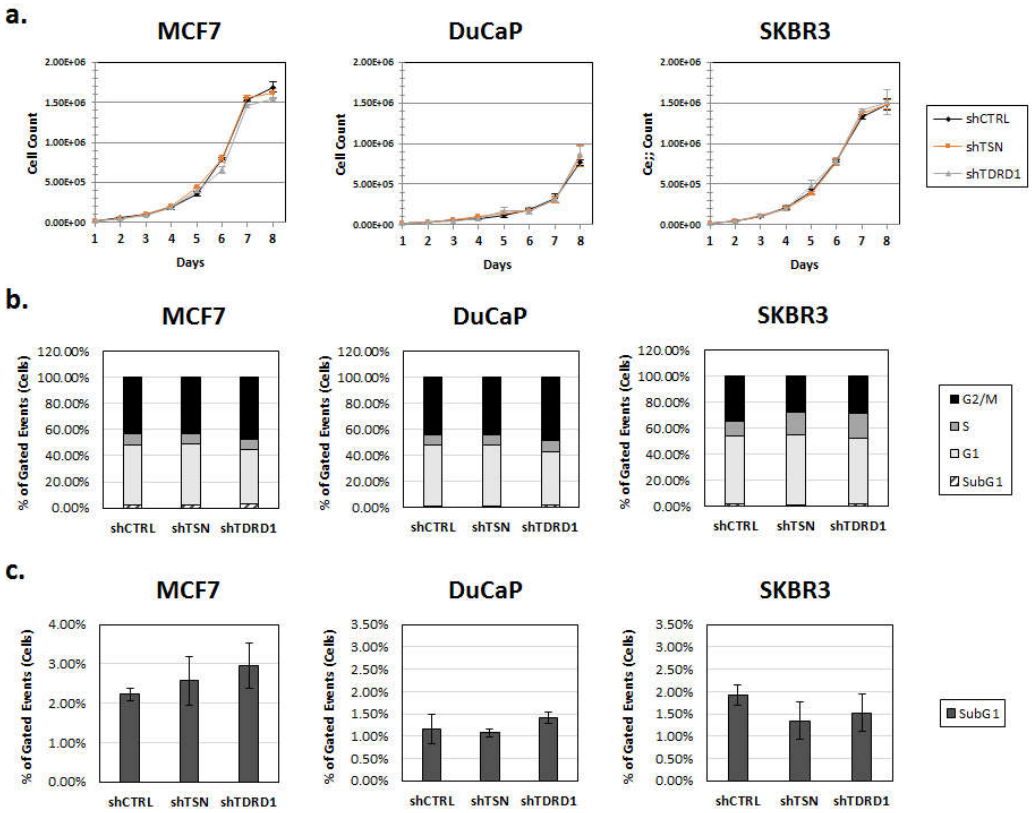


Figure 3.1 Characterization of stable reader knockdown cell lines.
(a) Growth curves of stable knockdown cell lines over an eight-day period as indicated. Data points are representative of mean average readings of biological replicates and error bars indicate standard deviation of cell count; (b) Asynchronous cell cycle profile distribution of cell lines at 80% confluence as indicated; (c) Percent of cells as indicated in Sub G_1 at 80% confluence; (b, c) Data points are representative of mean average readings of biological triplicates and error bars indicate standard deviation of readings. Differences in readings present no statistical significance based on single-factor ANOVA, $P<0.05$,.

3.3 Validation of RNA sequencing reveals deregulated candidate gene expression

As an initial inquiry into the possible effects of TSN and TDRD1 on E2F1 target gene expression, a previous member of the La Thangue laboratory conducted RNA sequencing analysis comparing the expression profiles of various stable TSN- and TDRD1-knockdown cell lines to wildtype MCF7, SKBR3 and DuCaP cells (provided by Chan, unpublished). This analysis was a fairly crude screen, limited by the lack of replicates, proper controls, and ribosomal RNA contamination. Thus, its results served merely as a conservative predictor and springboard for identifying genes of interest.

A preliminary scan of the deregulated genes in the analysis conducted revealed that more canonical E2F1 target genes such as p73, DHFR, E2F1-3, and cyclins A/B/E did not exhibit significant fold-change differences compared to control. However, reads

on these transcripts were also very low, and as such no definite conclusion could be made due to the limitations of the experiment (data not shown; Chan, unpublished).

From the initial screen, a small number of differentially regulated genes exhibiting a p-value less than 0.05 and an absolute $\log_2$ fold-change ($|\log_2 FC|$) of 1 or more were recommended for further analysis and validation (Table 3.1). The pool of genes recommended varied per cell type assessed, and were as such validated in the cellular context that they were found to be deregulated.

Table 3.1 List of deregulated genes returned by RNA-Seq (provided by Chan, 2015; unpublished)

	MCF7		SKBR3		DuCaP	
	shTDRD1	**shTSN**	**shTDRD1**	**shTSN**	**shTDRD1**	**shTSN**
Up-regulated	KLF2 MDC1	KLF2 MDC1	TCF19 CDC6 ZNF367 POLD1 PSMC3	TCF19 CDC6 SCAF1	KLF2 RAD54L	KLF2 IER2 ZNF524 WDR34 RAD54L
Down-regulated		SKP2	SYNGR4	SYNGR4	CASP8AP2 ZBED8	CASP8AP2

Notably, several of the candidates chosen demonstrate potential oncogenic and tumour suppressive activity (Table 3.2), while others have no reported association with cancer or relevant biological function. When taken in context with the respective expression patterns of these genes in TSN- and TDRD1- knockdown cells, several of the reported functions in the genes analysed may support the phenotype observed when the SDMA mark is depleted by PRMT5 knockdown or E2F1 mutation – that is, a preferred pro-apoptotic response rather than one of proliferation (Zheng et al. 2013). This is consistent with the hypothesis that TSN and TDRD1 may recognize this mark and carry out its downstream proliferative effects.

Table 3.2 Functional and biological relevance of deregulated target genes in cancer

	Name	**Function** (GenBank; Clark et al., 2016)	**Implications in Cancer**
KLF2	Kruppel-Like Factor 2	zinc finger transcription factor that regulates T-cell trafficking	• **tumour suppressor** that is epigenetically silenced in human cancers (Black et al. 2001; Taniguchi et al. 2012) o induction of cell quiescence (Black et al. 2001) o enhancement of DNA-damage-induced apoptosis (Wu & Lingrel 2004) o anti-angiogenesis (Bhattacharya et al. 2005) o suppression of growth mediated by KRAS (Fernandez-Zapico et al. 2011) and EGFR (Kannan-Thulasiraman et al. 2010)
MDC1	Mediator of DNA-Damage Checkpoint 1	required to activate the intra-S phase and G_2/M phase cell cycle checkpoints in response to DNA damage	• **oncogene** in cervical cancer (Yuan et al. 2012), and when mutated in lung cancer (Wang et al. 2014) • **tumour suppressor** in several hormone-regulated cancers: o breast cancer (Bartkova et al. 2007; Zou et al. 2015) o prostate cancer (C. Wang et al. 2015) • DNA damage response, cell cycle regulation and carcinogenesis (Stucki & Jackson 2004; Ozaki et al. 2015) • Radiosensitization (Z. Wang et al. 2015)
TCF19	Transcription Factor 19	G_1/S transition	• Promoter of proliferation and survival (Miao et al. 2013; Krautkramer et al. 2013)
CDC6	Cell Division Cycle 6	initiation of DNA replication	• **Oncogene** in various human cancers, as well as reports in neuroblastoma and prostate cancer (Gonzalez et al. 2006; Feng et al. 2008; Liu et al. 2014) • **Tumour suppressor** that is down-regulated in androgen-insensitive prostate cancer in an E2F1 dependent manner (Robles et al. 2002; Mallik et al. 2008; Jin & Fondell 2009)
ZNF367	Zinc Finger Protein 367	sequence-specific DNA binding transcription factor activity cell cycle	• Reported **tumour suppressor** functions, overexpressed in endocrine cancers, inhibits cancer progression (Jain et al. 2014)
SYNGR4	Synaptogyrin 4	integral membrane protein	• Patented as an **oncogenic marker** that promotes growth, survival and cell invasion in primary lung cancer (Nakamura et al. 2009)
SKP2	S-Phase Kinase-Associated Protein 2, E3 Ubiquitin Protein Ligase	phosphorylation-dependent ubiquitination	• **Oncogenic** and overexpressed in different forms of cancer (Gstaiger et al. 2001; Signoretti et al. 2002; Zhang & Wang 2006; Hershko 2008) o Deregulated proteolysis (Frescas & Pagano 2008) • Inactivation of Skp2 restricts cancer stem cell traits and cancer progression (Chan et al. 2013)
POLD1	Polymerase (DNA Directed), Delta 1, Catalytic Subunit	catalytic subunit of DNA polymerase delta DNA replication and repair	• **Tumour suppressor** with DNA repair capacity that is downregulated in and predisposes to familial colorectal cancer (Palles et al. 2013; Valle et al. 2014; Bellido et al. 2015; Nicolas et al. 2016)
PSMC3 /RPT5 /TBP-1	Proteasome 26S Subunit, ATPase, 3, 26S protease regulatory subunit 6A, TAT-Binding Protein 1	ATP/ubiquitin-dependent peptide cleavage Co-activates ligand-driven transcription mediated nuclear receptors	• Displays potential **tumour suppressor** capacity: • disables of oncogenic erbB receptor tyrosine kinases (Park et al. 1999) • Regulator of cellular proliferation (Sepe et al. 2011) • Responsible for proper protein turn-over; depleted in some tumour populations (Lam et al. 2002; Lee et al. 2011)

SCAF1	SR-Related CTD-Associated Factor 1	RNA binding and protein domain specific binding, pre-mRNA splicing	• Overexpressed and associated with poor prognosis in ovarian cancer (Scorilas et al. 2001; Leoutsakou et al. 2006b; Leoutsakou et al. 2006a) • Associated with colon cancer and leukaemia (Mathioudaki et al. 2004; Jang et al. 2008)
RAD54L	RAD54-Like	DEAD-like helicase superfamily homologous recombination and repair of DNA	• Frequently mutated in various cancers; (Matsuda et al. 1999; Leone et al. 2003; Li et al. 2006; Tong et al. 2015)
CASP8AP2	Caspase 8 Associated Protein 2	interaction with the death-effector domain (DED) of caspase 8 death-inducing signalling complex regulatory role in Fas-mediated apoptosis	• Associated with drug resistance and cell survival (Lee et al. 2012) • Promotes cellular survival in colorectal cancer cells (Hummon et al. 2012) • Low expression levels in paediatric acute lymphoblastic leukaemia (Jiao et al. 2012; Juarez-Velazquez et al. 2014; Cui et al. 2015)
ZBED8	Zinc Finger, BED-Type Containing 8, Transposon-derived Buster3 transposase-like protein	nucleic acid binding, RNA polymerase II transcription factor activity	
IER2	Immediate Early Response 2	regulation of neuronal differentiation transcription factor ell motility, seems to stimulate tumour metastasis	• Promotes tumour cell motility and metastasis in colorectal cancer (Neeb et al. 2012)
ZNF524	Zinc Finger Protein 524,	nucleic acid binding	
WDR34	WD Repeat Domain 34	cell cycle progression, signal transduction, apoptosis, and gene regulation	

Notably, the RNAseq data obtained from TSN- or TDRD1-knockdown cells further suggested that they exhibited very similar roles with respect to transcriptional regulation of the candidate genesdegrees of regulatory specificity.

The nature of the RNASeq data obtained required extensive validation to confirm its significance moving forward. To validate these findings, the mRNA expression levels of the candidate genes in their respective lentiviral-transduced stable TSN/TDRD1-knockdown cell lines were measured using qRT-PCR (Figure 3.2). Western blots of protein obtained in parallel to RNA extraction confirmed the knockdown of TSN and TDRD1 in the MCF7, SKBR3 and DuCaP cell lines (Figure 3.2 a, c, e).

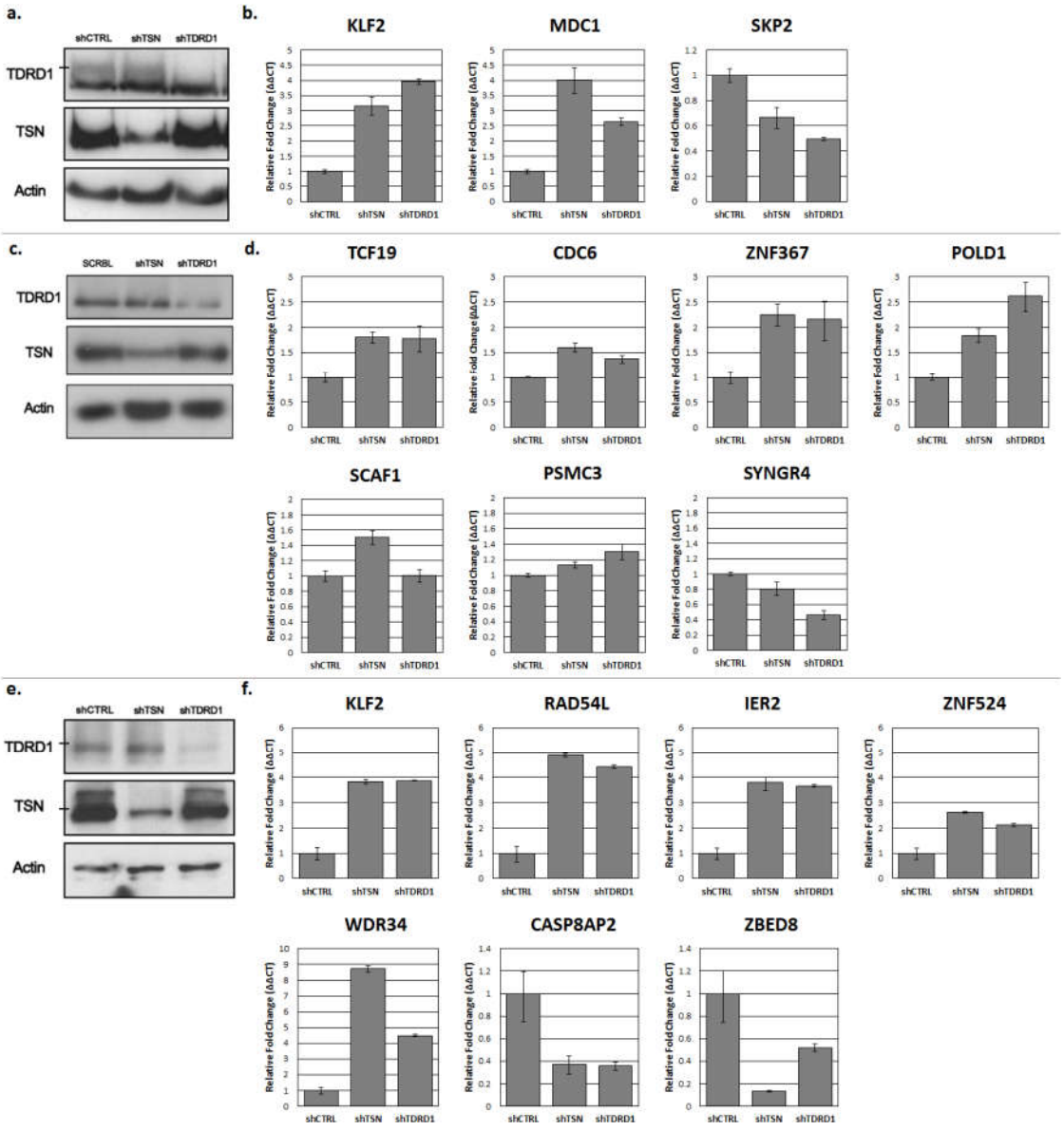


Figure 3.2 qRT-PCR validation of candidate genes obtained from RNA sequencing analysis.
(a, c, e) Western blot of 20 µg protein extracted from stable (a) MCF7, (c) SKBR3, and (e) DuCaP cell lines targeted with shRNA as indicated, and probed with antibodies as listed; (b, d, f) qRT-PCR analysis of cDNA obtained from (b) MCF7, (d) SKBR3, and (f) DuCaP cell lines targeted with shRNA as indicated. Expression levels of each gene listed are indicated in relative fold change (ΔΔCT), normalised to mRNA expression levels of actin and relative to shCTRL in each setup. Data points are representative of mean average readings of technical triplicates and error bars indicate lower and upper bounds of predicted ΔΔCT range based on the standard deviation of CT readings. (Representative data of n=3).

Accordingly, the results obtained from qRT-PCR analysis generally echo Chan's initial findings derived from the RNA sequencing data but show more conservative deregulation than originally predicted. In the MCF7 stable cell lines, MDC1 and KLF2 display upregulation in shTSN and shTDRD1 transduced cells compared to shCTRL

(non-targeting scrambled shRNA transduced cells), as predicted in Table 3.1. SKP2 is not only down-regulated upon TSN-knockdown but also when TDRD1 is knocked down (Figure 3.2b).

In the cellular context of SKBR3s, TCF19, CDC6, ZNF367, POLD1, and PSMC3 are all upregulated following knockdown of either TSN or TDRD1, despite only TCF19 and CDC6 displaying significant deregulation in both contexts in the previously described RNA sequencing results. SCAF1, as predicted shows only up-regulation in TSN-knockdown cells while remaining unperturbed in TDRD1-knockdown contexts. As predicted, SYNGR3 is down-regulated when either candidate reader is knocked down (Figure 3.2d). Interestingly, when KLF2 expression levels were tested once in SKBR3 cells, upregulation was also evident (data not shown), suggesting that KLF2 deregulation upon reader knockdown was not cell-type specific.

In DuCaP cells, KLF2, RAD54L, IER2, ZNF524 and WDR34 are all significantly up-regulated compared to control in both TSN- and TDRD1-knockdown cells, while CASP8AP2 and ZBED8 are down-regulated (Figure 3.2f).

Collectively, several genes with known tumour suppressor activity (KLF2, MDC1, CDC6, ZNF367, POLD1, PSMC3, RAD54L) were up-regulated upon TSN or TDRD1 knockdown, suggesting that TSN and TDRD1 play repressive roles in normal cellular contexts to prevent the expression of these genes and ultimately promote cellular proliferation. Notably, though some of these genes display dual oncogenic and tumour suppressive activity, in the relevant cellular contexts explored (breast and prostate cancer), these genes have been reported to play a more dominant tumour suppressive role and are silenced in these cancers. The de-repression of these genes under TSN or TDRD1 knockdown contexts support the growth-limiting and pro-apoptotic functions which are associated with the E2F1 ADMA mark and absence of the SDMA mark. Similarly, the

expression levels of several genes with proliferative and potentially oncogenic capacity (SYNGR4, SKP2, CASP8AP2) were stifled by the knockdown of TSN or TDRD1. Greater expression levels of these genes in normal conditions would potentially facilitate proliferative properties mediated by the recognition of the SDMA mark by TSN or TDRD1, further forming a mechanistic bridge between the two. Notably, several of the genes analysed are either reported E2F1 targets, or possess E2F1-promoter binding peaks in publicly available ENCODE data (Rosenbloom et al. 2013).

Thus, the results obtained from qRT-PCR analysis validate the findings of obtained from the original RNA sequencing screen and provide an interesting pool of genes on which more in-depth analysis can be conducted.

3.3 Transient knockdown experiments reflect similar transcriptional effects

While stable lentiviral transduced cell lines provided a good context for analysing the effects of TSN- and TDRD1 knock down, it was observed that knockdown was lost after several passages (data not shown). This presented the additional issue of potential population drift across different branched passages of the stable knockdown cells in culture. Furthermore, results obtained from stable cell lines may potentially reflect cellular conditions adapted to the stress of sustained depletion of either reader over several tissue culture passages, displaying compensatory mechanisms which drive aberrant gene expression. To better assess the immediate cellular response to reader depletion, and to confirm that results obtained from transient knockdown would similarly reflect the results obtained from stable shRNA knockdown cells, the levels of the selected genes were assessed after depletion of TSN and TDRD1 by transfected siRNA.

Optimal transfection conditions in MCF7, SKBR3 and DuCaP cells were first determined prior to embarking into larger scale transfections (Figure 3.3). Using siE2F1 oligonucleotide transfection as a basis, optimization experiments showed that MCF7 and

SKBR3 cells were amenable to siRNA transfection at concentrations as low as 12.5 nM, and that both commercial non-targeting control and non-targeting siGFP controls had minimal effect at all concentrations tested. DuCaP cells, however, only showed knockdown at siRNA concentrations of 50 nM, implying low transfection efficiency. Indeed, DuCaP cells were difficult to transfect, and did not show siRNA knockdown consistently throughout experiments. As such, transfections on MCF7 and SKBR3 cells were conducted at 12.5 nM siRNA concentrations, while 50 nM siRNA was used in DuCaPs. Additionally, siGFP was chosen as the control, mimicking the stress of lipofectamine transfection with a non-targeting siRNA.

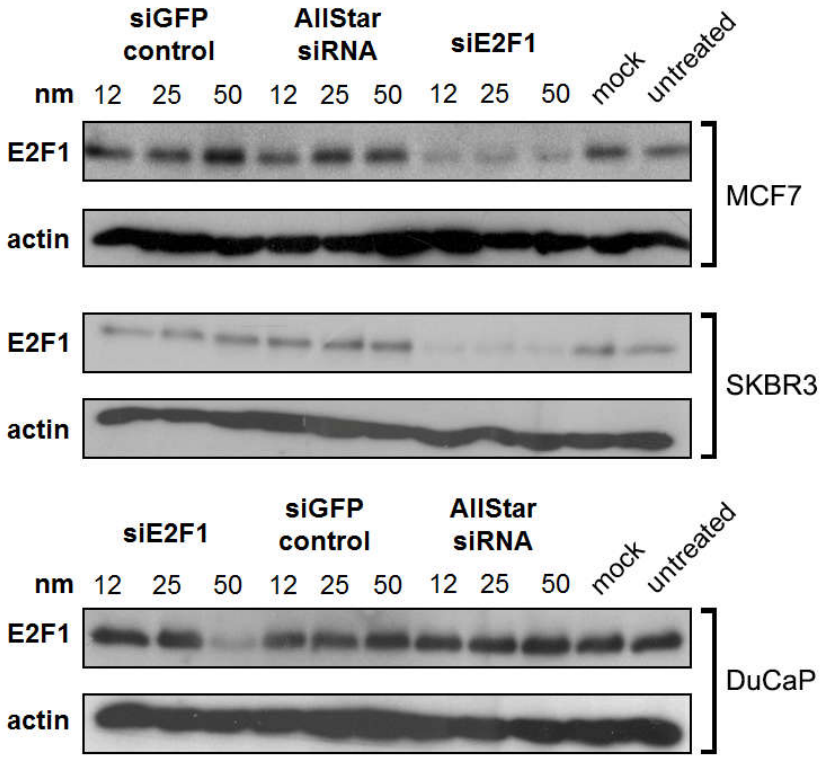


Figure 3.3 Optimization of siRNA transfection in different cellular backgrounds.
Western blot of 20 µg protein extracted from MCF7, SKBR3, and DuCaP cell lines targeted with E2F1-targeting siRNA at various concentrations as indicated, and probed with antibodies as listed

Under these optimised conditions, transient siRNA TSN- and TDRD1-knockdown cells were obtained and the mRNA expression levels of the returned target genes were measured using qRT-PCR (Figure 3.4).

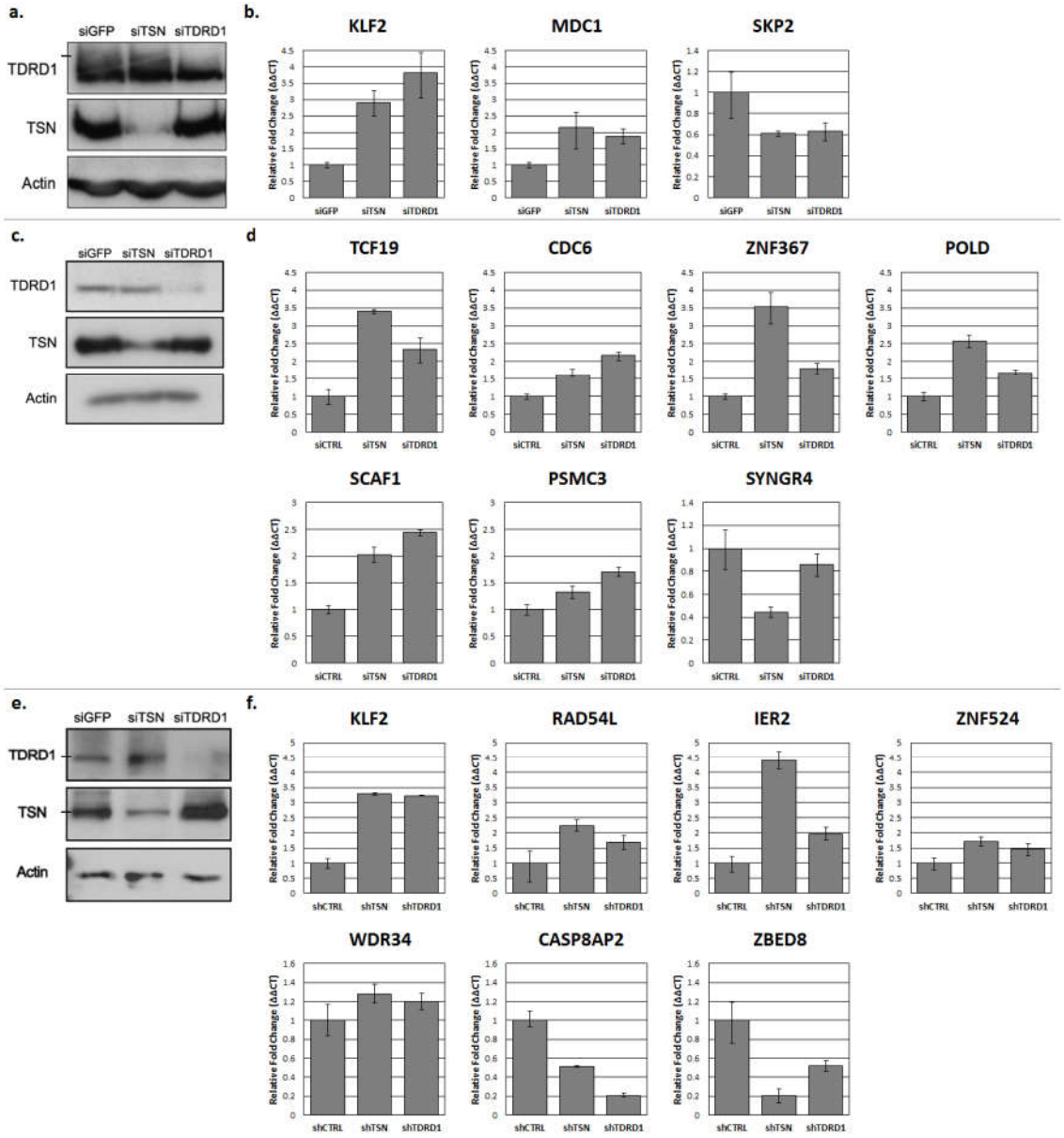


Figure 3.4 qRT-PCR analysis of candidate genes in transient siRNA knockdown contexts.
(a, c, e) Western blot of 20 µg protein extracted from (a) MCF7, (c) SKBR3, and (e) DuCaP cell lines targeted with siRNA as indicated, and probed with antibodies as listed; (b, d, f) qRT-PCR analysis of cDNA obtained from (b) MCF7, (d) SKBR3, and (f) DuCaP cell lines targeted with siRNA as indicated. Expression levels of each gene listed are indicated in relative fold change (ΔΔCT), normalised to mRNA expression levels of actin and relative to shCTRL in each setup. Data points are representative of mean average readings of technical triplicates and error bars indicate lower and upper bounds of predicted ΔΔCT range based on the standard deviation of CT readings. (Representative data of n=3)

Transient knockdown of TSN and TDRD1 resulted in similar trends in expression pattern as those observed in stable cell lines. KLF2 and MDC1 remained up-regulated in MCF7 cells, while SKP2 was shown to be down-regulated compared to siGFP control. In SKBR3 cells, TCF19, CDC6, ZNF367, POLD, SCAF1, and PSMC3 showed more prominent up-regulation, suggesting that transient knockdown context might show more pronounced deregulation than in stable cells in this context. Similarly, DuCaP cells maintained expression trends in all the genes assayed, but showed much more conservative deregulation, likely due to issues with transfection and knockdown efficiency in this cellular context.

As of yet, the experiments conducted cannot discount p73-induced apoptotic effects, however effects of p73 in the contexts tested were expected to be limited. During the timeframe used in both stable and transient knockdown experiments, limited apoptosis was observed within parallel setups tested for sub-G_1 population changes, and thus suggest transcriptional effects prior, but leading to, anti-proliferative activity observed by previous studies (Cho et al. 2012; Zheng et al. 2013)

By and large, the results obtained from the qRT-PCR analysis in both stable and transient knockdown cells validate the chosen genes from original RNA sequencing screen. Furthermore, the results suggest that TSN and TDRD1 play largely redundant roles, resulting in comparable trends in deregulation in the genes analysed.

3.4 E2F1 co-depletion experiments give insight into E2F1-associated regulation

TSN is known to have independent and multifaceted functions in RNA metabolism, and the extent of TDRD1 activity is unknown. It was thus necessary to confirm that the observed deregulation of candidate genes was achieved in an E2F1-dependent manner, and whether the observed changes in target gene expression were the

direct result of the functions of each reader in relation to E2F1-mediated transcriptional effects, or indirect mechanisms involving non-transcriptional functions of E2F1.

The promoters of all the genes initially validated in the previous chapter contain several candidate E2F1 consensus binding sites (5'-TTTSSCGS-3' or 5'-GGCGCAAA-3') or have documented ChIP peaks in publicly available ENCODE data (Bieda et al. 2006; Rabinovich et al. 2008; Cao et al. 2011; Rosenbloom et al. 2013). Furthermore, several of the genes returned, though not widely known, are established E2F1 targets. All the candidate genes thus have the potential capacity to be regulated by E2F1-dependent transactivational activity in response to TSN and TDRD1 reader effects.

While the deregulation observed in these candidate genes could be an E2F1 dependent effect, it could also be a by-product of reader depletion-induced stress, differences in transfection or siRNA efficiency, or an artefactual effect of TSN or TDRD1 that is unrelated to their E2F1 dependent functions. The simplest means to test these possibilities was to co-deplete E2F1 and to observe whether the observed deregulation persists. Thus qRT-PCR experiments to measure the mRNA expression of each gene candidate were repeated, this time co-depleting E2F1 in transient knockdown contexts (Figure 3.5, 3.6). Transient siRNA transfection offered a flexible and straightforward method to co-deplete E2F1 alongside the two readers in various combinations. At the same time, it allowed the immediate cellular response of the cells to be assessed, as opposed the previously generated stable cell lines, where cellular conditions adapted to the stress of sustained depletion could affect the capacity of cells to properly respond to additional knockdown treatments. One caveat to this RNAi system was to ensure consistent transfection efficiency especially when done in combinations of various siRNA species. As such, in order to draw proper conclusions from the data obtained, it was of utmost importance to ensure that the knockdown of each gene was achieved at a similar

level throughout the biological repeats. For the results obtained, a minimum of three biological replicates were prepared to ensure consistency throughout. While this was readily achievable in SKBR3 and MCF7 cells, DuCaP cells presented a substantial barrier to transfection or displayed limited response to the siRNA oligonucleotide transfected, and as such, the analysis was limited to SKBR3 and MCF7 backgrounds.

Co-depletion of E2F1 along with either TSN or TDRD1 via siRNA transfection was confirmed in Western blots of protein obtained in parallel to RNA extraction. In the co-depleted setups, E2F1 expression in MCF7 cells showed almost complete knockdown which is echoed in the measured E2F1 mRNA levels. More importantly, comparing single reader knockdown setups against E2F1 co-depletion setups showed results that were both expected of canonical E2F1 target genes, as well as results that were less straightforward to interpret (Figure 3.5).

In theory, canonical E2F1 targets should exhibit a decrease in mRNA expression levels in the absence of E2F1, reflecting the direct impact of E2F1 transactivation activity. This was observed in the case of CDC6, where E2F1 depletion resulted in a dramatic reduction of its mRNA levels. Interestingly, CDC6 was also up-regulated after TSN and TDRD1 knockdown in MCF7 cells, as it has been shown to be previously in SKBR3 cells. Upon E2F1 co-depletion with TDRD1, this up-regulation was reduced to less than the steady state levels observed in the siGFP control. This suggests that the up-regulation of CDC6 observed when TDRD1 was depleted was mediated by direct E2F1 transcriptional activity. In the case of E2F1 co-depletion with TSN, however, while CDC6 levels decreased significantly compared to single TSN knockdown cells, the steady state level of CDC6 remained higher than the siGFP control setup. This suggests that there is at the very least some level of E2F1 independent up-regulation occurring when TSN is absent. TSN possesses a variety of roles in post-transcriptional gene regulation,

particularly in the RNA-induced silencing complex (Gutierrez-Beltran et al. 2016b). In its absence, the observed increase in CDC6 levels could be attributed to the decreased RISC-mediated decay of the accumulating mRNA species or other indirect or E2F1-independent effects yet to be confirmed.

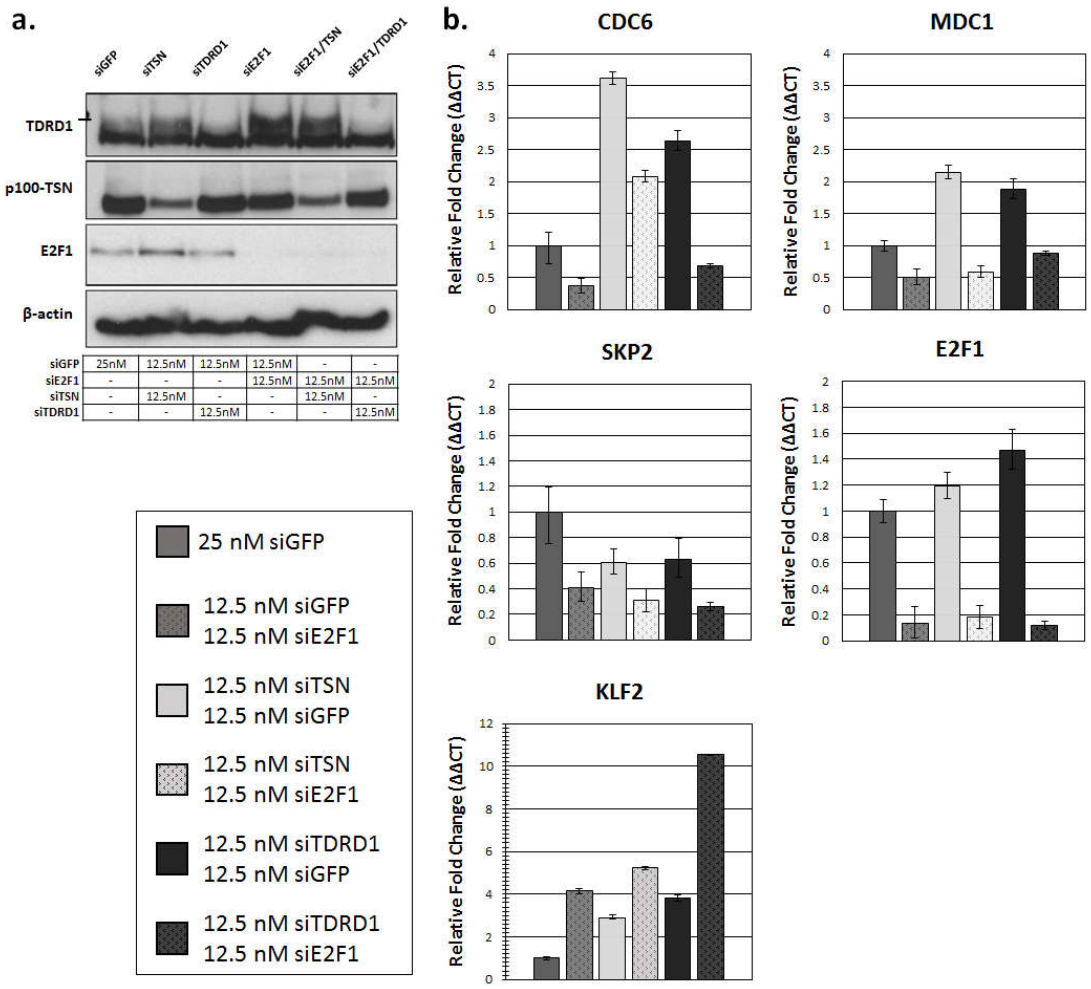


Figure 3.5 qRT-PCR analysis of MCF7 candidate genes in transient reader/E2F1 co-depletion experiments.
(a) Western blot of 20 µg protein extracted from MCF7 cells targeted with siRNA as indicated, and probed with antibodies as listed; (b) qRT-PCR analysis of cDNA obtained from MCF7 cells targeted with siRNA as indicated in (a). Expression levels of each gene listed are indicated in relative fold change (ΔΔCT), normalised to mRNA expression levels of actin, which were set to 1, and relative to shCTRL in each setup. Data points are representative of mean average readings of technical triplicates and error bars indicate lower and upper bounds of predicted ΔΔCT range based on the standard deviation of CT readings. (Representative data of n=3)

Using the trends of CDC6 deregulation in response to co-depletion treatment as a prototypic example of how a canonical E2F1 target gene should behave, it can be said that MDC1 and SKP2 behaved similarly to a canonical target upon E2F1 co-depletion but displayed distinct regulatory responses to TSN and TDRD1 knockdown. Like CDC6, MDC1 and SKP2 expression levels decreased across the board when E2F1 was co-depleted with control or reader knockdowns. Unlike CDC6 however, when E2F1 was co-depleted, MDC1 levels were reduced to equal or less than the steady state levels in the siGFP control, suggesting that the up-regulation observed upon both TSN or TDRD1 knockdown occurs in an E2F1-mediated manner.

In the case of SKP2, which responded to reader knockdown with down-regulated expression, the co-depletion of E2F1 further repressed its expression. This demonstrated that SKP2 is transcriptionally targeted by E2F1, which maintains its expression in normal cellular contexts. Moreover, it is possible that the normal levels of SKP2 expression are additionally maintained, or enhanced, by the presence of TSN, which constitutes an additive co-activator effect to that of E2F1. Thus, when TSN was lost, SKP2 levels decreased; and when E2F1 was further depleted, SKP2 was less likely to be expressed. Whether E2F1 and TSN cooperate in a direct manner to maintain SKP2 expression in normal cellular contexts is yet to be determined.

The deregulation of KLF2, on the other hand, was less straightforward to interpret as it did not behave like a canonical E2F1 transcriptional target. Like MDC1, KLF2 expression levels increased upon reader knockdown suggesting that the readers play a repressive role in its regulation. Interestingly, KLF2 levels were further increased upon E2F1 co-depletion. This points toward E2F1 playing a direct or indirect repressive role in KLF2 regulation.

In SKBR3 cells, TCF19, ZNF367 and CDC6 were chosen for further co-depletion analysis to narrow the scope of the study (Figure 3.6). E2F1 depletion in SKBR3 cells was less efficient than in MCF7, as demonstrated by Western blot and qRT-PCR analysis of E2F1 protein and mRNA expression respectively. Despite this, E2F1 co-depletion resulted in noticeable differences in candidate gene deregulation.

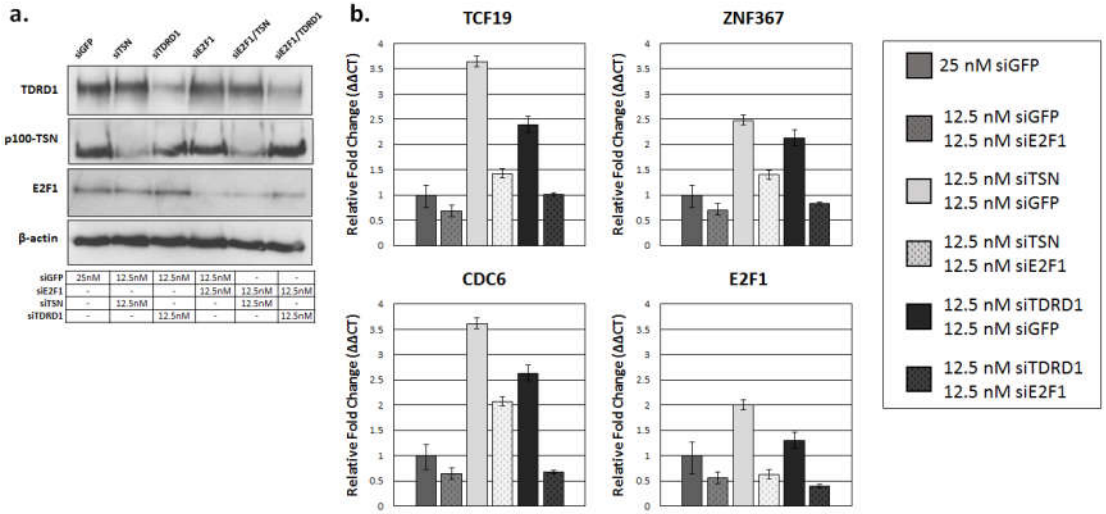


Figure 3.6 qRT-PCR analysis of SKBR3 candidate genes in transient reader/E2F1 co-depletion experiments.

(a) Western blot of 20 µg protein extracted from SKBR3 cells targeted with siRNA as indicated, and probed with antibodies as listed; (b) qRT-PCR analysis of cDNA obtained from SKBR3 cells targeted with siRNA as indicated in (a). Expression levels of each gene listed are indicated in relative fold change (ΔΔCT), normalised to mRNA expression levels of actin, which were set to 1, and relative to shCTRL in each setup. Data points are representative of mean average readings of technical triplicates and error bars indicate lower and upper bounds of predicted ΔΔCT range based on the standard deviation of CT readings. (Representative data of n=3)

As demonstrated previously in MCF7 cells, CDC6 behaved like a canonical E2F1 target in SKBR3 cells, suggesting that CDC6 deregulation upon reader and E2F1-knockdown may be a common mechanistic pathway in both cellular contexts. TCF19 and ZNF367 displayed very similar expression patterns to CDC6 in response to the various siRNA treatments, prompting the hypothesis that they are controlled by a common regulatory mechanism which likely involved E2F1 in a direct transcriptional fashion.

Moreover, the trends observed as a result of reader and E2F1 co-depletion were consistent across biological replicates (Figure 3.7), strengthening the correlation between knockdown, E2F1 activity and target gene deregulation.

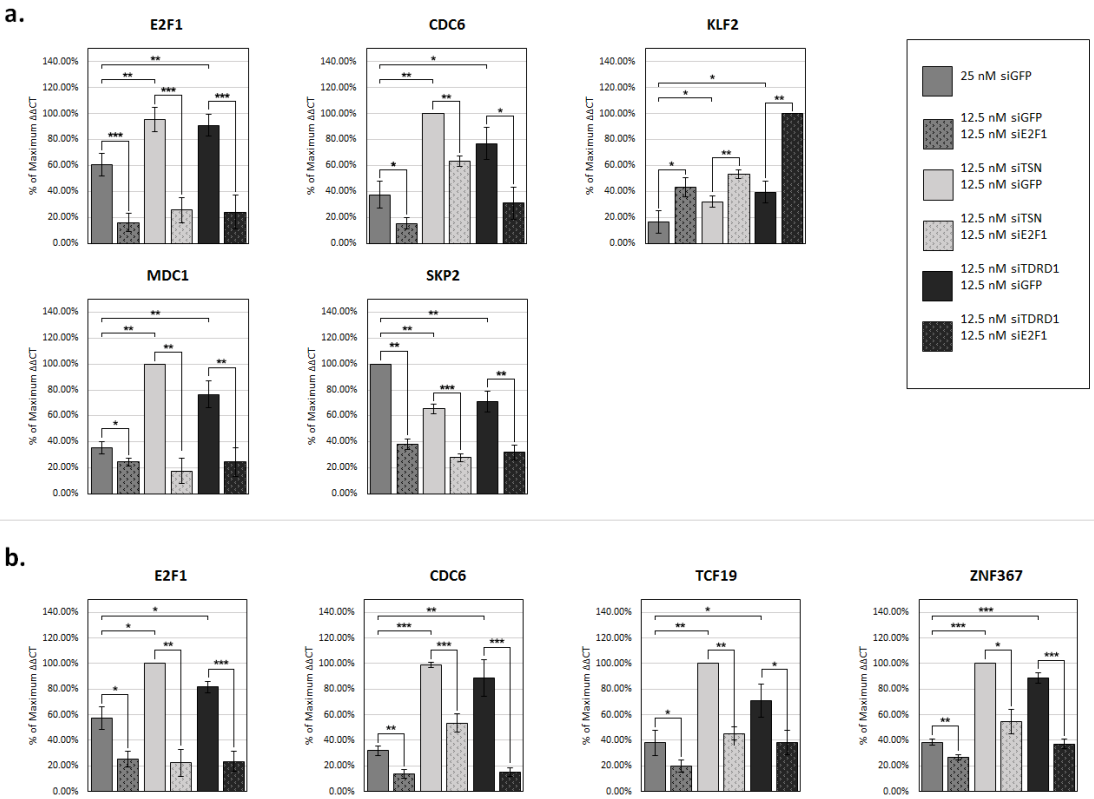


Figure 3.7 Reproducibility of co-depletion experiments across biological replicates
qRT-PCR analysis of co-depletion experiments conducted in biological replicates on (a) MCF7 and (b) SKBR3 cells in response to siRNA treatment as indicated (pooled data from n=3); Expression levels of each gene are indicated as mean values of percent maximum ΔΔCT obtained from the mean of technical triplicates of the three independent biological replicates. Error bars indicated standard deviation between the means obtained from each replicate. Statistical significance of deregulation was assessed pair-wise via two-tailed students' *t*-test as indicated (*p<0.05, **p<0.01, ***p<0.001).

3.5 Assessing E2F1 recruitment in response to TSN and TDRD1 knockdown

The candidate genes chosen all possess E2F1 consensus binding sites and documented E2F1 ChIP peaks **(**Appendix A2, Rosenbloom et al. 2015**)**, suggesting that changes in E2F1 recruitment to target promoters could play a direct role in facilitating the previously observed deregulation in response to reader knockdown.

To confirm reports of E2F1 binding to targets, chromatin immunoprecipitation of E2F1 was conducted. Only the promoters of candidate genes identified from MCF7 cells were assessed in the following experiments to narrow the scope of the study. Because

each gene constituted a distinct response to reader knockdown and E2F1 co-depletion, this allowed for a more comprehensive experimental scope to be covered at a smaller scale and within a shorter timeframe.

Using publicly available ENCODE data accessed from the UCSC Genome Browser (https://genome.ucsc.edu), ChIP primers were designed within areas of reported E2F1 binding and encompassing E2F1 consensus binding sequences (Appendix A2, Table 2.5). E2F1 ChIP activity on target promoters was then measured using qPCR amplification of chromatin fragments which immunoprecipitated with the E2F1 protein under reader knockdown conditions in MCF7 cells (Figure 3.8).

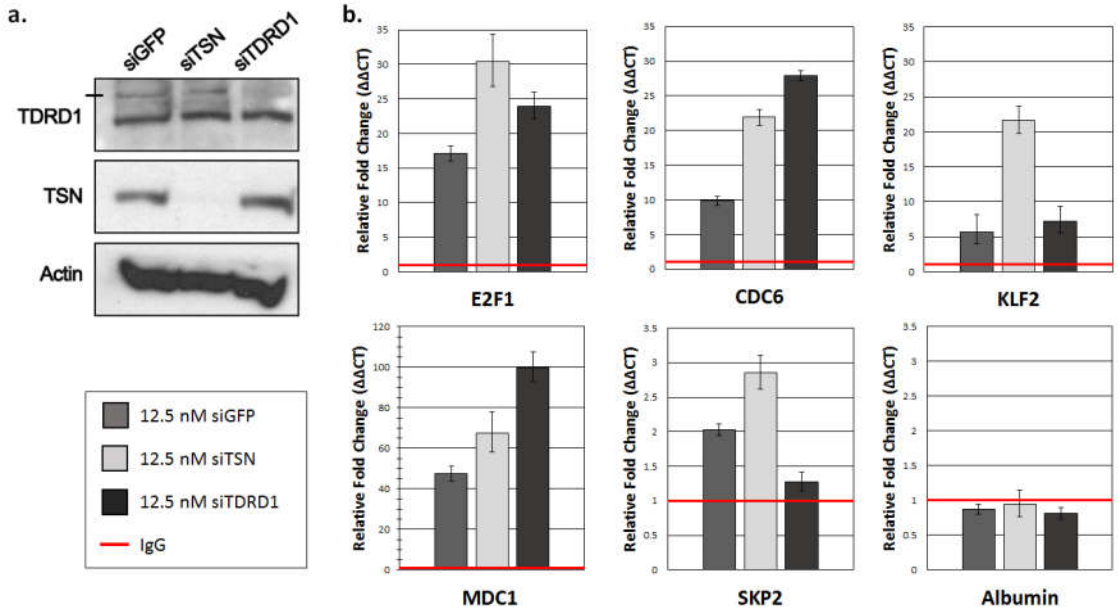


Figure 3.8 E2F1 ChIP on different target gene promoters assessed by qPCR.
(a) Western blot of 20 µg protein extracted from MCF7 cells targeted with siRNA as indicated, and probed with antibodies as listed; (b) qPCR analysis of immunoprecipitated chromatin fragments obtained after E2F1 pulldown from MCF7 cells targeted with siRNA as indicated. Expression levels of each gene listed are indicated in relative fold change (ΔΔCT), normalised to input and relative to IgG pulldown control expression levels (set to 1) in each setup indicated in red. Data points are representative of mean average readings of technical triplicates and error bars indicate lower and upper bounds of predicted ΔΔCT range based on standard deviation. (Representative data of n=3).

Western blot of cells harvested following siRNA transfection confirmed TSN and TDRD1 knockdown (Figure 3.8a). CDC6 and E2F1 served as positive controls for E2F1 recruitment and both showed at least 10-fold increase in promoter pulldown with E2F1 compared to IgG pulldown, whereas albumin served as a negative control, showing no

significant E2F1 ChIP activity its promoter. Comparatively, MDC1, KLF2 and SKP2 all showed significant levels of E2F1 ChIP on promoter regions, validating the observed E2F1 ChIP peaks on the UCSC Genome browser and confirming them as E2F1 targets in MCF7 cells.

Notably, CDC6 promoter pulldown was increased upon TSN and TDRD1 knockdown. This was also observed for the MDC1 promoter, echoing the hypothesis that regulatory effects on MDC1 and CDC6 were achieved using similar pathways of regulation. In the case of SKP2, contrasting effects of TSN and TDRD1 knockdown on promoter pulldown were observed. TSN knockdown resulted in increased ChIP activity, whereas TDRD1 knockdown reduced levels to below that of the siGFP control, coming close to levels of IgG pulldown. KLF2 showed a similar trend, wherein TSN knockdown dramatically enhanced pulldown, but demonstrated little to no change compared to the siGFP control when TDRD1 was depleted.

These trends were shown to be consistent throughout biological replicates, albeit at different scales depending on the efficiency of ChIP per replicate conducted (Figure 3.9). As such it can be inferred that related E2F1 binding to the promoters of the candidate genes chosen are affected consistently by reader knockdown.

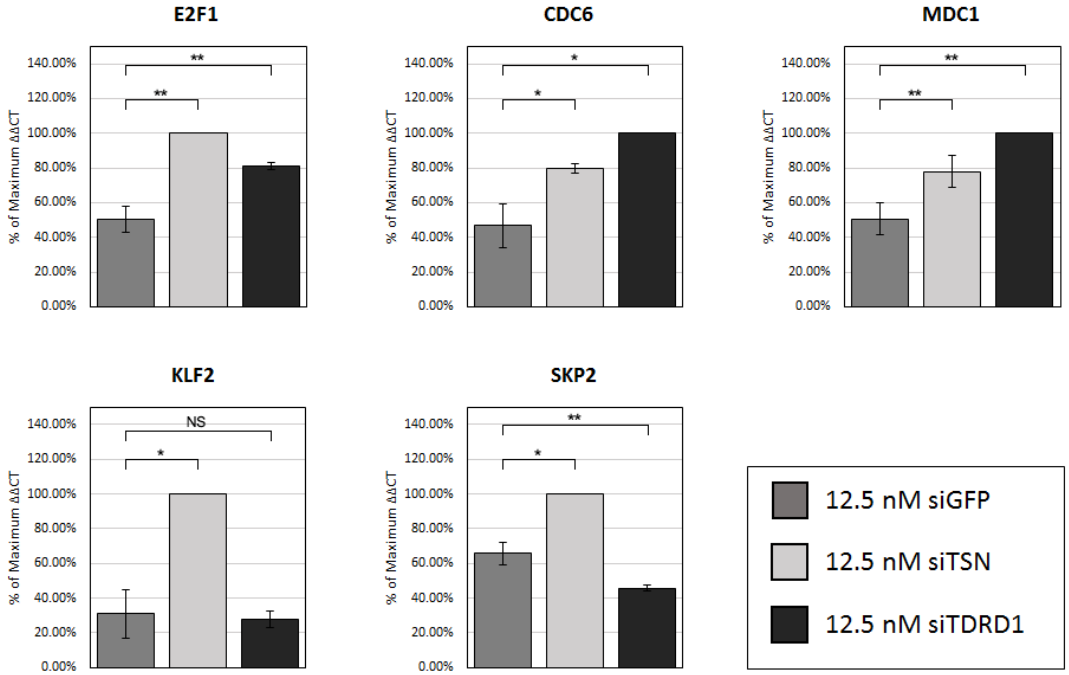


Figure 3.9 Reproducibility of E2F1 ChIP experiments across biological replicates
qPCR analysis conducted in biological replicates of immunoprecipitated chromatin fragments obtained from E2F1 pulldown on MCF7 cells targeted with siRNA as indicated (pooled data from n=3); Expression levels of each gene are indicated as mean values of percent maximum ΔΔCT obtained from the mean of technical triplicates of the three independent biological replicates. Error bars indicated standard deviation between the means obtained from each replicate. Statistical significance of deregulation was assessed pair-wise via two-tailed students' *t*-test as indicated (NS - not significant, *p<0.05, **p<0.01, ***p<0.001).

3.6 Assessing reader recruitment to target gene promoters

The ability of TSN and TDRD1 to recognize the SDMA mark on E2F1 raises the question of whether this binding facilitates reader recruitment to target gene promoters in an E2F1 dependent manner. Previous studies have demonstrated E2F1-dependent TSN recruitment to E2F1 target promoters (Zheng et al. 2013). It is possible then, that any changes in observed E2F1-dependent TSN or TDRD1 recruitment to the promoters of target genes may function cooperatively to bring about changes in transcriptional output. As such, chromatin immunoprecipitation of both TSN and TDRD1 on target gene promoters was attempted to see whether E2F1 presence on the candidate genes of interest facilitated similar recruitment of TSN or TDRD1 (Figure 3.10).

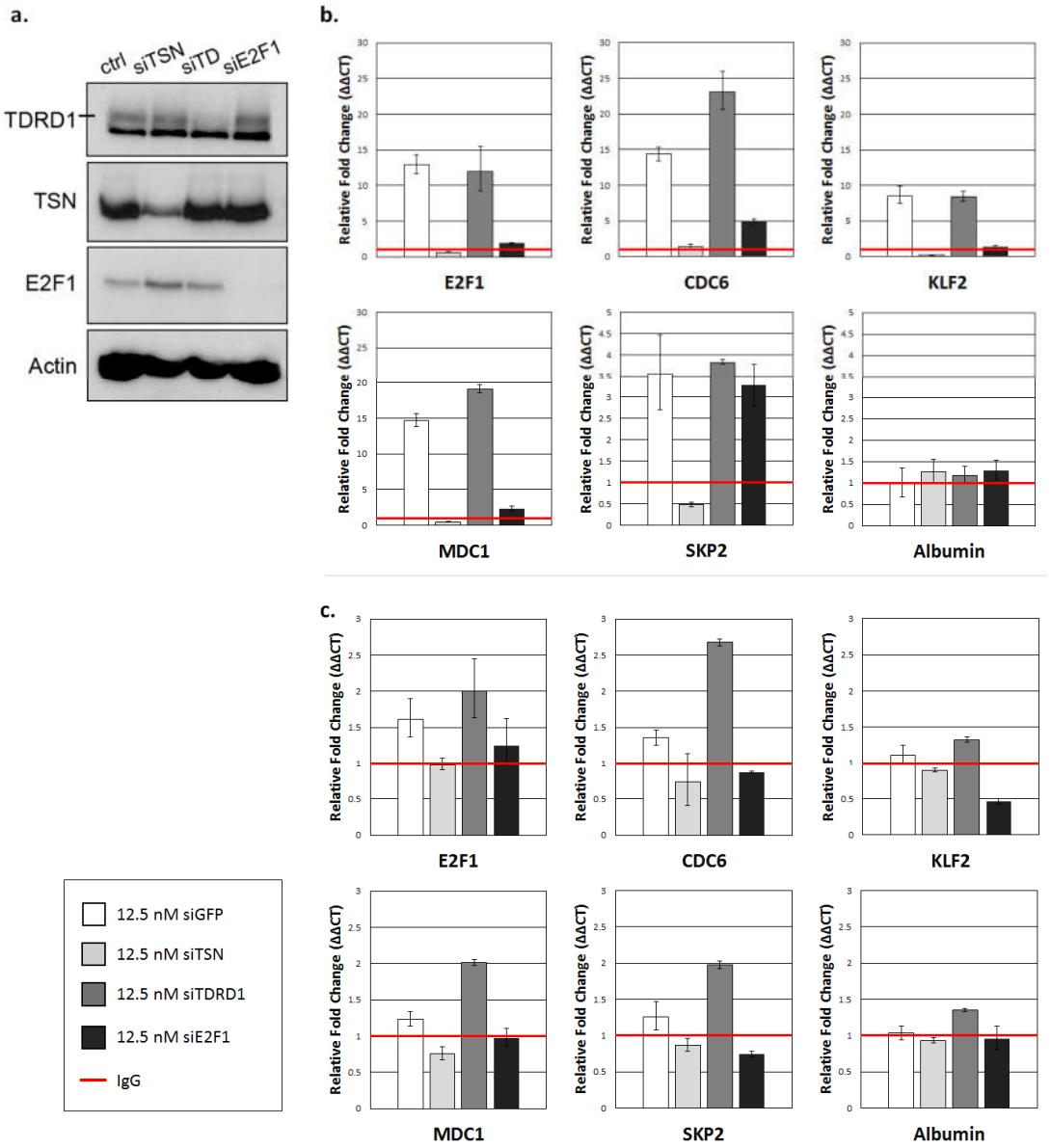


Figure 3.10 TSN and TDRD1 ChIP on different target gene promoters assessed by qPCR.
(a) Western blot of 20 µg protein extracted from MCF7 cells targeted with siRNA as indicated, and probed with antibodies as listed; (b, c) qPCR analysis of immunoprecipitated chromatin fragments obtained after (b) TSN, and (c) TDRD1 pulldown from MCF7 cells targeted with siRNA as indicated. Expression levels of each gene listed are indicated in relative fold change (ΔΔCT), normalised to input and relative to IgG pulldown control expression levels (set to 1) in each setup indicated in red. Data points are representative of mean average readings of technical triplicates and error bars indicate lower and upper bounds of predicted ΔΔCT range based on standard deviation. (Representative data of n=3).

Control, TSN, TDRD1 and E2F1 siRNA knockdown treatments were prepared and confirmed via Western blot (Figure 3.10a). TSN was successfully detected on the promoters of E2F1 and CDC6, which served as positive controls for TSN ChIP, showing at least 10-fold recruitment over the IgG control. Meanwhile no significant recruitment to albumin was observed (Figure 3.10b). ChIP activity on the promoter regions of KLF2,

MDC1 and SKP2 following TSN pulldown was evident, suggesting TSN recruitment on their promoters. Moreover, amplification was expectedly reduced in TSN knockdown setups, corresponding to decreased TSN expression levels. The expression levels of KLF2 and MDC1 promoters was notably reduced upon E2F1 knockdown, suggesting that TSN recruitment to these two genes was mediated by E2F1, much like CDC6 and E2F1. This was not the case for SKP2, as E2F1 knockdown had no significant effect on TSN pulldown of the SKP2 promoter. Interestingly, TDRD1 knockdown enhanced pulldown of MDC1 and CDC6 promoters, perhaps suggesting mutually exclusive or competitive binding between the two readers. Furthermore, the trends in promoter pulldown in response to siRNA treatment were consistent throughout biological replicates (Figure 3.11).

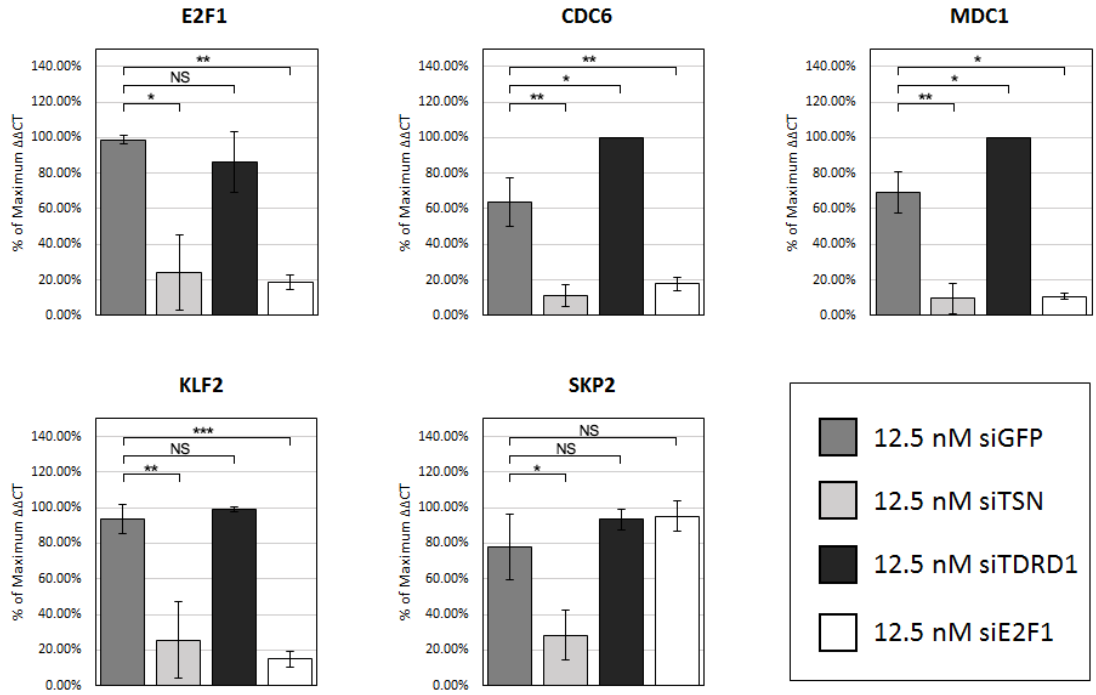


Figure 3.11 Reproducibility of TSN ChIP experiments across biological replicates
qPCR analysis conducted in biological replicates of immunoprecipitated chromatin fragments obtained from TSN pulldown on MCF7 cells targeted with siRNA as indicated (pooled data from n=3); Expression levels of each gene are indicated as mean values of percent maximum ΔΔCT obtained from the mean of technical triplicates of the three independent biological replicates. Error bars indicated standard deviation between the means obtained from each replicate. Statistical significance of deregulation was assessed pair-wise via two-tailed students' *t*-test as indicated (NS - not significant, *p<0.05, **p<0.01, ***p<0.001).

TDRD1 ChIP was less successful, showing minimal fold-change difference compared to the IgG control (Figure 3.9c). Surprisingly, ChIP signals were higher in setups transfected with siTDRD1, where TDRD1 expression was supposedly depleted. This indicated the possibility of non-specific pulldown by the TDRD1 antibody used. Indeed, a non-specific band was, and has been evident in several TDRD1 blots that is only detectible in MCF7 cells, perhaps accounting for the non-specific pulldown observed in these ChIP experiments.

All in all, ChIP experiments revealed E2F1 ChIP activity on all the candidate genes. In several cases, E2F1 recruitment was altered by the absence of TSN or TDRD1. Particularly, TSN knockdown appeared to enhance E2F1 recruitment to target promoters. Additionally, TSN was confirmed to be recruited to the target promoters assessed in both E2F1-dependent and -independent manners, shedding light on potential mechanistic differences in the transcriptional regulation of the genes assessed.

3.7 Discussion

While it has been established that the arginine methylation is able to switch the function of E2F1 between cellular proliferation and apoptosis, little is known about how such a switch occurs. The role of E2F1 in cell cycle progression relies heavily on its transactivation of various cell cycle-related genes which promote proliferation and prepare the cell for eventual division. However, E2F1 is also able to target various pro-apoptotic genes, and its ability to do so has been related to its abundance and half-life in cells. Indeed, extensive efforts have been undertaken to understand what drives the E2F1 bias between proliferative and apoptotic genes (Knezevic and Brash, 2004; Black et al., 2005; Johnson and DeGregori, 2006; Roworth et al., 2015).

The emergence of next generation whole-transcriptome sequencing capabilities has provided a far more comprehensive view of gene deregulation at the mRNA level than previously before in various cellular contexts and treatments.

In this study, RNA sequencing served as a powerful tool for elucidating proliferative or pro-apoptotic E2F1 transcriptional effects, and the known arginine dimethylation marks which promote either cellular proliferation or apoptosis served as reasonable experimental direction for its use in this study.

PRMT1 and PRMT5, the arginine methyltransferases responsible for the ADMA and SDMA mark on E2F1 respectively, have extensive roles in transcriptional and nuclear receptor co-activation, modulation of histone methylation, nucleocytoplasmic shuttling, and signal transduction (Blanc & Richard 2017). This makes it difficult to isolate E2F1-related transcriptional effects when either of them are depleted. Additionally, knockdown of either PRMT may be lethal and cause extensive cell death. Thus, elucidating the effects of the different E2F1 arginine dimethylation marks requires a less straightforward approach than depleting the transferases responsible for their deposition.

The generation of various E2F1 mutants containing arginine to lysine mutations at Arg109/111/113, the residues where ADMA and SDMA marks are deposited, has been achieved by a member of the La Thangue laboratory (Zheng et al. 2013). The mutation of Arg109 effectively inhibits the ADMA mark and the mutation of Arg111 and 113 inhibits the SDMA mark, allowing for more focused transcriptional effects to be explored by RNA sequencing analysis without the need for PRMT depletion. Indeed, extensive deregulation of E2F1-dependent genes has been reported (Roworth, unpublished). This is a current topic of interest in the La Thangue laboratory, but is merely supplemental to the actual scope of the current study.

The identification of readers of the different dimethylation marks provides an alternative perspective to their effects on E2F1-dependent transcriptional output. While readers of the ADMA mark deposited by PRMT1 have yet to be identified, two Tudor domain proteins have been identified that are able to recognize and bind to the SDMA mark: TSN and TDRD1. In theory, depletion of these readers would to some degree mimic the depletion of the SDMA mark, preventing it from being read, and thus resulting in the disruption of the proliferative function of E2F1-SDMA. Indeed, it has been shown that TSN depletion increases E2F1 levels and associated E2F1-dependent PARP cleavage, while decreasing colony formation (Zheng et al. 2013). Additionally, TSN possesses several established roles in RNA metabolism, a subset of which may potentially be E2F1-dependent (Gutierrez-Beltran et al. 2016a).

While information on the functions of TSN are expansive and far-reaching, little is known about the function of TDRD1 in somatic cells, and its activity in humans has generally been confined to the germline (Reuter et al. 2009; Mathioudakis et al. 2012b). Its over-expression in a number of hormone-sensitive cancers, however, suggests it may play an oncogenic role in these cellular contexts, which may or may not be E2F1-mediated (Boormans et al. 2013; Kacprzyk et al. 2013; Litovkin et al. 2014; Xiao et al. 2016). As such, the cellular backgrounds used provided an appropriate context for studying the roles of TSN and TDRD1 when both are expressed in cancer, and also provide both hormone-sensitive and insensitive backgrounds for testing.

The analysis of transcriptional effects upon depletion of TSN or TDRD1 in these cellular contexts presented a straightforward perspective to elucidating E2F1 proliferative effects mediated by TSN and TDRD1. This served as the motivation behind the RNA sequencing conducted by a member of the La Thangue laboratory (Chan, unpublished), but its significance was limited by the lack of replicates for each shRNA treatment and

despite ribosomal depletion treatments, the persistence of potential ribosomal RNA contamination. As such, the data was approached in a conservative manner, and extensive validation was conducted.

In-depth validation using qRT-PCR revealed that TSN and TDRD1 seem to play largely redundant roles resulting in comparable trends in penultimate mRNA expression of the genes chosen. Predictably, the effects observed in both transient and stable knockdown contexts were expected to be limited by the actual extent of TSN and TDRD1 knockdown. It is entirely possible that what limited protein is left of TSN or TDRD1 could fully compensate for the fraction lost. Furthermore, any differences in deregulation observed between cell lines could be a result of differences in transfection efficiency, or inefficacy of the siRNA oligonucleotides used alone or in combination in different cellular contexts. At the same time, the RNAi systems used for the majority of this study come with their own disadvantages, primarily concerning specificity and possible off-target effects (Boettcher & Mcmanus 2015). siRNA in particular has been shown to induce silencing of non-targeting mRNAs with limited sequence complementarity in a dosage dependent manner. As such, the methods used in these experiments were designed to minimise siRNA concentrations as much as possible.

The stable shRNA knockdown cell lines were originally created to circumvent inconsistencies of traditional siRNA transient knockdown. However, the stable shRNA knockdown cell lines used in the initial RNA sequencing screen posed additional concerns moving forward due to their polyclonal nature. As such, individual cells within each cell line can have varying levels of shRNA expression resulting from random integration of the lentiviral construct into the genome of the cell. This may account for the observed loss of knockdown over time, and thus also predisposes the cell lines to population drift over several passages.

Bearing these limitations in mind, the nature of the deregulation observed in the target genes analysed after initial validation revealed various pathways by which the proliferative effects of the recognition of the SDMA mark could be carried out. Such deregulation could facilitate the ultimate expression of oncogenic genes while silencing growth-limiting, checkpoint-activating and tumour-suppressing transcripts. A summary of the experimental findings can be found in Table 3.3.

Table 3.3 Summary of experimental findings on each target gene

	qRT-PCR				E2F1 ChIP		TSN ChIP	
	TSN KD	E2F1 Co-D	TDRD1 KD	E2F1-Co-D	TSN KD	TDRD1 KD	TDRD1 KD	E2F1 KD
CDC6	+	-	+	-	+	+	+	-
MDC1	+	-	+	-	+	+	+	-
KLF2	+	++	+	++	+	**n/e**	**n/e**	-
SKP2	-	- -	-	- -	+	-	**n/e**	**n/e**

*KD – knockdown; Co-D – co-depletion; + up-regulated vs control; - down-regulated vs control; **n/e** no significant effect

Assessment of candidate gene expression following co-depletion of TSN and TDRD1 with E2F1 suggested both direct E2F1 involvement in mediating the observed deregulation, as well as potential E2F1-independent reader activity.

As an example, expression patterns of canonical E2F1 target CDC6 in particular suggest direct E2F1-mediated gene transactivation in the regulatory effects of TSN and TDRD1. Upon TSN or TDRD1 knockdown, CDC6 mRNA levels rose substantially in both SKBR3 and MCF7 cells. This suggests that TSN and TDRD1 in wild-type MCF7 and SKBR3 play repressive roles which limit steady state levels of CDC6. Further co-depletion of E2F1 abrogated the up-regulation of CDC6 in response to TSN or TDRD1 knockdown in both cellular context assessed, suggesting that rather than independent TSN and TDRD1 biological activity, the up-regulation observed was mediated by E2F1-dependent activity. Moreover, such a response seemed to be in place for both oestrogen/progesterone receptor-positive (MCF7) and negative (SKBR3) breast cancers tested. The hypothesis of direct E2F1 involvement was further strengthened by the observation of enriched CDC6 promoter amplification upon E2F1 pulldown compared to

IgG in MCF7 cells. Such activity was further enhanced upon reader knockdown. This supports the notion that the role of E2F1 in relation to reader-dependent deregulation is one of direct promoter occupancy and gene transactivation, and that TSN and TDRD1 potentially act to limit E2F1 function in this regard.

This hypothesis could potentially apply to several of the candidate genes assessed, as in the case of MDC1 in MCF7s, and TCF19 and ZNF367 in SKBR3s. Conclusions cannot be made about the deregulation of genes observed in DuCaPs as co-depletion and ChIP experiments were not conducted in that cellular background.

A previous member of the La Thangue laboratory had previously shown that TSN and TDRD1 co-immunoprecipitate with E2F1 in vitro (Moehlenbrink, unpublished). It is possible that in the regulatory context discussed, interaction of TSN and TDRD1 with E2F1 could hinder its transcriptional activity, and in the absence of reader interaction, E2F1 transactivation is enhanced. While TDRD1 ChIP was unsuccessful, this study has shown that the promoter regions of CDC6 and MDC1 are pulled down with endogenous TSN protein, and that E2F1 depletion abrogates the observed ChIP activity. This supports the need for E2F1 to be present for TSN binding to target promoters to occur. Whether TSN and E2F1 are in a complex on the promoters of these target genes has yet to be confirmed by sequential ChIP experiments (ChIP-reChIP; Furlan-Magaril et al., 2009). Collectively, however, the data suggests that TSN and TDRD1 play a repressive role that is dependent on E2F1 transcription on these particular target genes.

SKP2 expression patterns in MCF7 cells present a less simplistic response to in TSN or TDRD1 depletion. Given the results of reader/E2F1 co-depletion experiments and the knowledge of SKP2 as a known E2F1 target, it can be hypothesised that normal levels of SKP2 expression are maintained by E2F1-dependent transcription and that TSN and TDRD1 could further enhance SKP2 expression in these cellular contexts, though not

necessarily in an E2F1-dependent manner. In the ChIP experiments conducted, TSN knockdown increased SKP2 promoter pulldown with E2F1, whereas TDRD1 knockdown had the inverse effect of decreasing pulldown compared to the siGFP control. Moreover, the SKP2 promoter region also immunoprecipitated with TSN. However, this was not found to be E2F1-dependent, as depletion of E2F1 showed insignificant effects on TSN ChIP activity. These results, compounded with the findings that TSN and TDRD1 depletion resulted in decreased SKP2 expression which was further repressed upon E2F1 co-depletion, initially seem contradictory. The decreased SKP2 expression in response to TSN could be either a cooperative effect between TSN and E2F1, or the additive effect of the individual co-activator effects of TSN and those of E2F1. Interestingly, the data obtained suggests that the regulation of SKP2 by TSN is likely independent of E2F1 transcription. Indeed, TSN has been shown to possess various transcriptional co-activator activities (Leverson et al. 1998; Yang et al. 2007; Liu et al. 2011), particularly in conjunction with signal transducers and activator of transcription (STATs) (Paukku et al. 2003; Välineva et al. 2006; Low et al. 2006). Accordingly, various studies have reported STAT-dependent regulation of SKP2 and STAT-binding to the SKP2 promoter (Furuhata et al. 2009; Huang et al. 2012). It is possible that TSN and E2F1 each regulate SKP2 expression through different pathways. This could explain the consequent increased E2F1 ChIP activity observed upon TSN-depletion as perhaps being due to reduced promoter occupancy of TSN, or a compensatory mechanism for the loss of transcriptional activation by the TSN-STAT complex. This could also explain why E2F1 depletion had no effect on TSN recruitment, as TSN was possibly not recruited by E2F1, but rather by STATs which bind to the SKP2 promoter. This suggests that TSN and E2F1 may each play independent roles in maintaining SKP2 expression, explaining the loss of SKP2 expression in their absence. However, TDRD1 regulatory activity on the SKP2 promoter

may still be E2F1-dependent, as TDRD1 depletion reduced E2F1 binding, though the results of the converse experiment of depleting E2F1 and assessing TDRD1 recruitment could not be confirmed. Unfortunately, none of the reported functions of TDRD1 explain the down-regulation of SKP2 observed upon its knockdown, and as such, it is more likely that it achieves such a regulatory function in a cooperative manner with other regulatory proteins or through indirect effects on SKP2 regulators.

Compared to the previously discussed regulation of target genes, the expression patterns of KLF2 observed after reader co-depletion of E2F1 were unusual. In MCF7 cells, KLF2 expression was enhanced upon TSN or TDRD1 knockdown. But unlike the genes which behaved like canonical E2F1 target CDC6, the co-depletion of E2F1 resulted in further enhancement of its expression. This suggests a potential repressive function for E2F1 in KLF2 gene regulation which is additive to the repressive roles that TSN and TDRD1 could potentially play individually. Studies which could potentially support a suppressive role for E2F1 on target promoters show that when bound to RB, E2F1 is able to able to form a complex between DNMT1 and HDAC1 to repress transcription in target promoters (Wolffe et al. 2000; J. William Harbour & Dean 2000). Indeed, examination of KLF2 on genome browser has revealed substantial binding peaks for histone 3 lysine 27 trimethylation (H3K27me3), a mark associated with repressed and inactive gene promoters. These coincide with recruitment peaks for various HDACs on the KLF2 promoter (Appendix A1). Though these findings are purely coincidental, if E2F1 is involved in the deposition of repressive marks on target promoters, the increased E2F1 recruitment to the KLF2 promoter in response to TSN depletion observed in this study could facilitate the upregulation of its transcript levels.

It is more likely, however, that the observed deregulation is an indirect effect of reader depletion. Although TSN was found to bind in an E2F1-dependent manner to the

KLF2 promoter, its function there is unclear. Independent of E2F1, TSN could also facilitate gene silencing and repression through its role in RISC-mediated RNA degradation in the context of KLF2 expression, and the depletion of E2F1 could indirectly affect RISC-mediated activity (Gutierrez-Beltran et al. 2016a). In the case of TDRD1, its known physiological roles somatic cells are limited. TDRD1 has been shown to act as a molecular scaffold between PIWI proteins and piRNAs to silence transposons in the germline (Gayatri & Bedford 2014). Interestingly, recent studies highlight emerging roles that PIWI proteins and piRNAs may play in somatic tissues (Ross et al. 2014). As such, TDRD1, when expressed inappropriately in cancer, may impact various epigenetic signatures by facilitating the formation of repressive complexes on chromatin through its methylarginine reading capacity and multiple Tudor domains. The loss of TDRD1, and thus the potential loss of such epigenetic repressor complexes, represent one explanation for the indirect up-regulation of KLF2 observed.

Interestingly, TDRD1 knockdown had the additional effect of increasing TSN binding to MDC1 and CDC6 promoters. However, due to the failure of TDRD1 ChIP, the converse could not be shown. If the corresponding depletion of TSN resulted in increased TDRD1 recruitment, it could be inferred that the two compete for E2F1 binding and thus alter each other's consequent recruitment, resulting in subsequent regulatory functions that would be exclusive of each other. While it has not been confirmed if the binding of TSN and TDRD1 to the E2F1 SDMA mark is mutually exclusive, competitive binding between the two which may limit their individual biological functions in conjunction with E2F1 could constitute another regulatory mechanism.

Knowledge of the chromatin binding activity of both E2F1 and the proteins responsible for the recognition of its SDMA mark, compounded with previous qRT-PCR

experiments, allow for hypothesis-driven speculations on the mechanisms of SDMA-reader-dependent transcriptional regulation of E2F1 target genes (Figure 3.12).

Further confirmatory experiments need to be conducted to fully affirm the accuracy of the hypothetical mechanisms proposed. TDRD1 ChIP would help confirm and assess TDRD1 binding to target promoters and E2F1 dependency. The confirmation of reader-competitive recognition of the SDMA would similarly benefit from TDRD1 ChIP experiments. Co-IP of readers and E2F1 under reader knockdown treatments would likewise be able to confirm the mutual exclusivity of reader binding. ChIP-reChIP experiments would be useful to determine the co-recruitment of E2F1 and TSN to target promoters in macromolecular complexes. It would also help confirm potential STAT-related TSN activity on the SKP2 promoter which may also influence E2F1 recruitment.

It may also be worthwhile to explore the other known biological functions of TSN, and to see whether any of these may potentially involve E2F1. Interestingly, recent findings in the La Thangue laboratory have shown for the first time that TSN enables E2F1 recruitment to RNA species (Roworth, unpublished). Notably, TSN plays a distinct role in RNA splicing through its interaction with protein and RNA components of the spliceosome via symmetrically dimethylated arginine in the spliceosome core (Yang et al. 2007; Gao et al. 2012; Jariwal et al. 2015; Gutierrez-Beltran et al. 2016b). It is possible that the interaction between TSN and E2F1 facilitated by the SDMA mark enables co-transcriptional splicing, influencing the relative expression of mRNA splice variants. It is known that splice variation accounts for differences in RNA stability and functional isoforms (Hamer & Leder 1979; ’t Hoen et al. 2011; Yap & Makeyev 2016). Thus, this represents another level of potential gene regulation which may bring about the proliferative phenotype associated with the E2F1 SDMA mark.

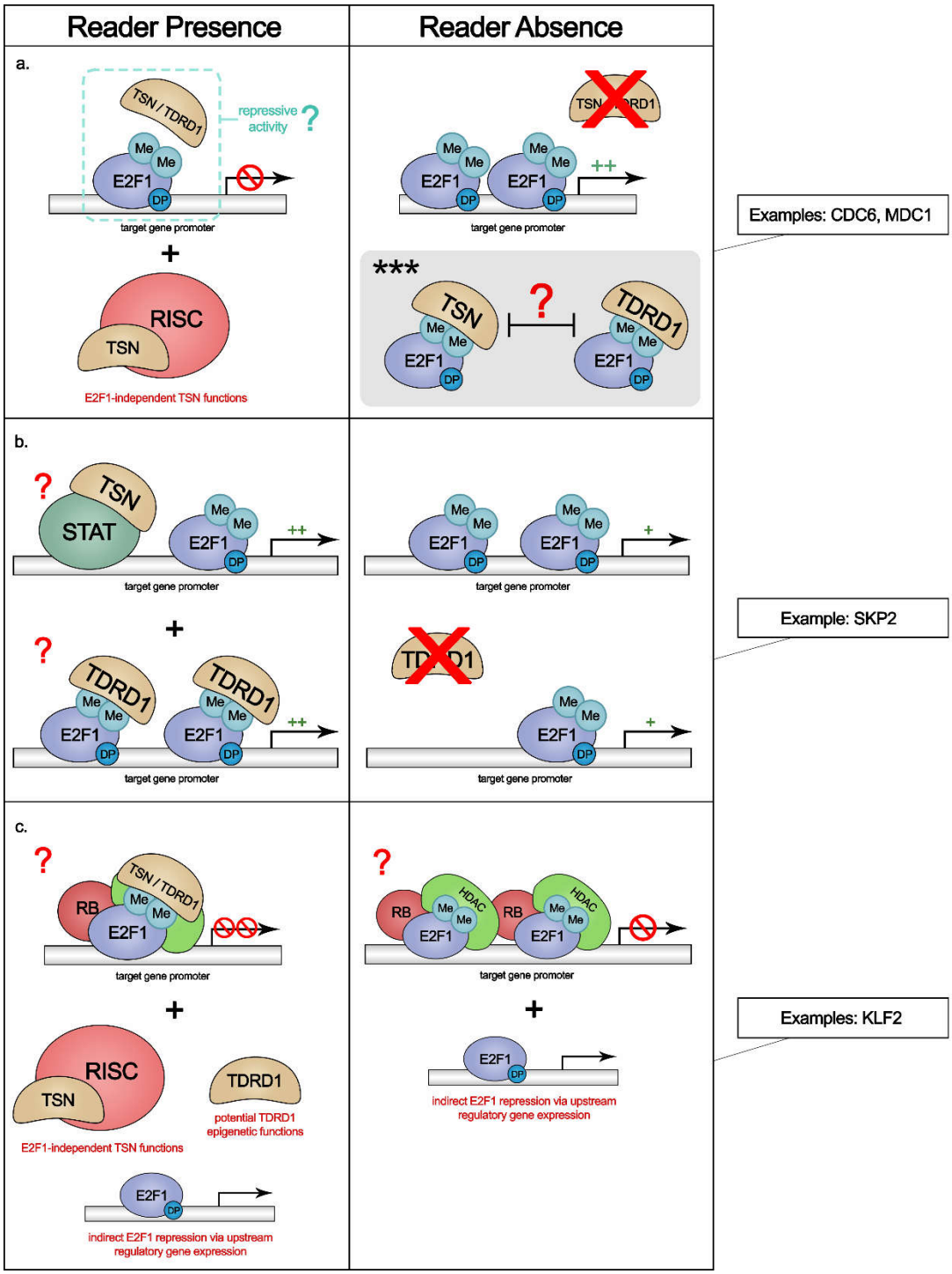


Figure 3.12 Hypothesised regulatory roles for TSN and TDRD1 in E2F1 target gene transcription.
(a) Readers may hinder E2F1-mediated transcription, through a yet to be confirmed repressive mechanism. TSN may also display E2F1-independent repressive activity. When readers are absent, E2F1 transcription proceeds as normal. ***Potential competitive TSN/TDRD1 binding may be apparent; (b) TSN could display co-activator activity independent of E2F1 while TDRD1 enhances E2F1-mediated transcription through an unknown mechanism. When TSN is absent, E2F1 recruitment increases possibly due to reduced STAT binding, resulting in less efficient gene activation. When TDRD1 is absent, E2F1 recruitment is decreased; (c) E2F1 may display repressor functions that are enhanced by the binding of TSN or TDRD1. Biological activities of TSN or TDRD1, and indirect E2F1 transcriptional activity may also contribute to gene silencing. In the absence of readers, E2F1 recruitment may compensate via its repressive role.

Chapter 4 : E2F1 plays a role in TSN-associated splicing activity

4.1 Introduction

It has now been demonstrated that TSN and TDRD1 participate in the transcriptional regulation of a variety of E2F1 target genes. Little is known about the other biological functions of TDRD1 which may potentially involve E2F1 activity. TSN, however, has extensive roles in RNA metabolism (Gutierrez-Beltran et al. 2016b). It has recently been demonstrated that E2F1 binds to target RNA species in a TSN-dependent manner (Roworth, unpublished). This raises the question of whether TSN is able to facilitate non-canonical roles of E2F1 outside of its activity as a transcriptional activator. Indeed, E2F1 has documented non-transcriptional roles in DNA repair, recruiting repair factors to sites of DNA damage, potentially through chromatin remodelling (Biswas & Johnson 2012). TSN, however, functions prominently in mRNA splicing through the interaction of its Tudor domain with the core components of RNA splicing machinery, such as Prp8, SmB, SmD1/D3 and SAM68 (Yang et al. 2007; Gao et al. 2012; Jariwal et al. 2015; Gutierrez-Beltran et al. 2016b). Thus, the interaction between TSN and the E2F1 SDMA mark may facilitate TSN recruitment to target promoters to perform co-transcriptional splicing. This adds yet another non-transcriptional role to the E2F1 functional repertoire.

Alternative splicing influences both RNA stability as well as the expression of functional isoforms of proteins. Several proteins with proliferative and apoptotic functions also possess splice variants that are less stable, or that when expressed, result in truncated non-functional proteins (Hamer & Leder 1979; ’t Hoen et al. 2011; Yap & Makeyev 2016). The preferential expression of splice variant species thus constitutes another level of regulation which could facilitate the proliferative effects of the E2F1 SDMA mark.

This chapter will focus on assessing the splice variant expression of the target genes chosen upon reader knockdown to assess potential TSN- and E2F1- dependent effects on alternative splicing.

4.2 Candidate genes possess several documented and predicted splice variants

To determine the potential extent of splicing in each MCF7 candidate gene, predicted and documented splice variant data was accessed from Ensembl release 88, genome assembly GRCh38.p10 (Aken et al. 2016) (Appendix B1).

The KLF2 (ENSG00000127528) entry listed two protein-coding transcript variants – one documented as the primary expressed transcript, and one predicted transcript. MDC1 (ENSG00000137337) was listed to have nine transcript variants – the documented primary protein-coding transcript, five other predicted protein-coding transcripts, and three predicted processed transcripts, of which two possessed retained introns. SKP2 (ENSG00000145604) was listed to have one primary protein-coding transcript and five other protein-coding transcripts, of which two have been documented to code for functional protein isoforms. In addition, three non-coding processed transcripts were listed, and were predicted to undergo non-sense mediated decay.

4.3 TSN mediates changes in candidate transcript variant expression

In order to narrow the scope of the study, selected variants which represented functional protein coding transcripts, non-functional protein-coding transcripts, and non-coding transcripts, were chosen for further study (Table 6.1). Primers were then designed on areas common to all reported transcripts to assess the total expression levels of the all the transcript variants of each candidate gene. Additionally, primers specific to each transcript variant were also designed (Figure 4.1)

Table 4.1 Representative candidate gene transcripts chosen for further study (Aken et al. 2016)

	Variant Name	Transcript ID	BP	Protein	Biotype	RefSeq
KLF2	KLF2-001	ENST00000248071.5	2018	355aa	Protein coding	NM_016270; NP_057354
	KLF2-002	ENST00000592003.1	564	78aa	Protein coding	-
MDC1	MDC1-001	ENST00000376406.7	7576	2089aa	Protein coding	NM_014641; NP_055456
	MDC1-008	ENST00000489540.1	1402	n/a	Retained intron	-
	MDC1-007	ENST00000492462.1	819	n/a	Retained intron	-
SKP2	SKP2-002	ENST00000274255.10	3759	424aa	Protein coding	NM_005983; NP_005974
	SKP2-202	ENST00000620197.4	3255	210aa	Protein coding	NM_001243120; NP_001230049
	SKP2-001	ENST00000274254.9	1537	410aa	Protein coding	NM_032637; NP_116026
	SKP2-009	ENST00000504386.1	574	80aa	Nonsense mediated decay	-

qRT-PCR was used to assess the expression of the different transcript variants relative to the total transcript expression levels of each gene (Figure 4.2). The total transcript expression levels of each gene were first assessed by normalising to the expression levels actin. These showed the same trends of deregulation observed earlier in the study upon transient knockdown of each reader and represented the general effects of reader knockdown on transcription. In order to account for the effects of changing steady state transcription in response to treatment, the individual transcript variant expression levels were then independently normalised to the raw total transcript expression of each gene before being assessed relative to the siGFP control. These changes thus constitute changes in splicing events, taking transcription level changes in response to treatment into account. Notably, changes in the expression levels of each transcript variant were observed upon TSN or TDRD1 knockdown and the trends of transcript variant deregulation upon treatments were consistent across biological setups (Figure 4.3)

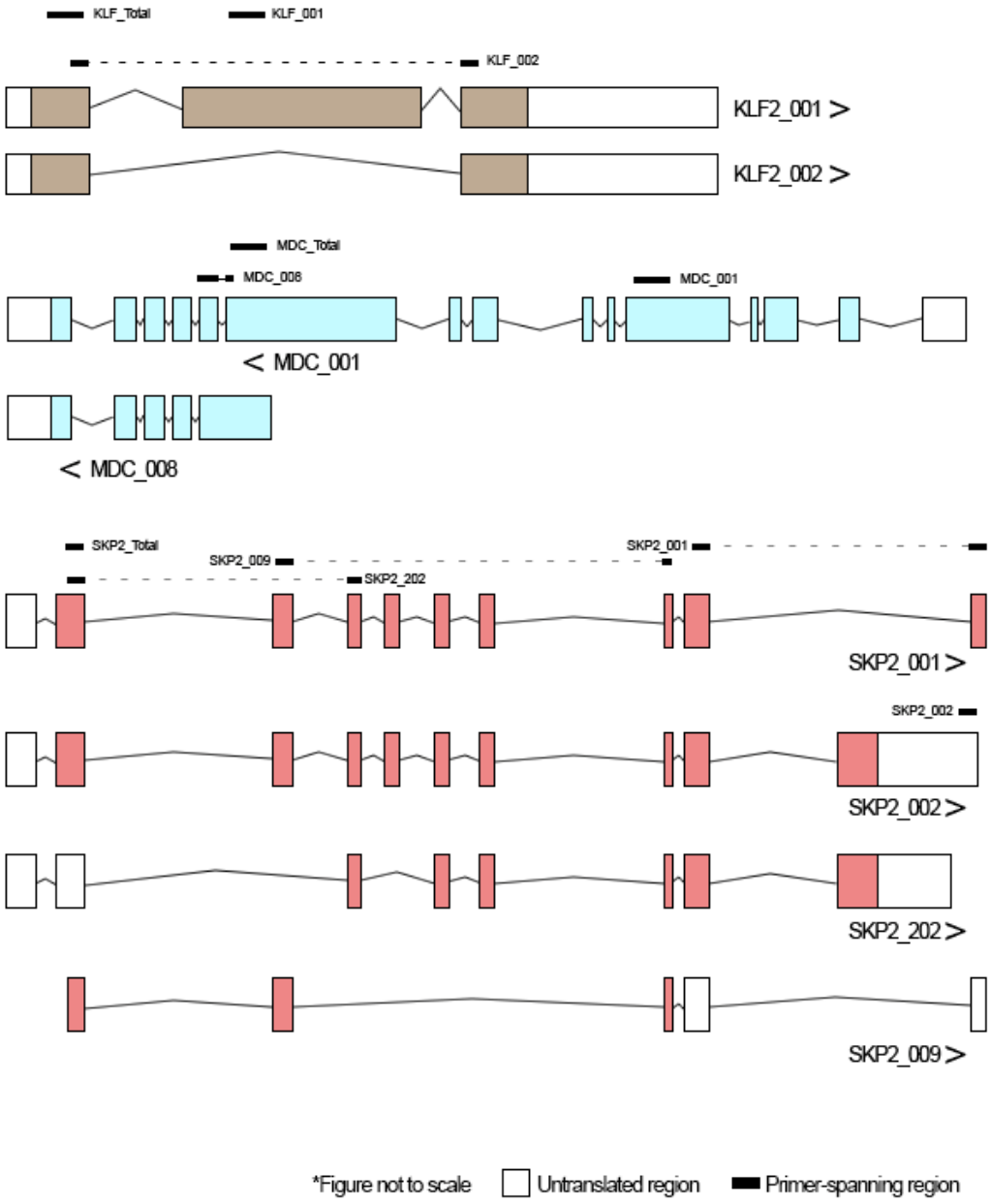


Figure 4.1 Exon-intron landscape of target genes and primer design

Loose depiction of the exon-intron landscapes of the candidate transcript variants of KLF2, MDC1 and SKP2. Exons are depicted as coloured blocks and introns are depicted as lines. Untranslated regions are marked in white blocks and primer spanning regions corresponding to predicted PCR products of each primer pair are depicted as black dashes.

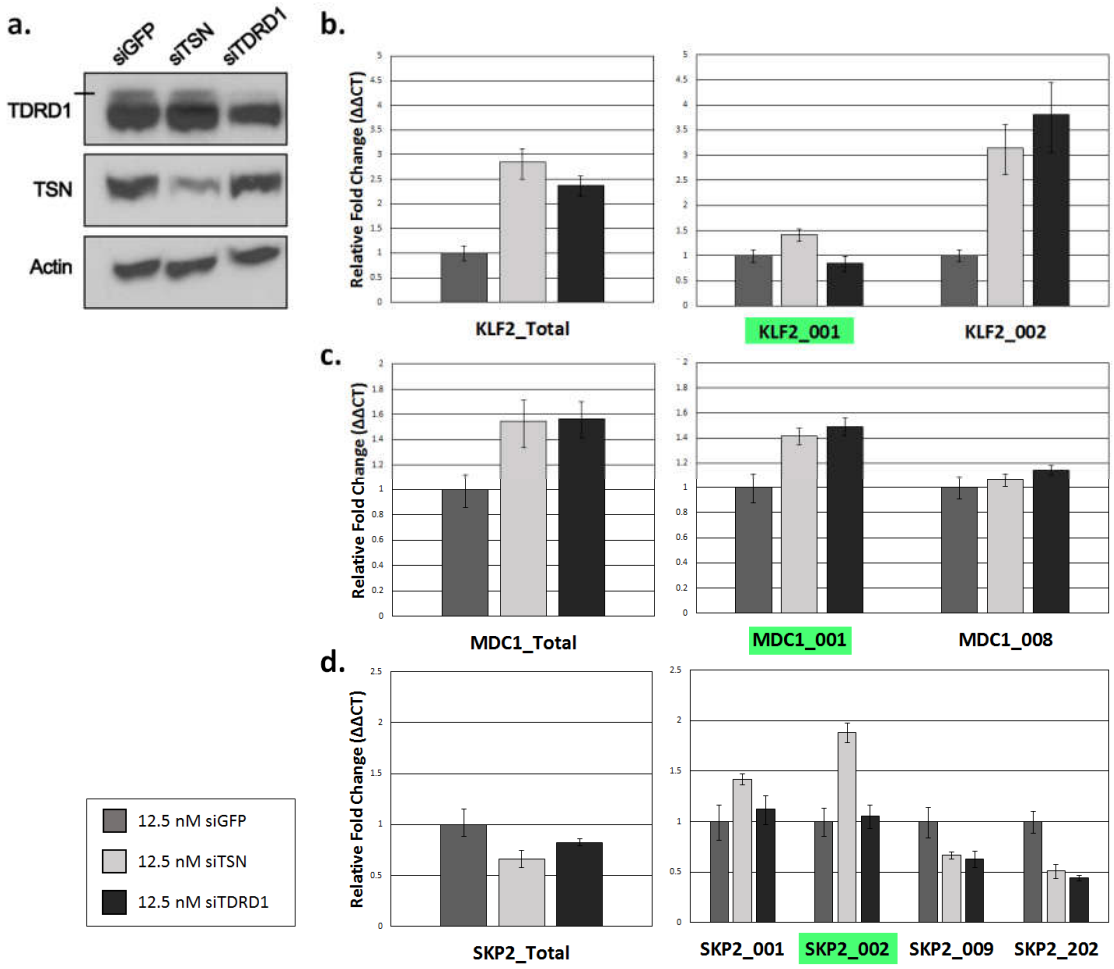


Figure 4.2 qRT-PCR analysis of candidate gene splice variant expression.
(a) Western blot of 20 µg protein extracted from MCF7 cells targeted with siRNA as indicated, and probed with antibodies as listed; (b, c, d) qRT-PCR analysis of cDNA obtained from MCF7 cells targeted with siRNA as indicated. Total transcript expression of (b) KLF2, (c), MDC1, and (d) SKP2 was first normalised to actin levels set to 1 in each setup, and expressed relative to the siGFP control. In a separate analysis, relative expression levels of each transcript variant were then normalised to the raw total transcript expression levels, which were set to 1, and assessed relative to the siGFP control in each setup. Expression levels of each gene listed are indicated in relative fold change (ΔΔCT). Data points are representative of mean average readings of technical triplicates and error bars indicate lower and upper bounds of predicted ΔΔCT range based on standard deviation. Transcripts highlighted in gene represent the primary protein-coding transcript (Representative data of n=3).

The expression levels of KLF2_001, the primary protein-coding transcript of KLF2 (O'Leary et al. 2016), increased upon TSN knockdown, but not upon TDRD1 knockdown. On the other hand, KLF2_002, a predicted variant which encodes a 78-amino acid product which lacks the functional $Cysteine_2/Histidine_2$ zinc finger motifs (Aken et al. 2016; O'Leary et al. 2016), exhibited non-reader specific up-regulation (Figure 4.2b).

In the case of MDC1, changes were observed in the expression of MDC1_001, the primary protein-coding transcript, as well as in MDC1_007, a predicted non-coding

transcript with a retained intron (Aken et al. 2016). While MDC1_001 expression increased in response to TSN and TDRD1 knockdown, MDC1_007 expression decreased following the same treatment. These changes were observed to be non-reader specific and likely do not constitute the individual splicing-related functions of TSN. No significant changes were observed in the expression of MDC_008, another non-coding transcript (Aken et al. 2016) (Figure 4.2c).

SKP2 transcript variants showed more prominent TSN-dependent changes in variant expression. TSN knockdown prompted the increased expression levels of SKP2_002, the primary coding transcript, and SKP2_001, which encodes an isoform of SKP2 with a distinct C-terminal region (O'Leary et al. 2016). TDRD1 knockdown, on the other hand, showed no significant effect on the expression of these transcripts. In contrast, the levels of SKP2_009, a transcript that is predicted to undergo non-sense mediated decay (Aken et al. 2016), and SKP2_202, a documented protein-coding transcript with a truncated N-terminal region and missing internal segments (O'Leary et al. 2016), showed decreased expression upon either TSN or TDRD1 knockdown (Figure 4.2d).

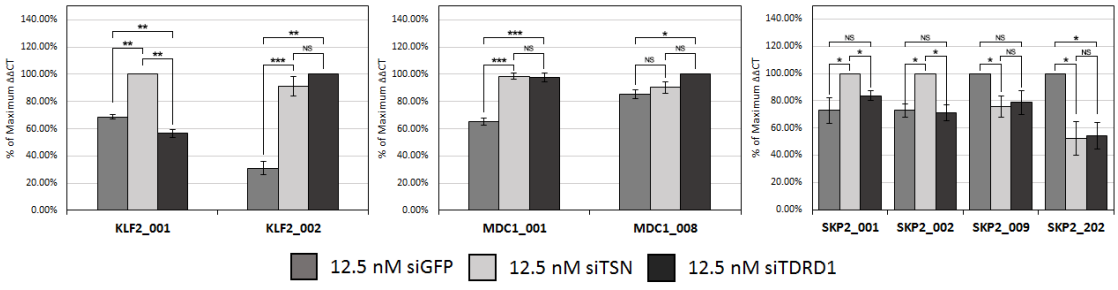


Figure 4.3 Reproducibility of splice variant expression experiments across biological replicates
qRT-PCR analysis of splice variant expression experiments conducted in biological replicates on MCF7 cells in response to siRNA treatment as indicated (pooled data from n=3); Expression levels of each gene are indicated as mean values of percent maximum ΔΔCT obtained from the mean of technical triplicates of the three independent biological replicates normalized against total transcript levels. Error bars indicated standard deviation between the means obtained from each replicate. Statistical significance of deregulation was assessed pair-wise via two-tailed students' *t*-test as indicated (NS – not significant, *p<0.05, **p<0.01, ***p<0.001).

4.4 E2F1 mediates TSN-associated changes in transcript expression

To determine whether the observed TSN knockdown-specific changes were mediated in an E2F1-dependent manner, E2F1 was co-depleted, and the effects on the selected transcripts were assessed (Figure 4.4).

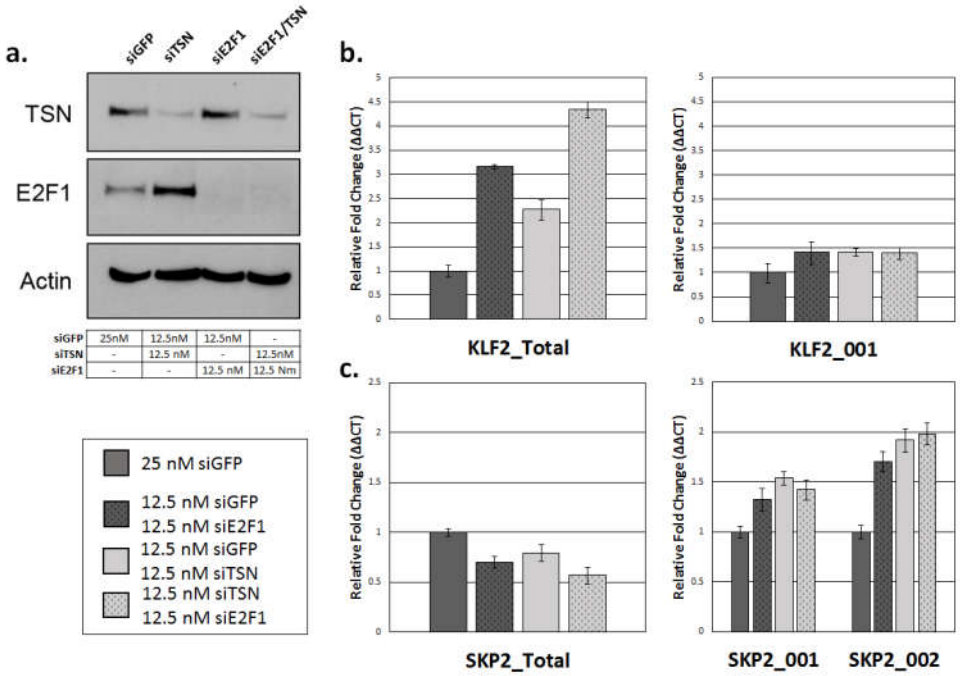


Figure 4.4 qRT-PCR analysis of candidate gene splice variant expression in TSN/E2F1 co-depletion experiments.
(a) Western blot of 20 µg protein extracted from MCF7 cells targeted with siRNA as indicated, and probed with antibodies as listed; (b, c) qRT-PCR analysis of cDNA obtained from MCF7 cells targeted with siRNA as indicated. Total transcript expression of (b) KLF2, and (c) SKP2 was first normalised to actin levels, set to 1 in each setup, and expressed relative to the siGFP control. Separately, relative expression levels of selected transcript variants were then normalised to the raw total transcript expression levels, which were set to 1, and assessed relative to the siGFP control in each setup. Expression levels of each gene listed are indicated in relative fold change (ΔΔCT). Data points are representative of mean average readings of technical triplicates and error bars indicate lower and upper bounds of predicted ΔΔCT range based on standard deviation. (Representative data of n=3).

Interestingly E2F1 knockdown mimicked the effects of TSN knockdown in KLF2_001, SKP2_001 and SKP2_002 transcripts, indicating that E2F1 plays a role in maintaining decreased expression of these transcript variants. Moreover, the co-depletion of both TSN and E2F1 had no significant effect on these transcripts. Moreover, these effects were consistent across replicates (Figure 4.5). Taken together, these results confirm a role for TSN in the differential splicing of E2F1-target genes. Furthermore, the results suggest that E2F1 mediates these effects in concert with TSN.

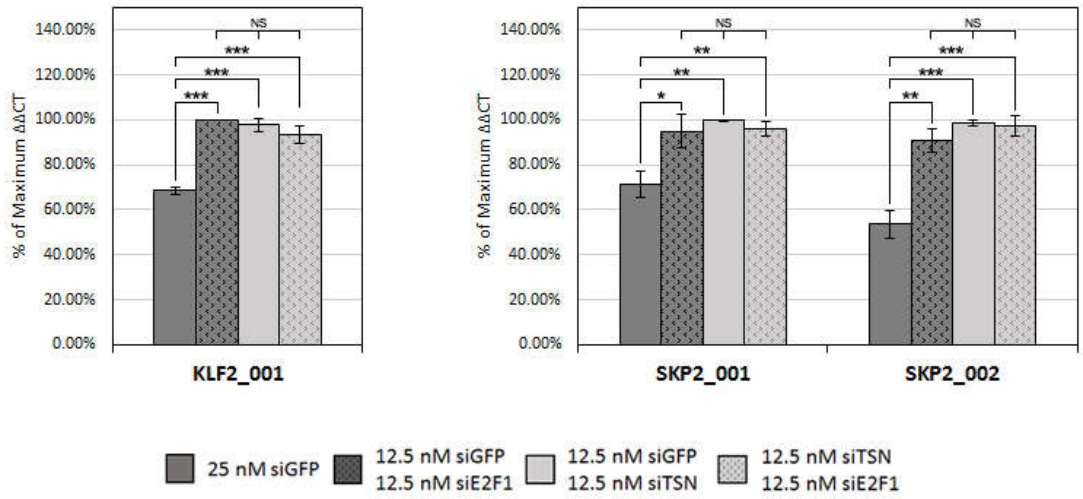


Figure 4.5 Reproducibility of E2F1 co-depletion experiments on splice variant expression across biological replicates

qRT-PCR analysis of the effects of E2F1 co-depletion on splice variant expression experiments conducted in biological replicates on MCF7 cells in response to siRNA treatment as indicated (pooled data from n=3); Expression levels of each gene are indicated as mean values of percent maximum ΔΔCT obtained from the mean of technical triplicates of the three independent biological replicates normalized against total transcript levels. Error bars indicated standard deviation between the means obtained from each replicate. Statistical significance of deregulation was assessed pair-wise via two-tailed students' t-test as indicated, and via single factor ANOVA collectively (NS – not significant, *p<0.05, **p<0.01, ***p<0.001).

4.5 Discussion

While this study has thus far improved our understanding of the role of TSN in regulating E2F1-dependent transcription, it has not explored the possible roles that E2F1 may play in the other known biological functions of TSN. It was recently demonstrated in the La Thangue lab that TSN enabled E2F1 binding to several RNA species (Roworth, unpublished). As such, there was reasonable evidence to believe that E2F1 may play a role in one or more of the RNA processing activities of TSN. Particularly, TSN plays a role in splicing through its interaction with small nuclear ribonucleoproteins (snRNPs) and several Sm proteins which facilitate spliceosome complex formation. It was hypothesized that TSN binding to E2F1 through its SDMA mark could facilitate the splicing of various E2F1 target genes. Indeed, current studies in the La Thangue lab have shown TSN-dependent recruitment of E2F1 to p73 and BCLX mRNA in U2OS and MCF7 cells, as well as concomitant changes in the expression of their respective splice

variants (Roworth and Kaya, unpublished). These effects on splicing have been observed to occur despite the lack of TSN recruitment to the promoters of these genes (Zheng et al. 2013).

Splice variation can account for the translation of functionally different isoforms of the same gene, as in the case with BCLX and p73 (Boise et al. 1993; Di et al. 2013; Logotheti et al. 2013). It may also cause differences in RNA stability which can lead to non-sense mediated decay or shorter mRNA half-life (Hamer & Leder 1979; Yap & Makeyev 2016). As such, alternative splicing constitutes another means by which the expression of genes may be regulated. This raised the question of whether the candidate genes chosen in this study could potentially be regulated by E2F1-mediated TSN splicing activity.

KLF2, MDC1 and SKP2 all possess predicted and documented transcript variants in the Ensembl database (Aken et al. 2016). To assess the extent of alternative splicing in these genes, the expression of a few selected primary functional protein coding transcripts, isoform coding transcript variants, and transcripts which are processed, but remain non-coding was assessed.

In this study, qRT-PCR was used to measure the expression of the different gene transcripts using primers specifically designed to amplify only one transcript variant. The design process was aided by exon and intron sequences annotated in the Ensembl database (Aken et al. 2016). The nature of qPCR and differing primer annealing efficiency limits analysis which compares expression between two different primer sets. To better assess the amplification data obtained, the total transcript expression levels were first normalised to actin. In an independent analysis, individual transcript variants were then normalised to the raw total expression levels. As such, total expression relative to actin, and individual transcript expression relative to the total have been reported separately. It is

important to note that comparisons can only be drawn within individual primer sets, between various knockdown treatments. Consequently, a higher fold change observed in one individual transcript compared to another does not necessarily indicate that it is expressed in higher amounts.

Transcript expression was measured in cells which exhibited both TSN and TDRD1 knockdown. Only TSN has been reported to play a role in pre-mRNA splicing, whereas TDRD1 is not yet known to have functions in this pathway. As such, to limit the analysis, only transcripts which exhibited TSN-specific deregulation were considered for further confirmatory analysis involving E2F1.

Of the transcripts assessed, KLF2_001, SKP2_001, and SKP2_002 showed deregulation only when TSN was knocked down. KLF2_001 constitutes the primary protein-coding transcript of the documented tumour suppressor KLF2. Thus, its up-regulation is consistent with the associated TSN knockdown phenotype.

In contrast, SKP2_001 and SKP2_002 are both transcripts documented to express oncogenic isoforms of SKP2. Indeed, both transcripts are overexpressed in breast cancer cell lines (Radke et al. 2005). The primary SKP2_002 transcript encodes for the SKP2 E3 ubiquitin ligase, which is responsible for the ubiquitin dependent repression of p53. SKP2_001, on the other hand, encodes for SKP2B, a cytosolic isoform with an altered C-terminus. SKP2B has been implicated in the degradation of prohibitin, and thus impinges on the regulation of the pRB pathway (Germain 2011; Foster et al. 2003). Their up-regulation is thus counter-intuitive to the established pro-apoptotic phenotype associated with TSN knockdown. Notably, however, total SKP2 levels remain decreased upon TSN knockdown. It is possible that the up-regulation of the two functional transcripts serve as a compensatory effect to drive expression of functional protein given the decreased total expression levels.

To confirm if these effects may potentially be indicative of a role for E2F1 in TSN-associate splice activity, the transcript levels of KLF2_001, SKP2_001, and SKP2_002 were measured following knockdown of E2F1. Notably, the knockdown of E2F1 resulted in similar deregulation to that observed upon TSN depletion. Moreover, the same deregulation was achieved when E2F1 and TSN were co-depleted. This highly suggests that E2F1 and TSN work in concert to maintain the transcript levels of these genes.

Further confirmatory experiments are required to confirm the cooperative interaction of TSN and E2F1 to bring about alternative splicing. Namely, RNA immunoprecipitation of TSN and E2F1 on the respective transcripts needs to be measured. The results obtained from this study are thus merely an initial indication of possible RNA metabolic functions of E2F1. Further work is currently being done on the role of E2F1 in TSN-associated splicing within the lab, and will likely be better able to establish this non-canonical role of E2F1.

Results showing altered expression of individual transcript variants could potentially account for differences RNA stability and ultimately, total protein expression levels of various genes. At the same time, details into preferential transcript expression that are not readily revealed by measuring total transcript levels of a gene can be assessed, and give insight into the ultimate functional consequence of the measured expression.

Ultimately, measuring the protein levels of the coded isoforms which result from splice variant translation presents a more functional assessment of alternative splicing. The assessment of protein isoform expression via Western blot, for example, can better demonstrate the regulatory roles that TSN and E2F1 may play in determining the outcome of alternative splicing and maintaining proliferative versus apoptotic effects. Thus, if

E2F1 does play a role in alternative splicing, this indicates yet another regulatory mechanism which can determine its overall biological output.

Chapter 5 : Discussion

5.1 TSN and TDRD1 as points of study

Prior to this study, it was established that the arginine methylation status of E2F1 could potentially mediate its opposing functions in cellular proliferation and apoptosis. The identification of mutually exclusive symmetric and asymmetric arginine dimethylation marks on E2F1, and the proliferative and apoptotic effects associated with each of them was a novel piece of insight into how the functional dichotomy of E2F1 was mediated (Cho et al. 2012; Zheng et al. 2013).

PRMT1 and PRMT5 were found to be responsible for the deposition of the ADMA and SDMA marks on E2F1 respectively, and initially they represented attractive points of interest to better isolate the molecular mechanism behind opposing E2F1 functions. However, as established post-translational modifiers, PRMT1 and PRMT5 possess extensive roles in transcriptional and nuclear receptor co-activation, modulation of histone methylation, nucleocytoplasmic shuttling, and signal transduction (Blanc & Richard 2017). As such, the isolated study of E2F1 arginine dimethylation-mediated activity by depleting PRMT1 and PRMT5 could be confounded by the deregulation of a variety unrelated pathways. Moreover, knockdown of PRMTs may be lethal and cause extensive cell death.

Further study has since identified two readers of the proliferative SDMA mark: TSN and TDRD1. As readers of the E2F1 dimethylation mark, they constitute a functional bridge between the post-translational modification of E2F1 and its resulting biological function. As such, the absence of TSN or TDRD1 mimics the absence of the SDMA mark itself. Illustrating this theoretical connection, TSN depletion, like PRMT5 depletion, increases E2F1 half-life and associated apoptotic markers (Zheng et al. 2013). It was hypothesised that depletion of TDRD1, yet another reader of the SDMA mark,

would have similar effects. Thus, TSN and TDRD1 lend themselves toward the exploration of E2F1-dependent biological effects.

Moreover, TSN and TDRD1 are overexpressed in several cancers, supporting their role in a proliferative phenotype (Jariwal et al. 2015; Boormans et al. 2013). This additionally stresses the importance of understanding their E2F1-dependent functions in the context of human malignancy. Indeed, to implicate TSN and TDRD1 as potential targets for treatment in cancer, a better understanding of their independent and E2F1-dependent roles must be reached. The need for two readers, of the SDMA mark, for example, may allude to mutually exclusive pathways of action, or protective functional redundancy to promote an oncogenic phenotype. As such, the roles of TSN and TDRD1 as readers of a proliferative post-translational modification on E2F1 are a relevant and interesting area of research. This study thus aimed to explore the E2F1-dependent effects of TSN and TDRD1 on target mRNA expression in order to expand our knowledge on how the proliferative effects of SDMA on E2F1 are mediated.

5.2 Roles for TSN and TDRD1 in E2F1-dependent transcriptional regulation

The analysis of transcriptional effects upon depletion of TSN or TDRD1 provided a straightforward approach to elucidating their role in E2F1-mediated proliferative effects. Guided by an initial RNA sequencing screen, the deregulated expression of several now confirmed E2F1 target genes was validated by qRT-PCR analysis. Several of these genes possess proliferative functions, while others function in pro-apoptotic pathways (Table 3.2). Specifically, the expression of several genes which possessed documented tumour suppressive activity was enhanced upon TSN or TDRD1 knockdown, suggesting that these genes are suppressed by TSN or TDRD1 to perhaps mediate a proliferative phenotype. Further supporting a proliferative role for TSN and

TDRD1, the expression of genes with oncogenic capacity was repressed upon their depletion.

The co-depletion of E2F1 with TSN and TDRD1 highlighted E2F1-dependent effects on candidate gene expression. In MCF7 and SKBR3 cells, CDC6 served as a prototypic example to explain the observed up-regulation upon TSN or TDRD1 knockdown and its subsequent loss after E2F1 co-depletion. ChIP experiments confirmed changes in direct binding of E2F1 to the CDC6 promoter in response to reader depletion, suggesting direct roles for E2F1 in transcriptional regulation of these genes in concert with TSN and TDRD1. Contextualising this data with the finding of E2F1-dependent recruitment of TSN to their promoters, it is possible that TSN binding to promoters could be facilitated by the E2F1 SDMA mark. This binding could then facilitate context-dependent limitation of normal E2F1 transcriptional activity. In addition to CDC6, several of the genes tested (MDC1, TCF19, ZNF367) displayed similar regulatory responses to E2F1-reader co-depletion, suggesting the same regulatory mechanism governing their expression in MCF7 and SKBR3 cells, although further confirmatory ChIP is necessary.

In the regulation of KLF2, TDRD1 and TSN were suggested to play repressive roles in cooperation with E2F1, as both individual reader knockdown, as well as E2F1 knockdown result in increased KLF2 expression levels. ChIP experiments similarly supported the role of TSN in stabilising E2F1-associated repression in the KLF2 promoter. The absence of TSN promoted increased E2F1 binding to the KLF2 promoter, perhaps as a compensatory mechanism for maintaining repressive regulation. Furthermore, it was shown that TDRD1 had little impact on E2F1 ChIP activity, and thus may exert its regulatory effects in a manner independent of E2F1 function. While these effects are more likely to be indirect effects of E2F1 through its transactivation of other

regulatory proteins, or unrelated effects of independent reader function, studies have shown that E2F1 is able to exert repressive effects in a direct manner when bound to RB, by recruiting chromatin remodelling complexes to facilitate the silencing of gene expression (Wolffe et al. 2000). TSN and TDRD1 may somehow influence the repressive capacity of E2F1 and potentially help mediate more efficient silencing of this genetic locus, independently or cooperatively.

Interestingly, the ChIP analysis also brought to light possible E2F1-independent effects of TSN. Results showed increased recruitment of E2F1 to the SKP2 promoter in the absence of TSN. Moreover, E2F1-independent recruitment of TSN to the SKP2 promoter was also observed. This made it apparent that the regulatory effects of TSN on SKP2 observed upon its depletion could instead be mediated by its individual functions, rather than through cooperative co-activator effects with E2F1. It was proposed that STAT-mediated recruitment could account for TSN presence on the SKP2 promoter, and that this could similarly enhance SKP2 expression as an additive effect to E2F1-mediated transactivation (Furuhata et al. 2009; Huang et al. 2012).

Given the additional information provided by the data obtained in this study, a more detailed understanding of the roles that TSN and TDRD1 play as readers of the proliferative SDMA mark of E2F1 has been attained. Indeed, E2F1 exerts its biological outcome primarily through its regulation of the expression of a variety of target genes. Examining TSN and TDRD1 in the context of E2F1 function has revealed selective suppression of certain pro-apoptotic and anti-proliferative genes, while contributing to the expression of genes with proliferative effects and oncogenic capacity (Table 3.1, 3.2). It has previously been reported, for example, that TSN is able to bind to the promoters of E2F1, cyclin E, CDC6 and DHFR, all genes associated with proliferative signalling, but not to the promoters of p73 and APAF-1, which are associated with apoptosis. Moreover,

the ChIP activity observed on the promoters of proliferative genes was abrogated after depletion of PRMT5 or E2F1 (Zheng et al., 2013). This constitutes an acting model for the molecular mechanism that brings about the proliferative phenotype promoted by the E2F1 SDMA mark, wherein the SDMA mark biases E2F1 toward the activation of pro-proliferative genes and suppression of pro-apoptotic genes. The proposed hypothetical regulation of the genes chosen in this study both support this functional model, but also hints at deeper underlying complexity in the biological effects of E2F1 symmetric arginine dimethylation.

In the cellular context of oestrogen receptor-positive breast cancer from which the MCF7 cell line is derived, MDC1 acts as tumour suppressor by enhancing oestrogen-receptor mediated transactivation, and its depletion promotes proliferative and oncogenic properties of breast cancer. (Bartkova et al. 2007; Zou et al. 2015). Additionally, MDC1, as part of the DNA damage checkpoint machinery, prevents chromosome instability and maintains genetic integrity. It furthermore stands at the crossroad between cell survival and induced cell death in response to DNA damage through its regulation of p53 (Stucki & Jackson 2004; Nakanishi et al. 2007; Yuan et al. 2012; Ozaki et al. 2015).

KLF2 is also an established tumour suppressor that is silenced in several human malignancies (Black et al. 2001; Taniguchi et al. 2012). Its roles in inducing cell quiescence, enhancing DNA damage-induced apoptosis, anti-angiogenesis, and KRAS/EGF4-mediated growth suppression make it a prime target for silencing to promote sustained proliferation (Black et al. 2001; Wu & Lingrel 2004; Bhattacharya et al. 2005; Kannan-Thulasiraman et al. 2010; Fernandez-Zapico et al. 2011).

In contrast, SKP2 is an established oncogene whose inactivation restricts cancer progression (Gstaiger et al. 2001; Signoretti et al. 2002; Hershko 2008; Kitagawa et al. 2008). As such, repression of the tumour suppressive roles of KLF2 and MDC1, and

enhancement of SKP2 expression by the concerted activity of TSN, TDRD1 and E2F1 support SDMA as a post-translational mark biasing E2F1 toward proliferation.

On the other hand, CDC6, though known to have tumour suppressive properties in prostate cancer (Robles et al. 2002; Mallik et al. 2008; Jin & Fondell 2009), largely acts as an oncogenic marker in breast cancer, contributing to poor prognosis and increased cellular proliferation (Kornegoor et al. 2012; Booher et al. 2012). As it stands, the regulation of KLF2, MDC1 and CDC6 in the proposed model supresses their expression when the SDMA mark persists and is read by TDRD1 or TSN. The repression of a tumour suppressors like MDC1 and KLF2 supports the proliferative outcome which is associated with the symmetric dimethylation mark of E2F1, however, the suppression of an oncogene like CDC6 suggests deeper underlying complexity. Perhaps, in some cellular contexts, the repression of CDC6 sustains a more proliferative phenotype as it does in prostate cancer, but further study is required to understand the seemingly contradictory regulation of this gene by E2F1 and TSN. It is likely that the role of TSN and TDRD1 in determining the biological outcome of E2F1-related activity runs deeper than mere direct effects on transcriptional activity, but also constitutes complex indirect effects mediated by the downstream activities and altered expression of E2F1 target genes. Indeed, there is much we have yet to discover about the intricacies of what determines the predominant biological function of E2F1.

Despite this, the sum of TSN- and TDRD1- associated regulation of E2F1 target genes contributes to the proliferative phenotype associated with the SDMA mark (Figure 5.1). By and large, there is evidence of redundant functions for TSN and TDRD1 in this regard. The pathways and mechanisms which are employed by each reader to achieve this common regulatory activity, however, are likely to differ from each other. This is likely due to their molecular structure, known biological activity and demonstrated recruitment

to the target genes assessed. This study has uncovered potential roles of TSN and TDRD1 in stabilising the repressive direct or indirect activity of E2F1 by a yet unknown mechanism, enhancing E2F1 transcriptional activity, and alternative splicing are al. This is demonstrated by the measured expression levels of the candidate genes assessed in this study, as well as the regulation of their functional and non-functional transcript variants. The expression patterns of the genes assessed, as well as the cellular context from which they were taken all support the proliferative functions of E2F1 associated with the SDMA mark.

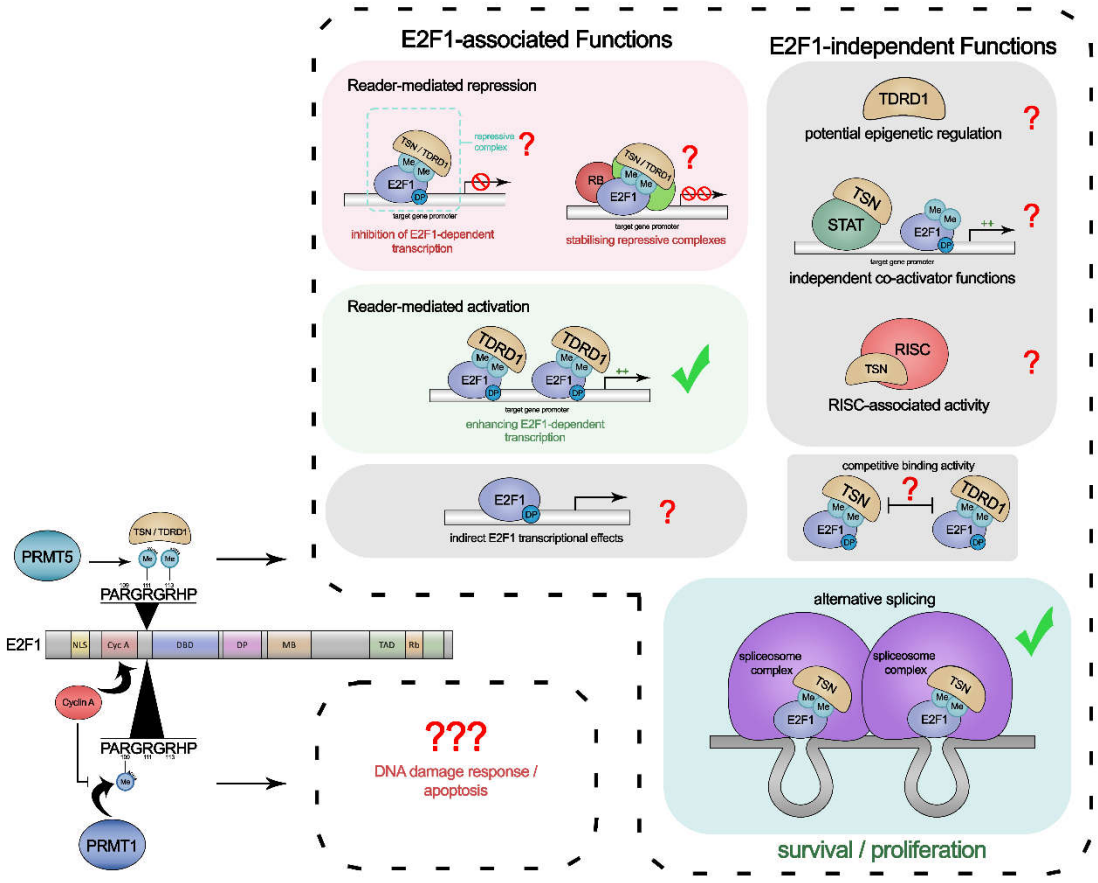


Figure 5.1 Model: Roles of TSN and TDRD1 in mediating the SDMA-associated E2F1 proliferative phenotype.
TSN and TDRD1 have been established to participate in gene regulation through a variety of E2F1-dependent and -independent activities. TSN and TDRD1 may thus regulate E2F1 target genes in cooperation with E2F1 through the formation and stabilisation of repressive complexes, and the enhancement of E2F1 transcriptional activity. Indirect E2F1 transcriptional effects, individual co-activator functions and RNA processing activities, as well as potential epigenetic regulation may also account for regulatory functions observed. Furthermore, E2F1 has been implicated in TSN-associated alternative splicing. This presents a potential mechanistic model for mediating the E2F1 proliferative phenotype. Mechanisms that have been confirmed by the study are marked with checks whereas speculated mechanisms are annotated with question marks.

5.3 Alternative splicing analysis reveals roles for E2F1 in TSN splicing activity

In addition to demonstrating roles for TSN and TDRD1 in the regulation of the transcriptional activity of E2F1, this study also revealed potential roles for E2F1 in TSN-mediated splicing. Recent findings in the La Thangue laboratory have demonstrated TSN-dependent recruitment of E2F1 to p73 and BCLX mRNA (Roworth and Kaya, unpublished). Given this reported activity, the alternative splicing of the candidate genes chosen was explored.

Interestingly, certain transcript variants of KLF2 and SKP2 showed TSN-specific deregulation of transcript expression, and these were further confirmed to be E2F1-dependent. Alternative splicing, with its ability to influence RNA stability, RNA half-life and protein isoform translation, may thus constitute another regulatory mechanism by which E2F1 could carry out its opposing biological functions, independent of its transcriptional functions. The further implication that this potential role for E2F1 in RNA processing is mediated by TSN, an established reader of its symmetric dimethylation mark, is another area worth further consideration in future studies.

5.4 Limitations of the study

The sum of the experiments conducted thus far give compelling evidence for regulatory roles of TSN and TDRD1 in E2F1-dependent transcriptional output, as well as novel E2F1 functions in pre-mRNA splicing. However, the conclusions that can be made based on the data obtained from this study alone are limited. While speculations for the mechanistic roles for TSN and TDRD1 can be formulated from the results obtained, further supplementary studies and more meticulous methods of assessment are necessary to confirm its accuracy. Additionally, these experiments were conducted in established cancer cell lines which have likely sustained severe genetic alteration, and which possess multiple deregulated pathways. As such, the results obtained from these studies do not

constitute physiological functions in normal cells. It would be just as informative to explore these mechanisms in normal cells to understand how they may contribute to oncogenesis and transformation.

It is also necessary to qualify that the experiments conducted in this study were done in the context of RNAi-mediated gene silencing. Incomplete knockdown, off-target effects, and the polyclonal nature of the cells used, all limit the results obtained from these experiments (Boettcher & Mcmanus 2015). The employment of CRISPR-Cas9 systems and the use of monoclonal cell lines could potentially yield more pronounced and accurate effects on E2F1 target genes. The La Thangue laboratory is currently in the process of generating TSN- and TDRD1-CRISPR-knockouts in MCF7, SKBR3, and DuCaP cells to better supplement these findings.

The failure of the attempted TDRD1 ChIP also constitutes a severe limitation in the conclusions that can be made concerning its role in transcriptional regulation. There was reasonable evidence to believe that the antibody used in this procedure displayed non-specific binding to other protein species. As such, continued optimisation, as well as the use of better antibodies may be implemented to successfully ChIP TDRD1 to target promoters. Moving forward, information on the E2F1-dependent binding of TDRD1 to promoters can provide more detail to the proposed model, and perhaps confirm competitive mechanisms between TDRD1 and TSN for E2F1 binding and recruitment.

5.5 Recommendations

Further confirmatory experiments may help verify the accuracy of the proposed model for TSN and TDRD1 function. As mentioned earlier, successful TDRD1 ChIP is essential to better grasp whether the regulatory role it plays is E2F1 dependent, and whether TDRD1 can be recruited to the promoters of E2F1 target genes. Additionally, the sequential chromatin immunoprecipitation of readers and E2F1 can confirm possible

co-recruitment and complex interaction at the promoters of target genes. Furthermore, co-immunoprecipitation experiments of TSN and TDRD1 with E2F1 upon knockdown of either reader may potentially verify whether their recognition of the SDMA mark is mutually exclusive.

With regard to the potential role of E2F1 in TSN-associated splicing activity, individual and sequential RNA immunoprecipitation of TSN and E2F1 on target mRNA can confirm their involvement in the processing of various alternative splice variants. Additionally, small molecule inhibition of RISC loading may supplement experimental findings, discounting E2F1-independent roles of TSN in RNA interference via the RISC (Tan et al. 2012).

The scope of the study can be further extended in terms of the cellular conditions tested. For example, the results obtained upon reader and E2F1 knockdown may further be supplemented by results from conducting corresponding rescue experiments involving inducible overexpression of either reader or introduction of exogenous reader protein. Furthermore, as TSN and TDRD1 have been shown to play largely redundant roles in the ultimate expression of E2F1 target genes, the effects of a double TSN/TDRD1 knockdown or knockout can be explored. Double-knockdown treatments remove the possibility of buffering roles for one reader in the absence of the other, and may give new insights into the extent of their biological redundancy in the context of E2F1 function.

While the role of TDRD1 has been demonstrated to be redundant to that of TSN thus far, the observation that TDRD1 is only expressed in the soma in contexts of hormone sensitive cancer hints at more prominent effects in response to hormone depletion or stimulation (Boormans et al. 2013). Indeed, previous research conducted in the laboratory has demonstrated increased apoptosis upon hormone depletion when TDRD1 is knocked down in MCF7 and DuCaP cells, but not when TSN is depleted

(Chan, unpublished). Thus, more conclusive data regarding TDRD1-specific functions may be obtained under additional hormone stimulation or deprivation. Furthermore, the suggested role of TDRD1 in chromatin remodelling and gene silencing presents a potential point of interest.

With regard to the analysis of E2F1-associated TSN effects, experiments can be conducted using small molecule treatments that inhibit RISC loading and spliceosome assembly to minimise the confounding E2F1-independent effects of TSN in RNA metabolism (Tan et al. 2012; Bonnal et al. 2012).

Moreover, the availability of various E2F1 cell lines possessing mutations in Arg111 and 113, the sites of deposition for the SDMA mark, constitutes another perspective for testing effects on E2F1 transcriptional readout (Zheng et al. 2013). These mutants functionally mimic the loss of the readers in a more confined context which limit artefactual deregulation that results from reader (TSN/TDRD1) or writer (PRMT1/5) knockdown. Thus, the use of these cell lines can better confirm if the effects on the target genes selected are directly mediated by the E2F1 SDMA mark through any of the models proposed.

Global effects may also be assessed by repeating the RNA sequencing analysis initially conducted, using monoclonal CRISPR cell lines with complete TSN or TDRD1 knockout. Furthermore, proper controls and triplicate samples should be prepared and treated to enrich for poly-A species, and to minimise ribosomal RNA contamination.

Ultimately, it is necessary to confirm whether the deregulation of the candidate genes at the mRNA level result in measurable changes in protein expression and effects on biological assays. At the same time, assessment of known functional isoforms of the proteins using specific antibodies would be a better indicator of the ultimate functional effects of TSN and TDRD1 knockdown on E2F1-associated effects.

5.6 Conclusions and Implications - Understanding E2F1 in the context of arginine methylation and cancer

PRMT5 has been implicated in various aspects of tumorigenesis and is upregulated in several human malignancies, cancer cell lines and patient samples (Chan-Penebre et al. 2015). The mechanisms of PRMT5 cell-transformation have yet to be fully clarified, though its physiological roles extend heavily into cell-death, cell cycle progression, growth, and proliferation via its methylation of various target substrates (Karkhanis et al. 2011). Indeed, arginine methylation is involved in numerous cellular pathways. The interplay of PRMTs with their regulatory elements and substrates affect the arginine methylation landscape in a robust but tightly controlled manner. It is becoming increasingly apparent that PRMTs, as well their respective demethylases are targets for oncogenic expression in various cancers, despite the lack of genetic mutations detectible in malignancies (Yang & Bedford 2013; Poulard et al. 2016). Multiple clues have led researchers to believe that PRMTs and their post translational modifications play substantial roles in mediating enzyme-substrate switching, signal transduction, miRNA regulation, subcellular compartmentalisation, gene transcription and mRNA splicing – all of which can additively affect tumorigenic properties of cancer. Identifying specific pathways involved in each of the functional branches of arginine methylation in cancer improves our understanding of how it can be better targeted for treatment.

This study further implicates the E2F1 pathway as a target of arginine methylation which plays a significant role in promoting various tumorigenic properties. Specifically, this study has contributed to our understanding of how E2F1 is able to mediate proliferative branch of its opposing effects through symmetric arginine dimethylation deposited by PRMT5. This further expands our knowledge of how PRMT5 inhibitors can be specifically administered a to limit the proliferative effects of E2F1 in particular.

Zooming in on the proliferative functions of E2F1 and determining what dictates their manifestation also allows for a better understanding of E2F1 as an oncogene or tumour suppressor itself. Several factors involved in the E2F1 proliferative pathway may all constitute potential targets for cancer treatment, but only if the tumour suppressive properties of E2F1 are kept intact. This study forms part of the foundation for the potential clinical use of several already developed PRMT5 inhibitors to specifically target the proliferative activity of E2F1, potentially allowing the use of such treatments in combination with drugs that target other cancer pathways. In fact, E2F1-dependent effects of PRMT5 inhibitors are currently being studied in the La Thangue lab (Jin, unpublished). Further studies to better elucidate the details of the involvement of TSN and TDRD1 in E2F1 proliferative effects may thus be able to implicate them as candidates for targeted cancer treatment downstream of the arginine methylation mark, improving the repertoire of targeted cancer treatments.

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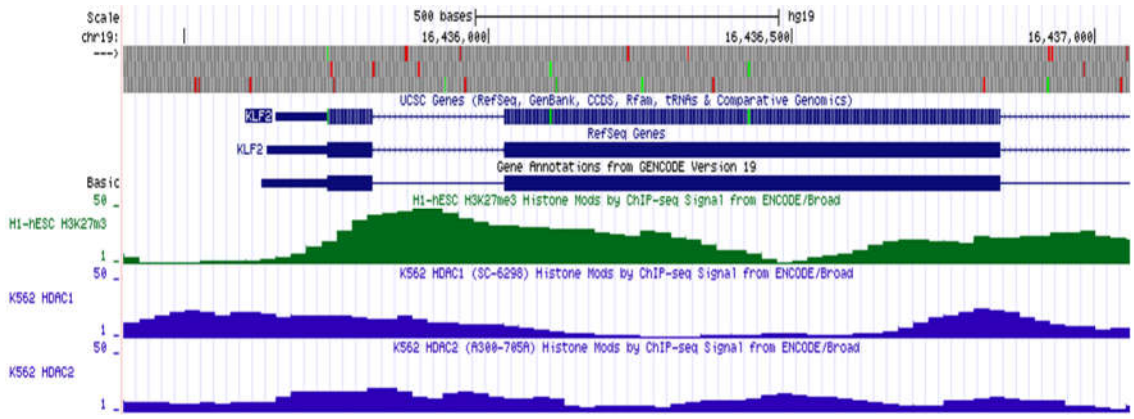
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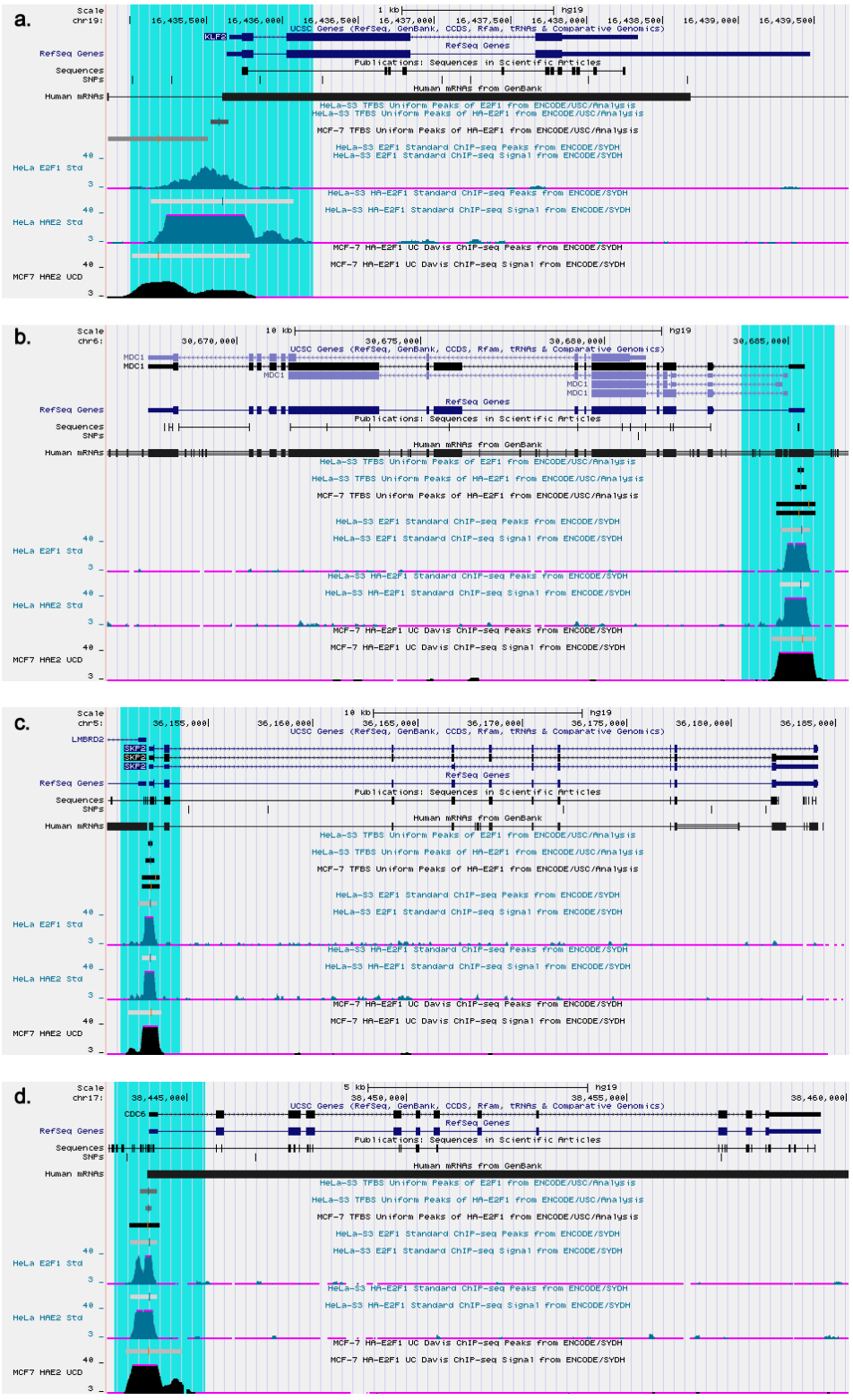
functional genomics in human cells. *Nature*, 509(7501), pp.487–491. Available at: http://dx.doi.org/10.1038/nature13166.

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Appendix A. UCSC Genome Browser Tracks

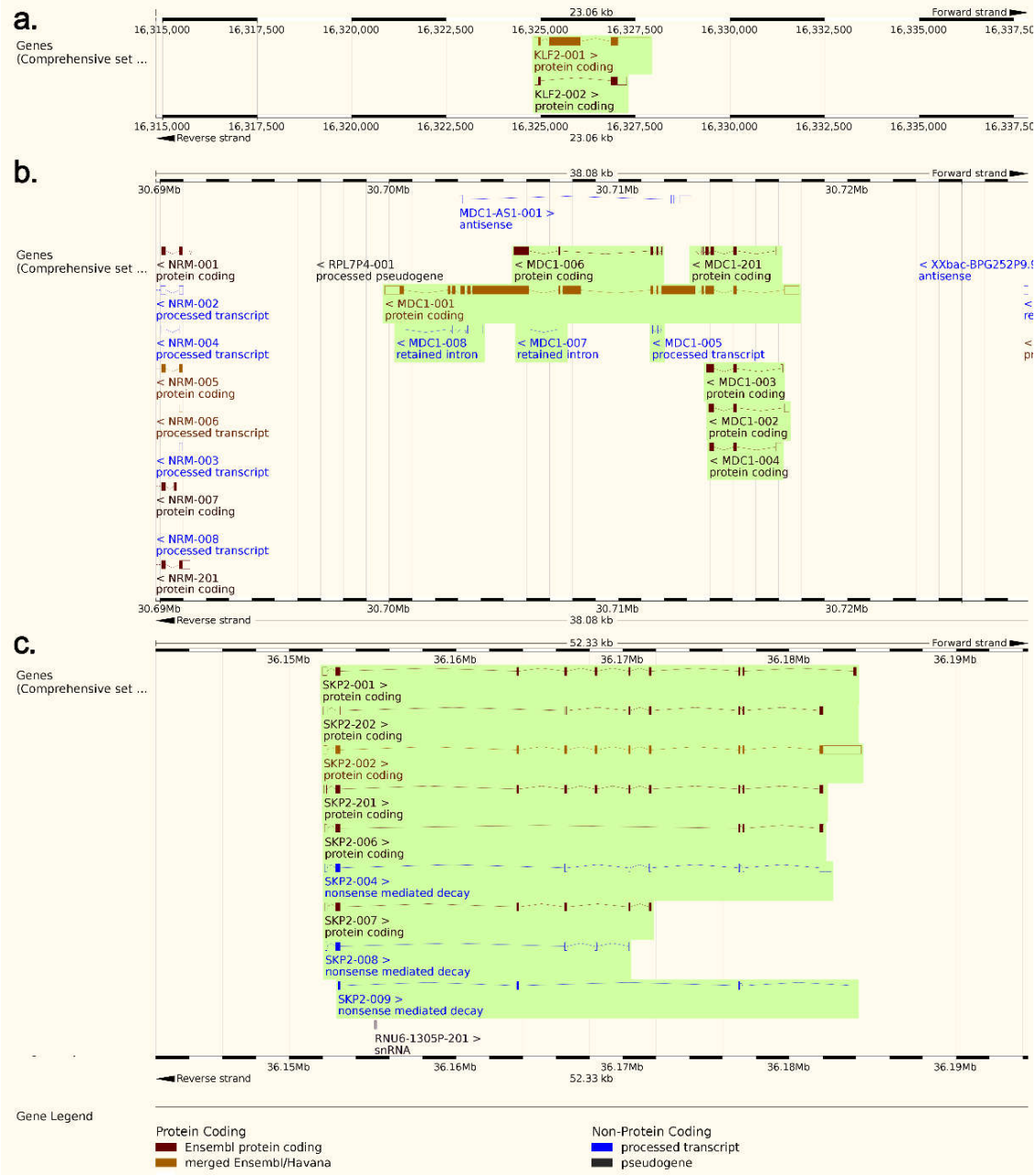


Appendix A1. Repressive histone marks and HDAC recruitment to the KLF2 promoter.
ChIP data acquired from the ENCODE/Broad Institute Release 3 show increased H3K27me3 inactivation marks on the KLF2 gene, as well as recruitment of HDACs on the KLF2 promoter in different cell lines (Rosenbloom et al. 2013).



Appendix A2. E2F1 ChIP peaks on target gene promoters.
Predicted areas of E2F1 binding on the promoters of (a) KLF2, (b) MDC1, (c) SKP2 and (d) CDC6 are highlighted in teal based off publicly available ENCODE data (Rosenbloom et al. 2013).

Appendix B. Encode transcript variant tables



Appendix B1. Candidate gene Ensembl transcript tables.
Predicted and documented transcript variants of (a) KLF2, (b) MDC1, and (c) SKP2 accessed from Ensembl release 88, genome assembly GRCh38.p10. Red and yellow variants represent predicted and document protein-coding transcripts; Blue indicates processed transcripts with no documented protein products; Black indicates processed pseudogenes (Aken et al. 2016).