IDENTIFICATION AND FUNCTIONAL CHARACTERISATION OF NOVEL APC/C INTERACTING PROTEINS

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A thesis submitted to the

University of Birmingham

for the degree of

DOCTOR OF PHILOSOPHY

School of Cancer Sciences

CRUK Institute for Cancer Studies

The Medical School

University of Birmingham

April 2009

ABSTRACT

The anaphase promoting complex /cyclosome (APC/C) is a multi-protein E3 ubiquitin ligase complex that regulates cellular proliferation through its ability to target essential cell cycle regulators such as cyclin A, cyclin B, securin and S-phase kinase-associated protein 2 (SKP2) for proteasomal-dependent degradation. APC/C substrates and coactivators are aberrantly expressed in many cancers. It is thought that the APC/C can also regulate cellular proliferation by controlling p21 and cell division cycle 6 homologue (CDC6) transcription. It is therefore of great interest to study other cellular proteins that interact with the APC/C as these proteins may also be important in cell cycle regulation and hence may enhance our understanding of the molecular basis of cancer. Therefore, the aim of my study was to identify novel APC/C interacting proteins through mass spectrometric analysis of APC/C subunit 7 (APC7) immunoprecipitates and go on to examine the functional significance of these interactions.

I identified six novel APC7 interacting proteins, and decided to focus on two of these interactions. Initially I examined the interaction between APC7 and transcriptional intermediary factor 1γ (TIF1γ), a transcriptional repressor previously reported to be aberrantly expressed in cancer. Data presented in this study demonstrate that the APC/C and TIF1γ cooperate to regulate mitotic progression. Notably TIF1γ displays *in vivo* interactions with APC/C subunits 1-8, the APC/C's mitotic coactivator CDC20 and mitotic substrate cyclin A. Moreover, TIF1γ depletion by RNAi arrests cells in a metaphase-like state characterised by elevated levels of APC/C substrates, cyclin A, cyclin B and CDC20. In support of a role for TIF1γ in regulating mitotic progression by directly targeting the APC/C, cells treated with TIF1γ-specific siRNA exhibit reduced APC/C E3 ubiquitin ligase activity. This work defines TIF1γ as a novel mitotic regulatory protein essential for APC/C function and suggests that loss of TIF1γ may compromise genomic integrity by allowing the mis-segregation of chromosomes.

I also investigated the functional relationship between APC7 and the nuclear factor 90/45 heterodimer (NF90/NF45), given that all of these proteins function as transcription factors. Results obtained demonstrate that APC5 and APC7 bind directly to NF90 *in vitro* and also form complexes with NF90 *in vivo*. It appears that APC/C interaction with NF90 is important in the regulation of interleukin-2 (IL-2) and tumour

neucrosis factor (TNF) transcription, as exogenous APC5 and APC7 cooperate with NF90/NF45 to transactivate IL-2 and TNF promoter constructs. Lastly endogenous APC5 and APC7 bind to the IL-2 promoter *in vivo* while endogenous APC5 represses TNF transcription *in vivo*. Given that TNF and IL-2 stimulate cellular proliferation data presented here suggests that the APC/C might control cellular proliferation by regulating TNF and IL-2 transcription.

AKNOWLEDGEMENTS

I would firstly like to thank my supervisors, Andy Turnell and Roger Grand, for their perseverance and help over my many years at Birmingham. Having known them for almost a third of my life they seem to have become almost a surrogate family complete with tantrums and arguments, but also many highlights. I have learnt a great deal from Andy who has always been bristling with lots of good ideas, excellent technical advice and an encyclopaedic knowledge of all things APC/C. Roger has been extremely kind and helpful throughout my time here, lending tools and even money to go on a date with, he has also been a constant source of knowledge from science to the arts right through to details of obscure historical figures.

I would also like to thank past and present members of my lab and the institute. In particular I would like to thank Rachel Bruton for her friendship, advice and occasional nagging (for my own good of course). A special mention should also go to Andy Blackford, my link to the aristocracy, who has provided me with practical help, was a source of constant enthusiasm and just a thoroughly decent co-worker and friend, all in all his Belgium finishing school did a fine job. Then there is Grant, yet to master the intricacies of appropriate after shave application, who has first and foremost been an extremely good friend and just good fun to be around. I would also like to thank him for indulging my wilder scientific fantasies, providing me with encouragement and insight, and not complaining too much about me taxing his reagents; Grant's group have always been helpful and stimulating company, in particular Kelly provided me with lots of technical advice and a constant stream of interesting hair cuts. In addition I would like to thank Phil Byrd for his willingness to offer help and advice and always being a pleasure to be around. I am also grateful to the present crop of students in the lab who have greatly enhanced my life since arriving and are a much welcome daily distraction.

I would also like to thank my footballing pals who have also given me many years of comradeship, provided me with an escape from the realities of PhD life and most importantly have put up with my wayward shooting skills. A special mention should go to Si Vass who aside from being an excellent house mate and friend provided me with constructive criticism for parts of this manuscript, and also Shimmers who I am

extremely grateful to for always being a good mate, providing me with lots of amusement and help with the mass spec.

I am grateful for the financial support given by CRUK. Some of the work in this thesis could not have been carried out without kind gifts of reagents from Peter N Kao, Sirio Dupont, Stefano Picollo, and Frank Raucher

I would like to thank my parents for their support and kindness over the years and letting me follow my own path. Lastly I am tremendously appreciative of Lisa who has had to put up with many weekends and evenings without the pleasure of my company during the process of writing this thesis, we will have plenty of time in the future to spend together!

“We talk about it for twenty minutes and then we decide I was right.”

On dealing with a player who disagrees.

Brian Clough

“I have not failed. I've just found 10,000 ways that won't work.”

Thomas Edison

“The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact.”

Thomas Huxley

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LIST OF ABBREVIATIONS

Δ	denotes removal of protein sequence
Ad VA RNAII	adenovirus viral associated RNAII
ADAR1	adenosine deaminase acting on RNA
AP-1	activator protein-1
APC/C	anaphase promoting complex/cyclosome
APL	acute promyelocytic leukemia
APS	ammonium persulfate solution
ARRE-2	antigen receptor response element-2
ATF4	activating transcription factor 4
ATP	adenosine triphosphate
ATTC	American Tissue Culture Collection
BAX	BCL2 –associated X
BCL2	B-cell lymphoma 2
BSA	bovine serum albumin
C-terminal	carboxy-terminal
CAK	CDK activating kinase
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CDC	cell division cycle
CDH1	CDC20-homologue protein 1
CDK	cyclin-dependent kinase
CDT1	CDC10 –dependent transcript 1
CENP-F	centromere protein F

CEP290	centrosomal protein 290kDa
ChIP	chromatin immunoprecipitation
CHOP	CCAAT enhancer binding homologous protein
CIN8p	chromosome instability 8p
CRE	cAMP response element
CREB	CRE binding protein
CsA	cyclosporin A
CtBP1	C-terminal binding protein 1
D-box	destruction box
DAPI	4’,6-diamidino-2-phenylindole
DMEM-Hepes	Dulbecco’s Modified Eagles Hepes buffered Medium
DMSO	dimethyl-sulphoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA protein kinase
$DNA\text{-}PK_{cs}$	catalytic subunit of DNA-PK
dNTP	deoxynucleotide triphosphate
DP-1	dimerisation partner 1
DSB	double strand break
dsRBM	double stranded RNA binding motif
dsRNA	double stranded ribonucleic acid
DUBs	deubiquitinating enzymes
DZF	dsRBM and zinc finger associated motif
E. coli	*Escherichia coli*
E1A	adenovirus early region 1A
E3ORF3	adenovirus early region 3 open reading frame 3

ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eEF1A	eukaryotic elongation factor 1A
EMI1	early mitotic inhibitor 1
ER	estrogen receptor
ERG	E26 oncogene homolog
ESI	electrospray ioniser
FCS	foetal calf serum
FITC	fluorerscein isothiocyanate
GST	glutathione S-transferase
GTF	general transcription factor
GTP	guanosine triphosphate
HA	hemagglutinin
HAT	histone acetyl transferase
HEF1	enhancer of filamentation 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hnRNP P2	heterogeneous ribonucleoprotein P2
HOXC10	homeobox C10
HP1	heterchromatin protein 1
HPLC	high-performance liquid chromatography
HPV	human papilloma virus
IL-2	interleukin-2
IL-2R	IL-2 receptor
IP	immunoprecipitation
IR	ionising radiation

IRES	internal ribosome entry site
ID2	inhibitor of differentiation 2
IgG	immunoglobulin G
ILF3	interleukin enhancer binding factor 3
Inr	initiator sequence
IPTG	isopropyl β-D-1-thiogalactopyranoside
IVT	*in vitro* translation/translation
Kb	kilobase
KDa	kilodalton
KRAB	Krüppel associated box
LB	Luria Broth
LMP2	large multifunctional protease 2
LMP7	large multifunctional protease 7
LTR	long terminal repeat
MAPK	mitogen-activated protein kinase
MCC	mitotic checkpoint complex
MCM	minichromosome maintenance
MDC1	mediator of DNA damage checkpoint 1
MDM2	murine double minute 2
MECL1	multicatalytic endopeptidase complex-like 1
MHC I	major histocompatibility complex I
mix1	mix1 homeobox
Mr	*morula*
mRNA	messenger ribonucleic acid
MS/MS	tandem mass spectrometer

N-terminal	amino-terminal
NEBD	nuclear envelope breakdown
NEK2A	NIMA-related kinase 2
NETN	NP40, EDTA, tris and sodium chloride
NF45	nuclear factor 45
NF90	Nuclear factor 90
NFAT1	nuclear factor of activated T-cells 1
NFκB	nuclear factor kappa B
NHEJ	non-homologous DNA-end-joining
NIMA	never in mitosis gene A
NLP	ninein-like protein
NLS	nuclear localisation signal
NMDAR	N-methyl-Daspartate receptor
ORC1	origin recognition complex subunit 1
PA28	proteasome activator 28
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pH3	phosphohistone H3
PHD	plant homeobox domain
PI-3K	phosphoinositide 3-kinase
PKC	protein kinase C
PKR	protein kinase R
PMA	phorbol 12-myristate 13-acetate
PML	promyelocytic leukaemia

PML-NBs	PML nuclear bodies
PR	progesterone receptor
PTC	papillary thyroid carcinomas
R-SMADs	receptor-SMADs
RAR	retinoic acid receptor
RB	retinoblastoma
RBCC	RING finger, B-boxes, and a coiled-coil domain
RET	rearranged during transfection
RID	nuclear receptor interaction domain
RING	really interesting new gene
Rnasein	RNAse inhibitor
RNA	ribonucleic acid
RNAi	RNA interference
RPMI-1640	Roswell Park Memorial Institute 1640 medium
RSV	Rous sarcoma virus
RT	room temperature
RTS	Rubinstein Taybi syndrome
RXR	retinoid X receptor
SAC	spindle assembly checkpoint
SCC1	sister chromatid cohesion 1
SCF	skp cullin F-box
SDS	sodium dodecyl sulfate
SDS-PAGE	dodecyl sulphate-polyacrylamide gel electrophoresis
SDW	sterile distilled water
SKP	S phase kinase-associated protein

SMAD	similar to Mothers against decapentaplegic homolog
SNON	ski-related novel protein N
SP-10	S-locus protein 10
SRY-box2	sex determining region Y-box 2
SUMO	small ubiquitin like modifier
SWI/SNF	switch/sucrose nonfermentable
TAP	transporter associated with antigen processing
TBE	Tris, Boric acid and EDTA
TBP	TATA-binding protein
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween 20
TCA	trichloroacetic acid
TCR	T-cell receptor
TE	tris EDTA
TEMED	(N, N, N', N'-tetramethyl-ethylenediamine)
TGF-β	transforming growth factor-β
TLS	translocated in liposarcoma
TNF	tumour neucrosis factor
TNFR	TNF receptor
TPR	tetratrico peptide repeat
TRIM	tripartite motif
tRNA	transfer RNA
TSS	TIF1 signature sequence
UBC	ubiquitin-conjugating enzyme
USP44	ubiquitin-specific protease 44

VDR	vitamin D3 receptor
WB	Western blotting
XKID	*Xenopus* chromokinesin kid

CHAPTER 1

INTRODUCTION

1.1. Cancer

Cancer is a disease that arises as a result of damage to the pathways that regulate cellular proliferation and death. Damage to these pathways is caused by genetic lesions that facilitate a gain in function of oncogenes and inactivation of tumour suppressors. There is a large amount of evidence to indicate that tumourigenesis is a multi-step process, requiring an accumulation of multiple mutations in order for a healthy cell to progress in to a malignant tumour (Vogelstein and Kinzler 1993; Hartwell and Kastan 1994; Hahn and Weinberg 2002). Despite the fact that there are more than 100 distinct types of cancer, and a vast array of cancer cell genotypes, all human cancers display common phenotypes of self sufficiency in growth signalling, insensitivity to anti-growth signalling, the ability to evade apoptosis, the unlimited ability to replicate, and enhanced angiogenesis and metastasis (Hanahan and Weinberg 2000; Hahn and Weinberg 2002; Hahn and Weinberg 2002).

1.1.1. Oncogenes

Oncogenes are mutated derivatives of proto-oncogenes, which are involved in regulating proliferation, differentiation and apoptosis in normal cells. Oncogene activation occurs as a result of point mutations, chromosomal translocations and amplification of proto-oncogenes. Mutation of just one proto-oncogene allele produces a dominant gain in function, which contributes to uncontrolled cellular proliferation and tumour development (Land, Parada et al. 1983; Konopka, Watanabe et al. 1985; Bishop 1987). Identification of the first oncogene began with work by Rous almost a century ago, which demonstrated that cancer could be triggered as a result of viral infection (Rous 1983). Subsequent study led to the identification of the transforming gene *v-src* from the Rous sarcoma virus (RSV) as the causative agent (Hanafusa, Halpern et al.

1977; Fung, Crittenden et al. 1983; Brickell 1992). *v-src* is generated as a result of viral recombination with the cellular (*c*)-*src* (Robinson 1982; Wyke 1983; Yaciuk and Shalloway 1986). c-src functions as a non receptor tyrosine kinase and transduces signals from plasma membrane receptors to the ras mitogen-activated protein kinase (MAPK) pathway in order to elicit cellular proliferation (Courtneidge 1994). Further studies have identified *c-src* as a *bona fide* oncogene, as increases in levels of its expression and kinase activity of its protein product have been observed in human cancers (Jacobs and Rubsamen 1983; Cartwright, Kamps et al. 1989; Budde, Ke et al. 1994; Muthuswamy, Siegel et al. 1994; Mao, Irby et al. 1997)

1.1.1.1. Ras

The *ras* proto-oncogene was the first example of a naturally occurring mutation in a human cancer, and has since been shown to be mutated in 25% of all human tumours (Hanahan and Weinberg 2000). The ras protein family consists of Harvey [Ha]-ras, Kirsten [K]-ras and Neuroblastoma [N]-ras, all of which share a common intrinsic guanosine triphosphatase (GTPase) activity, allowing them to modulate cellular signalling cascades that stimulate cellular proliferation. Ras achieves this through various means such as activation of the raf pathway, which permits cells to enter G1, inactivating retinoblastoma (RB) in order to enable progression into S phase, and triggering the phosphoinositide 3-kinase (PI3-K) pathway to inhibit apoptosis(Coleman, Marshall et al. 2004). Mutations in *ras* that render the GTPase activity of ras constitutively active have been identified in lung (Hilbe, Dirnhofer et al. 2004; Huber and Stratakis 2004), colon (Westra, Plukker et al. 2004), breast and pancreatic cancers (D'Cruz, Gunther et al. 2001; Xiong 2004).

1.1.2. Tumour suppressors

Tumour suppressors are proteins that generally act to repress cellular proliferation and/or enhance apoptosis, and are activated in response to cellular stresses such as DNA damage (Nelson and Kastan 1994), activation of oncogenes and hypoxia (Lowe and Ruley 1993; Graeber, Osmanian et al. 1996). Unlike oncogenes that are activated during tumourigenesis, tumour suppressors must be inactivated in order for cancer to develop. Using the prototypical tumour suppressor RB as a model, Knudson developed his 'two hit hypothesis', which states that loss of both alleles must occur in order for inactivation of tumour suppressors to occur (Knudson 1971).

1.1.2.1. p53

p53 is a particularly significant example of a tumour suppressor as it is mutated in more than 50% of all human cancers (Soussi and Beroud 2001). These mutations predominantly affect p53's sequence specific DNA binding domain (Hainaut and Hollstein 2000), and therefore prevent p53 from activating transcription of genes required for G1 and G2 cell arrest, apoptosis and differentiation (Kastan, Zhan et al. 1992; Agarwal, Agarwal et al. 1995; Aloni-Grinstein, Schwartz et al. 1995; Paules, Levedakou et al. 1995). Although many of the mutations that occur in p53 adhere to the Knudson 'two hit hypothesis', it is unusual amongst tumour suppressors in that some p53 mutations produce a dominant negative effect, therefore mutation of just one p53 allele can lead to inactivation of all cellular p53 and hence contribute to tumourigenesis. This is attributed to the fact that p53 is active only as a tetramer, therefore a mutation in just one p53 subunit is sufficient to affect activity of the entire p53 tetramer (Shaulian, Zauberman et al. 1992; Shaulian, Zauberman et al. 1993) (Halazonetis and Kandil 1993; Unger, Mietz et al. 1993).

1.1.2.2. CBP/p300

The cyclic adenosine monophosphate (cAMP) response element (CRE) is found in the regulatory regions of many genes. Following production of cAMP the CRE is bound by the CRE binding protein (CREB), which then recruits the CREB binding protein (CBP), facilitating transcriptional activation (Chrivia, Kwok et al. 1993). CBP shares a number of functional domains with p300, which allow these proteins to function as transcriptional coactivators. Notably, CBP and p300 contain an acetyltransferase domain that allows these proteins to acetylate histone tails in order to produce a relaxed DNA conformation suitable for transcription. CBP and p300 are required for transcriptional activation of genes that stimulate cellular growth, proliferation, differentiation and apoptosis. (Goodman and Smolik 2000; Iyer, Ozdag et al. 2004). For instance, CBP/p300 can cooperate with p53 to activate transcription of the murine double minute 2 (MDM2), $p21^{CIP1}$ and BCL2 –associated X (BAX) promoters in order to induce cell cycle arrest and apoptosis (Avantaggiati, Ogryzko et al. 1997; Gu, Shi et al. 1997; Lill, Grossman et al. 1997). Interestingly CBP/p300 can also regulate the DNA binding ability of p53 by directly acetylating p53 (Gu and Roeder 1997; Sakaguchi, Herrera et al. 1998; Dornan, Shimizu et al. 2003).

There is mounting evidence to support a role for p300 and CBP as tumour suppressors, which is consistent with their ability to regulate transcription of genes involved in cellular growth, proliferation, differentiation and apoptosis. For instance chromosomal translocation of p300 and CBP is evident in cancer (Borrow, Stanton et al. 1996) (Giles, Dauwerse et al. 1997; Chaffanet, Gressin et al. 2000; Kitabayashi, Aikawa et al. 2001; Panagopoulos, Fioretos et al. 2001). Moreover CBP monoallelic inactivation is the genetic basis for Rubinstein Taybi syndrome (RTS), a disease characterised by

pleiotropic developmental abnormalities and an increased incidence of malignancies (Miller and Rubinstein 1995; Petrij, Giles et al. 1995).

1.2. Gene expression

Despite containing an identical genome, each individual cell within any multicellular organism can achieve specialisation by modulating the expression of a subset of its genes. Eukaryotic gene expression is a multistage process that begins with transcription of messenger ribonucleic acid (mRNA) and includes pre-mRNA processing, mRNA export, and culminates in the translation of mRNA into a functional protein product.

1.2.1. RNA polymerase II dependent transcription

Transcription is modulated by specific binding of transcriptional activators and repressors to their cognate DNA binding sequences within cellular gene promoters (Banerji, Rusconi et al. 1981; de Villiers and Schaffner 1981; Jones and Tjian 1985; Hiller, Hengstler et al. 1988). Transcriptional activators recruit coactivator proteins that include histone acetyl transferases (HATs) such as CBP/p300, and nucleosome remodelling complexes such as switch/sucrose nonfermentable (SWI/SNF). These coactivator proteins alter chromatin structure in order to allow access to RNA polymerase II and subsequent mRNA synthesis (Brown, Lechner et al. 2000; Gibbons 2005). In contrast, access to DNA by RNA polymerase II can be prevented by binding of transcriptional repressors to cellular gene promoters. Transcriptional repressors generally function by recruiting corepressors such as histone deacetylases and DNA methylases in order to promote a closed DNA conformation that is therefore inaccessible to RNA polymerase II (Stallcup 2001; Hellebrekers, Griffioen et al. 2007).

RNA polymerase II is unable to recognise its target promoters directly, and therefore must associate with the general transcription factors (GTFs) TFIIB, D, E, F and H in order to form a preinitiation complex, and associate with the core promoter elements: the TATA box and the initiator sequence (Inr) (Weis and Reinberg 1992). The multi-component factor TFIID binds directly to the TATA box via its TATA-binding protein (TBP) and also binds to TFIIB, which recruits RNA polymerase II to the promoter (Matsui, Segall et al. 1980; Li, Flanagan et al. 1994; Leuther, Bushnell et al. 1996; Cosma, Panizza et al. 2001). The intrinsic helicase activity of the multi subunit TFIIH unwinds DNA, while its kinase activity that is conferred by cyclin-dependent kinase 7 (CDK7) initiates transcription by phosphorylating RNA polymerase II (Lu, Zawel et al. 1992; Tirode, Busso et al. 1999; Seroz, Perez et al. 2000). An additional multi-component protein factor known as mediator, is essential for RNA polymerase II dependent transcription *in vivo,* and bridges the interaction between transcriptional activators and the preinitiation complex (Flanagan, Kelleher et al. 1991; Thompson, Koleske et al. 1993; Kim, Bjorklund et al. 1994; Myers, Gustafsson et al. 1998).

1.3. Transition through the cell cycle occurs in a cyclin-CDK dependent manner

Cyclins are a group of proteins that regulate the orderly progression of cells through the cell cycle in conjunction with CDKs and are named on the basis of the cyclical periodic nature of their expression (see Fig 1.1 for a summary of cyclin-CDK activity throughout the cell cycle). They all share a common cyclin box that mediates their ability to bind, activate, and hence direct CDK activity (Minshull, Pines et al. 1989; Pines and Hunter 1989; Kobayashi, Stewart et al. 1992; Lees and Harlow 1993). Activation of CDKs also requires their phosphorylation by the CDK activating kinase (CAK), which comprises

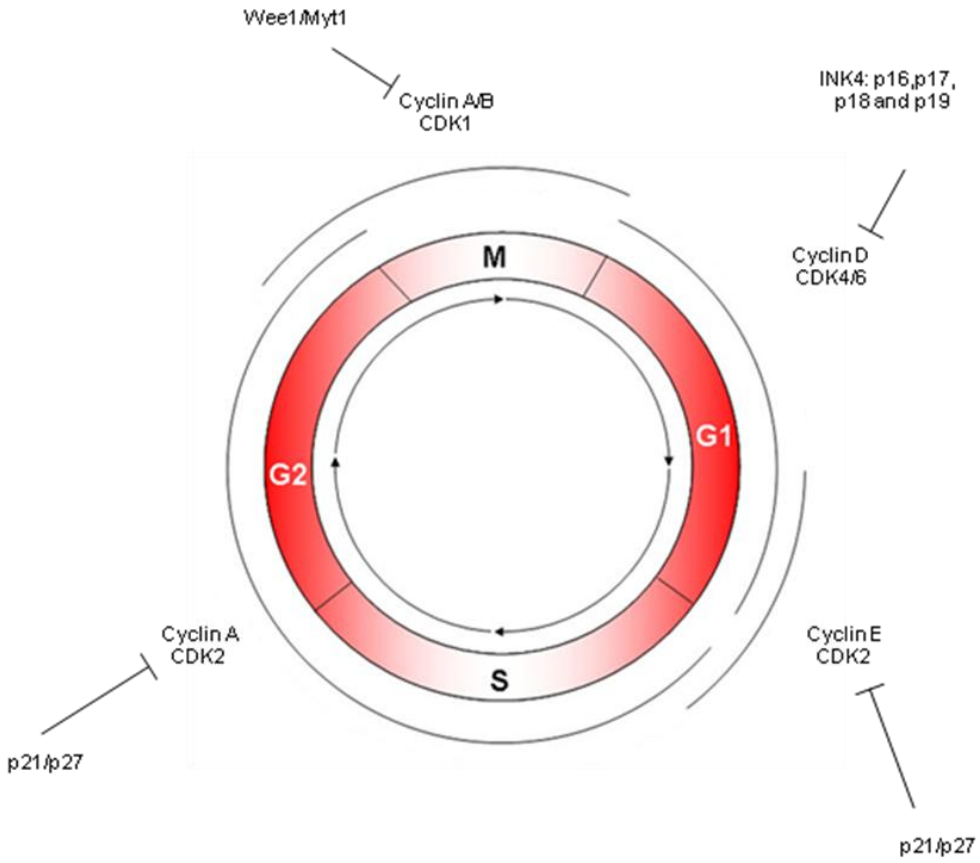


Fig 1.1. Cyclin-CDK activity throughout the cell cycle
Block arrows indicate inhibition. This figure was designed using references from section 1.3

CDK7 and cyclin H (Solomon, Lee et al. 1992; Poon, Yamashita et al. 1993; Fisher and Morgan 1994; Matsuoka, Kato et al. 1994), and the removal of an inhibitory phosphorylation by CDC25 (cell division cycle) phosphatases. The activation of cyclin-CDK complexes is regulated by CDC25A during the G1-S and G2-M transitions, whereas CDC25B and CDC25C function during the G2-M transition (Sadhu, Reed et al. 1990; Millar, Blevitt et al. 1991; Honda, Ohba et al. 1993; Dunphy 1994; Strausfeld, Fernandez et al. 1994; Lammer, Wagerer et al. 1998; Donzelli, Squatrito et al. 2002; Mailand, Podtelejnikov et al. 2002).

1.3.1. Cyclin D

Entry into the cell cycle is stimulated by activation of proto-oncogenes such as *ras* and *myc*, which essentially enhance the formation of cyclin D-CDK4 and cyclin D-CDK6 complexes (Matsushime, Ewen et al. 1992; Meyerson and Harlow 1994; Albanese, Johnson et al. 1995; Yu, Ciemerych et al. 2005). The activity of cyclin D-CDK4 or CDK6 complexes can be inhibited by members of the INK4 protein family of CDK inhibitors, which comprises $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$ (Sherr 1996; Pavletich 1999). Cyclin D-CDK4/6 complexes predominantly function through phosphorylation of RB family proteins, thereby relieving repression of E2F responsive genes required for cell cycle progression, which notably includes cyclin E (Ohtani, DeGregori et al. 1995; Geng, Eaton et al. 1996; Harbour and Dean 2000; Classon and Harlow 2002). In addition to enhancing transcription of cyclin E, cyclin D-CDK4/6 complexes further enhance the activity of cyclin E-CDK2 complexes by binding to and thus titrating out the CDK2 inhibitors $p27^{KIP1}$ and $p21^{CIP1}$ (Dulic, Lees et al. 1992; Koff, Giordano et al. 1992; el-Deiry, Tokino et al. 1993; Gu, Turck et al. 1993; Xiong, Hannon et al. 1993; Sherr and Roberts 1995). Significantly, binding of $p16^{INK4A}$ to

cyclinD-CDK4/6 complexes has been demonstrated to induce dissociation of the CDK inhibitors $p27^{KIP1}$ and $p21^{CIP1}$, thereby enhancing G1 arrest by inhibiting the kinase activity of CDK2 (Jiang, Chou et al. 1998; McConnell, Gregory et al. 1999).

1.3.2. Cyclin E

Increasing levels of cyclin E during G1 result in formation of cyclin E-CDK2 complexes that are required to negotiate the restriction point in G1, after which the cell is committed to proliferation (Ohtsubo and Roberts 1993; Ohtsubo, Theodoras et al. 1995). It is thought that cyclin E-CDK2 stimulates G1 progression through activation of E2F dependent transcription by further enhancing RB family phosphorylation (Lundberg and Weinberg 1998; Harbour, Luo et al. 1999) and by directly phosphorylating E2F(Morris, Allen et al. 2000). Significantly, cyclin E-CDK2 is thought to mediate transcription of E2F responsive genes required for DNA synthesis, such as *thymidine kinase* (Chang, Huang et al. 1995), *b-myb* and *cyclin A* (DeGregori, Kowalik et al. 1995; Ohtani 1999). In addition, cyclin E-CDK2 organises the assembly of replication complexes containing CDC6 (Furstenthal, Kaiser et al. 2001; Coverley, Laman et al. 2002). Lastly cyclin E-CDK2 also initiates replication of centrosomes (Hinchcliffe, Li et al. 1999; Lacey, Jackson et al. 1999; Matsumoto, Hayashi et al. 1999), which are required during mitosis in order to anchor bi-polar mitotic spindles and facilitate correct chromosomal segregation. Defects in chromosomal segregation can result in a change in the number of chromosomes in daughter cells, which is known as aneuploidy. Interestingly aneuploidy occurs in many solid tumours and can contribute to tumourigenesis as it can cause daughter cells to receive extra copies of oncogenes or lose tumour suppressors (D'Assoro, Lingle et al. 2002; Fukasawa 2005).

1.3.3. Cyclin A

Cyclin A is expressed at the onset of S phase and through its association with CDK2 directly activates DNA synthesis (Girard, Strausfeld et al. 1991; Bashir, Horlein et al. 2000). Cyclin A-CDK2 complexes down regulate E2F transcription during S phase through phosphorylation of E2F's transcription binding partner DP-1 (dimerisation partner 1) (Krek, Xu et al. 1995). Cyclin A also forms a complex with CDK1 in G2, which may facilitate entry in to mitosis (Pagano, Pepperkok et al. 1992), as formation of cyclin A-CDK1 complexes might be important for nuclear envelope breakdown, chromosomal condensation and to stimulate nuclear import of cyclin B (Gong, Pomerening et al. 2007). The activity of cyclin A-CDK complexes can be inhibited by $p27^{KIP1}$ and $p21^{CIP1}$ or ablated as a result of cyclin A destruction. Cyclin A destruction occurs during nuclear envelope breakdown (NEBD) in response to ubiquitylation of cyclin A by the anaphase promoting complex/cyclosome (APC/C) (Dawson, Roth et al. 1995; Sigrist, Jacobs et al. 1995; Sudakin, Ganoth et al. 1995; Furuno, den Elzen et al. 1999; den Elzen and Pines 2001) (Sherr and Roberts 1999).

1.3.4. Cyclin B

Cyclin B transcription/translation is activated during late S phase and the protein exerts its effects by binding exclusively to CDK1 *in vivo* (Pines and Hunter 1989; Riabowol, Draetta et al. 1989). During early G2, cyclin B-CDK1 activity is inhibited by wee1 and myt1 kinases (Parker and Piwnica-Worms 1992; Mueller, Coleman et al. 1995). Removal of an inhibitory phosphate group from CDK1 by CDC25 phosphatases results in cyclin B-CDK1 activation and aids mitotic entry (Lammer, Wagerer et al. 1998; Mailand, Podtelejnikov et al. 2002) (Millar, Blevitt et al. 1991; Strausfeld, Fernandez et

al. 1994). Significantly, cyclin B-CDK1 stimulates nuclear lamin breakdown, through phosphorylation of lamin subunits and induces the dissociation of caldesmon from actin microfilaments, thereby permitting cell rounding (Dessev, Iovcheva-Dessev et al. 1991; Yamashiro, Chern et al. 2001). Also, consistent with its recruitment to chromatin, kinetochores and centrosomes during mitosis (Bentley, Normand et al. 2007; Chen, Zhang et al. 2008), cyclin B-CDK1 regulates a number of factors known to be associated with these structures. For instance cyclin B-CDK1 inhibits RNA poll II and III transcription by phosphorylating TFIIB (Hartl, Gottesfeld et al. 1993; Gottesfeld, Wolf et al. 1994; Leresche, Wolf et al. 1996). Furthermore cyclin B-CDK1 initiates centrosomal segregation by phosphorylating EG5, which is a kinesin-related motor protein required for bipolar spindle formation (Blangy, Lane et al. 1995; Mayer, Kapoor et al. 1999). Lastly, cyclin B-CDK1 initiates mitotic exit through activation of the APC/C (an E3 ubiquitin ligase) (Kraft, Herzog et al. 2003), which targets cyclin B and the inhibitor of sister chromatid separation, securin, for destruction (Dawson, Roth et al. 1995; Sigrist, Jacobs et al. 1995; Sudakin, Ganoth et al. 1995; Cohen-Fix, Peters et al. 1996; Kim, Fong et al. 2007).

1.4. The ubiquitin-proteasome system

Eukaryotic cells possess two major systems for protein degradation. The first to be characterised was the lysosomal system, which is chiefly concerned with the degradation of membrane associated proteins and extracellular proteins taken up by endocytosis. The predominant form of nonlysosomal protein degradation is mediated by the ubiquitin-proteasome system, which is responsible for >80% of cellular protein degradation (Lee and Goldberg 1998; Glickman and Ciechanover 2002; Dalton 2004) (refer to Fig 1.2 for an overview of the ubiquitin-proteasome pathway).

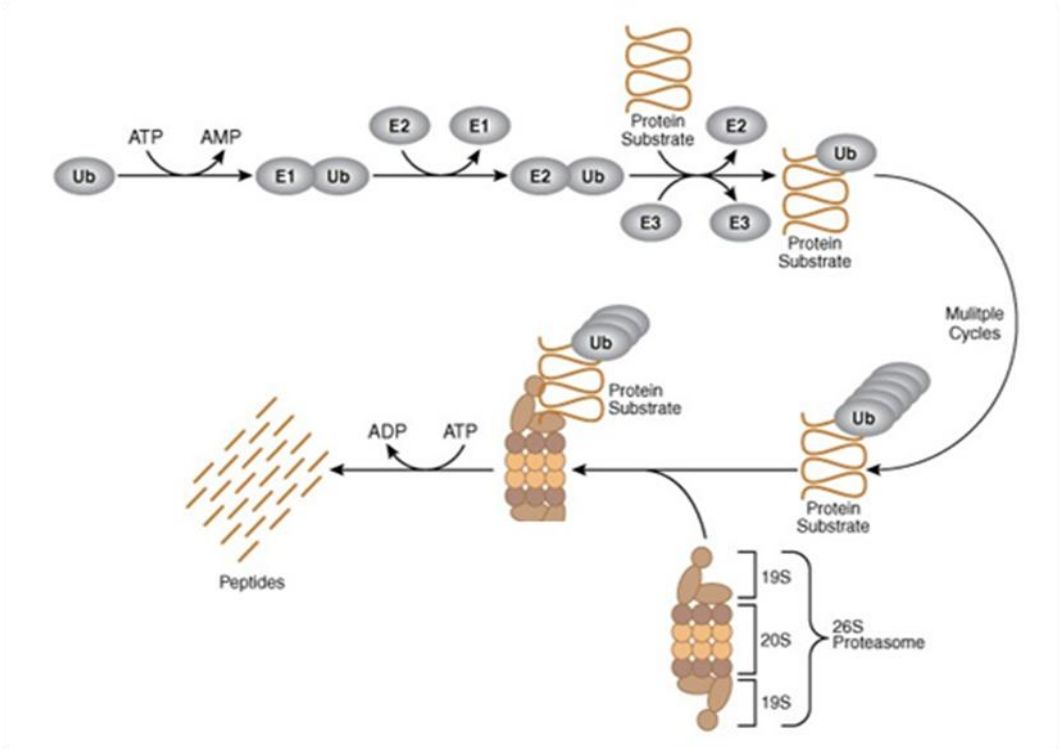


Fig1.2. The ubiquitin proteasome pathway
Ubiquitylation is dependent on ATP hydrolysis and occurs via a three-step mechanism that involves an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme and an E3 ubiquitin ligase enzyme. Polyubiquitylated protiens are recognised and degraded by the 26S proteasome (adapted from http://www.cellsignal.com/reference/pathway/Ubiquitin_Proteasome.html)

Protein degradation by the ubiquitin-proteasome pathway requires a protein to be post-translationally modified by the attachment of a polyubiquitin chain so that it can be recognised and degraded by the 26S proteasome. Protein ubiquitylation is mediated by an enzymatic cascade that consists of an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme (UBC) and an E3 ubiquitin ligase. Initially, the E1 binds to ubiquitin, and activates its C-terminal glycine residue through the formation of a high-energy thiol ester intermediate (E1~S~ubiquitin). Secondly the activated ubiquitin is transferred to an E2 enzyme. Lastly transfer of the ubiquitin from the E2 to a specific substrate is mediated by an E3 enzyme (Ciechanover and Schwartz 1998; Burger and Seth 2004). Formation of a polyubiquitin chain is mediated through attachment of additional ubiquitin molecules to any one of seven lysines within the ligated ubiquitin and may be enhanced by the presence of an E4 ubiquitin-elongation enzyme (Pickart 2001; Kuhlbrodt, Mouysset et al. 2005). Attachment of Lys(11)-, Lys(29)- and Lys(48)-linked polyubiquitin causes a protein to be targeted for degradation, whereas lysine (6)- or lysine (63)-linked polyubiquitin can regulate functions other than protein degradation, such as modulating protein activity or sub-cellular localisation (Hochrainer and Lipp 2007). Removal of ubiquitin from protein substrates is controlled by deubiquitinating enzymes (DUBs), providing a further layer of complexity in the regulation of protein proteolysis. DUBs also function to maintain cellular levels of free ubiquitin and remove liberated ubiquitin from the 26S proteasome (Nijman, Luna-Vargas et al. 2005)

The proteolytic component of the 26S proteasome is known as the 20S proteasome and has a barrel-like structure made up of two outer α-rings comprising seven α-type subunits, and two inner β-rings made up of seven β-type subunits. The proteolytic activity of the 20S proteasome is conferred by its β1, β2 and β5 subunits, which display

intrinsic energy independent chymotrypsin-, trypsin- and caspase-like activity respectively. Untimely degradation of proteins is prevented by constriction of the outer α rings of the 20S proteasome, which is relieved after binding of the 19S regulatory particle (RP, also called proteasome activator 700 (PA700)) to one or both ends of the 20S proteasome to form the 26S proteasome (Groll, Bajorek et al. 2000; Heinemeyer, Ramos et al. 2004; Rechsteiner and Hill 2005). The 19S RP facilitates protein degradation by recognising ubiquitylated proteins, cleaving ubiquitin from its substrate and unfolding proteins in preparation for their degradation by the 20S proteasome (DeMartino 2005). Given its role in controlling protein stability, and hence activity, it is unsurprising therefore that the 26S proteasome has been implicated in numerous cellular processes such as cell cycle control, signal transduction, transcription, and programmed cell death (Weissman 1997; Hershko and Ciechanover 1998; Glickman and Ciechanover 2002)

Formation of the proteasome variant known as the immunoproteasome is induced by Interferon-γ (IFN-γ) treatment (Tanaka 2009). The predominant function of the immunoproteasome is to produce antigenic peptides for presentation by the major histocompatibility complex I (MHC I) pathway (Khan, van den Broek et al. 2001; Teoh and Davies 2004), although a large number of antigens presented by the MHCI pathway are produced by the 26S proteasome (Tanaka 2009). The immunoproteasome differs from the 26S proteasome in that its 20S proteasome component (20Si) is thought to be bound and activated by the proteasome activator 28 (PA28, also known as the 11S regulator) a heteroheptamer consisting of PA28α and PA28β subunits (Preckel, Fung-Leung et al. 1999; Rechsteiner, Realini et al. 2000; Yawata, Murata et al. 2001; Rivett and Hearn 2004). Also, the β1, β2 and β5 of the 20S proteasome, which confer proteolytic activity are replaced in the 20Si with distinct subunits known as β1i (LMP2),

β2i (MECL1), and β5i (LMP7). This results in increased chymotrypsin- and trypsin-like activity, which favour the production of peptides with C-terminal hydrophobic and basic amino acids. The resulting peptides are more suited to interaction with the peptide binding pockets of MHCI pathway molecules (Murata, Yashiroda et al. 2009; Tanaka 2009). Immunoproteasomes are particularly enriched at the endoplasmic reticulum and are thought to be located in close proximity to TAP (transporter associated with antigen processing) to aid delivery of antigens to MHC class I molecules, which are found in the lumen of the endoplasmic reticulum (ER) (Brooks, Murray et al. 2000). Interestingly, aberrant immunoproteasome activity has been implicated in a number of diseases, such as multiple myeloma (Altun, Galardy et al. 2005) and also in diseases that are characterised by abnormal immune responses including rheumatoid arthritis and inflammatory bowel disease (Egerer, Martinez-Gamboa et al. 2006; Visekruna, Joeris et al. 2006; Fitzpatrick, Small et al. 2007). It is hoped that development of specific immunoproteasome inhibitors might be effective for treatment of these conditions.

1.5. The APC/C

The APC/C is a multi-component E3 ubiquitin ligase that coordinates temporal progression through the cell cycle, as a result of its ability to target cell cycle regulatory proteins for degradation. The APC/C regulates proteolysis by catalysing the formation of polyubiquitin chains on its substrates in order to promote their degradation by the 26S proteasome. APC/C-mediated degradation inactivates effector proteins such as cyclin A and cyclin B, or inhibitory proteins such as securin and geminin (see Table 1.1 for a list of APC/C substrates) (Peters 1999). In general APC/C substrates are defined by the presence of a D-box (minimal sequence is RXXL) or KEN-box (Glotzer, Murray et al. 1991; King, Glotzer et al. 1996; Pfleger and Kirschner 2000), although distinct

Table 1.1. Cell cycle related APC/C substrates (adapted from Acquaviva and Pines 2006)

Protein	Timing of destruction	APC/C	Function
cyclin A	Pro-metaphase	CDC20	Mitotic regulator
NEK2A	Pro-metaphase	CDC20	Centrosome regulator
HOXC10	Pro-metaphase	CDC20	Transcription factor
cyclin B	Metaphase	CDC20	Mitotic regulator
securin	Metaphase	CDC20	Separase inhibitor
CDC20	Anaphase	CDH1	APC/C activator
CDH1	G1	CDH1	APC/C activator
UBCH10	G0/G1		APC/C cofactor
cyclin B3	Anaphase	CDH1	Mitotic/meiotic reg
PLK1	Anaphase	CDH1	Mitotic regulator
aurora A	Anaphase	CDH1	Mitotic regulator
aurora B	Anaphase/telophase	CDH1	Mitotic regulator
survivin	Anaphase*	CDH1	Localises Aurora B
ndc10	Anaphase*	CDH1	Kinetochore
SLK1	Anaphase*	CDH1	Spindle component
ase1/prc1	Anaphase*	CDH1	Spindle component
KIP1	Anaphase*	CDH1	Spindle motor
anillin	Anaphase*	CDH1	Cytokinesis regulator
ECT2	Anaphase*	CDH1	Cytokinesis regulator
geminin	Anaphase*	CDH1	DNA replication
hsl1	G1	CDH1	Swe1 inhibitor
SKP2	G1	CDH1	SCF component
CKS1	G1	CDH1	CDK cofactor
SNON	G1	CDH1	Inhibitor of TGF-β

* Post anaphase, not yet determined *in vivo*

destruction motifs have been identified in proteins such as chromosome instability 8p (CIN8p) (Hildebrandt and Hoyt 2001), aurora A (Littlepage and Ruderman 2002), origin recognition complex subunit 1 (ORC1) (Araki, Wharton et al. 2003), *Xenopus* chromokinesin kid (XKID) and spo13 (Castro, Vigneron et al. 2003) (Sullivan and Morgan 2007). A recent report reveals the existence of an additional substrate motif known as a TEK-box that is also found in ubiquitin and facilitates ubiquitin nucleation and subsequent chain elongation (Jin, Williamson et al. 2008).

1.5.1. Factors required for APC/C ubiquitin ligase activity

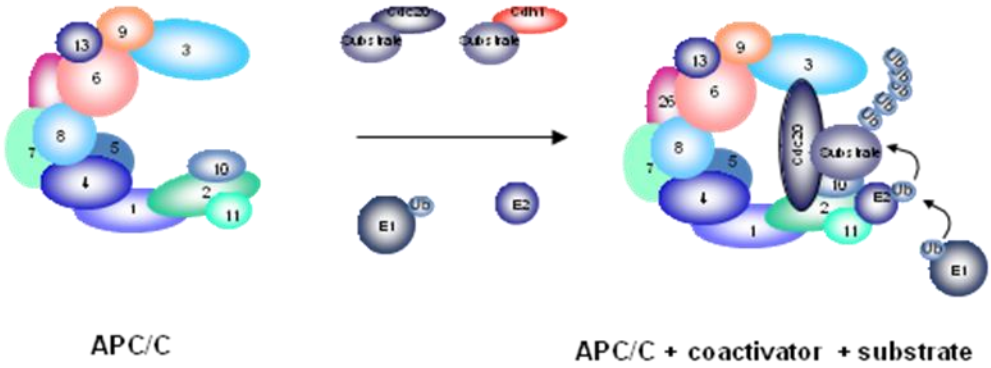
Amongst the E3 ubiquitin ligases the APC/C is extremely complex comprising at least 11 subunits in vertebrates and 13 in yeast (see Table 1.2 for a summary of mammalian APC/C components and Fig 1.3 for a depiction of their organisation). However, APC/C ligase activity, albeit with greatly reduced substrate specificity, can be reconstituted *in vitro* in the presence of just APC2 and APC11 in conjunction with an E1 ubiquitin activating enzyme and an E2 ubiquitin conjugating enzyme (Gmachl, Gieffers et al. 2000; Yoon, Feoktistova et al. 2002; Vodermaier, Gieffers et al. 2003). Significantly, APC2 contains a cullin domain and APC11 contains a really interesting new gene (RING) finger motif (Zachariae, Shevchenko et al. 1998; Gmachl, Gieffers et al. 2000), which are both commonly found in other E3 ubiquitin ligases such as SKP (S phase-associated protein) cullin F-box (SCF) E3 ubiquitin ligases (Pickart 2001). It is thought that association of the cullin and RING-finger domains is important for recruitment of E2 enzymes (Gmachl, Gieffers et al. 2000; Tang, Li et al. 2001; Vodermaier, Gieffers et al. 2003). In order to catalyse substrate ubiquitylation *in vivo* the APC/C requires a universal E1 enzyme, and either one of the E2 enzymes UBCH4 or UBCH10, and must generally be bound by one of its coactivators CDC20 or CDC20-homologue protein 1

Table 1.2. Subunits and coactivators of the mammalian APC/C (adapted from van Leuken 2008 and Peters 1999).

Subunits	Predicted size	Functional Aspects
APC1	216	Mitotic phosphosites; scaffold?
APC2	94	Cullin
APC3/CDC27	92	TPR; mitotic phosphosites
APC4	92	Connector?
APC5	85	Connector?
APC6/CDC16	72	TPR; mitotic phosphosites
APC7	63	TPR; mitotic phosphosites
APC8/CDC23	69	TPR; mitotic phosphosites
APC10	21	Processivity factor
APC11	11	RING-E3
APC13	9	Stability factor?
CDC26	10	Stability factor?
Coactivators		
CDC20	55	WD40 repeats; substrate binding
CDH1	55	WD40 repeats; substrate binding

(CDH1) (Aristarkhov, Eytan et al. 1996; Yu, King et al. 1996; Peters 1999). The coactivators are thought to bind to APC/C subunits, which contain tetratrico peptide repeat (TPR) domains, through their C-boxes (DRF/YIPXR) and an IR dipeptide (Schwab, Neutzner et al. 2001; Passmore, McCormack et al. 2003; Vodermaier, Gieffers et al. 2003). It is thought that binding of CDH1 or CDC20 causes a conformational change in the APC/C that is associated with the activation of the APC/C's ligase activity (Dube, Herzog et al. 2005). In addition, CDC20 and CDH1 also contain a WD40 repeat, which is believed to mediate binding to substrate destruction motifs (Kraft, Vodermaier et al. 2005).

A)



B)

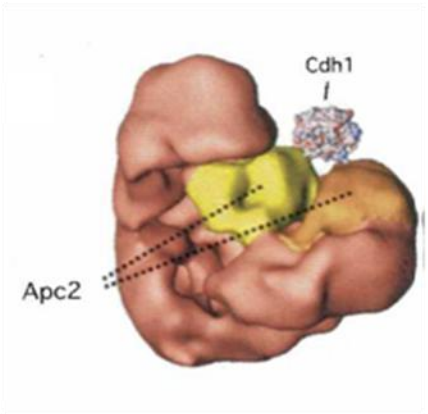


Fig 1.3. The APC/C is a multicomponent E3 ubiquitin ligase
(A) The APC/C is activated after association with its coactivators CDC20 and CDH1, which bind to substrates via their WD40 domains.APC1 functions as a scaffold for assembly of the catalytic and structural blocks of the APC/C. The catalytic domain consists of the cullin APC2, RING finger APC11 and the processitivity factor APC10. The structural block contains the tetratrico peptide repeat (TPR) containing subunits APC3/cdc27, APC6/cdc16, APC7 and APC8/CDC23. APC4 and APC5 may connect the catalytic block to the TPR block. The TPR subunit APC7 is only found in mammalian APC/C and its exact position within the APC/C is unknown (adapted from van Leuken 2008) {van Leuken, 2008 #880}. (**B**) The electron microscope structure of the human APC/C, with APC2 located by immuno-gold staining {Thornton, 2006 #881}.

1.5.2. Sequential formation of mitotic APC/C^{CDC20} and APC/C^{CDH1}

The APC/C is activated during mitosis and remains active until late G1, as a result of interaction with its coactivators CDC20 and CDH1, which function in a cell cycle dependent manner (see Fig 1.4 for an overview of APC/C activity and regulation during the cell cycle). Despite the fact that CDC20 is synthesised during S and G2 phases, it can only associate with, and activate, the APC/C efficiently after the APC/C has been phosphorylated by CDK1 on multiple subunits such as APC3 and APC1 in prophase (Kraft, Herzog et al. 2003). APC/C^{CDC20} complexes mediate degradation of cyclins A and B, and securin, therefore allowing sister chromatid separation, and subsequent mitotic exit (Dawson, Roth et al. 1995; Sigrist, Jacobs et al. 1995; Sudakin, Ganoth et al. 1995; Cohen-Fix, Peters et al. 1996). As a result of cyclin B degradation at the metaphase-anaphase transition CDK1 activity is ablated and can no longer maintain inhibitory phosphorylation of CDH1 (Jaspersen, Charles et al. 1999; Listovsky, Zor et al. 2000), allowing formation of APC/C^{CDH1} complexes that remain active until late G1 (Fang, Yu et al. 1998; Kramer, Gieffers et al. 1998; Zachariae,

Schwab et al. 1998; Jaspersen, Charles et al. 1999). Significantly, APC/C^{CDH1} facilitates mitotic exit by mediating degradation of proteins such as CDC20 (Prinz, Hwang et al. 1998; Shirayama, Zachariae et al. 1998), Polo-like kinase 1 (PLK1) and the aurora kinases A and B (Shirayama, Zachariae et al. 1998; Littlepage and Ruderman 2002; Lindon and Pines 2004; Nguyen, Chinnappan et al. 2005). In addition APC/C^{CDH1}enables prereplication complex (preRC) formation during M and early G1 by targeting the replication licensing inhibitor geminin for degradation (McGarry and Kirschner 1998; Li and Blow 2005).

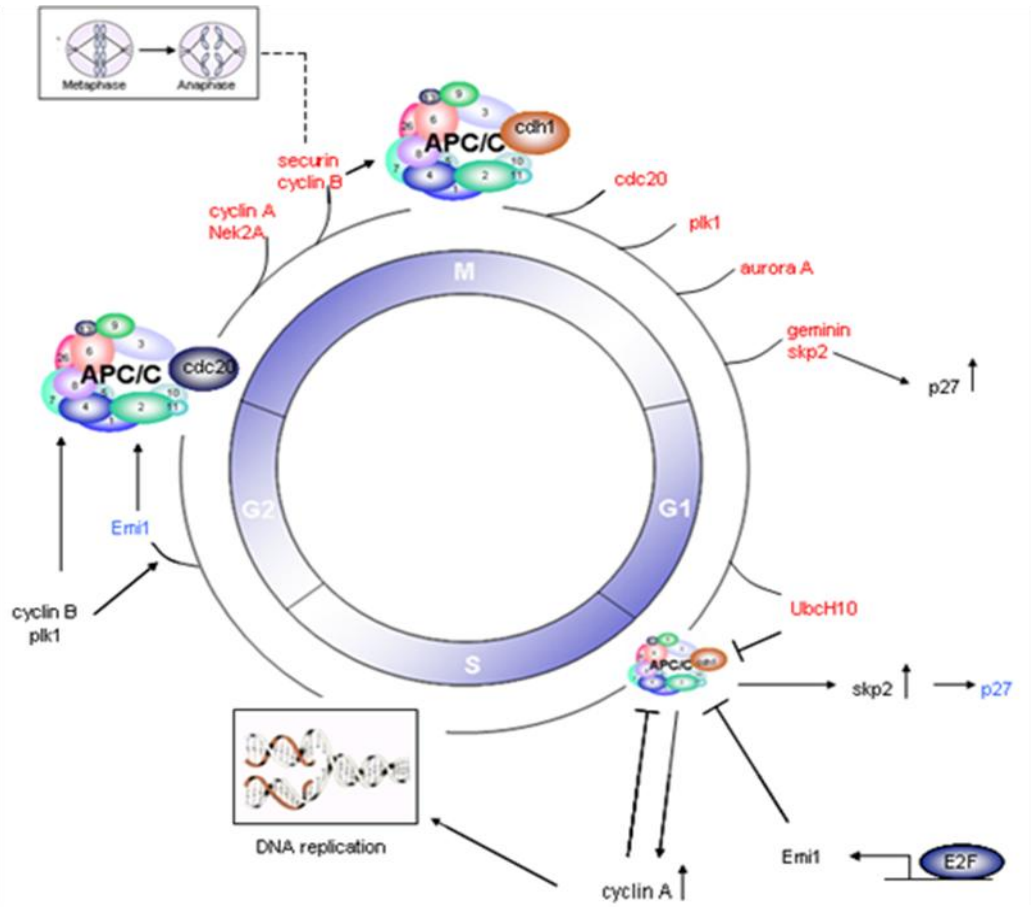


Fig 1.4. APC/C E3 ubiquitin ligase activity is regulated through cell cycle dependent binding of its coactivators CDC20 and CDH1.
APC/C^{CDC20} is active from prometaphase until the metaphase-anaphase transition. At this point cyclin B degradation prevents the maintenance of an inhibitory phosphorylation upon cdh1, therefore APC/C^{CDH1} is activated and facilitates mitotic exit. APC/C^{CDH1} remains active until late G1 until it targets UBCH10 for degradation. This allows accumulation of cyclin A, which further enforces APC/C^{CDH1} inactivation by directing CDK dependent phosphorylation of CDH1. APC/C^{CDH1} inhibition is further enhanced as a result of E2F dependent transcription of the APC/C inhibitor EMI1. In addition accumulation of the APC/C substrate Skp2 facilitates degradation of the CDK inhibitor $p27^{KIP1}$, which in conjunction with cyclin A accumulation allows transition into S phase and subsequent DNA replication. APC/C^{CDC20} becomes active during prometaphase as a result of $SCF^{h\text{-}TRCP}$ dependent EMI1 degradation, which is elicited by the creation of phosphodegron in EMI1 by cyclin B- cdk and PLK1 kinase activity. APC/C substrates are highlighted in red; proteins degraded by SCF complexes are highlighted in blue. Block arrows indicate inhibition; while normal arrows show positive events; bent arrows indicate transcription. Proteins followed by small vertical arrows denote instances of protein accumulation.

1.5.3. Chromosome segregation during mitosis requires cyclin B and securin degradation

Prophase, prometaphase and metaphase constitute the early phases of mitosis and are chiefly concerned with bipolar chromosome attachment. Anaphase is characterised by the separation of sister chromatids, and the initiation of their migration to opposite poles of the cell, known as chromosome segregation. Untimely chromosome segregation and possible subsequent aneuploidy (Orr, Bousbaa et al. 2007), is prevented by a ring-like multisubunit complex called cohesin that persists at the centromere until anaphase (Gerton 2005; Losada and Hirano 2005). Cohesin maintains sister chromatid cohesion until the spindle assembly checkpoint (SAC) is inactivated. At which point the cohesin component, sister chromatid cohesion 1 (SCC1) is cleaved by separase allowing sister chromatids to migrate to opposite poles (Uhlmann, Lottspeich et al. 1999; Uhlmann, Wernic et al. 2000; Hauf, Waizenegger et al. 2001). Separase is rendered inactive prior to metaphase through binding of its inhibitor securin (also known as pituitary tumor transforming gene (PTTG)) and due to inhibitory phosphorylation by cyclin B-CDK1 (Stemmann, Zou et al. 2001; Tfelt-Hansen, Kanuparthi et al. 2006). Therefore the metaphase-anaphase transition cannot occur until securin and cyclin B are degraded by the 26S proteasome in late metaphase as a result of their polyubiquitylation by APC/C^{CDC20} (see Fig 1.5 for an overview of APC/C dependent chromosome segregation) (Dawson, Roth et al. 1995; Sigrist, Jacobs et al. 1995; Sudakin, Ganoth et al. 1995; Cohen-Fix, Peters et al. 1996).

1.5.4. Chromosome segregation is inhibited by the spindle checkpoint

Given that APC/C^{CDC20} is activated during prophase, it is important to prevent the untimely cleavage of securin and cyclin B, in order to block early chromosome

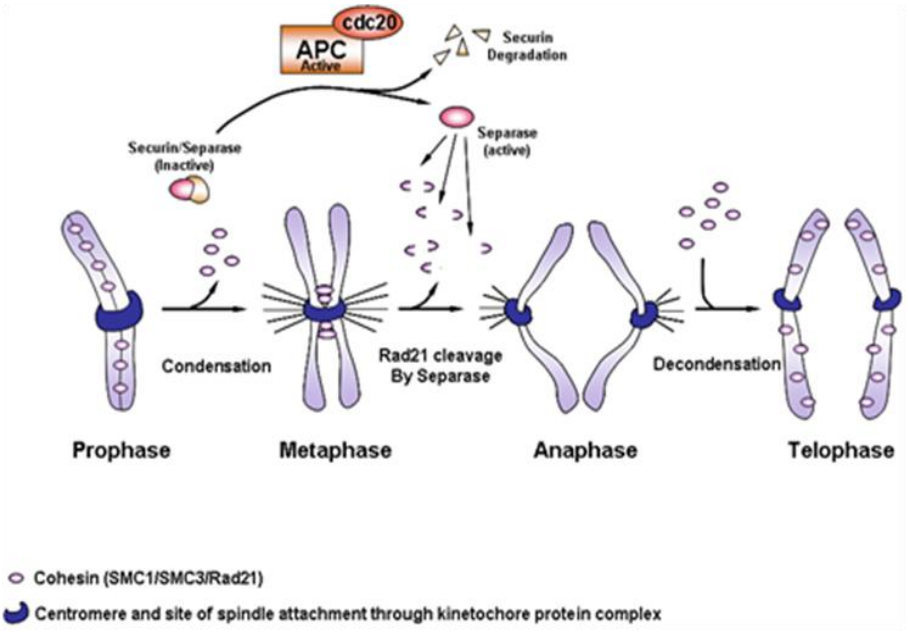


Fig 1.5. Chromosome segregation is controlled by the APC/C
Following DNA replication sister chromatids are connected by cohesin along the length of the chromatids. When the chromosomes condense in prophase all cohesin apart from that which is restricted to the centromere and the surrounding pericentric domain dissociates from the chromosomes. The remaining cohesin prevents chromosome separation until the SAC has been satisfied. Inactivation of the SAC in late metaphase facilitates APC/C activation, which then targets the seperase inhibitor securin for degradation. Active seperase can then cleave the cohesin subunit Rad21 (SCC1), allowing each sister chromatid to migrate to opposing poles of the cell and progression into anaphase. At the end of mitosis in telophase when the chromosomes decondense cohesin reassociates with the chromosomes along their entire length. It is therefore thought that cohesin is important for chromosome condensation/decondensation. This figure was provided by Dr G Stewart.

segregation, and possible aneuploidy. Activation of the SAC prevents early chromosome separation by selectively inhibiting APC/C^{CDC20} dependent proteolysis of cyclin B and securin (den Elzen and Pines 2001; Geley, Kramer et al. 2001). The APC/C mediates degradation of NIMA (never in mitosis gene A)-related kinase 2 (NEK2A), homeobox C10 (HOXC10) and cyclin A during early mitosis despite SAC activation. The mechanisms that allow this to occur are poorly understood. However, despite the fact that CDC20 activity is inhibited by the SAC, CDC20 is notably still required for activation of APC/C ligase activity towards these early mitotic, checkpoint independent substrates, suggesting the existence of multiple APC/C populations (den Elzen and Pines 2001; Geley, Kramer et al. 2001; Gabellini, Colaluca et al. 2003; Hayes, Kimata et al. 2006; Wolthuis, Clay-Farrace et al. 2008).

The SAC is activated in the presence of misaligned chromosomes and unattached kinetochores, and is mediated by the mitotic checkpoint complex (MCC), which comprises mitotic arrest deficient 1(MAD1), CDC20, MAD2, budding uninhibited by benzimidazoles R1 (BUBR1) and BUB3 (Sudakin, Chan et al. 2001). Significantly, direct interactions between CDC20, MAD2 and BUBR1 account for the majority of APC/C inhibition in arrested cells (Fang, Yu et al. 1998; Hwang, Lau et al. 1998; Fang 2002; Braunstein, Miniowitz et al. 2007). Interestingly, MAD2 and BUBR1 are thought to enforce the spindle checkpoint by catalysing CDC20 degradation, as they appear to mediate APC/C dependent ubiquitylation of CDC20 (Pan and Chen 2004; King, van der Sar et al. 2007; Nilsson, Yekezare et al. 2008; Ge, Skaar et al. 2009). In contrast two other studies reveal that the APC/C can also promote SAC inactivation, as ubiquitylation of CDC20 by the APC/C actually prevents interaction between CDC20 and MAD2. Consistent with this, depletion of the ubiquitin specific protease 44

(USP44) that antagonises CDC20 ubiquitylation *in vivo* results in SAC failure (Reddy, Rape et al. 2007) (Stegmeier, Rape et al. 2007).

Aurora B also plays a key role in regulating the spindle checkpoint, as it aids recruitment of MCC components to the kinetochore and promotes MCC activation and correct chromosome alignment by severing microtubules that are not attached to kinetochores properly (Tanaka, Rachidi et al. 2002; Ditchfield, Johnson et al. 2003; Hauf, Cole et al. 2003).

1.5.5. Regulation of the APC/C during interphase

During G1, PC/C^{CDH1} inhibits CDK activity and therefore prevents early passage into S phase; indeed ablation of CDH1 expression leads to an enrichment of cells in S phase (Sudo, Ota et al. 2001; Bashir, Dorrello et al. 2004; Wei, Ayad et al. 2004). APC/C^{CDH1} prevents early S phase entry by targeting the F-box protein SKP2 for destruction, which results in elevated levels of the CDK inhibitor $p27^{KIP1}$ (Bashir, Dorrello et al. 2004; Wei, Ayad et al. 2004). In addition APC/C^{CDH1} also inhibits CDK activity by maintaining cyclin A instability during G1 (Hsu, Reimann et al. 2002; Havens, Ho et al. 2006).

APC/C^{CDH1} activity is inhibited during late G1 and S phases through mechanisms that are intricately linked to cyclin-CDK activity. During late G1 when all APC/C^{CDH1} substrates have been degraded, liberated APC/C^{CDH1} negatively regulates its own ability to ubiquitylate substrates by targeting the E2 UBCH10 for degradation (Yamanaka, Hatakeyama et al. 2000; Rape and Kirschner 2004). Cyclin A-CDK accumulation further enforces the inactivation of APC/C^{CDH1} by mediating phosphorylation of CDH1, which prevents association between the APC/C and CDH1 (Lukas, Sorensen et al. 1999; Rape and Kirschner 2004). E2F-dependent transcription at the G1-S transition,

presumably mediated by cyclin E-CDK2 complexes, results in expression of the early mitotic inhibitor 1 (EMI1), which also inhibits the formation of the APC/C^{CDH1} complex during S and G2 phases (Hsu, Reimann et al. 2002). In addition, inhibition of APC/C^{CDH1} activity is further enforced during S phase and G2 by the E3 ubiquitin ligase SCF^{Roc1}, which targets CDH1 for degradation (Benmaamar and Pagano 2005). This multifaceted approach to APC/C^{CDH1} inhibition enables the accumulation of the APC/C^{CDH1} substrate CDC20 during S and G2 phases. However, untimely formation of APC/C^{CDC20} complexes is also inhibited by EMI1 during this period (Reimann, Freed et al. 2001), therefore preventing the premature destruction of APC/C substrates. Complete inhibition of APC/C activity is maintained until EMI1 is targeted for degradation by the E3 ubiquitin ligase $SCF^{h\text{-}TRCP}$, as a result of PLK1 and CDK1 dependent phosphorylation of EMI1. Indeed stabilisation of EMI1 or ablation of $SCF^{h\text{-}TRCP}$ activity prevents activation of APC/C^{CDC20} and arrests cells in prometaphase (Guardavaccaro, Kudo et al. 2003; Margottin-Goguet, Hsu et al. 2003; Hansen, Loktev et al. 2004).

1.5.6. APC/C^{CDH1} regulates DNA replication licensing and initiation

Formation of prereplication complexes (preRCs) often referred to as replication licensing, requires that CDC6 and chromatin licensing and DNA replication factor 1 (CDT1) cooperate with the origin of replication complex (ORC) to load minichromosome maintenance (MCM) factors 2–7 on to origins of replication (Machida, Hamlin et al. 2005). During S phase preRC formation and subsequent rereplication at freshly liberated origins of replication is prevented through APC/C^{CDH1} inhibition, which allows accumulation of the APC/C substrates and licensing inhibitors cyclin A and geminin. Cyclin A-CDK2 complexes prevent preRC formation by

stimulating nuclear export of CDC6 and by promoting degradation of CDT1. Geminin prevents preRC formation by inhibiting interaction between CDT1 and MCM complexes (Sivaprasad, Machida et al. 2007). It is not until anaphase when APC/C^{CDH1} is reactivated that geminin is degraded and replication licensing can occur (see Fig 1.6 for an overview of APC/C dependent preRC formation) (McGarry and Kirschner 1998; Li and Blow 2005).

1.5.7. APC/C^{CDH1} induces quiescence

Most study of the APC/C has so far been directed towards understanding its role in promoting cellular proliferation, however APC/C^{CDH1} also carries out important functions during the induction and maintenance of quiescence or G0. This was first suggested by the presence of APC/C^{CDH1} in differentiated mouse and human brain sections, while CDC20 expression, which is required for cellular division was absent (Gieffers, Peters et al. 1999). More recently APC/C^{CDH1} has been found to be active during G0 in neurons where it maintains cyclin B instability, and therefore prevents re-entry into G1 and subsequent apoptosis (Almeida, Bolanos et al. 2005). A number of neurodegenerative diseases such as Alzheimer's are characterised by stimulation of N-methyl-D-aspartate receptors (NMDARs) (Parameshwaran, Dhanasekaran et al. 2008) (Fan and Raymond 2007), which are thought to enhance cyclin B1 accumulation by stimulating CDH1 phosphorylation, therefore rendering the APC/C inactive, resulting in neuronal apoptosis and neurodegeneration (Maestre, Delgado-Esteban et al. 2008). The APC/C is also required to maintain quiescence in murine hepatocyte cells, as conditional inactivation of APC2 forces them to re-enter the cell cycle and go on to arrest in metaphase, which ultimately results in liver failure (Wirth, Ricci et al. 2004).

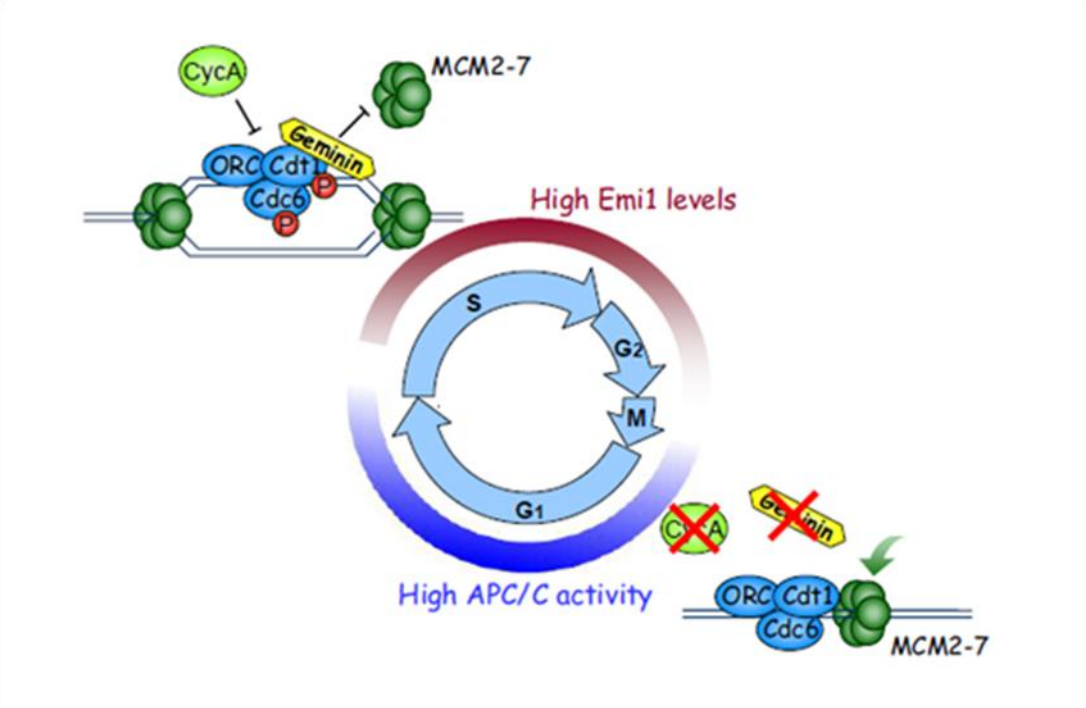


Fig 1.6. The APC/C prevents rereplication by regulating preRC formation

During late mitosis and G1, APC/C activity is high and enables preRC formation by preventing the accumulation of geminin and cyclin A. At the G1/S transition the APC/C is inactivated by EMI1, which allows accumulation of geminin and cyclin A. Geminin prevents preRC formation and therefore rereplication, as geminin associates with cdt1, preventing CDT1 from recruiting MCM to the origins of replication. In addition cyclin A/CDK complexes also prevent preRC formation by phosphorylating CDT1 in order to elicit its degradation and also by phosphorylating CDC6 to stimulate its nuclear export. In late G2 EMI1 is degraded, facilitating APC/C activation and subsequent geminin and cyclin A degradation, which allows a new round of preRC formation (taken from Sivaprasad, U., Y. J. Machida, et al. 2007).

1.5.7.1. APC/C^{CDH1} initiates quiescence in response to TGF-β signalling

Activation of transforming growth factor-β (TGF-β) receptors following binding by TGF-β results in phosphorylation and hence activation of the protein similar to mothers against decapentaplegic homolog 2 (SMAD2) and SMAD3 (also known as receptor-SMADs (R-SMADs)). Phosphorylated SMAD2 and SMAD3 then associate with SMAD4 and translocate into the nucleus where they regulate a program of transcription that is generally considered to inhibit cellular proliferation and induce differentiation (Attisano and Wrana 2000; Massague, Seoane et al. 2005). Interestingly, TGF-β treatment also promotes interaction between APC/C^{CDH1} and SMAD3, which is important for degradation of ski-related novel protein N (SNON) and subsequent induction of quiescence (Wan, Liu et al. 2001). Degradation of the transcriptional repressor SNON enhances transcription of the CDK inhibitors $p15^{INK4B}$ and $p21^{CIP1}$, and as a result promotes quiescence (Wu, Glickstein et al. 2007). It appears that APC/C^{CDH1} can also transduce TGF-β signals by stimulating degradation of the human enhancer of filamentation 1 (HEF1), as HEF1 interacts with CDH1, SMAD3 and APC10 while SMAD3 has previously been shown to mediate HEF1 degradation in response to TGF-β (Nourry, Maksumova et al. 2004). It is thought that HEF1 is targeted for degradation by APC/C^{CDH1} as it has a role in attenuating SMAD3 gene responses (Liu, Elia et al. 2000).

1.5.7.2. Interaction between APC/C^{CDH1} and RB is important for quiescence

RB prevents cell cycle progression by repressing transcription of E2F-responsive genes that are important for cellular proliferation (Classon and Harlow 2002). However, RB can also mediate cell cycle exit, differentiation and senescence independently of its capacity to inhibit transcription. It achieves this by enhancing the degradation of the F-box protein SKP2, and as a result promotes the accumulation of the SKP2 substrate, and

CDK inhibitor $p27^{KIP1}$ (Alexander and Hinds 2001; Ji, Jiang et al. 2004; Thomas, Johnson et al. 2004). Interestingly, a recent study by Binne and Dyson (2007) reveals that RB mediates SKP2 degradation by recruiting APC/C^{CDH1}. Significant observations made by the authors demonstrate that APC/C subunits associate with RB during cell cycle exit, that RB precipitates APC/C ligase activity and that knockdown of CDH1 significantly attenuates the ability of RB to induce cell cycle-arrest. (Binne, Classon et al. 2007).

1.5.7.3. APC/C^{CDH1} couples axon growth and quiescence

The APC/C regulates axon growth, as knockdown of CDH1 expression or over-expression of APC/C inhibitors such as EMI1 increases the rate and final length of axon growth (Konishi, Stegmuller et al. 2004). It appears that the ability of the APC/C to limit axonal growth is coupled to its capacity to induce a G0 state. For instance, APC/C^{CDH1} dependent degradation of SNON is not only required for cell cycle exit, but also occurs in quiescent neuronal cells in response to basal TGF-β stimulation in order to prevent axon growth (Stegmuller, Konishi et al. 2006; Stegmuller, Huynh et al. 2008). In addition, APC/C dependent degradation of the inhibitor of differentiation 2 (ID2) is also required for inhibition of axonal growth (Lasorella, Stegmuller et al. 2006). Deregulation of ID2 expression can prevent quiescence while eptopic expression of ID2 can stimulate quiescent cells to re-enter the cell cycle (Lasorella, Boldrini et al. 2002; Kowanetz, Valcourt et al. 2004; Baghdoyan, Lamartine et al. 2005; Chaudhary, Sadler-Riggleman et al. 2005). These findings may be of particular importance for treatment of spinal injuries, as localised inactivation of CDH1 in damaged neuronal tissue may be sufficient to force neurons back into their growth phase.

1.5.7.4. APC/C^{CDH1} controls differentiation

In addition to its role in regulating cell cycle exit and cell growth, the APC/C also appears to control differentiation. For instance, inactivation of CDH1 inhibits lens differentiation, which is characterised by a reduction in the levels of the CDK inhibitors p21^{CIP1} and p15^{INK4B} (Wu, Glickstein et al. 2007), suggesting that CDH1 facilitates differentiation by stimulating cell cycle exit, and not by directly regulating differentiation. However the APC/C does appear to regulate differentiation independently of cell cycle effects in *Drosophila*, as inactivation of the APC2 homologue *morula* (*Mr*) and various APC/C subunits in *C. elegans* doesn't force cells to re-enter G1, but does result in an increase in the number of glutamate receptors in the post synaptic neuron (Juo and Kaplan 2004; van Roessel, Elliott et al. 2004). Its seems likely that the APC/C may regulate glutamate receptor recycling by direct monoubiquitylation, as monoubiquitylation has been shown to be important for endocytosis and recycling of plasma membrane receptors while APC/C inactivation increases the number of glutamate receptors (Hicke 2001) (Juo and Kaplan 2004).

1.5.8. The APC/C functions in concert with CBP and p300

Work from our lab demonstrates that the transcriptional coactivators CBP and p300 associate with the APC/C *in vivo*, and that CBP and p300 display a direct interaction with the E1A homology domains of APC5 and APC7 *in vitro*. These interactions are important for the APC/C's novel function as a transcriptional regulator, as APC5 and APC7 stimulate CBP auto-acetylation, and therefore enhance the ability of CBP to acetylate histone H4 and hence stimulate transcription. In light of this it seems likely that APC5 and APC7 may also modulate p300's function as a transcriptional regulator in a similar fashion.

A number of observations also from our lab suggest that the APC/C may regulate the G1-S transition by modulating transcription of a panel of genes in conjunction with p300. Significantly, APC5 and APC7 enhance $p21^{CIP1}$ transcription in response to ionising radiation (IR) and also display a physiological interaction with the $p21^{CIP1}$ promoter. This implies that the APC/C can inactivate CDK activity by enhancing expression of $p21^{CIP1}$ in response to DNA damage and hence arrest cells in G1. Moreover APC5 and APC7 may also be able to regulate E2F dependent progression into S phase, which is suggested by the ability of APC5 and APC7 to potentiate E2F-1 dependent transcription of the CDC6 promoter. Lastly, ablation of p300 expression results in an enrichment of cells in G1, suggesting that deregulation of APC/C-p300 target genes might interfere with processes required for G1 progression.

Work from our lab also demonstrates that CBP, in contrast to p300, mediates polyubiquitylation of the APC/C's early mitotic substrates and therefore is important for progression into anaphase. Specifically, CBP immunocomplexes are capable of polyubiquitylating cyclin B *in vitro*, and are therefore associated with APC/C E3 ubiquitin ligase activity. Moreover, ablation of CBP expression results in the stabilisation of cyclin B and other APC/C substrates *in vivo*, while also causing a substantial delay in mitotic progression. These observations could be attributed to the ability of CBP to function as an E4 ubiquitin-elongation enzyme (Grossman, Deato et al. 2003), which may enhance the formation of polyubiquitin chains upon APC/C substrates. Alternatively, there are many instances in which CBP has been demonstrated to alter protein function by direct acetylation (Yang 2004), suggesting the intriguing possibility that CBP might contribute to activation of APC/C^{CDC20} ubiquitin ligase activity by directly acetylating APC/C subunits.

1.5.9. The role of the APC/C during tumourigenesis

Aberrant chromosomal segregation can often give rise to aneuploidy, which is commonly observed in malignant tumours (Castedo and Kroemer 2004). Given that deregulation of the APC/C can give rise to incorrect chromosomal segregation, it is somewhat surprising that there are few reports of altered APC/C subunit expression in human tumours. These include deleterious mutations in APC6 and APC8 identified in colon cancers and reduced APC7 expression in breast cancer (Wang, Moyret-Lalle et al. 2003; Park, Choi et al. 2005). However, there is mounting evidence to suggest that the majority of APC/C pathway deregulation during tumourigenesis occurs due to mutation of APC/C regulatory molecules and over-expression of APC/C substrates. For instance, a recent report shows that the APC/C coactivators CDC20 and CDH1 are over-expressed in a number of malignancies (Singhal, Amin et al. 2003; Lehman, Tibshirani et al. 2007; Kidokoro, Tanikawa et al. 2008). Interestingly, an absence of CDH1 expression correlates with accumulation of SKP2 and a decrease in the level of $p27^{KIP1}$ in breast, colon, and rectal cancers (Fujita, Liu et al. 2008; Fujita, Liu et al. 2008).

Over-expression of securin has also been observed in tumours originating from pituitary (Pei and Melmed 1997), thyroid (Heaney, Nelson et al. 2001) ovary (Puri, Tousson et al. 2001), breast and central nervous system tissues (Solbach, Roller et al. 2004; Chamaon, Kirches et al. 2005). Securin over-expression in tumours and in cell lines results in an increase in aneuploidy and genomic instability (Mu, Oba et al. 2003; Yu, Lu et al. 2003; Kim, Pemberton et al. 2005; Kim, Ying et al. 2007). Over-expression of securin is believed to contribute to tumourigenesis by preventing chromosome segregation, resulting in asymmetrical cytokinesis and subsequent aneuploidy (Yu, Lu et al. 2003). Somewhat paradoxically, inactivation of securin has also been implicated

in aneuploidy, as it appears that securin is also required for separase activation (Jallepalli, Waizenegger et al. 2001).

The APC/C regulator and substrate PLK1, is also over expressed in many cancers such as melanoma and non-small-cell lung cancer (Wolf, Elez et al. 1997) (Strebhardt, Kneisel et al. 2000). Significantly cells in which PLK1 is over expressed display defects in cytokinesis, which manifests as potentially tumourigenic multi-nuclei (Mundt, Golsteyn et al. 1997). Given that PLK1 mediates degradation of the APC/C inhibitor EMI1, it is tempting to speculate that PLK1 over-expression may result in premature EMI1 destruction and hence contribute to early activation of APC/C^{CDC20}, which could result in aberrant chromosome separation and aneuploidy (Hansen, Loktev et al. 2004).

1.5.10. Substrate recruitment requires cooperation between the APC/C and its coactivators

Classically, substrate binding to the APC/C was believed to be entirely mediated through the APC/C's coactivators CDC20 and CDH1; indeed substrate binding to the APC/C is directly proportional to the amount of coactivator present (Passmore and Barford 2005). However, current opinion favours the view that substrate binding is a cooperative process, requiring interaction between substrate and both the APC/C and either CDC20 or CDH1. In support of this notion, it is thought that the APC/C subunit APC10 can also mediate substrate interaction, as ablation of its expression prevents stable binding of the APC/C to its substrates (Passmore, McCormack et al. 2003). In addition the APC/C binds to the D-box of cyclin B in the absence of CDC20 and CDH1 (Yamano, Gannon et al. 2004), and securin in the absence of CDC20 (Eytan, Moshe et al. 2006). However a notable exception to this rule is provided by the mitotic kinase NEK2A, which can bind to the APC/C completely independently of CDC20 via an MR

dipeptide, although association of CDC20 with the APC/C is still required for NEK2A degradation (Hayes, Kimata et al. 2006).

1.5.11. Regulation of APC/C activity by factors intrinsic to the substrate

In addition to direct regulation of the APC/C and its coactivators by factors such as phosphorylation, degradation, and binding of inhibitors, it appears that the ability of the APC/C to mediate protein ubiquitylation is also regulated by factors that are intrinsic to the substrate. For instance, APC/C^{CDC20} recognises substrates that contain two D-boxes, whereas APC/C^{CDH1} preferentially recognises substrates that contain a D-box and a KEN box (Pfleger and Kirschner 2000; Burton, Tsakraklides et al. 2005).

The ordering of substrate degradation is thought to be determined by the rate at which a substrate can be ubiquitylated. For instance, CDC20, PLK1 and aurora A are preferentially multi-ubiquitylated *in vitro* in a manner that corresponds to the order of their degradation *in vivo* (Lindon and Pines 2004). It is thought that this is dependent on whether substrates are polyubiquitylated in a processive single event or distributively over multiple rounds of APC/C binding. As a result of this distributive substrates would have a longer half life due to the prolonged period of time that they are exposed to DUBs and competition by processive substrates. It has been suggested that the fashion in which a substrate is modified may be attributed to the affinity of the APC/C for its destruction motifs or the density of its lysine residues (Rape, Reddy et al. 2006).

Substrate modification can also be regulated by spatial separation of the APC/C and its substrates. For example in *Drosophila melanogaster* embryos only a small fraction of the total cellular pool of cyclin B is associated with the mitotic spindles and is accessible to the APC/C, while the majority found in the cytoplasm remains stable (Clute and Pines 1999). Also, maintenance of yeast fin1 phosphorylation by CDK1 until

metaphase prevents its association with the spindle and hence the APC/C, while its dephosphorylation by CDC14 causes it to migrate to spindle poles where it is degraded by the APC/C (Woodbury and Morgan 2007).

Substrate phosphorylation has also been demonstrated to directly inhibit ubiquitylation of substrates. For example, CDC6 is no longer targeted for degradation by the APC/C after its G1 phosphorylation by cyclin E-CDK2 on residues that lie adjacent to its D-box (Mailand and Diffley 2005).

1.6. Cytokines

Cytokines are a large group of secreted proteins and glycoproteins that allow inter-cellular communication between haemopoietic and non-haemopoietic cells. They are secreted in response to a wide range of stimuli which include infection, inflammation, invasion, injury, irradiation and other cytokines. The list of cytokines is constantly evolving with more than 191 identified to date (Tracey and Cerami 1993; Peters 1996; Tayal and Kalra 2008). Each cytokine exerts its effects by binding to its cognate cell surface receptors, which then activate various cellular signalling cascades (Tracey and Cerami 1993; Peters 1996; Tayal and Kalra 2008).

1.6.1. TNF

The cytokine tumour necrosis factor (TNF) was originally identified on the basis of its ability to promote tumour regression and has more recently been demonstrated to stimulate destruction of tumour vasculature (Old 1988; Weichselbaum, Kufe et al. 2002). However, subsequent studies have also defined TNF as a tumour promoter (Rosen, Goldberg et al. 1991; Suganuma, Okabe et al. 1999; Rivas, Carnevale et al. 2007). For instance, TNF can enhance proliferation of human papilloma virus (HPV)

infected carcinoma cells, as it enhances CDK activity through up regulation of cyclin A and cyclin B, and down regulation of $p21^{CIP1}$ and $p27^{KIP1}$. Other TNF functions include lymphocyte and leukocyte activation and migration, inflammation, fever, apoptosis (Tracey, Lowry et al. 1988; Fiers 1991; Vassalli 1992; Vandenabeele, Declercq et al. 1995).

1.6.1.1. TNF transcription

Levels of TNF are tightly regulated at the level of transcription through binding of sub-sets of transcription factors to the TNF promoter in a cell type and stimulus dependent manner (Falvo, Uglialoro et al. 2000). TNF transcription can be initiated by a diverse range of factors such as lipopolysaccharide (Economou, Rhoades et al. 1989), PMA (phorbol 12-myristate 13-acetate) treatment (Hensel, Mannel et al. 1987; Richards, Dennert et al. 1989) and T-cell receptor (TCR) ligation (Sung, Bjorndahl et al. 1988). The TNF promoter is activated by transcription factors such as activator protein-1 (AP-1) (Rhoades, Golub et al. 1992), nuclear factor kappa B (NF-κB), (Yao, Mackman et al. 1997), Ets1 (Kramer, Wiegmann et al. 1995), and nuclear factor of activated T-cells 1 (NFAT1) (Oum, Han et al. 2002). In addition, CBP and p300 appear to fulfil important roles as activators of the TNF promoter. For example, the TNF promoter contains a cyclic-AMP response element (CRE), which is required for induction of TNF transcription (Tsai, Jain et al. 1996).

1.6.2. IL-2

Activation of TCRs or ligation of cytokine receptors such as the IL-2 receptor (IL2-R) results in IL-2 secretion and expression of IL-2 receptors (Lenardo, Chan et al. 1999). The predominant function of IL-2 is to stimulate proliferation of CD4+ and CD8+ T-

cells, and to provide protection from apoptosis (Miyazaki, Liu et al. 1995). Interestingly, mice deficient in IL-2 signalling display auto-immunity (Schorle, Holtschke et al. 1991; Sadlack, Merz et al. 1993), which is currently attributed to their inability to produce T regulatory cells (T_{reg}), which are important for recognising self reactive T-cells (Almeida, Legrand et al. 2002).

1.6.2.1. IL-2 transcription

The region that extends -300bp from the start site in the IL-2 promoter is sufficient for maximal activation of IL-2 transcription (Jain, Loh et al. 1995), and is activated by transcription factors such as octamer-1 (oct-1) (Kamps, Corcoran et al. 1990; Hentsch, Mouzaki et al. 1992), NF-κB, AP-1 (Brabletz, Pietrowski et al. 1991; Jain, McCaffrey et al. 1992), NFAT1 (Shaw, Utz et al. 1988) and CREB (Solomou, Juang et al. 2001; Solomou, Juang et al. 2001).

Transcriptional regulation of the IL-2 promoter is partially dependent on its antigen receptor response element-2 (ARRE-2) (a model for activation of the IL-2 promoter from the ARRE-2 is displayed in Fig 1.7). The ARRE-2 is prebound by a transcriptional regulatory complex that functions as a repressor in resting T-cells and undergoes expansion into a transcriptional activator after T-cell activation (Shaw, Utz et al. 1988; Randak, Brabletz et al. 1990). It is thought that the ARRE-2 transcriptional regulatory complex induces IL-2 transcription following recruitment of NFAT1, which undergoes nuclear translocation in response to dephosphorylation by the calcium dependent phosphatase calcineurin following T-cell activation (Flanagan, Corthesy et al. 1991; McCaffrey, Luo et al. 1993; McCaffrey, Perrino et al. 1993). T-cell activation also results in protein kinase C (PKC) dependent upregulation of fos and jun, which dimerise and associate with the AP-1 binding site in the 3' region of the ARRE-2, therefore

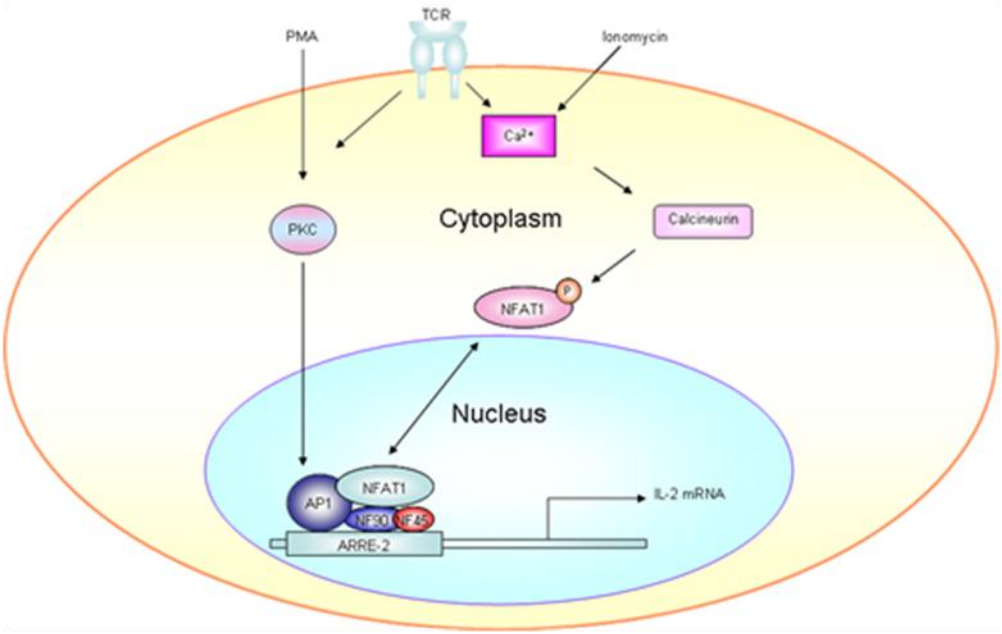


Fig 1.7. A model for activation of the IL-2 promoter from the ARRE-2 by NF90/NF45

Upon T-cell receptor (TCR) ligation or stimulation with PMA and ionomycin NFAT1 is dephosphorylated by the calcium dependent phosphatase calcinuerin. Dephosphorylated NFAT1 then translocates into the nucleus where it binds to the ARRE-2 through interaction with AP-1 and NF90/NF45, therefore activating IL-2 transcription.

stabilising the interaction between the ARRE-2 and NFAT1 (Jain, McCaffrey et al. 1992; Boise, Petryniak et al. 1993; Northrop, Ullman et al. 1993).

A number of observations suggest that the ARRE-2 transcriptional regulatory complex may also contain nuclear factor 90 (NF90) and its binding partner nuclear factor 45 (NF45). For instance NF90/NF45 display similar properties to NFAT1 such as the ability to bind to the ARRE-2, activate the IL-2 promoter upon T-cell stimulation and sensitivity to the NFAT1 transcriptional inhibitors FK506 and cyclosporin A (CsA) (Flanagan, Corthesy et al. 1991; Corthesy and Kao 1994; Kao, Chen et al. 1994; Aoki, Zhao et al. 1998). Recent work reveals that NF90 is associated with the IL-2 promoter *in vivo* and that targeted disruption of NF90 in mice results in severe T-cell lymphocytopenia and an impairment of ARRE-2 driven IL2 transcription (Shi, Godfrey et al. 2007; Shi, Qiu et al. 2007). In addition Ku70 and Ku80 display a capacity to bind to the ARRE-2 element in *vitro*, while Ku70 and Ku80 cooperate with NF90/NF45 to regulate the IL-2 promoter *in vitro* (Aoki, Zhao et al. 1998; Ting, Kao et al. 1998). Significantly Ku70 and NF90 demonstrate dynamic binding to the IL2 promoter *in vivo* in response to PMA and ionomycin stimulation; in this instance a reduction of Ku70 binding and concomitant increase in binding of NF90 to the IL-2 promoter correlates with activation of IL2 transcription, therefore suggesting that Ku70 functions to repress ARRE-2 dependent transcription in unstimulated cells (Shi, Qiu et al. 2007).

1.7. NF90/NF45

Nuclear factor 90 (NF90) and its binding partner nuclear factor 45 (NF45) were originally identified due to their ability to bind to, and regulate transcription from the antigen receptor response element-2 (ARRE-2) found within the interleukin-2 promoter (IL-2) (Corthesy and Kao 1994). Subsequent work revealed that NF90/NF45 also

regulate transcription from the proliferating cell nuclear antigen (PCNA) promoter and the S-locus protein 10 (SP-10) promoter *in vitro* (Reichman, Parrott et al. 2003; Ranpura, Deshmukh et al. 2007). NF90/NF45 also mediate gene expression by regulating splicing (Saunders, Perkins et al. 2001; Zhou, Licklider et al. 2002; Leung, Andersen et al. 2003), mRNA export, mRNA stabilisation (Shim, Lim et al. 2002; Shi, Zhao et al. 2005), and translation (Ting, Kao et al. 1998; Xu and Grabowski 1999). Recent work also demonstrates that NF90/NF45 is important for mitotic exit and may regulate DNA synthesis (Guan, Altan-Bonnet et al. 2008).

1.7.1. Structural features of NF90/NF45

NF90 and NF45 display homology in their dsRBM (double stranded RNA binding motif)-and zinc finger associated motif (DZF), which has been proposed to mediate their DNA binding activity (Shi, Zhao et al. 2005), while NF45 has limited similarity to the prokaryotic transcription factor σ-54 and to human DNA topoisomerase I (Gralla 1991; Merino, Madden et al. 1993).

1.7.2. The NF90 family

The NF90 family comprises five proteins that are generated by differential splicing of the interleukin enhancer binding factor 3 (ILF3) locus, giving rise to proteins with an apparent molecular mass of 90KDa and 110KDa (see Fig 1.8 for an overview of NF90 family structure). These proteins are essentially identical apart from some minor polymorphisms and the addition of a C-terminal GQSY-rich region in NF110, which mediates binding to the splicing factors survival motor neuron (SMN) and translocated in liposarcoma (TLS) (Duchange, Pidoux et al. 2000; Saunders, Perkins et al. 2001; Reichman, Parrott et al. 2003). Interestingly the GQSY-rich region enhances

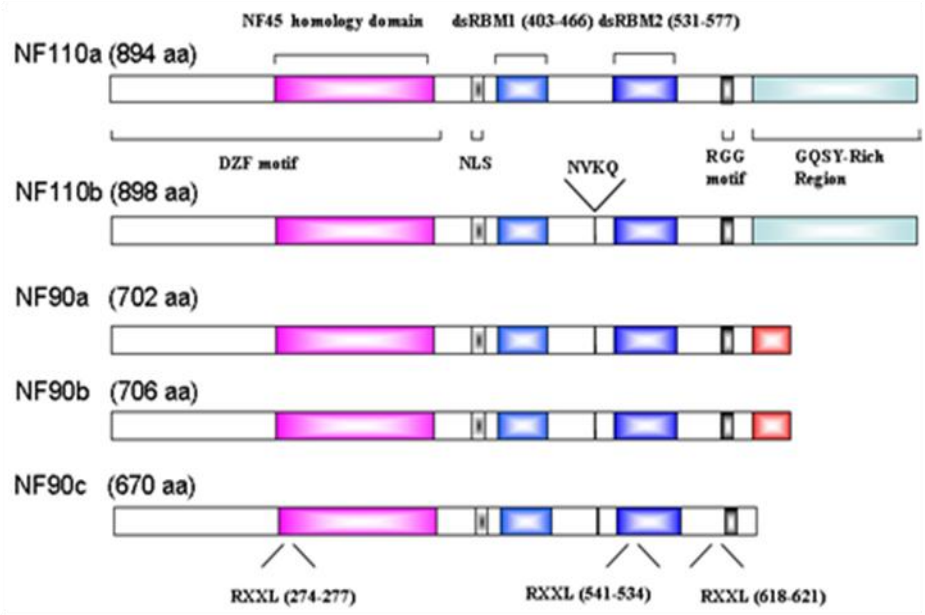


Fig 1.8. A linear representation of the NF90 family

DZF, dsRBM and zinc finger associated motif; NLS, nuclear localisation signal; dsRBM, dsRNA binding motif; RGG, RGG rich motif ; RXXL, putative D-boxes (adapated from Reichman 2003).

the ability of NF110 to activate the PCNA promoter *in vitro*, which may be due to the enhanced DNA binding conferred by the GQSY-rich region (Reichman, Parrott et al. 2003). Members of the NF90 family all contain dsRBMs that allow them to bind to mRNA, small dsRNA regulatory molecules such as small NF90-associated RNA (SNAR) and adenovirus VA RNAII (Liao, Kobayashi et al. 1998; Langland, Kao et al. 1999; Parrott and Mathews 2007). NF90 family proteins may also be capable of binding to RNA through a RGG motif (Patel, Vestal et al. 1999). The dsRBMs of NF90 facilitate binding to other proteins of the dsRBM family such as protein kinase R (PKR) and adenosine deaminase acting on RNA (ADAR1), by utilising dsRNA molecules as binding intermediates to bridge the interaction (Parker, Fierro-Monti et al. 2001; Nie, Ding et al. 2005). The dsRBMs found in NF90 family proteins appear to be important for transcription, as mutation of the dsRBMs, so that they cannot bind dsRNA, results in a reduction in the transcriptional activity of NF90 family members (Reichman and Mathews 2003).

1.7.3. NF90/NF45 and post transcriptional RNA regulation

The presence of dsRBMs and an RGG motif might be important for the ability of NF90 to regulate pre-mRNA and mRNA processing. Indeed NF90/NF45 might play an important role in splicing, as they have been identified as components of the splicesosome in proteomic studies (Zhou, Licklider et al. 2002; Leung, Andersen et al. 2003), while NF110 binds to the splicing factor TLS and associates with spliced RNA (Saunders, Perkins et al. 2001). In addition to its putative role in splicing, NF90 also regulates mRNA stability and nuclear export. For example, NF90 binds to IL-2 mRNA and facilitates its nuclear export and stabilisation (Shim, Lim et al. 2002). Moreover

NF90 mediates cell cycle exit and differentiation through binding to, and stabilization of, $p27^{KIP1}$ and myoD mRNA (Shi, Zhao et al. 2005).

The initial observation that NF90 interacts with the elongation initiation factor 2 subunits alpha, beta, and gamma suggested that NF90 also functions in translation (Ting, Kao et al. 1998). Subsequent work defined NF90 as a *bona fide* translation factor as it was shown to inhibit acid beta-glucosidase translation in liver (Xu and Grabowski 1999). A recent study by Vumbaca *et al.* 2008 also demonstrates that NF90 is required to load vascular endothelial growth factor (VEGF) mRNA onto polyribosomes (Vumbaca, Phoenix et al. 2008).

1.8. The TRIM/RBBC family

The TRIM/RBCC family is composed of at least 66 proteins to date, which all share a common N-terminal region that is composed of a RING finger, B-boxes, and a coiled-coil domain (RBCC), also known as a tripartite motif (TRIM) (Nisole, Stoye et al. 2005). Its most notable member promyelocytic leukaemia (PML), is required for formation of PML nuclear bodies (PML-NBs), which are involved in a number of diverse cellular processes, including cellular senescence, apoptosis and DNA repair (Le, Yang et al. 1996; Wang, Delva et al. 1998; Wang, Ruggero et al. 1998) (Mirzoeva and Petrini 2001; Carbone, Pearson et al. 2002). In acute promyelocytic leukemia (APL) the N-terminal region of PML that encompasses the TRIM/RBCC domain, which is essential for PML nuclear body formation, fuses with the retinoic acid receptor (RAR) (de The, Lavau et al. 1991) (Pandolfi, Grignani et al. 1991; Fagioli, Alcalay et al. 1998), precluding PML body formation and negating its growth suppressive properties (Grignani, Ferrucci et al. 1993; Rogaia, Grignani et al. 1995). Interestingly, there are a number of other observations that demonstrate the importance of the TRIM/RBCC

motif in the regulation of cellular growth. Specifically, the N-terminal TRIM/RBCC of transcriptional intermediary factor 1 alpha (TIF1α) and TIF1γ are fused to the RET receptor tyrosine kinase domain in papillary thyroid carcinomas (PTC) (Klugbauer and Rabes 1999). Moreover, the TRIM/RBCC domain is essential for targeting of TIF1α into PML-associated tracks during adenovirus infection. It has been proposed that the adenovirus protein E3ORF3, sequesters TIF1α into PML-associated tracks in order to modulate its function as a regulator of hormone induced growth suppression, therefore creating an environment that is permissive to viral replication (Yondola and Hearing 2007).

1.8.1. The transcriptional intermediary factor 1 (TIF1) family

The TIF1 family of proteins α, β, γ and δ exhibit ~30% identity at the amino acid level, while TIF1α and TIF1γ exhibit the highest degree of identity at 50% (see Fig 4.1 for a linear depiction of TIF1γ)(Venturini, You et al. 1999; Yan, Dolle et al. 2004). The TIF1 proteins display identical arrangement of their TRIM/RBCC domains, TIF1 signature sequence (TSS), plant homeobox domain (PHD) and bromodomains, suggesting that they share common functions. It has been demonstrated that the PHD domain of TIF1β is required for TIF1β SUMOylation (Mascle, Germain-Desprez et al. 2007). Indeed the PHD domain is structurally similar to the PML RING finger (Capili, Schultz et al. 2001), which is suggested to function as an E3 SUMO ligase (Quimby, Yong-Gonzalez et al. 2006). The bromodomain of TIF1α is required for binding to nucleosomes *in vitro* (Remboutsika, Yamamoto et al. 2002), indicating that it may be necessary for the TIF1 family to regulate transcription *in vivo*.

1.8.1.1. The TIF1 family regulates transcription

TIF1α, TIF1β and TIF1γ have all been demonstrated to function as transcriptional repressors when tethered to DNA, and this function is thought to be dependent on their TSS motif (Le Douarin, Nielsen et al. 1996; Moosmann, Georgiev et al. 1996; Venturini, You et al. 1999). TIF1β associates with the Krüppel associated box (KRAB) domain of human zinc finger proteins (Friedman, Fredericks et al. 1996; Kim, Chen et al. 1996), a motif that is one of the most widely distributed transcriptional repression domains, suggesting that TIF1β functions as a universal co-repressor. Moreover, TIF1α, TIF1β and TIF1δ all interact with heterochromatin protein 1 (HP1) subunits that are located on transcriptionally inactive heterochromatin (Le Douarin, Nielsen et al. 1996; Khetchoumian, Teletin et al. 2004). Furthermore TIF1α can bind to and regulate the AF-2 transcription activation domain of retinoid X (RXR), retinoic acid (RAR), vitamin D3 (VDR), estrogen (ER), and progesterone (PR) nuclear receptors (Le Douarin, Nielsen et al. 1996; vom Baur, Zechel et al. 1996; Beckstead, Ortiz et al. 2001). However, despite the ability of TIF1β and TIF1γ to heterodimerise with TIF1α (Peng, Feldman et al. 2002; Germain-Desprez, Bazinet et al. 2003; Teyssier, Ou et al. 2006), studies have, as yet, been unable to demonstrate that these interactions are important for the ability of TIF1α to regulate nuclear receptor function (Le Douarin, Nielsen et al. 1997; Venturini, You et al. 1999).

1.8.2. TIF1γ regulates cellular proliferation, differentiation and contributes to tumourigenesis

TIF1γ was first proposed to regulate cellular proliferation and differentiation, as a result of its fusion to the rearranged during transfection (RET) receptor tyrosine-kinase domain in papillary thyroid carcinomas (PTC) (Klugbauer and Rabes 1999). More

recent work in zebra fish concludes that the *TIF1γ* ortholog *mon* is required for differentiation of erythroid progenitor cells and posterior mesenchymal cells (Ransom, Bahary et al. 2004). In addition to this, work by He and colleagues (2006) reveals that TIF1γ mediates differentiation of hematopoietic, mesenchymal, and epithelial cells in response to TGF-β signalling. TGF-β is generally considered to inhibit cellular proliferation and induce differentiation via SMAD 2/3-SMAD4 dependent transcription (Attisano and Wrana 2000; Massague, Seoane et al. 2005) However, He *et al.* (2006) show that TGF-β signalling also induces complex formation between activated SMAD 2/3 and TIF1γ. Significantly, knockdown of TIF1γ impedes differentiation of CD34+ haematopoietic progenitors, but has no effect on the growth-inhibitory action of TGF-β, which is shown to be mediated by SMAD4 (He, Dorn et al. 2006).

Ectodermin, the *Xenopus* homologue of TIF1γ mediates ectoderm formation during embryogenesis. Importantly, ectodermin expression is highly enriched at the blastula animal pole, an ectoderm precursor, where it functions to attenuate the mesoderm inducing activity of TGF-β during embryogenesis. Indeed injection of ectodermin mRNA into developing *Xenopus* embryos enhances the expression of the ectoderm marker SRY (sex determining region Y)-box 2 while also reducing levels of the prospective mesoderm markers mix1 and mixer. In this instance ectodermin appears to inhibit TGF-β signalling by functioning as a ubiquitin ligase for the TGF-β signal transducer SMAD4, as ectodermin knock down reduced the amount of hemagglutinin (HA)-ubiquitin SMAD4 conjugates while ectodermin over-expression decreases the levels of SMAD4 and increases the formation of HA-ubiquitin SMAD4 conjugates (Dupont, Zacchigna et al. 2005). It is thought that ectodermin's RING domain is important for its ubiquitin ligase activity, as mutation of RING domain residues that have previously been shown to inhibit interaction between SMAD ubiquitination

regulatory factor 1 (SMURF1) and the ubiquitination machinery (Zhu, Kavsak et al. 1999), appears to impair ectodermin function. This is demonstrated by over-expression of an ectoderm RING domain mutant, which in contrast to the wild type (*wt*) protein, is unable to enhance the formation of HA-ubiquitin SMAD4 conjugates (Dupont, Zacchigna et al. 2005). Also injection of mRNA encoding the ectodermin RING mutant into developing embryos fails to inhibit mesoderm formation, whereas injection of *wt* ectodermin prevents mesoderm induction (Dupont, Zacchigna et al. 2005).

Work by Dupont and Piccolo (2005) also reveals that TIF1γ enhances cellular proliferation in adult cells by interfering with the TGF-β-SMAD4 antiproliferative axis. For instance treatment with TIF1γ siRNA enhances the ability of TGF-β and activin, a member of the TGF-β super family, to induce growth arrest. Consistent with this, TIF1γ knockdown cells treated with activin display increased expression of $p21^{CIP1}$, which crucially mediates the antiproliferative effects of TGF-β (Nicolas and Hill 2003).

In accordance with its role in enhancing proliferation, aberrant TIF1γ expression appears to contribute to tumourigenesis. For instance TIF1γ expression is enriched in the nuclei of the proliferating crypt regions of normal colon samples, whereas staining of colonic adenomas and adenocarcinomas reveals that TIF1γ becomes noncompartmentalized and over expressed. It is thought that TIF1γ promotes tumourigenesis by attenuating the TGF-β response in tumour cells, as C32 colorectal cancer cells become more sensitive to TGF-β growth arrest following TIF1γ depletion. It appears that TIF1γ inhibits growth arrest in these cells through SMAD4 degradation, as TIF1γ depletion in SMAD4 null tumour lines has no effect on cell growth (Dupont, Zacchigna et al. 2005).

Although work by He et al. (2006) and Dupont et al. (2005) shares common ground in that both studies demonstrate that TIF1γ is involved in regulating TGF-β signalling, the

authors differ in the actual mechanism by which TIF1γ achieves this. Dupont et al. (2005) argue that TIF1γ attenuates the TGF-β response by targeting SMAD4 for degradation. In contrast He *et al* (2006) dispute that TIF1γ is a SMAD4 ubiquitin ligase, as they were unable to demonstrate TIF1γ as an ubiquitin ligase, and maintain that TIF1γ doesn't actually bind to SMAD4, but instead competes with SMAD4 for binding to SMAD2/3 in order to mediate differentiation. Whether one of these models dominates remains to seen.

1.9. Aims

The APC/C is a large multicompoment E3 ubiquitin ligase that mediates cell growth, quiescence and progression through mitosis. The APC/C controls the cell cycle through its ability to post-translationally modify important cell cycle regulators with polyubiquitin chains, resulting in their proteasomal-dependent degradation (Kanayama, Tamura et al. 1992; Sudakin, Ganoth et al. 1995; Peters 2002; Skaar and Pagano 2008). However, the precise function of individual APC/C subunits remains unclear. Thus in order to further explore the subtleties of APC/C regulation and discern the functions of individual APC/C subunits the aim of the first part of my study was to identify novel APC7 interacting proteins by mass spectrometery. APC7 was chosen due to the availability an excellent antibody generated for a previous project in our laboratory and also because APC7 is found only in vertebrates (Han, Kim et al. 2008), suggesting additional APC/C functions in vertebrates. The second aim of my project was to determine the functional role of novel APC7 interacting proteins.

CHAPTER 2

MATERIALS AND METHODS

2.1. Molecular biology techniques

2.1.1. Bacterial strains

Strain	Genotype
BL21-codon Plus (DE3)-RIL (Stratagene)	*E. coli* B F^- *ompT hsdS* (r_B- m_B-) *dcm*$^+$ Tet^r *gal* λ (DE3) *end*A Hte [*arg*U *ile*Y *leu*W Cam^r] DH5α
DH5α (Invitrogen)	*sup*E44, *Δlac*U169 (ϕ80 *lac*ZΔM15) *hsd*R17 *rec*A1 *end* A1 *gyr*A96 *thi*-1 *rell*A1

2.1.2. Media

Luria Broth (LB): 10g/L bactotryptone (Difco), 5g/L bacto-yeast extract (Difco) and 10g/L NaCl (pH 7.2), and was sterilised prior to use.

LB-agar plates: LB was supplemented with 1.5% agar and sterilised. Prior to pouring ampicillin was added to a concentration of 100µg/ml.

2.1.3. Antibiotics

Ampicillin was made up as a stock solution of 100mg/ml in sterile distilled water (SDW); filter sterilised through a 0.2µm filter and stored at 4^{o}C.

2.1.4. Enzymes

All enzymes were obtained from Roche unless otherwise stated. ABI PRISM® BigDye™ Terminator v 3.0 Cycle Sequencing Ready Reaction kit with ampli Taq DNA Polymerase was purchased from Applied Biosystems, Perkin Elmer.

2.1.5. Oligonucleotides

Oligonucleotides were custom synthesised by Alta Biosciences Ltd, The University of Birmingham.

2.1.6. Measuring DNA concentration

DNA was diluted 50 fold in SDW and 50µl was added to a quartz micro-cuvette. The absorbance was measured using an Eppendorf photometer at wave lengths of 260 and 280nm

2.1.7. Transformation of Bacteria

Transformation of DNA constructs into BL21 and DH5α competent cells was performed as follows (DNA constructs are displayed in Table 2.2). The cells were initially thawed on ice and 25µl aliquots were then combined with approximately 50ng of DNA, and mixed with gentle pipetting. Following incubation on ice for 30 minutes samples were heat shocked at 42^{o}C for 1 minute and cooled on ice for 5 minutes. After addition of 250µl of LB, the transformation mixture was incubated at 37^{o}C for 1 hour at 220rpm in a shaking incubator. Cells were then pelleted at 13000rpm for 1 minute, resuspended in 100µl of LB broth and spread onto L-agar plates containing ampicillin. After drying, the plates were incubated over night at 37^{o}C and analysed the next morning.

2.1.8. Small scale preparation of DNA

Plasmid DNA was extracted using a Qiagen miniprep kit as per the instructions supplied by the manufacturer. Briefly, a single colony containing the desired plasmid was used to

inoculate 5mls of LB broth supplemented with 100µg/ml of ampicillin, and incubated overnight at 37^{o}C with shaking. Following centrifugation at 3000rpm, cell pellets were re-suspended in 250µl of P1 buffer and transferred to a 1.5ml microfuge tube. Cells were then lysed for 5 minutes after addition of 250µl P2 buffer. The lysis reaction was neutralised by 350µl of buffer N3. After centrifugation at 13000rpm for 10 minutes the supernatant was transferred into QIAprep column and adsorbed by centrifugation at 13000rpm for 1 minute. Following washing with of 750µl of buffer PE and centrifugation, the DNA was eluted into a fresh sterilised 1.5ml microfuge tube by addition of 50µl of sterilised distilled water (SDW) to the column and subsequent centrifugation. The DNA solution was then stored at -20^{o}C.

2.1.9. Large scale preparation of DNA

DNA was purified using a Genelute HP plasmid maxiprep kit (Sigma) following the protocol supplied. In short, 10ml of LB containing 100µg/ml of ampicillin was inoculated with a single colony of bacteria containing the desired plasmid, and left at 37^{o}C for 9 hours at 220rpm in a shaking incubator. The culture was then used to inoculate a 250ml flask of LB containing 100µg/ml of ampicillin, and grown over night as per the initial culture. The following morning the cells were pelleted at 6000rpm for 10 minutes at 4^{o}C, and resuspended in 12mls of resuspension solution and lysed by mixing with 12mls of lysis solution. The solution was allowed to clear for 3-5 minutes and the reaction was stopped by addition of 8mls of neutralisation solution. The resulting cell lysis suspension was immediately combined with 9mls of binding solution and added to the barrel of a filter syringe and left to stand for 5 minutes. Meanwhile a binding column was primed for adsorption of DNA by addition of 12ml of binding solution and centrifugation at 3000 x g for 2 minutes. The contents of the syringe filter

were then added to the binding column and centrifuged at 3000 x g for 2 minutes. Following washing with wash solutions 1 and then 2, DNA was collected by addition of 3mls of elution solution to the binding column and centrifugation. The DNA was then precipitated with 0.7 volumes of room temperature isopropanol and 0.1 volumes of 3M Sodium Acetate pH 5.5 at 13000 x g for 30 minutes at 4 ^{o}C. DNA pellets were washed in 70% ethanol, centrifuged as above for 10 minutes, air dried, resuspended in 100-200µl of SDW and stored at -20 ^{o}C.

2.1.10. PCR of gene sequences flanked by restriction sites

10-100ng of template DNA was mixed with 25pmol of the appropriate forward and reverse primers containing the desired restriction sites (see Table 2.1), 5µl of 10X buffer, 1µl of dNTPs (10mM), 1µl of *pfu* DNA polymerase (2.5 U/µl) (Promega) and SDW up to total volume of 50µl. DNA polymerase was added to sample reactions after 5minutes at 95^{o}C in thermal cycler. The reaction solutions were mixed and placed in the thermal cycler (GeneAmp PCR Systems 9600) and subjected to the following conditions: 1 cycle of 95^{o}C for 5 minutes, 55^{o}C for 2 minutes, 72^{o}C for 2 minutes; 15-30 cycles of 95^{o}C for 1 minute, 55^{o}C for 30 seconds, 72^{o}C for 1minute/Kb; and 1 cycle of 72^{o}C for 7 minutes. The resulting DNA products were resolved by agarose gel electrophoresis and purified using a Qiagen gel extraction kit as detailed below.

2.1.11. Real time PCR

16-HBE cells were treated with the appropriate siRNAs as described in section 2.3.2. 3 days later the cells were either mock treated or stimulated for 2.5 hours with 20ng/ml of PMA and 2µM ionomycin in DMEM-HEPES-supplemented with 8% FCS. Next, total RNA was purified using an RNAeasy kit (Qiagen) as per the manufacturers instructions.

Following elution in 30µl of RNase free water any remaining DNA was digested by addition of 3 µl of 10X DNA digestion buffer and 1µl of DNase (Ambion) followed by incubation at 37^{o}C for 20 minutes. The reaction was quenched by addition of 3µl of inactivating reagent (Ambion) and RNA was repurified using an RNAeasy kit (Qiagen) following the manufacturers instructions.

cDNA synthesis was performed using the Promega IMProm-II reverse transcription system. Each reaction contained 400ng of RNA template, 4µl of 5X reaction buffer, 0.5mM dNTPs, 20units of recombinant RNase inhibitor, 1µl of reverse transcriptase, 1µl of random primer and was made up to 20µl with nuclease free water. The reverse transcription reaction was then transferred to a thermal cycler (GeneAmp PCR Systems 9600) and subjected to a temperature of 25^{o}C for 5 minutes followed by incubation at 42 ^{o}C for 1 hour. The reverse transcriptase was then inactivated at 70 ^{o}C for 15 minutes in preparation for realtime-PCR (RT-PCR).

The RT-PCR was carried out using 10ng of cDNA and 0.4µM of TNF primers or 2.5ng of cDNA and 0.1µM β-actin primers as a normalising control, and 14ul of mastermix made up to 20 µl with nuclease free water. Alternatively, a no template control reaction was carried out with each primer set. A standard curved was generated using 1ng, 0.5ng, 0.2ng, 0.05ng and 0.01ng of mock treated control cell cDNA with either 0.4µM TNF primers or 0.1µM β-actin primers and 14ul of mastermix again made up to 20µl with nuclease free water. Each reaction was carried out in triplicate. The reactions were then transferred to an ABI PRisim 7700 sequence detection system and cycled at the following conditions: 95 ^{o}C for 10 minutes, and 40 cycles of 95 ^{o}C for 15 seconds and 60 ^{o}C for 1 minute.

Table 2.1 Primers for generating restriction sites flanking coding regions

Gene	Primer	Sequence
NF45	Forward	5'GTCATGAATTCATGAGGGGTGACAGAGGCCGTGGT 3'
	Reverse	5'ATTGCCTCGAGTTAGGCTCCAGTCTTTCCCTTGGG 3'
NF90	Forward	5'GTCATGAATTCATGCGTCCAATGCGAATTTTTGTGA 3'
	Reverse	5'GTCATGAATTCTCATGTAGCCTCCATGGTTGGCGCC 3'
NF90 Fragment 1	Forward	5'GTCATGAATTCATGCGTCCAATGCGAATTTTTGTGA 3'
	Reverse	5'GTTACGCGGCCGCATAGGTGGTGCTTGGTGGGATCTG 3'
NF90 Fragment 2	Forward	5'GTCATGAATTCGCCATTACGCCCATGAAACGCCCA 3'
	Reverse	5'GTTACGCGGCCGCTACCACTGGGGCAGGGGCCACCAC 3'
NF90 Fragment 3	Forward	5'GTCATGAATTCGAAGCTGTCTCCACCCCTAGTGCG 3'
	Reverse	5'GTTACGCGGCCGCTCATGTAGCCTCCATGGTTGGCGC 3'
NF90 dsRBM1	Forward	5'GTCATGAATTCGCTATGAATGCCCTGATGCGGTTGA 3'
	Reverse	5'GTTACGCGGCCGCCATGTCCTGTAACACCTTAACGGCC 3'
NF90 dsRBM2	Forward	5'GTCATGAATTCAAGAACCCAGTCATGGAGCTGAACG 3'
	Reverse	5'GTTACGCGGCCGCCTTTTCTAGGGCAGCAAGAGCAGCG 3'
TIF1γ Fragment 1	Forward	5'GTCATGGATCCATGGCGGAAAACAAAGGCGGCGGC 3'
	Reverse	5'ATTGCCTCGAGCTGTCTTTCCTTTGTAACATTCTC 3'
TIF1γ Fragment 2	Forward	5'GAAATGGATTCATGAAGTTACTACAGCAGCAGAAT 3'
	Reverse	5'ATTGCCTCGAGCTGATCAGATTTGAAACTAAGACT 3'

2.1.12. Cloning

2.1.12.1. Generation of DNA coding regions flanked by restriction sites

To facilitate cloning, gene sequences with 5' and 3' flanking restriction sites were generated by PCR as described above. Alternatively, coding sequences were excised from a vector by restriction enzyme digest for subcloning.

2.1.12.2. Restriction digest

The digest was carried out for 3 hours at 37^{o}C with the appropriate restriction enzymes, in a volume of 50µl that contained 500ng-2µg of DNA, 5µl of 10x buffer, and 25 units of each restriction enzyme. DNA was then gel purified as detailed below.

2.1.12.3. Ligation

The gene of interest was cloned in to pcDNA 3.1 for *in vitro* transcription/translation (IVT) and *in vivo* over-expression or pGEX 4T-1 in order to generate GST-fusion proteins (see Table 2.2 for all constructs made for this study). Ligation reactions were incubated at 16 ^{o}C overnight and contained 3µl of 10X buffer, 1 unit of T4 DNA ligase, 500ng-2µg of digested insert, and 20ng of vector cut with complementary restriction enzymes, made up to a total volume of 30µl with SDW. The following day the reactions were heated at 65^{o}C for 15 minutes then placed on ice for 5 minutes, mixed with 25µl of DH5α cells, and transformed as previously described. DNA from the resulting bacterial colonies was purified using a Qiagen miniprep kit and sequenced.

2.1.13. Agarose gel Electrophoresis

Analysis of DNA was performed on agarose gels prepared with 50ml of TBE (100mM Tris,100mM boric Acid, 2mM EDTA, pH 8.3), 1% w/v agarose and ethidium bromide

was added to a final concentration of 0.5µg/ml. Samples were diluted 1/6 with 6X gel buffer [30%v/v of glycerol, 0.25% w/v of bromophenol blue and 0.25% w/v of xylene cyanol FF in SDW] and then loaded. Gel electrophoresis was performed in 1X TBE at 60V for approximately 40 minutes, depending on the size of the DNA to be analysed. DNA was visualised by exposure to UV light.

2.1.14. Gel extraction of DNA

In order to purify DNA generated by PCR or subjected to digestion with restriction enzymes, samples were first resolved by agarose gel electrophoresis. The DNA of interest was excised and the DNA was purified using QIAquick Gel extraction kit (Qiagen) as per the manufactures instructions.

2.1.15. Sequencing

Sequencing reactions were carried out in order to validate the DNA sequence of coding regions generated by PCR. Each reaction was carried out using a kit obtained from ABI and consisted of: 1µl of terminator ready reaction mix (Big Dye™ terminator V3.0), 4µl of 5X buffer, 200ng-500ng of DNA, 5pmol of primer and made up to 20µl with SDW (see Table 2.3 for primers used). Reactions were placed in a thermal cycler (GeneAmp PCR Systems 9600) for the sequencing reaction. A total of 25 cycles were performed using the following conditions: 96^{o}C for 10 seconds, 50^{o}C for 5 seconds, and 60^{o}C for 4 minutes. The PCR products were precipitated by adding 80µl of 75% v/v isopropanol for 30 minutes at room temperature (RT) and pelleted at 13000rpm for 20 minutes in a centrifuge. The pellet was then rinsed with 200µl of 75% v/v isopropanol and centrifuged for a further 5 minutes. The residual isopropanol was removed by air drying at a temperature of 90 ^{o}C. Following addition of 10µl of formamide the mixture

was heated for 5 minutes at 95^{o}C, quenched on ice, and briefly centrifuged before loading on the ABI Prism 337 Automated DNA sequencer (PE Applied Biosystems). Sequences were analysed using the NCBI nucleotide-nucleotide BLAST analysis obtained online at http://www.ncbi/nlm/nih.org.

Table 2.2. Gene expression constructs used in this study

Gene	Vector	Application	Source
NF45 (aa 1-390)	pGEX 4T-1 pcDNA 3.1	GST-fusion protein production *In vivo* over-expression	PCR from cDNA
NF90 (aa 1-671)	pGEX 4T-1 pcDNA 3.1	GST-fusion protein production *In vivo* over-expression	PCR from cDNA
NF90 Fragment 1 (aa 1-366)	pGEX 4T-1	GST-fusion protein production	PCR from cDNA
NF90 Fragment 2 (aa 367-501)	pGEX 4T-1	GST-fusion protein production	PCR from cDNA
NF90 Fragment 3 (aa 502-671)	pGEX 4T-1	GST-fusion protein production	PCR from cDNA
NF90 dsRBM1 (aa 403-466)	pGEX 4T-1	GST-fusion protein production	PCR from cDNA
NF90 dsRBM2 (aa 531-592)	pGEX 4T-1	GST-fusion protein production	PCR from cDNA
TIF1γ Fragment 1 (aa 1-392)	pGEX 4T-1	GST-fusion protein production	PCR from cDNA
TIF1γ Fragment 2 (aa 393-800)	pGEX 4T-1	GST-fusion protein production	PCR from cDNA
APC5 (aa 1-755)	pcDNA 3.1	*In vivo* over-expression and IVT	Dr A Turnell

APC7 (aa 1-565)	pcDNA 3.1	*In vivo* over-expression and IVT	Dr A Turnell
ΔAPC7 (aa 1-146-433-565)	pcDNA 3.1	*In vivo* over-expression	Dr A Turnell

Table 2.3. Primers utilised for sequencing

Primer	Sequence
pcDNA3 forward	5'ACTCACTATAGGGAGACCCAAGC 3'
pcDNA3 reverse	5'GCAACTAGAAGACACAGTCGAGG 3'
pGEX4T1 forward	5'GGGCTGGCAAGCCACGTTTGGTG 3'
pGEX4T1 reverse	5'CCGGGAGCTGCATGTGTCAGAGG 3'

2.1.16. Production and purification of GST fusion proteins

pGEX 4T-1 expression constructs containing the coding sequence of interest down stream of a region that encodes glutathione S-transferase (as displayed in Table 2.2), were transformed into BL21 cells and plated out as previously described. The following day two colonies were used in order to inoculate 20mls of LB broth containing 100μg/ml of ampicillin, the starter culture was incubated overnight at 37^{o}C with shaking at 200rpm. This was then used to inoculate 500mls of LB medium in the presence of 100μg/ml ampicillin and grown at 37ºC for 2.5 hours with shaking at 200rpm. Once the bacteria had reached an appropriate density (A600 = 0.6-0.8), the culture was cooled to 30^{o}C and incubated for further 3 hours in the presence of 0.02% w/v isopropyl β-D-1-

thiogalactopyranoside (IPTG) (Sigma). The bacterial cells were then pelleted at 6000rpm for 10 minutes at 4^{o}C and stored at -80^{o}C.

In order to purify the GST-fusion proteins, the bacterial cell pellet was lysed in 20mls of lysis buffer containing 1X PBS, 1mM EDTA (pH 8) and 1% v/v Triton X-100. The lysate was then sonicated 3 times on ice for 45 seconds, at two minute intervals. The lysate was then centrifuge for 20 minutes at 20000rpm at 4^{o}C, the resulting supernatant was transferred to a fresh tube and centrifuged again as before. The supernatant was then mixed with 2mls of a solution containing 1:1 lysis buffer and glutathione-agarose beads, and rotated at 4^{o}C for 2 hours. The beads were then centrifuged at 3000rpm and washed in lysis buffer 3 times and twice with wash buffer (1X PBS and 1mM EDTA pH 8.0). In order to elute the GST-fusion proteins, the glutathione-agarose beads were incubated with 2ml of 20mM glutathione and 50mM Tris solution pH 8.0 again at 4^{o}C for 2 hours with rotation. Following centrifugation the supernatant containing eluted GST-fusion protein was transferred to dialysis tubing, which was previously hydrated in a warm solution of 3% $NaHCO_3$, 2mM EDTA pH 8.0 for 20 minutes to chelate metal ions. This was followed by overnight dialysis at 4^{o}C in 2L of 25mM Tris pH 8.0, 100mM NaCl, 1mM dithiothreitol (DTT) (Sigma) and 10% v/v glycerol. The dialysed GST-fusion protein was transferred to a new tube and its protein concentration was determined by Bradford assay and quantified against a standard curve. The purity of the proteins was assessed by SDS-PAGE electrophoresis and Coomassie blue staining. The protein was stored in aliquots at −80^{o}C.

2.1.17. *In vitro* transcription/translation (IVT)

Eukaryotic in vitro translation was carried out using a Promega TNT T7/SP6 coupled rabbit reticulocyte or wheat germ lysate system as per the manufacturer's instructions.

Briefly the reaction consisted of 25µl reticulocyte or wheat germ lysate, 2µl reaction buffer, 1µl T7 or SP6 RNA polymerase, 1µl of 1mM amino acid mixture minus methionine, 2µl of [35S]-methionine (1000Ci/mmol at 10mCi/ml; Amersham Biosciences). 0.5µl RNasin ribonuclease inhibitor (40U/µl), 1µg DNA template and nuclease free water to a final volume of 50µl. The reaction was incubated at 30^{o}C for 90 minutes, centrifuged and then stored at -80^{o}C.

2.1.18. Luciferase reporter gene assays

Typically, 0.5-2µg of the relevant DNA constructs were transfected into HCT116 cells using Lipofectamine 2000 according to manufacturer's instructions (see Table 2.2 for the expression constructs used). The total amount of DNA added was kept equal by adding 'empty' pcDNA3.1 vector to all transfected cells. After 48 hours the cells were washed twice in cold saline and lysed in 400µl of lysis reagent for 30 minutes at RT. Cell debris was then pelleted by centrifugation at 13000rpm for 5 minutes. 20µl samples, of equivalent protein concentration, were mixed with 100µl of luciferase assay reagent. Light emission was measured immediately using a Berthold luminometer. Cell lysis and luciferase reagents were purchased from Promega.

2.2. Tissue culture techniques

2.2.1. Tissue culture media and solutions

All tissue culture reagents were presterilised and purchased from Invitrogen unless otherwise stated. All reagents were stored at 4^{o}C and pre-warmed to 37^{o}C unless otherwise specified. Cell lines were maintained in Roswell Park Memorial Institute 1640 medium (RPMI-1640), or Dulbecco's Modified Eagle's HEPES buffered Medium

(DMEM-HEPES) supplemented with 2mM L-glutamine. Before use, all media were supplemented with 8% foetal calf serum (FCS) unless otherwise stated.

2.2.2. Maintenance of cell cultures

Cells were grown in humidified incubators in a 95% air and 5% carbon dioxide environment at 37^{o}C. Adherent cell lines were maintained in DMEM-HEPES. To subculture adherent cells, medium was removed when cells reached confluency, and the cells were then washed twice in PBS. 1ml of trypsin solution was added to the cells, which were then incubated at 37^{o}C as necessary. Detachment of the adherent cells was confirmed by microscopy, after which cells were washed with media containing FCS to inactivate the trypsin and centrifuged for 4 minutes at 1400 rpm. The cells were re-suspended in DMEM-HEPES complete media and re-plated to the required density. Suspension cells were grown in 75cm^2 tissue culture flasks and were subcultured by removing 50% of the cell culture volume and mixing 1:1 with RPMI-1640.

2.2.3. Cell lines

The cells used in this study, are summarized in Table 2.4

Table 2.4. Human cell lines used in this study

Name	Description	ATCC number
HCT116	Colon carcinoma cell line expressing *wt* p53	CCL -247
16HBE	Bronchial epithelial cells expressing SV40 large T-antigen	Gift from Dr C Stellato
A549	Small cell lung carcinoma cell line	CCL -185
Jurkat T-	Thymus cells expressing SV40 large T-antigen	TIB-152

cells		
HeLa	Cervical carcinoma cell line expressing human papilloma virus (HPV-18)	CCL-2.2

2.2.4. Cryopreservation of cells

Cells were trypsinised, pelleted by centrifugation at 1400 rpm for 4 minutes. Each confluent plate was divided into 5 aliquots and each aliquot re-suspended in 1ml of cold DMEM-HEPES complete with 10% v/v dimethyl-sulphoxide (DMSO). The cells were stored in sterile ampoules, and placed in the neck of liquid nitrogen containers or in freezing boxes at -80^{o}C over night, allowing cooling at a controlled rate of 1^{o}C/minute. The ampoules were then transferred to liquid nitrogen storage tanks.

2.2.5. Recovery of cells from liquid nitrogen

To recover cells from nitrogen, cell suspensions were thawed quickly in a 37^{o}C waterbath followed by immediate dropwise transfer to a centrifuge tube containing 10ml of culture medium. The cells were then pelleted at 1400rpm and re-suspended in the appropriate fresh culture medium and re-plated and incubated at 37^{o}C.

2.2.6. Nocodazole release

A549 cells were grown to 90% confluence and incubated at 37^{o}C overnight with DMEM-HEPES supplemented with 8% FCS and nocodazole at a concentration of 1µg/ml. Mitotic cells were then harvested by shakeoff and washed twice in DMEM-HEPES containing 8%FCS. After resuspension in DMEM-HEPES containing 8%FCS the cells were then plated out and harvested at the appropriate time.

2.3. Cell Biology Techniques

2.3.1. Transient transfection of mammalian cells using Lipofectamine 2000

Twenty four hours prior to transfection HCT116 cells were plated onto a 6cm tissue culture dish and incubated at 37ºC in a CO_2 incubator until the cells were 90% confluent. Transfection solution was prepared by incubating 10 µl of Lipofectamine 2000 transfection reagent (Invitrogen) with 90µl of serum-free DMEM-HEPES, for five minutes at room temperature. 1-2µg of plasmid DNA was diluted into 100µl of serum free DMEM-HEPES medium. Then the transfection reagent and DNA mixture were combined, mixed gently and incubated at room temperature for 30 minutes to allow DNA-liposome complexes to form. Meanwhile, cultured cells were washed with 2mls of DMEM-HEPES. For each transfection, 3.8mls of DMEM-HEPES containing 8% FCS was added into the transfection mixture and overlaid onto the rinsed cells. The cells were then incubated with the transfection mixture at 37ºC. Transfected cells were harvested at appropriate time after transfection and assayed for protein expression or reporter gene activity. The DNA plasmids used for transfection in this study are listed in Table 2.2.

2.3.2. Transient down regulation of gene expression by small interfering RNAs

Purified, annealed double stranded 21-mer RNA oligonucleotides, with dTdT overhangs were custom synthesised by Ambion and predesigned non-silencing control siRNA was also purchased from Ambion (all siRNAs used are displayed in Table 2.5). 8µl of the desired siRNA (40µM) was mixed with 20µl of Oligofectamine (Invitrogen) in 972µl of Optimem medium and left for 30 minutes. 1ml of the siRNA solution was added to

HeLa cells grown to 35% confluence on 6cm tissue culture dishes and washed twice with 4mls of Optimem medium. The following day 3mls of DMEM-HEPES supplemented with 12% FCS was added to each dish. Cells were typically harvested 60 hours post transfection.

Table 2.5. siRNAs used in this study

Target protein	siRNA	Sense sequence
Non-silencing	Negative Control #1 siRNA	Proprietary
NF45	NF45	5'GCACCUGAUGAAACUUCCUtt 3'
NF90	NF90	5'CGGGAAGAUAUCACACAGAtt 3'
TIF1γ	TIF1γ_1	5'CCUGCAUCUAGAAAGUGAAtt 3'
TIF1γ	TIF1γ_2	5'GCGACUGAUUACUUUCCAGtt 3'

2.3.3. Flow cytometric analysis

Flow cytometry was used to asses the cell cycle distribution of HeLa cells transfected with non-silencing and TIF1γ-specific siRNA, which are displayed in Table 2.5. Cells were harvested by trypsinisation 72 hours after transfection, washed twice with ice- cold PBS and resuspended in 3ml of PBS. Cells were fixed by adding 7mls of ice-cold ethanol whilst vortexing to avoid cell clumping and stored at -20 ºC.

On the day of analysis cells were pelleted at 1600rpm for 5 minutes and washed in PBS twice, permeabilised in ice cold PBS with 0.25% Triton X-100 for 15 minutes. After washing with 10mls of PBS with 1% BSA, cells were incubated with 100µl 1% w/v BSA containing an anti-phosphohistone H3 (Ser10) polyclonal antibody for 1.5 hours at room temperature. Samples were then washed twice with 1ml of 1% w/v BSA in PBS and resuspended in 100µl 1% w/v BSA in PBS containing fluorescein isothiocyanate

(FITC)-conjugated-anti-Rabbit antibody, and left in a light resistant container for 30 minutes (antibody dilutions used for this procedure are displayed in Table 2.6). After washing with 1ml of PBS with 1% w/v BSA and then 1ml of PBS, cells were resuspended in 1 ml of PBS containing propidium iodide (25μg/ml) and 0.1mg/ml of RNAse A, and left at RT in a light resistant container for a further 30 minutes. Cell cycle analysis was carried out using a Coulter XL-MCL flow cytometer (Beckman-Coulter).

2.4. Protein chemistry techniques

2.4.1. Preparation of cultured adherent cell samples for protein analysis by Western blot analysis

Tissue culture medium was removed and cells were washed twice in 5ml of cold 0.9% saline. Cells were lysed in buffer containing 9M urea, 50mM Tris (pH 7.3) and 0.15M β-mercaptoethanol. After incubation at 4^{o}C for 15 minutes with gently rocking, cells were detached from the tissue culture dish with a plastic scraper. Harvested cells were sonicated for 10sec on ice and clarified by centrifugation at 13000 rpm for 20 minutes at 4^{o}C. The supernatant was transferred to a fresh centrifuge tube for protein determination or storage at −80ºC.

2.4.2. Protein determination by Bradford assay

A small volume (2-10μl) of the protein sample was carefully diluted and mixed with 1ml of Bradford reagent (BioRad). After 15 minutes the absorbance was measured in a spectrophotometer at λ 595 nm against the distilled water blank. Protein concentrations were determined by comparison with a standard calibration curve prepared from known quantities of bovine serum albumin (0-10μg).

2.4.3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Immunoprecipitates, GST pull-down samples or 50µg of whole cell lysates were electrophoresed on 12% polyacrylamide gels that included ultra pure Biorad acrylamide solution (30% w/v acrylamide, 0.8% N, N-bis acrylamide), 0.1M Tris,0.1M bicine (N, N-bis [2-hydroxy-ethyl-glycine]) pH 8.3, 0.1% w/v SDS and 0.3% v/v TEMED (N, N, N', N'-tetramethyl-ethylenediamine) in a total volume of 50ml. After the addition of 300µl of 10% ammonium persulfate solution (APS), the gel was poured into the apparatus (Hoefer) and left to polymerise. A well-forming comb was inserted into the gel before polymerisation occurred. Once the gel had set the comb was removed, the wells rinsed with distilled water and immersed in running buffer (0.1M bicine, 0.1M Tris pH 8.3, 0.1% w/v SDS). Samples were diluted in an equal volume of Laemmli sample buffer (25% v/v glycerol, 62.5mM Tris pH 6.8, 5% β-mercaptoethanol, 2% w/v SDS and 0.01% w/v bromophenol blue), boiled for 5minutes and applied to the gel. Pre-stained molecular weight markers were also applied to the gel. The samples were typically electrophoresed overnight, at 12mA in running buffer.

2.4.4. Staining of polyacrylamide gels

After electrophoresis, gels were stained in 0.1% w/v Coomassie brilliant blue R-250 (Sigma) in methanol/glacial acetic acid/water (3:1:6 v/v) on a shaker for 20 minutes at room temperature, and subsequently destained in methanol/glacial acetic acid/water (3:1:6 v/v) as required.

2.4.5. Staining of proteins on nitrocellulose membranes

To evaluate even loading and efficient transfer of proteins after electroblotting, nitrocellulose filters were stained with Ponceau S stain (0.1% w/v Ponceau S (Sigma) and 3% v/v trichloroacetic acid (TCA)) for a few minutes. After visualization of protein bands, the dye on the membrane was washed off with distilled water and the proteins on it were subjected to immunoblot analysis.

2.4.6. Detection of radioactive proteins on gels

Following electrophoresis, gels were stained with Coomassie blue as previously described. The gel was then soaked in the Amplify™ reagent (Amersham Bioscience) for 2 hours with agitation. Amplify™ reduces exposure times for gels containing [35S]-labelled samples. The gel was dried under vacuum at 80ºC for 1 hour and exposed for autoradiography at −20ºC.

2.4.7. GST-pulldown

The binding capacity of [^{35}S]-labelled APC5 and APC7 for GST-NF45, GST-NF90 and various GST-NF90 fragments was assayed by GST-pulldown. Typically, 20µg of GST-fusion protein was incubated with 20µl of [^{35}S]-labelled protein on ice for 1 hour. Volumes were then equalised by addition of low salt buffer (50mM Tris pH 7.4, 0.15M NaCl and 1% NP-40), and protein complexes were isolated by incubation with 20µl glutathione-agarose beads for 1 hour with rotation at 4^{o}C. Beads were then washed with 1ml of low salt buffer 3 times. GST-protein complexes were then eluted with 80µl of buffer containing 20mM glutathione and 50mM Tris pH 8.0. Lastly the eluate was mixed with Laemmli sample buffer and boiled for 5 minutes in preparation for SDS-PAGE.

2.4.7.1. Treatment of GST-pulldowns with RNase V1

Prior to elution as described above, glutathione-agarose beads were washed twice with RNase V1 digestion buffer (Ambion) and resuspended in the 30 µl of the same buffer containing 6U of RNase V1 (Ambion). The samples were then incubated at 37°C for 25 minutes and the glutathione-agarose beads containing the still bound proteins were centrifuged and washed twice in 500µl of low salt buffer.

2.4.8. In-gel digestion of proteins for analysis by mass spectrometry

Following Coomassie staining, protein bands of interest were excised with a sterile scalpel and placed in a sterile 1.5ml microfuge tube and washed twice in 400µl of wash solution 1 (50% v/v acetonitrile and 50mM ammonium bicarbonate) for 45 minutes at 37^{o}C on a shaker. After removal of the wash buffer the gel slices were dried in a DNA-Mini vacuum centrifuge for 30 minutes. The gel slice was then treated with 250µl of reduction solution (10% v/v acetonitrile, 50mM ammonium bicarbonate and 50mM DTT) at 56^{o}C in a water bath for 1 hour. The supernatant was then removed and replaced with alkylating solution (10% v/v acetonitrile, 50mM ammonium bicarbonate and 200mM iodoacetamide) and left for 30 minutes at room temperature in total darkness. After 3 washes with 500µl of wash buffer 2 (10% v/v acetonitrile and 40mM ammonium bicarbonate) at room temperature for 15 minutes, the gel slice was dried in DNA-Mini vacuum centrifuge for 1hour. In order to digest any fixed proteins the gel slice was re-hydrated with 20µl of 12.5µg/ml modified trypsin (Sigma) in wash buffer 2 at room temperature for 1 hour. In order to immerse the gel slice, a further 20µl of wash buffer 2 was added and the sample was incubated overnight with shaking at 37^{o}C. The next day the supernatant was removed and stored in a 1.5ml eppendorf tube. The gel

slice was then twice incubated with 25µl of 50% acetonitrile and 0.1% formic acid for 30 minutes at 37^{o}C with shaking. Following centrifugation the supernatant was removed and pooled with the tryptic supernatant from the previous step and stored at -20^{o}C until mass spectrometric analysis.

2.5. Immunological techniques

2.5.1. Antibodies

All antibodies utilised in this study, including their application, dilution, species and source are displayed in Table 2.6.

Table 2.6. Antibodies used in this study

Antibody	Antigen	Application	Dilution	Species	Source
TIF1γ78	TIF1γ	WB	1:500	Rabbit	Made in conjunction with Eurogentec
TIF1γ98	TIF1γ	WB IP	1:500 1:10	Rabbit	Made in conjunction with Eurogentec
αNF45	NF45	WB IP	1:1000 1:25	Rabbit	Prof P Kao

αNF90	NF90	WB IP	1:1000 1:25	Rabbit	Prof P Kao
DRBP76	NF90	ChIP	1:20	Mouse	BD Transduction Labs
AF3.1	APC3	IF IP	1:500 1:25	Mouse	CRUK
#4	APC5	WB IP ChIP	1:2000 1:25 1:160	Mouse	Dr A Turnell
#33	APC5	ChIP	1:160	Mouse	Dr A Turnell
197	APC7	WB IP ChIP	1:1000 1:25 1:160	Rabbit	Dr A Turnell
198	APC7	ChIP	1:160	Rabbit	Dr A Turnell
B-5-1-2	α-Tubulin	IF	1:5000	Mouse	Sigma
Phospho-histone H3 (ser10)	Phospho-histone H3 (ser10)	IF FACs	1:1000 1:100	Rabbit	Cell signalling
Anti-Mouse-HRP	Mouse IgG	WB	1:1000	Goat	DAKO
Anti-Rabbit-HRP	Rabbit IgG	WB	1:2000	Swine	DAKO

Alexa fluor® 488 anti-Rabbit	Rabbit IgG	FACs	1:50	Donkey	Invitrogen
Alexa fluor® 546 anti-Rabbit	Rabbit IgG	IF	1:1000	Goat	Invitrogen
Alexa fluor® 488 anti-Mouse	Mouse IgG	IF	1:1000	Goat	Invitrogen
Anti-FLAG M2	FLAG	WB	1:1000	Mouse	Sigma

2.5.2. Western blotting

Following electrophoresis, cell lysates were electrophoretically transferred to a nitrocellulose membrane. A blotting cassette was set up containing a piece of nitrocellulose membrane pre-soaked in blotting buffer (0.05M Tris, 0.19M glycine and 20% v/v methanol). The membrane was placed onto a sheet of 3MM Whatman filter paper on a blotting pad and then the gel was placed onto nitrocellulose membrane. The gel was then overlaid with another sheet of pre-soaked 3MM Whatman filter paper and a second blotting pad. The blotting cassette was placed in a Hoefer transblot electrophoresis apparatus that was filled with blotting buffer with the nitrocellulose facing the anode. Blotting was carried out for 6-7 hours at 34 volts. After transfer, the membrane was stained with Ponceau S stain as previously described, in order to visualise the transferred proteins. The membrane was then washed for 10 minutes in Tris-buffered saline with Tween 20 (TBS-T) (0.1% v/v Tween™ 20, 8g/L Sodium

Chloride and 0.02M Tris pH 7.6) to remove the Ponceau S stain. The nitrocellulose membrane was incubated on an orbital shaker for 1 hour at room temperature in blocking agent (5% w/v skimmed dried milk, in PBS) to block non-specific binding sites. Primary antibodies were diluted in TBS-T with 5% w/v skimmed milk, and added to the blots in polythene bags, which were heat sealed and incubated for 2 hours at room temperature or overnight at 4ºC on a rocking platform. The membrane was rinsed and washed with 3 X 10 minute washes in TBS-T. Blots were then incubated with the appropriate secondary antibody conjugated to horseradish peroxidise, which was diluted in TBS-T with 5% w/v skimmed milk and incubated for 2h at room temperature with gentle agitation. After a further five 15 minute washes in TBS-T the antigen-antibody complex was visualised using the enhanced chemiluminescence (ECL) detection reagent (Amersham). The membranes were soaked in 1:1 mixture of ECL detection solution for one minute. The membranes were wrapped, protein side up, in a Saran wrap sheet and exposed to X-ray film for an appropriate period of time. Exposure times, ranging from a few seconds to 5 minutes were used, depending on the protein being detected or primary antibody.

2.5.3. Immunoprecipitation

Cells were lysed for 15 minutes at 4^{o}C in 0.5mls of immunoprecipitation (IP) buffer containing 50mM Tris pH 7.4, 0.825M NaCl and 1% v/v NP-40. Alternatively cells were lysed in NETN buffer (50mM Tris [pH 7.5], 150mM NaCl, 0.5mM EDTA, and 0.5% v/v NP40). Cell lysates were centrifuged at 35,000 rpm for 20 minutes at 4^{o}C. Protein complexes were then immunoprecipitated by adding an appropriate antibody, typically 20µg, and mixed by rotation for two hours at 4^{o}C. The resulting protein-antibody complexes were then mixed for a further hour with 20µl of packed protein G

agarose beads (Sigma). Immunocomplexes bound to the beads were then spun and washed three times with 1ml of the relevant IP buffer, prior to resuspending in Laemmli sample buffer and boiling for 5 minutes in preparation for SDS-PAGE.

2.5.4. Immunofluorescence

Cells were grown on 12-well glass slides to densities of around 1-2 x 10^4cells/well. Slides were washed twice in PBS, fixed in 4% w/v paraformaldehyde in PBS for 8 minutes and washed again in PBS. Acetone extraction was then carried out for 15 minutes at -20^{o}C. Cells were rehydrated in PBS and blocked in 10% FCS in PBS for 1 hour. Primary antibodies were diluted to the appropriate concentration in 0.1% FCS in PBS, and were incubated with the cells for 2 hours. Cells were then incubated with secondary antibody diluted in 0.1% FCS in PBS for 1 hour in a light shielded container. Slides were then washed three times in PBS, before being mounted in Vectashield Mounting Medium (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI), and protected with glass coverslips. Cells were viewed on a Zeiss Axioskop microscope, and z-Layer images (0.2-µm horizontal sections) were recorded with Openlab v3.0.9 Biovision software package (Improvision). Single, equivalent z-layers were merged to show colocalisation.

2.5.5. Analysis of the mitotic distribution by immunofluorescence microscopy following RNAi

Two days post siRNA treatment as previously described, cells were trypsinised and plated on to poly-L-Lysine treated 12 well slides so that they reached a density of around 1-2 x 10^4cells/well the following day, at which point slides were fixed in 100% methanol at -20^{o}C for 8 minutes. Following fixation slides were washed in PBS three

times and blocked in 10% FCS in PBS for 1 hour. Slides were then washed in PBS and each well was incubated with α-tubulin and phospho-histone H3 antibodies in 50µl of PBS containing 2% FCS for 1 hour. Next, the slides were washed with PBS three times; each well was then incubated with 50µl of 2% FCS in PBS containing the appropriate secondary antibodies for 1 hour in a light proof container. Slides were then washed three times in PBS, before being mounted in Vectashield Mounting Medium (Vector Laboratories) containing DAPI, and protected with glass coverslips. Cells were viewed on a Nikon eclipse E600 and images were processed with the associated software.

2.5.6. Chromatin immunoprecipitation (ChIP)

In order to identify factors that bind to the IL-2 promoter in Jurkat T-cells, ChIP assays were performed as follows. Four million Jurkat T-cells in RPMI-1640 were either mock treated or stimulated with 20ng/ml of PMA and 2µM ionomycin for 6 hours at 37^{o}C. DNA was then crosslinked to any associated proteins by addition of 1% w/v formaldehyde and incubation at 37 ^{o}C for 10 minutes. The cells were then pelleted at 1000rpm, washed twice in PBS, resuspended in 400µl of lysis buffer (50mM HEPES pH7.5, 140mM NaCl, 1% v/v Triton X-100 and 1mM EDTA) and left on ice for 4 hours. Lysates were sonicated 3 times on ice at 1 minute intervals in order to shear the DNA to approximately 500 base pairs. Addition of 100units of EcoRI and HindIII, and 45µl of 10X NEB buffer 2 and overnight incubation at 37^{o}C completed DNA fragmentation. Next 30µl was removed from each sample for use as an input control and stored at -20 ^{o}C. Lysates were centrifuged at 2500rpm for 1 minute and pre-cleared with 20µl of protein A-Sepharose for 2 hours at 4^{o}C with rotation. After centrifugation at 2500rpm for 1 minute the supernatant was removed and incubated overnight with the appropriate antibodies at 4^{o}C with rotation (see Table 2.6). The following day

immunocomplexes were precipitated by addition of 60µl of protein A-Sepharose and rotation at 4^{o}C overnight. Beads were then washed with 500µl wash buffer 1 (50mMTris pH7.5, 500mM NaCl, 0.1%NP-40), 500µl wash buffer 2(50mMTris pH7.5, 0.1% NP-40) and 500µl TE buffer (20mM Tris pH8.0, 1mM EDTA).

Protein-DNA complexes were eluted overnight at 65^{o}C in 200 µl of extraction buffer (1%SDS in TE), and incubated with 100µg of proteinase K at 50^{o}C for 1 hour. The DNA was recovered with a Qiagen PCR purification Kit as per the manufacturers instructions and eluted in 50µl of water. DNA was isolated form the input sample by mixing 30µl of the DNA solution with 80µl of extraction buffer and heating overnight at 65^{o}C, incubating with 50µg of proteinase K at 50^{o}C for 1 hour and recovered with a Qiagen PCR purification Kit and eluted in 30µl of water.

PCR was carried out using FastStart Taq DNA polymerase and primers for amplification of the IL-2 promoter, which are displayed in Table 2.7. Briefly 8µl of DNA derived from ChIP was added to a reaction mix containing 2.5 µl of 10X PCR reaction buffer containing $MgCl_2$, 0.5 µl of dNTPs (10mM), 1µl of 5pmol/µl of forward and reverse primers, 5µl of GC-rich solution, 0.2µl of FastStart Taq DNA polymerase and 8.8µl of SDW. Cycling conditions were as follows: initial denaturation at 95 ^{o}C for 4minutes, followed by 35 cycles of denaturation at 95 ^{o}C for 30s, annealing at 59^{o}C for 30s, and extension at 72 ^{o}C for 45s, and a final extension at 72 ^{o}C for 7 minutes. PCR products were visualised by running the entire reaction volume on an agarose gel containing ethidium bromide.

Table 2.7. IL-2 promoter primers used for PCR analysis of immunoprecipitated chromatin

Promoter	Primer	Sequence
IL-2	Forward	$^{-317}$ 5'GAGTTACTTTTGTATCCCCACCCCC 3'$^{-292}$
	Reverse	^{33} 5'CCTGTACATTGTGGCAGGAGTTGAGG 3'^{58}

2.5.7. Ubiquitylation assay

A549 cells were washed in cold saline twice, lysed in APC/C lysis buffer (20mM Tris pH 7.5, 100mM NaCl, 20mM β-glycerophosphate, 5mM $MgCl_2$, 1mM NaF, 0.2% NP-40, 10% glycerol, 0.5mM DTT) for 20mins and centrifuged at 35,000 rpm for 20 minutes at 4^{o}C. Lysates were then incubated with 20µg of anti-APC3 antibody for 2 hours with rotation at 4^{o}C. The resulting protein-antibody complexes were then mixed for a further hour with 20µl of packed protein G agarose beads (Sigma). Immunocomplexes bound to the beads were then spun and washed three times with 1ml of APC/C lysis buffer. The ligase reaction was carried in a total of 10µl, containing 4µl of protein G agarose beads (Sigma) with bound APC3 and 50mM Tris (pH 7.5), 2mM $MgCl_2$, 2mM ATP, 15mM creatine phosphate, 350U/ml of creatine phosphokinase, 15µg/ml ubiquitin aldehyde, 80µg/ml rabbit E1 (Biomol), 50µgml of E2 enzymes UBC4, UBC5 and UBCH10, and either [35S]-cyclin A or [35S]-cyclin B. The reactions were incubated at 37 ^{o}C for 30 minutes before addition of Laemmli sample buffer and then boiled for 5 minutes in preparation for SDS-PAGE.

2.6. Statistical tests

A two tailed T-test was used to determine the statistical significance of all bar charts in this manuscript. The T-test is used to determine whether the difference between two means is significant. The test equation is displayed below; x-bar, mean. $\sigma_{d,}$ the standard deviation of the difference between the means.

$$T = \frac{\text{x-bar}^1 - \text{x-bar}^2}{\sigma_d}$$

CHAPTER 3

MASS SPECTROMETRIC ANALYSIS OF APC7 IMMUNOPRECIPITATES

3.1. Introduction

The APC/C is a multi-component E3 ubiquitin ligase that mediates ubiquitylation of proteins in order to elicit their degradation by the 26S proteasome (Kanayama, Tamura et al. 1992; Sudakin, Ganoth et al. 1995). The APC/C coordinates temporal progression through the cell cycle in conjunction with its coactivators CDC20 and CDH1 (van Leuken, Clijsters et al. 2008) (refer to section 1.5 for an overview of the APC/C).

Although the mechanisms underlying APC/C action are largely understood, little is known about how APC/C activity is modulated or how individual subunits contribute to APC/C function. Therefore, I decided to identify novel APC7 binding proteins using a mass spectrometry approach, in the hope that further study of APC7 and its binding partners may give new insight into the subtleties of APC/C regulation and function.

3.2. Results

3.2.1. Mass spectrometric determination APC7 immunoprecipitates

In order to determine novel APC7 binding proteins by mass spectrometric analysis, I initially performed an IP from A549 cells using an anti-APC7 polyclonal antibody. The APC7 antibody was generated using full length APC7. Given that this APC7 polyclonal antibody should recognise multiple APC7 epitopes it seems likely that this antibody is capable of immunoprecipitating all APC7 interacting proteins. Bound proteins were eluted, separated by SDS-PAGE and protein bands were detected by Coomassie R250 blue staining (Fig. 3.1). Individual protein bands were then excised and digested with modified trypsin. The resulting peptides were separated by high-performance liquid chromatography (HPLC) and analysed using an electrospray ioniser (ESI) coupled to tandem array mass spectrometery (MS/MS). It should be noted that mass spectrometric

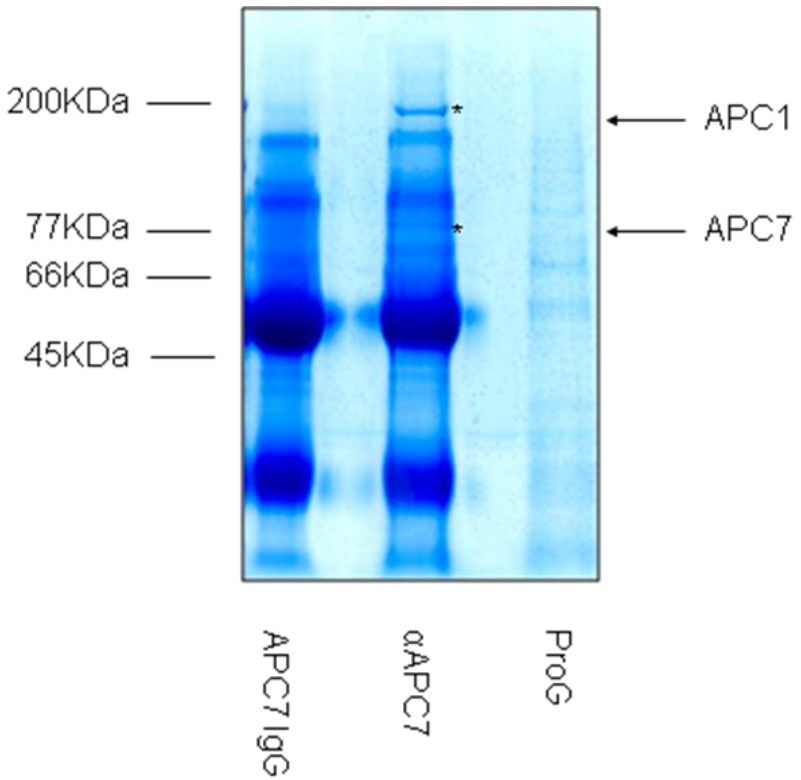


Figure 3.1. Separation of APC7 immunoprecipitates by SDS-PAGE. 20µg of anti-APC7 polyclonal Antibody was incubated with 4mg of A549 cellular extract prepared using HiLo lysis buffer. APC7 and bound proteins were immunoprecipitated using Protein G-Sepharose beads. The beads were then washed in lysis buffer and complexes were then eluted by addition of Laemmli sample buffer and incubation at 100°C for 5 minutes. Eluted proteins were resolved by SDS-PAGE and subjected to Coomassie R250 blue staining. Protein bands were then excised, digested with modified trypsin and analyzed using a ESI mass-spectrometer. *, denotes APC1 and APC7 as indicated.

data, like other methods of identifying protein-protein interactions is not entirely definitive and therefore any novel interactions identified must be corroborated by more traditional means such as co-IP and/or GST-pulldown. The results presented in this chapter were obtained over a number of separate experiments and a great deal of work went into the optimisation of the protocol as the departmental mass spectrometry facility was at this time in its infancy. In particular a number of gel staining methods were examined and steps to minimise false positives generated by non-specific binding were also investigated.

3.2.2. Detection of known APC7 binding proteins by mass spectrometric analysis

The initial objective was to demonstrate the efficacy of the mass spectrometer by identifying peptides from APC7, and other APC/C components and interacting proteins. The data obtained confirmed the presence of six APC7 peptides (Fig. 3.2) and resulted in detection of five peptides from APC8 (Fig. 3.3), three peptides from APC6 (Fig. 3.4), and five peptides from APC3 (Fig 3.5). Also four peptides were detected from the E2 ubiquitin conjugating enzyme UBCH10, a protein that is essential for the E3 ubiquitin ligase function of the APC/C (Hershko, Ganoth et al. 1994). Taken together these results provide overwhelming evidence to support the validity of this technique and its potential to identify novel APC7-interacting proteins.

3.2.3. Detection of novel APC7 binding proteins by mass spectrometric analysis

Interestingly peptides from six proteins previously unknown to bind to APC7 were also detected (Table 3.1 summarises the data obtained in this chapter), however the mass spectrometer generally identified fewer peptides from these novel interacting proteins when compared to known APC7 binding proteins. This may suggest that these novel

Table 3.1. Summary of APC7 interacting proteins identified by mass spectrometric analysis

APC/C components and known interacting proteins	**Number of peptides**	**Coverage of protein**
APC7	6	8%
APC3	5	5.8%
APC6	3	5.6%
APC8	5	9%
UBCH0	4	23.5%
Novel APC7 interacting proteins		
NF45	3	9.5%
TIF1γ	2	3.3%
eEF1A	2	4.3%
CEP290	2	1%
Ku70	3	8%
TLS	3	7.4%

APC7 interacting proteins are present in sub-stoichiometric amounts, binding to only a fraction of the total cellular pool of APC7.

3.2.3.1. Detection of Nuclear Factor 45 (NF45) peptides in APC7 immunoprecipitates by mass spectrometric analysis

Mass spectrometric analysis of APC7 immunoprecipitates resulted in the identification of three peptides from NF45 (Fig. 3.7). NF45 is always found *in vivo* as a heterodimer with members of the NF90 family. Both proteins were originally identified on the basis of their ability to bind to the antigen receptor response element-2 (ARRE-2) in the proximal IL-2 promoter in T-cells. Subsequent experiments defined NF90/NF45 as essential components of the ARRE-2 regulatory complex (Corthesy and Kao 1994; Kao, Chen et al. 1994), which activates IL-2 transcription in response to ligation of the T-cell receptor or treatment with stimuli such as PMA and ionomycin. NF90/NF45 also functions in translation (Xu, Busald et al. 2000), post-transcriptional mRNA stabilization and export (Shim, Lim et al. 2002; Shi, Zhao et al. 2005).

3.2.3.2. Detection of transcriptional intermediary factor 1 gamma (TIF1γ) peptides in APC7 immunoprecipitates by mass spectrometric analysis

Two peptides were identified from TIF1γ (Fig. 3.8), which is part of a family that is defined by the presence of an N-terminal TRIM/RBCC domain (Reymond, Meroni et al. 2001). This is composed of a RING finger, two B-boxes, and a coiled-coil domain (RBCC), and is also known as a tripartite motif (TRIM). The coiled coil region mediates TIF1γ homo-oligomerisation, hetero-oligomerisation with TIF1α and is thought to be important for targeting proteins to discrete cellular compartments (Peng, Feldman et al. 2002). The RING domain functions as a Smad4 E3 ubiquitin ligase

(Dupont, Zacchigna et al. 2005). There is evidence to suggest that TIF1γ functions as a transcriptional repressor (Venturini, You et al. 1999), which may be important for the ability of TIF1γ to mediate erythroid differentiation and embryonic development (Ransom, Bahary et al. 2004; Dupont, Zacchigna et al. 2005). A more detailed analysis of TIF1γ function can be found in section 1.8.

3.2.3.3. Detection of eukaryotic elongation factor 1A (eEF1A) peptides in APC7 immunoprecipitates by mass spectrometric analysis

My study also resulted in the identification of two peptides from eEF1A (Fig. 3.9). It has been demonstrated that eEF1A is required for the recruitment of aminoacyl-tRNA to the elongating ribosome, quality control of freshly synthesised proteins (Hotokezaka, Tobben et al. 2002) and promoting degradation of defective proteins by the proteasome (Chuang, Chen et al. 2005). There is also evidence to suggest that eEF1A interacts with the actin cytoskeleton and this may be important for its function in translation (Gross and Kinzy 2007). Given that eEF1A is highly homologous to a centrosomal protein in sea urchin eggs (Kuriyama, Savereide et al. 1990; Owen, DeRosier et al. 1992), it seems likely that eEF1A may also contribute to spindle pole function during mitosis.

3.2.3.4. Detection of centrosomal protein 290kDa (CEP290) peptides in APC7 immunoprecipitates by mass spectrometric analysis

Interaction between APC7 and CEP290 is suggested by the identification of two CEP290 peptides in APC7 immunoprecipitates (Fig. 3.10). Very little is known about the functions of CEP290, although it has been demonstrated as a centrosomal component (Sayer, Otto et al. 2006) and is also localised to the connecting cilium of retinal photoreceptors, an interaction that is important for retinal maintenance (Chang,

Khanna et al. 2006). Analysis of the amino acid sequence of CEP290 revealed the presence of 13 putative coiled-coil domains, an ATP/GTP binding site motif A (phosphate-binding loop or P-loop) that facilitates binding of motor proteins to DNA, a nuclear localisation signal and an acidic coiled-coil found in centrosomal proteins and required for microtubule organization. Interestingly these motifs are also present, but arranged differently, in centromere protein F **(**CENP-F), a protein that is important for chromosome segregation (Zhou, Wang et al. 2005). CEP290 also appears to function as a transcription factor, as it activates activating transcription factor 4 (ATF4) dependent transcription, and may antagonize the ability of CENP-F to inhibit activation of transcription by ATF4 (Sayer, Otto et al. 2006).

3.2.3.5. Detection of Ku70 peptides in APC7 immunoprecipitates by mass spectrometric analysis

Mass spectrometric analysis of APC7 immunoprecipitates also resulted in the detection of three peptides from Ku70 (Fig3.11), a component of the Ku heterodimer that also contains Ku80. It has been demonstrated that Ku is the regulatory subunit of DNA protein kinase (DNA-PK), which also comprises a catalytic subunit DNA-PK_{cs} (Carter, Vancurova et al. 1990; Collis, DeWeese et al. 2005). DNA-PK is crucial for repairing double strand breaks (DSBs) by non-homologous DNA-end-joining (NHEJ) and also functions during V(D)J recombination (Chen, Peterson et al. 1996; Errami, Smider et al. 1996). Intriguingly, DNA-PK also regulates the IL-2 promoter in cooperation with NF90/NF45 (Aoki, Zhao et al. 1998; Shi, Qiu et al. 2007). In addition Ku functions independently of DNA-PK in order to prevent chromosomal instability, by regulating telomere maintenance (Bailey, Meyne et al. 1999; Samper, Goytisolo et al. 2000). Ku also regulates the initiation of DNA replication (Novac, Matheos et al. 2001),

transcription, and protein expression by regulating internal ribosome entry site (IRES) dependent translation (Kuhn, Stefanovsky et al. 1993; Silvera, Koloteva-Levine et al. 2006).

3.2.3.6. Detection of translocated in liposarcoma (TLS) peptides in APC7 immunoprecipitates by mass spectrometric analysis

A total of three peptides were identified from TLS (Fig 3.12). This protein was first identified as an oncogenic fusion protein that contains the N-terminal half of TLS with full length CCAAT enhancer binding homologous protein (CHOP) (Crozat, Aman et al. 1993), and subsequently with the C-terminal half of v-ets erythroblastosis virus E26 oncogene homolog (ERG) (Ichikawa, Shimizu et al. 1994). However the peptides identified in this study originate from the C-terminal portion of TLS that is absent in the TLS oncogenic fusions, suggesting that APC7 may bind to wild type protein. TLS is identical to the heterogeneous ribonucleoprotein P2 (hnRNP P2), and as such can bind to RNA, regulate RNA splicing and may also be involved in mRNA export (Calvio, Neubauer et al. 1995). TLS is also thought to function as a transcription factor, as it can enhance nuclear factor kappa B (NFκB) mediated transactivation (Uranishi, Tetsuka et al. 2001). Lastly TLS mediates binding of homologous DNA (Baechtold, Kuroda et al. 1999) and TLS-deficient mice show an increased sensitivity to ionizing radiation due to chromosome pairing defects (Kuroda, Sok et al. 2000).

A)

```
   1 mnvidhvrdm aaaglhsnvr llssllltms nnnpelfspp qkyqllvyha dslfhdkeyr
  61 navskytmal qqkkalskts kvrpstgmsa stpqsqclps eievkykmae cytmlkqdkd
 121 aiaildgips rqrtpkinmm lanlykkagr erpsvtsyke vlrqcplald ailgllslsv
 181 kgaevasmtm nviqtvpnld wlsvwikaya fvhtgdnsra isticslekk sllrdnvdll
 241 gsladlyfra gdnknsvlkf eqaqmldlyl ikgmdvygyl laregrledv enlgcrlfni
 301 sdqhaepwvv sgchsfyskr ysralylgak aiqlnsnsvq alllkgaalr nmgrvqeaii
 361 hfreairlap crldcyegli ecylasnsir eamvmannvy ktlganaqtl tllatvcled
 421 pvtqekaktl ldkaltqrpd yikavvkkae llsreqkyed giallrnala nqsdcvlhri
 481 lgdflvavne yqeamdqysi alsldpndqk slegmqkmek eesptdatqe edvddmegsg
 541 eegdlegsds eaaqwadqeq wfgmq
```

Bi)

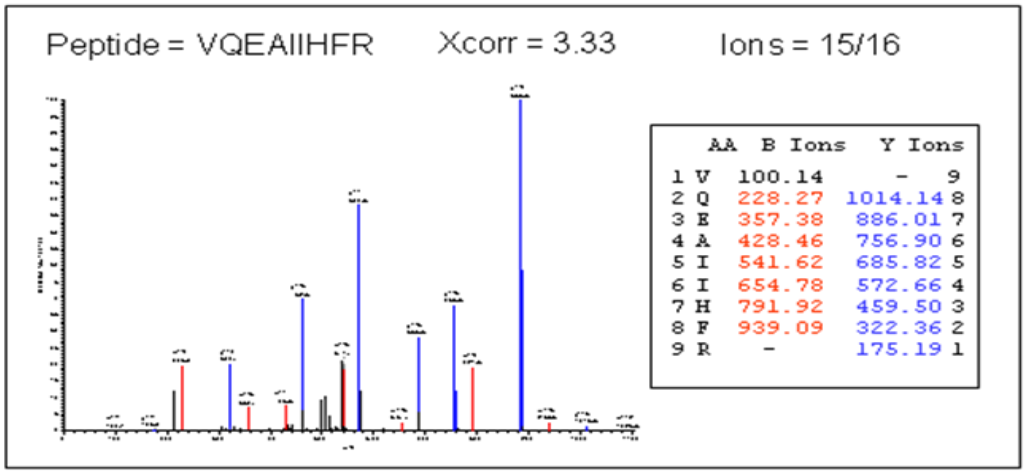
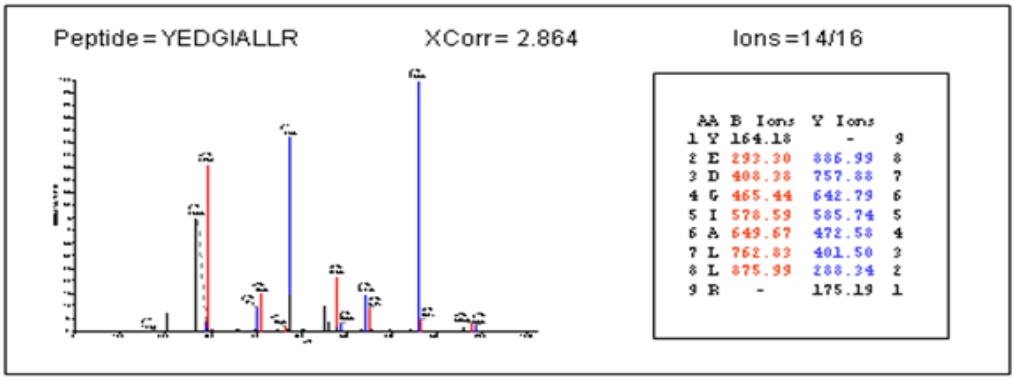


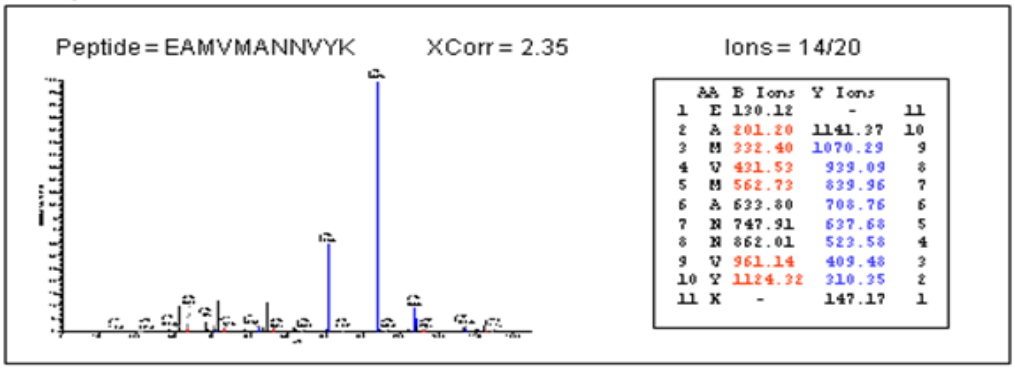
Figure 3.2A and Bi. ESI-mass spectrometric determination of APC7 peptides isolated from APC7 immunoprecipitates

(A) Six peptides from the APC7 protein were identified by mass spectrometry (highlighted in red, and in blue when two peptides overlap). (B) A mass spectrogram and table illustrating the ions generated from the APC7 peptide VQEAIIHFR by ESI-MS/MS is shown. Fifteen out of a possible sixteen B (red) and Y ions (blue) were identified. B ions contain the C-terminus of a fragmented amide bond, whereas Y ions retain the N-terminus. The peptide generated an Xcorr of 3.33. The Xcorr expresses the correlation between the experimental data and the theoretical data. Any Xcorr >2 suggests the presence of a given peptide with a high degree of certainty.

Bii)



Biii)



Biv)

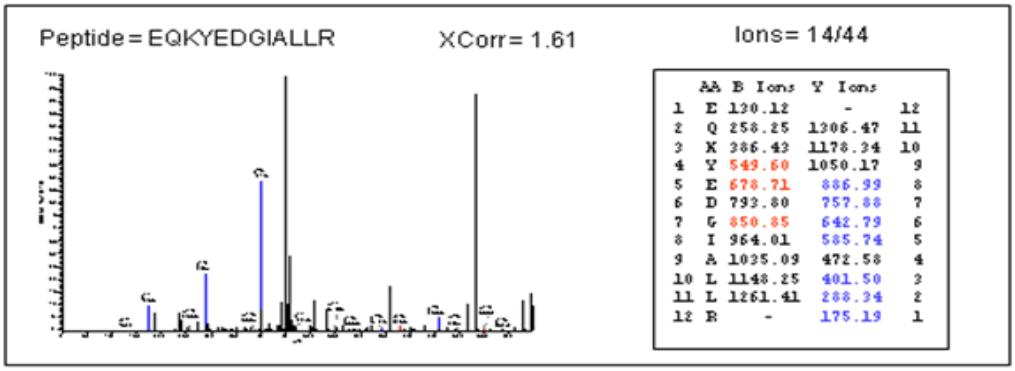
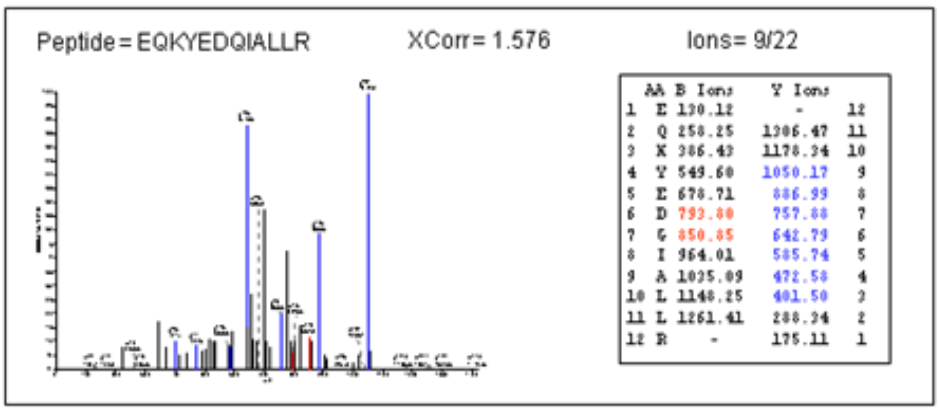


Figure 3.2Bii-iv. ESI-mass spectrometric determination of APC7 peptides isolated from APC7 immunoprecipitates

(Bii) A mass spectrogram and table illustrating the ions generated from the APC7 peptide YEDGIALLR by ESI-MS/MS is shown. **(Biii)** A mass spectrogram and table illustrating the ions generated from the APC7 peptide EAMVMANNVYK by ESI-MS/MS is shown. **(Biv)** A mass spectrogram and table illustrating the ions generated from the APC7 peptide EQKYEDGIALLR by ESI-MS/MS is shown.

Bv)



Bvi)

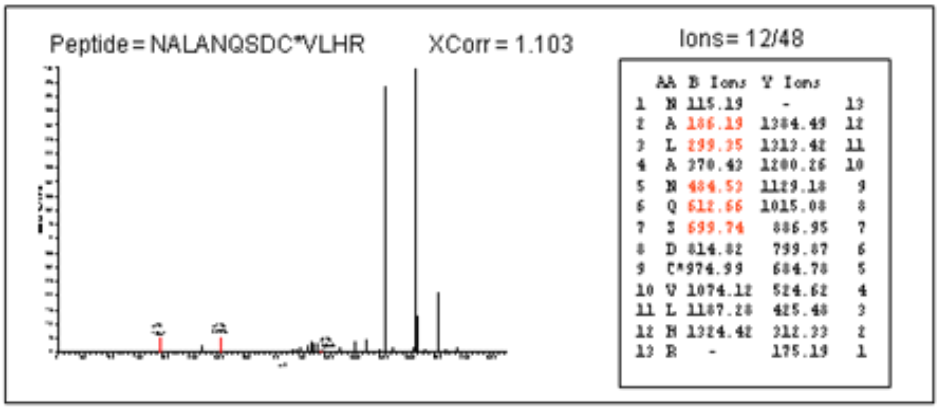


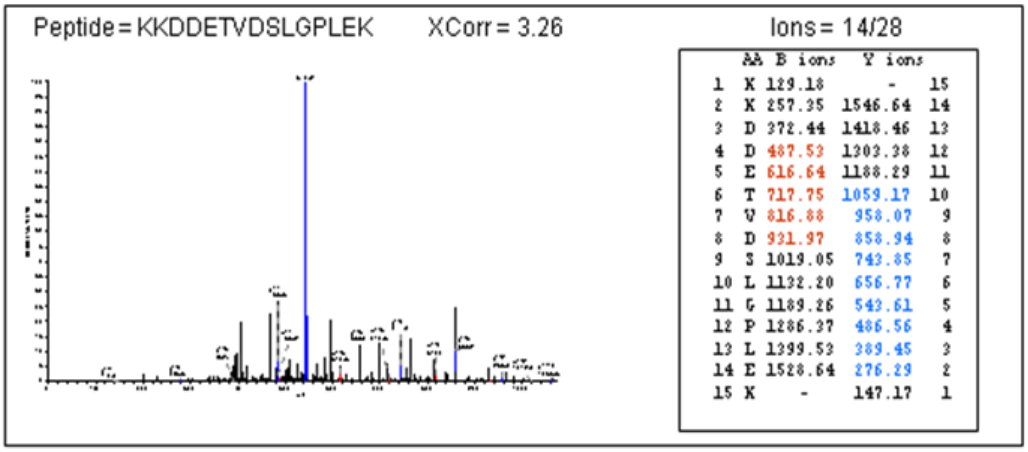
Figure 3.2Bv-vi. ESI-mass spectrometric determination of APC7 peptides isolated from APC7 immunoprecipitates

(Bv) A mass spectrogram and table illustrating the ions generated from the APC7 peptide EQKYEDQIALLR by ESI-*MS/MS* is shown. **(Bvi)** A mass spectrogram and table illustrating the ions generated from the APC7 peptide NALANQSDC*VLHR (* denotes alkylation) by ESI-*MS/MS* is shown.

A)

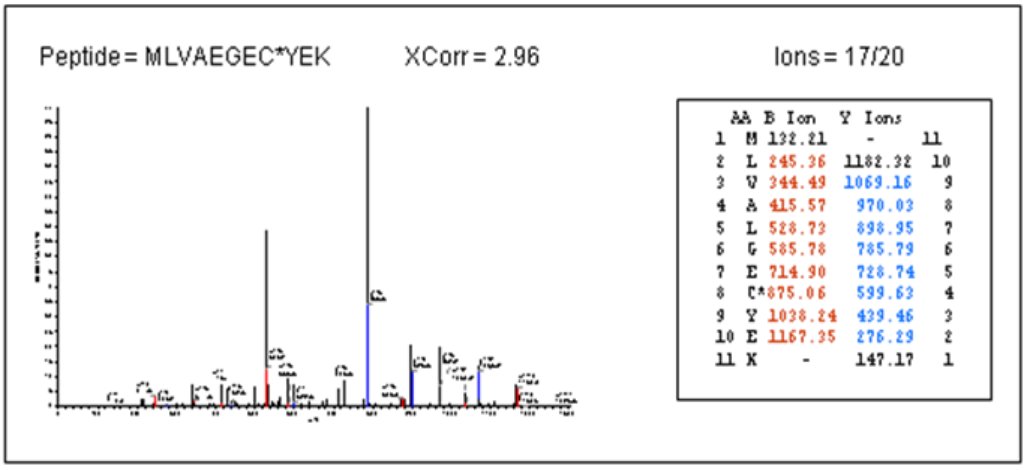
```
   1 mvpvavtaav  apvlsinsdf  sdlreikkql  lliagltrer  gllhsskwsa  elafslpalp
  61 laelqppppi  teedaqdmda  ytlakayfdv  keydraahfl  hgcnskkayf  lymysrylsg
 121 ekkkddetvd  slgplekgqv  knealrelrv  elskkhqare  ldgfglylyg  vvlrkldlvk
 181 eaidvfveat  hvlplhwgaw  lelcnlitdk  emlkflslpd  twmkefflah  iytelqliee
 241 alqkyqnlid  vgfskssyiv  sqiavayhni  rdidkalsif  nelrkqdpyr  ienmdtfsnl
 301 lyvrsmksel  sylahnlcei  dkyrvetccv  igmyyslrsq  hekaalyfqr  alklnprylg
 361 awtlmgheym  emkntsaaiq  ayrhaievnk  rdyrawyglg  qtyeilkmpf  yclyyyrrah
 421 qlrpndsrml  valgecyekl  nqlveakkcy  wrayavgdve  kmalvklakl  heqlteseqa
 481 aqcyikyiqd  iyscgeiveh  leestafryl  aqyyfkcklw  deastcaqkc  cafndtreeg
 541 kallrqilql  rnqgetptte  vpapfflpas  lsanntptrr  vfplnlssvt  p
```

Bi)



	AA	B ions	Y ions	
1	K	129.18	-	15
2	K	257.25	1546.64	14
3	D	372.44	1418.46	13
4	D	487.53	1303.38	12
5	E	616.64	1188.29	11
6	T	717.75	1059.17	10
7	V	816.88	958.07	9
8	D	931.97	858.94	8
9	S	1019.05	743.85	7
10	L	1132.20	656.77	6
11	G	1189.26	543.61	5
12	P	1286.37	486.56	4
13	L	1399.53	389.45	3
14	E	1528.64	276.29	2
15	K	-	147.17	1

Bii)

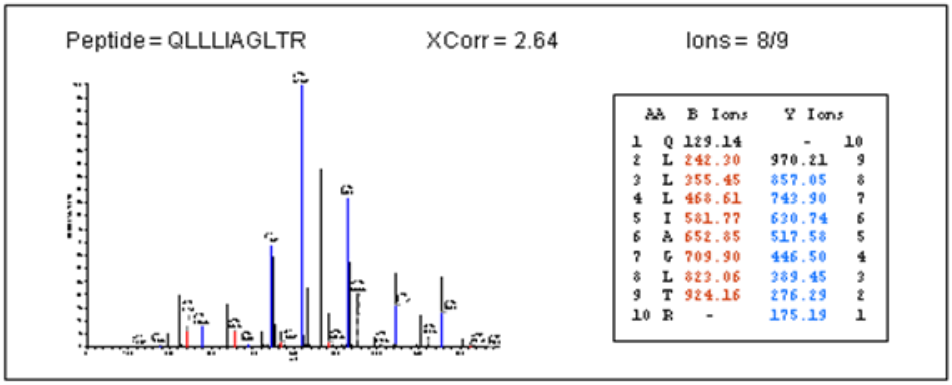


	AA	B Ion	Y Ions	
1	M	132.21	-	11
2	L	245.36	1182.32	10
3	V	344.49	1069.16	9
4	A	415.57	970.03	8
5	L	528.72	898.95	7
6	G	585.78	785.79	6
7	E	714.90	728.74	5
8	C*	875.06	599.63	4
9	Y	1038.24	439.46	3
10	E	1167.35	276.29	2
11	K	-	147.17	1

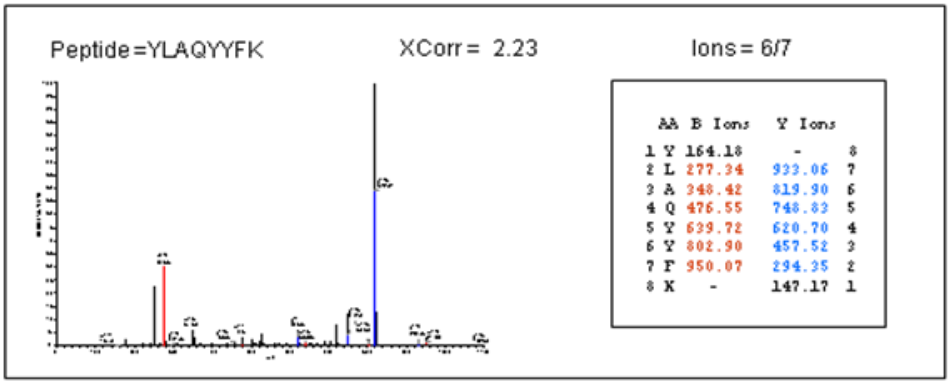
Figure 3.3A and Bi-ii. ESI-mass spectrometric determination of APC8 peptides isolated from APC7 immunoprecipitates

(A) five peptides from APC8 were identified by mass spectrometry (highlighted in red). **(Bi)** A mass spectrogram and table illustrating the ions generated from the APC8 peptide KKDDETVDSLGPLEK by ESI-MS/MS is shown. Fourteen out of a possible twenty eight B (red) and Y ions (blue) were identified. B ions contain the C-terminus of a fragmented amide bond, whereas Y ions retain the N-terminus. The peptide generated an Xcorr of 3.26. **(Bii)** A mass spectrogram and table illustrating the ions generated from the APC8 peptide MLVAEGEC*YEK (* denotes alkylation) by ESI-MS/MS

Biii)



Biv)



Bv)

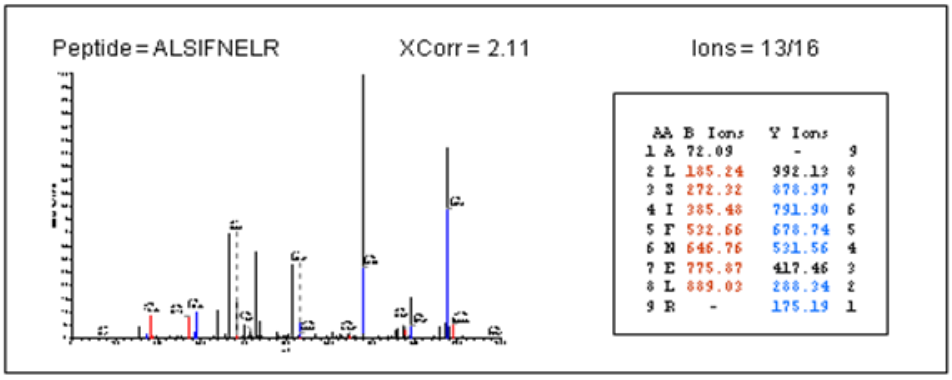


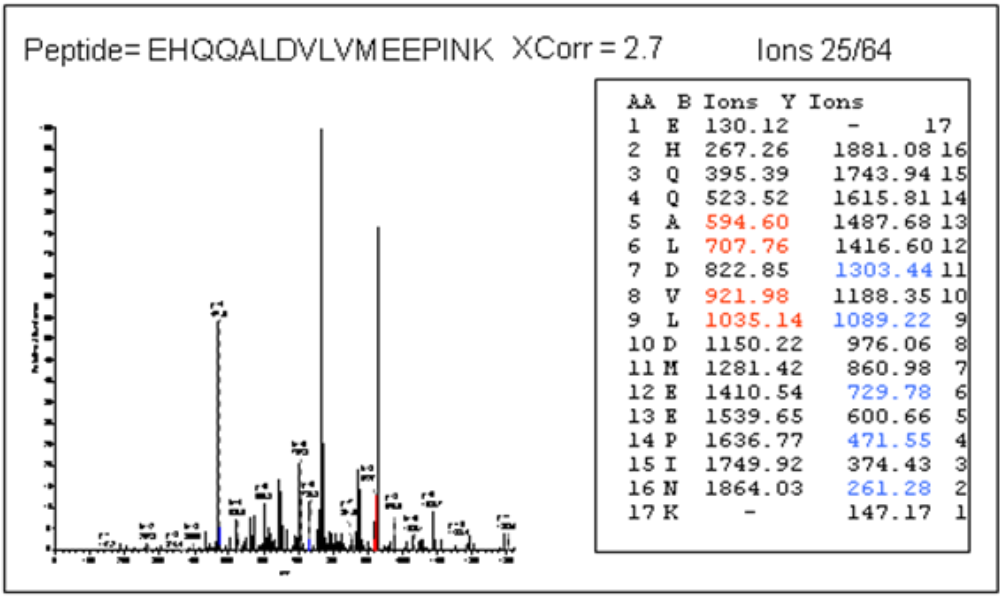
Fig 3.3Biii-v. ESI-mass spectrometric determination of APC8 peptides isolated from APC7 immunoprecipitates

(Biii) A mass spectrogram and table illustrating the ions generated from the APC8 peptide MLVAEGECYEK by ESI-MS/MS is shown. **(Biv)** A mass spectrogram and table illustrating the ions generated from the APC8 peptide YLAQYYFK by ESI-MS/MS is shown. **(Bv)** A mass spectrogram and table illustrating the ions generated from the APC8 peptide ALSIFNELR by ESI-MS/MS is shown.

A)

```
   1 mnlerlrkrv rqyldqqqyq salfwadkva slsreepqdi ywlaqclylt aqyhraahal
  61 rsrkldklye acrylaarch yaakehqqal dvldmeepin krlfekylkd esgfkdpssd
 121 wemsqssiks sicllrgkiy daldnrtlat ysykealkld vycfeafdll tshhmltaqe
 181 ekelleslpl sklcneeqel lrflfenklk kynkpsetvi pesvdglqen ldvvvvslaer
 241 hyyncdfkmc ykltsvvmek dpfhasclpv higtlvelnk anelfylshk lvdlypsnpv
 301 swfavgcyyl mvghkmehar rylskattle ktygpawiay ghsfaveseh dqamaayfta
 361 aqlmkgchlp mlyigleygl tnnsklaerf fsqalsiape dpfvmhevgv vafqmgewkt
 421 aekwfldale kikaignevt vdkweplmm lghvcrklkk yaealdyhrq alvlipqnas
 481 tysaigyihs lmgnfenavd yfhtalglrr ddtfsvtmlg hciemyigds eayigadikd
 541 klkcydfdvh tmktlkniis ppwdfrefev ekqtaeetgl tpletsrktp dsrpsleetf
 601 eiemnesdmm letsmsdhst
```

Bi)

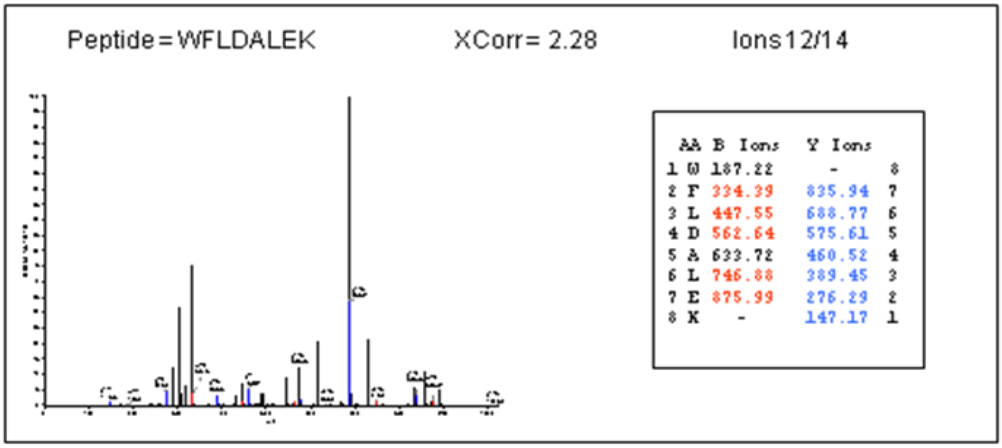


AA		B Ions	Y Ions	
1	E	130.12	-	17
2	H	267.26	1881.08	16
3	Q	395.39	1743.94	15
4	Q	523.52	1615.81	14
5	A	594.60	1487.68	13
6	L	707.76	1416.60	12
7	D	822.85	1303.44	11
8	V	921.98	1188.35	10
9	L	1035.14	1089.22	9
10	D	1150.22	976.06	8
11	M	1281.42	860.98	7
12	E	1410.54	729.78	6
13	E	1539.65	600.66	5
14	P	1636.77	471.55	4
15	I	1749.92	374.43	3
16	N	1864.03	261.28	2
17	K	-	147.17	1

Figure 3.4A and Bi. ESI-mass spectrometric determination of APC6 peptides isolated from APC7 immunoprecipitates

(A) Three peptides from APC6 were identified by mass spectrometry (highlighted in red). (**Bi**) A mass spectrogram and table illustrating the ions generated from the APC7 peptide EHQQALDVLVMEEPINK by ESI-MS/MS is shown. Twenty five out of a possible sixty four B (red) and Y ions (blue) were identified. B ions contain the C-terminus of a fragmented amide bond, whereas Y ions retain the N-terminus. The peptide generated an Xcorr of 2.7.

Biii)



Biv)

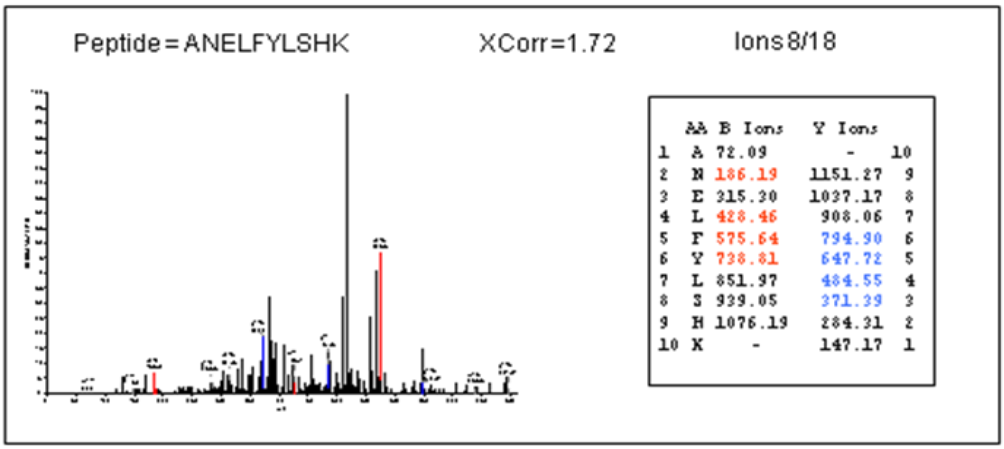


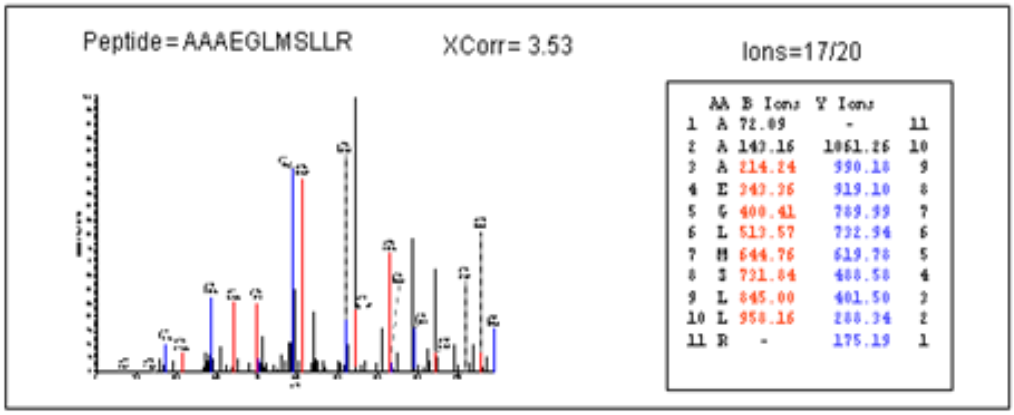
Figure 3.4Biii-iv. ESI-mass spectrometric determination of APC6 peptides isolated from APC7 immunoprecipitates

(A) A mass spectrogram and table illustrating the ions generated from the APC6 peptide WFLDALEK by ESI-MS/MS is shown. (B) A mass spectrogram and table illustrating the ions generated from the APC6 peptide ANELFYLSHK by ESI-MS/MS is shown.

A)

```
   1 mtvlqepvqa aiwqalnhya yrdavflaer lyaevhseea lfllatcyyr sgkaykayrl
  61 lkghscttpq ckyllakccv dlsklaegeq ilsggvfnkq kshddivtef gdsacftlsl
 121 lghvycktdr lakgsecyqk slslnpflws pfeslceige kpdpdqtfkf tslqnfsncl
 181 pnscttqvpn hslshrqpet vltetpqdti elnrlnless nskyslntds svsyidsavi
 241 spdtvplgtg tsilskqvqn kpktgrsllg gpaalspltp sfgilpletp spgdgsylqn
 301 ytntppvidv pstgapskks varigqtgtk svfsqsgnsr evtpilaqtq ssgpqtsttp
 361 qvlsptitsp pnalprrssr lftsdssttk enskklkmkf ppkipnrktk sktnkggitq
 421 pnindsleit kldssiiseg kistitpqiq afnlqkaaaa glmsllremg kgylalcsyn
 481 ckeainilsh lpshhyntgw vlcqigrayf elseymqaer ifsevrrien yrvegmeiys
 541 ttlwhlqkdv alsvlskdlt dmdknspeaw caagncfslq rehdiaikff qraiqvdpny
 601 ayaytllghe fvlteeldka lacfrnairv nprhynawyg lgmiyykqek fslaemhfqk
 661 aldinpqssv llchigvvqh alkksekald tlnkaividp knplckfhra svlfanekyk
 721 salqeleelk qivpkeslvy fligkvykkl gqthlalmnf swamdldpkg annqikeaid
 781 krylpddeep itqeeqimgt desqessmtd addtqlhaae sdef
```

Bi)



Bii)

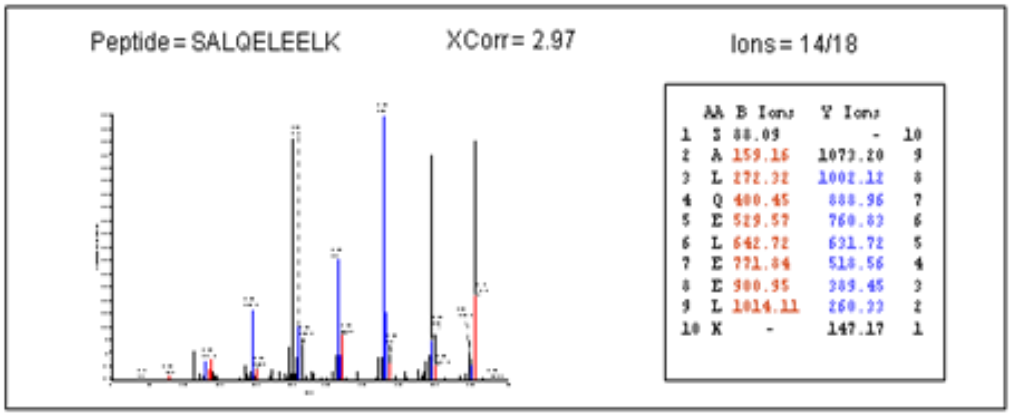
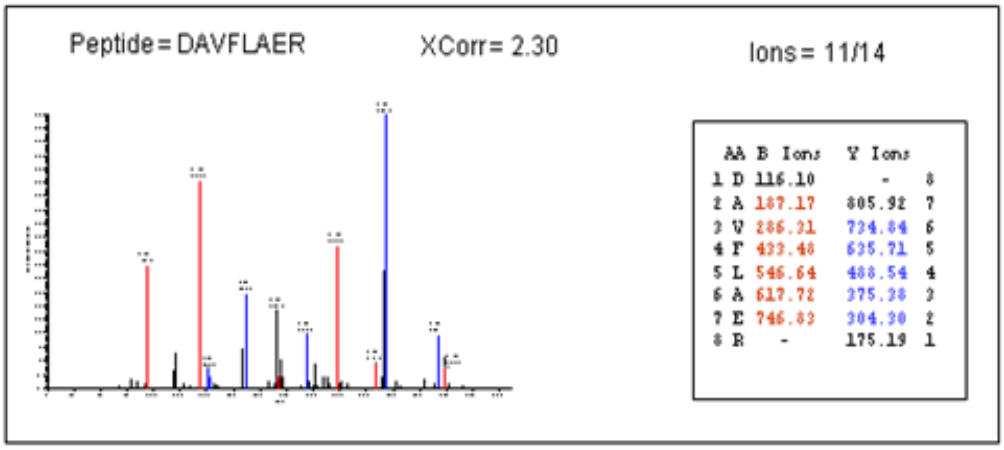


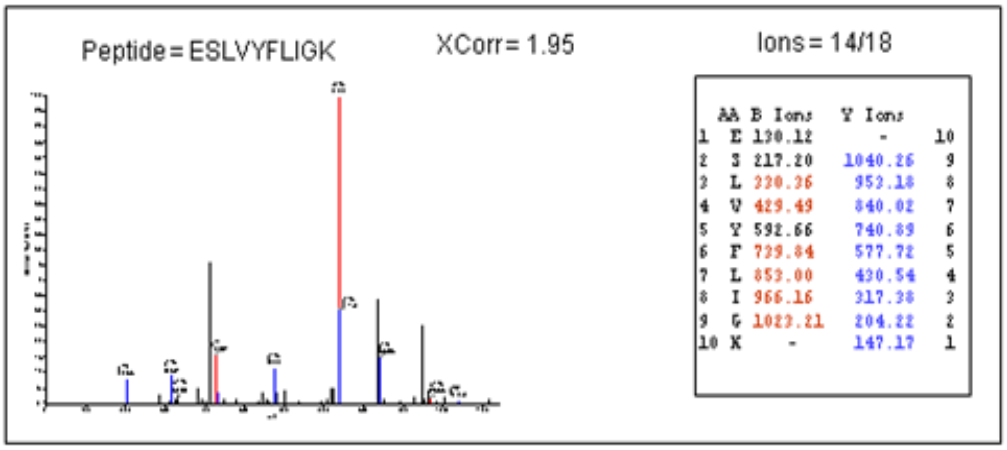
Figure 3.5A and Bi-ii. ESI-mass spectrometric determination of APC3 peptides isolated from APC7 immunoprecipitates

(A) Five peptides from APC3 were identified by mass spectrometry (highlighted in red). (**Bi**) A mass spectrogram and table illustrating the ions generated from the APC3 peptide AAAEGLMSLLR by ESI-MS/MS is shown. Seventeen out of a possible twenty B (red) and Y ions (blue) were identified. B ions contain the C-terminus of a fragmented amide bond, whereas Y ions retain the N-terminus. The peptide generated an Xcorr of 3.53 . (**Bii**) A mass spectrogram and table illustrating the ions generated from the APC3 peptide SALQELEELK by ESI-MS/MS is shown.

Biii)



Biv)



Bv)

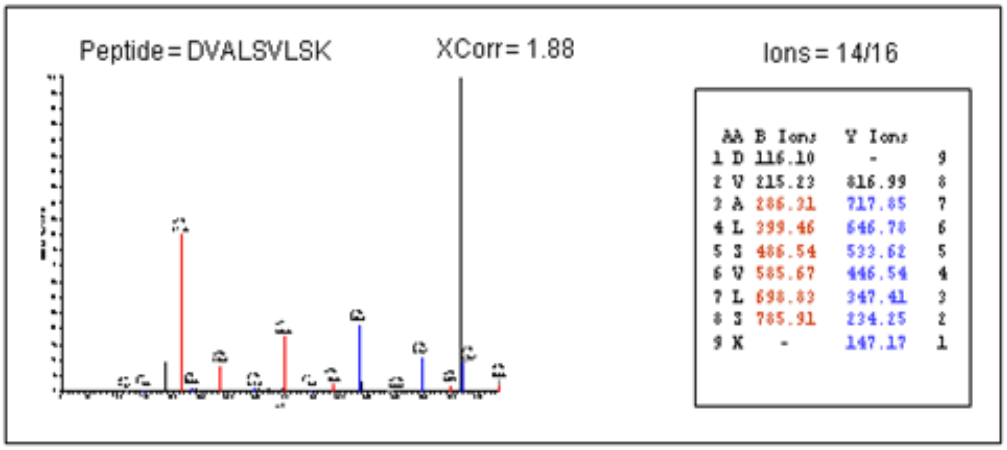


Figure 3.5Biii-v. ESI-mass spectrometric determination of APC3 peptides isolated from APC7 immunoprecipitates

(Biii) A mass spectrogram and table illustrating the ions generated from the APC3 peptide DAVFLAER by ESI-MS/MS is shown. **(Biv)** A mass spectrogram and table illustrating the ions generated from the APC3 peptide ESLVYFLIGK by ESI-MS/MS is shown. **(Bv)** A mass spectrogram and table illustrating the ions generated from the APC7 peptide DVALSVLSK by ESI-MS/MS is shown.

A)

```
  1 masqnrdpaa tsvaaarkga epsggaargp vgkrlqqelm tlmmsgdkgi safpesdnlf
 61 kwvgtihgaa gtvyedlryk lslefpsgyp ynaptvkflt pcyhpnvdtq gnicldilke
121 kwsalydvrt illsiqsllg epnidsplnt haaelwknpt afkkylqety skqvtsqep
```

Bi)

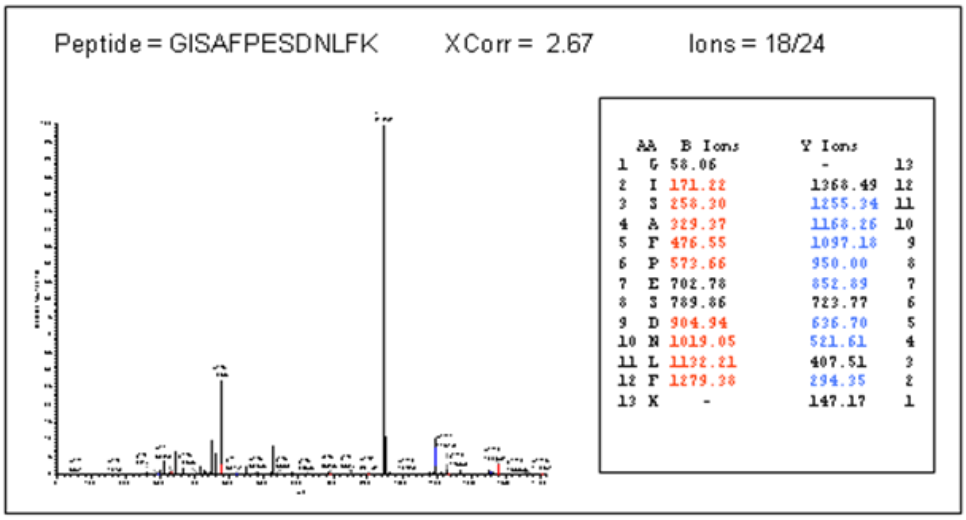
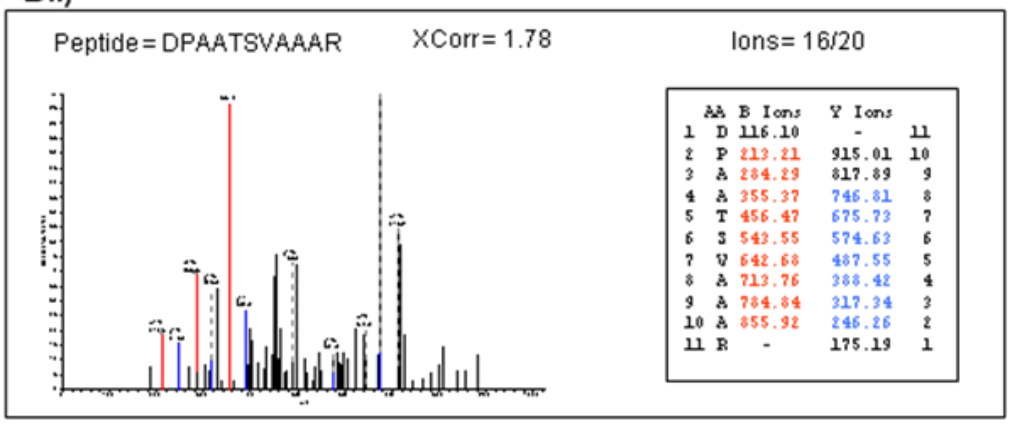


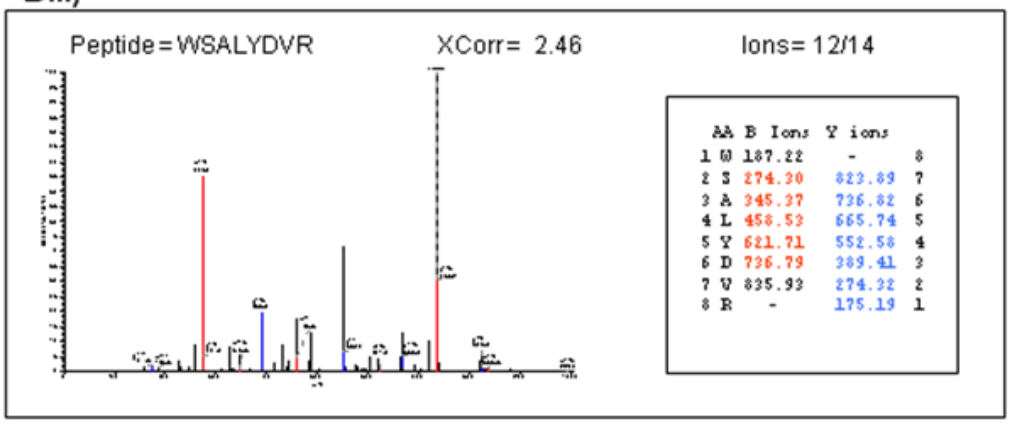
Figure 3.6A and Bi. ESI-mass spectrometric determination of UBCH10 peptides isolated from APC7 immunoprecipitates

(A) Four peptides from UBCH10 were identified by mass spectrometry (highlighted in red). **(Bi)** A mass spectrogram and table illustrating the ions generated from the UBCH10 peptide GISAFPESDNLFK by ESI-MS/MS is shown. Eighteen out of a possible twenty four B (red) and Y ions (blue) were identified . B ions contain the C-terminus of a fragmented amide bond, whereas Y ions retain the N-terminus. The peptide generated an Xcorr of 2.67.

Bii)



Biii)



Biv)

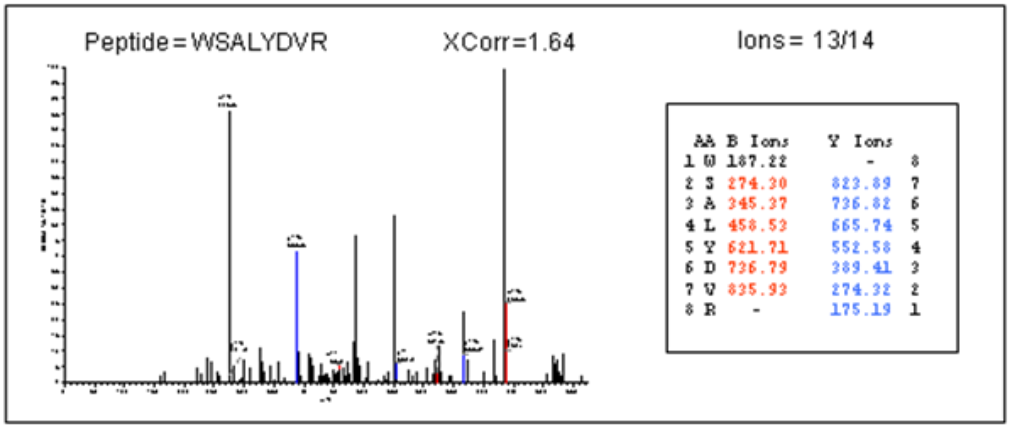


Figure 3.6Bii-iv. ESI-mass spectrometric determination of UBCH10 peptides isolated from APC7 immunoprecipitates

(Bii) A mass spectrogram and table illustrating the ions generated from the UBCH10 peptide DPAATSVAAAR by ESI-MS/MS is shown. **(Biii)** A mass spectrogram and table illustrating the ions generated from the UBCH10 peptide WSALYDVR by ESI-MS/MS is shown. **(Biv)** A mass spectrogram and table illustrating the ions generated from the UBCH10 peptide WSALYDVR by ESI-MS/MS is shown.

A)

```
   1 mrgdrgrgrg grfgsrggpg ggfrpfvphi pfdfylcema fprvkpapde tsfseallkr
  61 nqdlapnsae qasilslvtk innvidnliv apgtfevqie evrqvgsykk gtmttghnva
 121 dlvvilkilp tleavaalgn kvveslraqd psevltmltn etgfeisssd atvkilittv
 181 ppnlrkldpe lhldikvlqs alaairharw feenasqstv kvlirllkdl rirfpgfepl
 241 tpwildllgh yavmnnptrq plalnvayrr clqilaaglf lpgsvgitdp cesgnfrvht
 301 vmtleqqdmv cytaqtlvri lshggfrkil gqegdasyla seistwdgvi vtpsekayek
 361 ppekkegeee eenteeppqg eeeesmetqe
```

Bi)

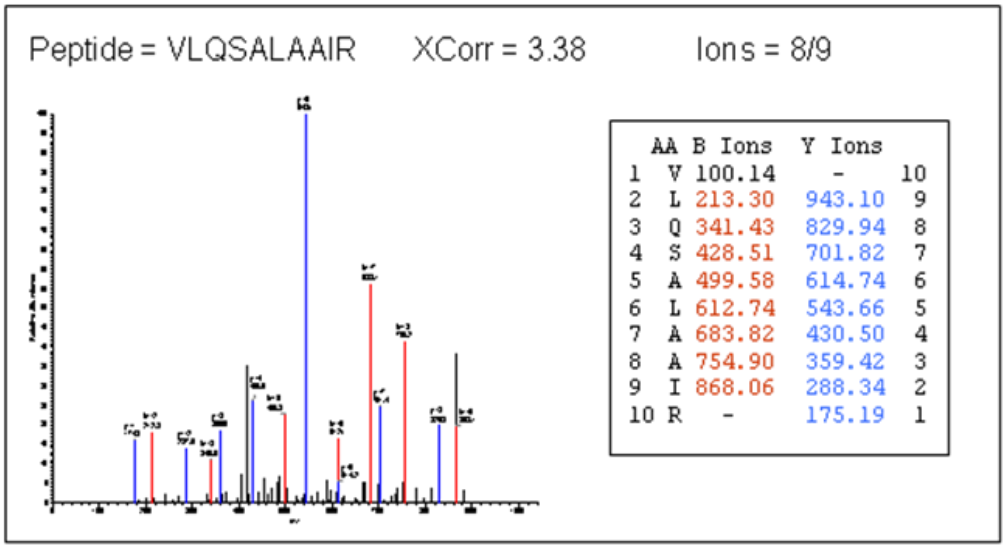
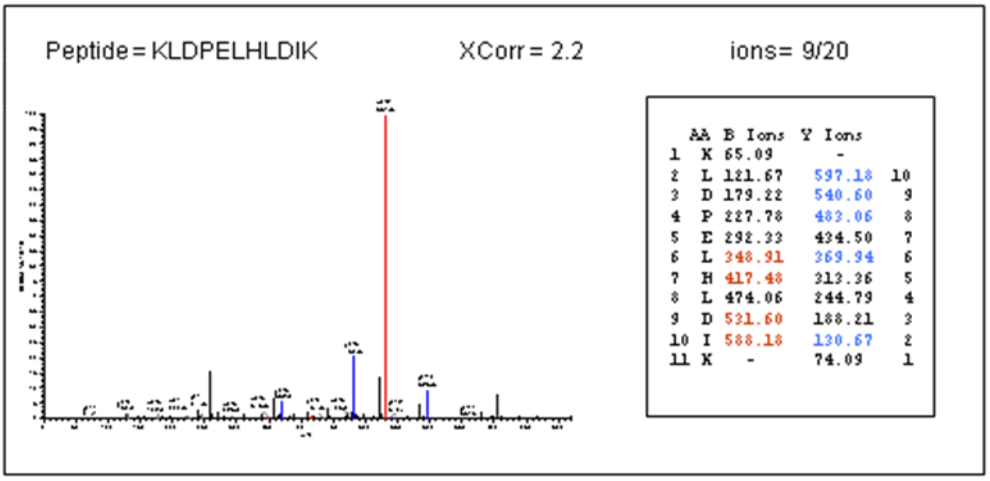


Figure 3.7A and Bi. ESI-mass spectrometric determination of NF45 peptides isolated from APC7 immunoprecipitates

(A) Three peptides from NF45 were identified by mass spectrometry (highlighted in red, and blue when two peptides are adjacent). **(Bi)** Eight out of a possible nine B (red) and Y ions (blue) were identified from the NF45 peptide: KLDPELHLDIK is shown. The peptide generated an Xcorr of 3.38.

Bii)



Biii)

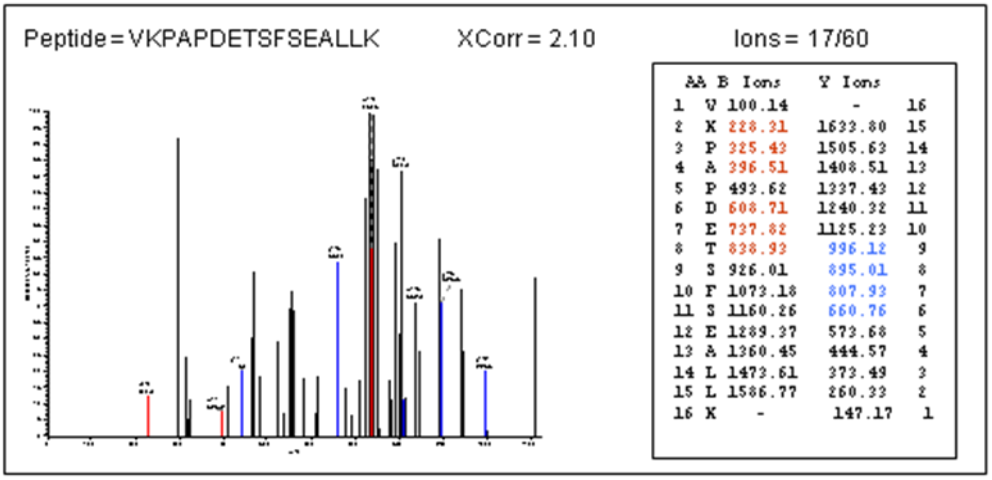


Figure 3.7Bii-iii. ESI-mass spectrometric determination of NF45 peptides isolated from APC7 immunoprecipitates

(Bii) A mass spectrogram and table illustrating the ions generated from the NF45 peptide VLQSALAAIR by ESI-MS/MSis shown. **(Biii)** A mass spectrogram and table illustrating the ions generated from the NF45 peptide VKPAPDETSFSEALLK by ESI-MS/MS is shown.

A)

```
   1 maenkgggea esggggsgsa pvtagaagpa aqeaepplta vlveeeeeeg gragaeggaa
  61 gpddggvaaa ssgsaqaass paasvgtgva ggavstpapa pasapapgps agpppgppas
 121 lldtcavcqq slqsrreaep kllpclhsfc lrclpeperq lsvpipggsn gdiqqvgvir
 181 cpvcrqecrq idlvdnyfvk dtseapsssd ekseqvctsc ednasavgfc vecgewlckt
 241 cieahqrvkf tkdhlirkke dvsesvgasg qrpvfcpvhk qeqlklfcet cdrltcrdcq
 301 llehkehryq fleeafqnqk gaienllakl lekknyvhfa atqvqnrike vnetnkrveq
 361 eikvaiftli neinkkgksl lqqlenvtke rqmkllqqqn ditglsrqvk hvmnftnwai
 421 asgsstally skrlitfqlr hilkarcdpv paangairfh cdptfwaknv vnlgnlvies
 481 kpapgytpnv vvgqvppgtn hisktpgqin laqlrlqhmq qqvyaqkhqq lqqmrmqqpp
 541 apvptttttt qqhprqaapq mlqqqpprli svqtmqrgnm ncgafqahqm rlaqnaarip
 601 giprhsgpqy smmqphlqrq hsnpghagpf pvvsvhntti nptspttatm ananrgptsp
 661 svtaielips vtnpenlpsl pdippiqled agsssldnll sryisgshlp pqptstmnps
 721 pgpsalspgs sglsnshtpv rppstsstgs rgscgssgrt aektslsfks dqvkvkqepg
 781 tedeicsfsg gvkqektedg rrsacmlssp essltpplst nlhleselda laslenhvki
 841 epadmnesck qsglsslvng kspirslmhr sariggdgmn kdddpnedwc avcqmggdll
 901 ccekcpkvfh ltchvptlls fpsgdwictf crdigkpeve ydcdnlqhsk kgktaqglsp
 961 vdqrkcerll lylychelsi efqepvpasi pnyykiikkp mdlstvkkkl qkkhsqhyqi
1021 pddfvadvrl ifkncerfne adsevaqagk avalyfedkl teiysdrtfa plpefeqeed
1081 dgevtedsde dfiqprrkrl ksderpvhik
```

B)

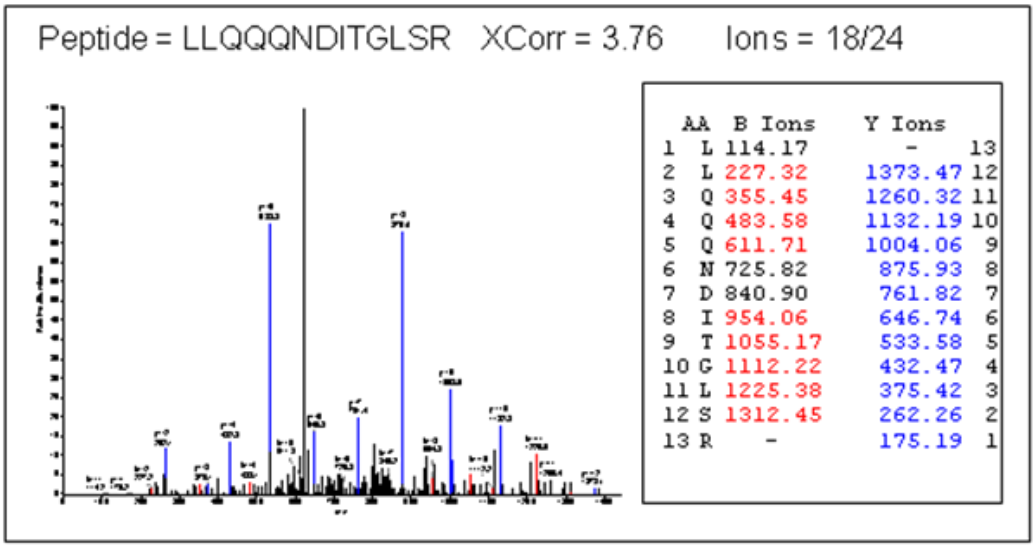


Figure 3.8A and Bi. ESI-mass spectrometric determination of TIF1γ peptides isolated from APC7 immunoprecipitates

(A) Two peptides from TIF1γ were identified by mass spectrometry (highlighted in red). **(Bi)** A mass spectrogram and table illustrating the ions generated from the TIF1γ peptide LLQQQNDITGLSR by ESI-MS/MS is shown. Eighteen out of a possible twenty four B (red) and Y ions (blue) were identified. The peptide generated an Xcorr of 3.33.

Biii)

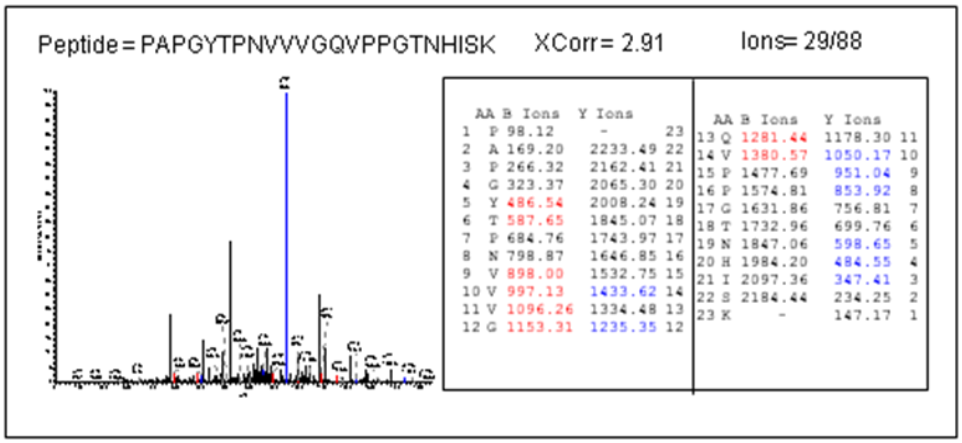


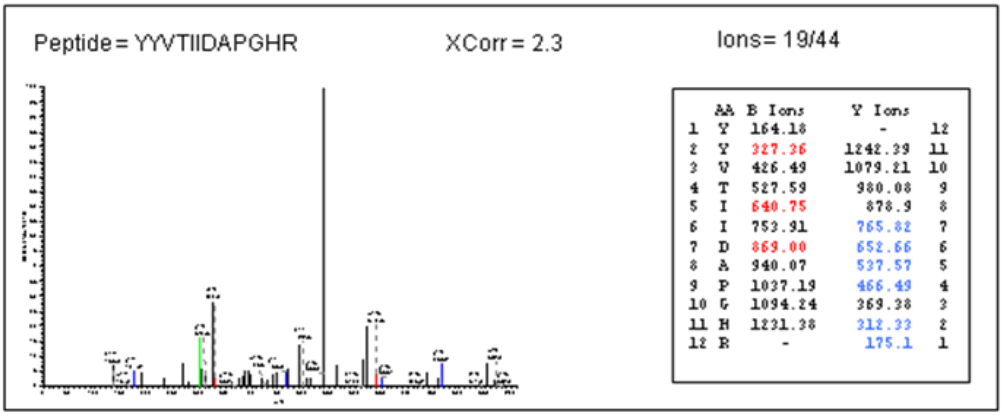
Figure 3.8Bii ESI-mass spectrometric determination of TIF1γ peptides isolated from APC7 immunoprecipitates

(A) A mass spectrogram and table illustrating the ions generated from the TIF1γ peptide PAPGYTPNVVVGQVPPGTNHISK by ESI-MS/MS is shown.

A)

```
   1 mgkekthini  vvighvdsgk  stttghliyk  cggidkrtie  kfekeaaemg  kgsfkyawvl
  61 dklkaererg  itidislwkf  etskyyvtii  dapghrdfik  nmitgtsqad  cavlivaagv
 121 gefeagiskn  gqtrehalla  ytlgvkqliv  gvnkmdstep  pysqkryeei  vkevstyikk
 181 igympdtvaf  vpisgwngdn  mlepsanmpw  fkgwkvtrkd  gnasgttlle  aldcilpptr
 241 ptdkplrlpl  qdvykiggig  tvpvgrvetg  vlkpgmvvtf  apvnvttevk  svemhheals
 301 ealpgdnvgf  nvknvsvkdv  rrgnvagdsk  ndppmeaagf  taqviilnhp  gqisagyapv
 361 ldchtahiac  kfaelkekid  rrsgkkledg  pkflksgdaa  ivdmvpgkpm  cvesfsdypp
 421 lgrfavrdmr  qtvavgvika  vdkkaagagk  vtksaqkaqk  ak
```

Bi)



Bii)

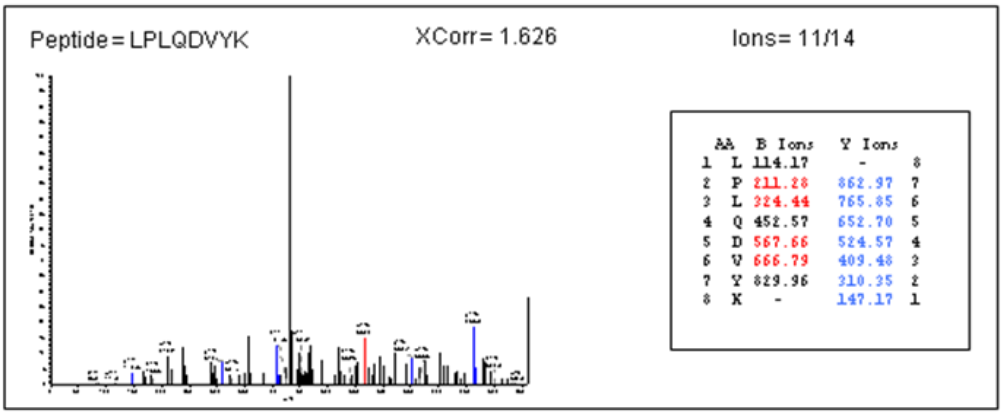


Figure 3.9. ESI-mass spectrometric determination of eukaryotic translation elongation factor 1 alpha 1 (eEF1A) peptides isolated from APC7 immunoprecipitates

(A) Two peptides from eEF1A were identified by mass spectrometry (highlighted in red). **(Bi)** A mass spectrogram and table illustrating the ions generated from the eEF1A peptide YYVTIIDAPGHR by ESI-MS/MS is shown. Nineteen out of a possible fourty four B (red) and Y ions (blue) were identified. The peptide generated an Xcorr of 2.3. **(Bii)** A mass spectrogram and table illustrating the ions generated from the eEF1A peptide LPLQDVYK by ESI-MS/MS is shown.

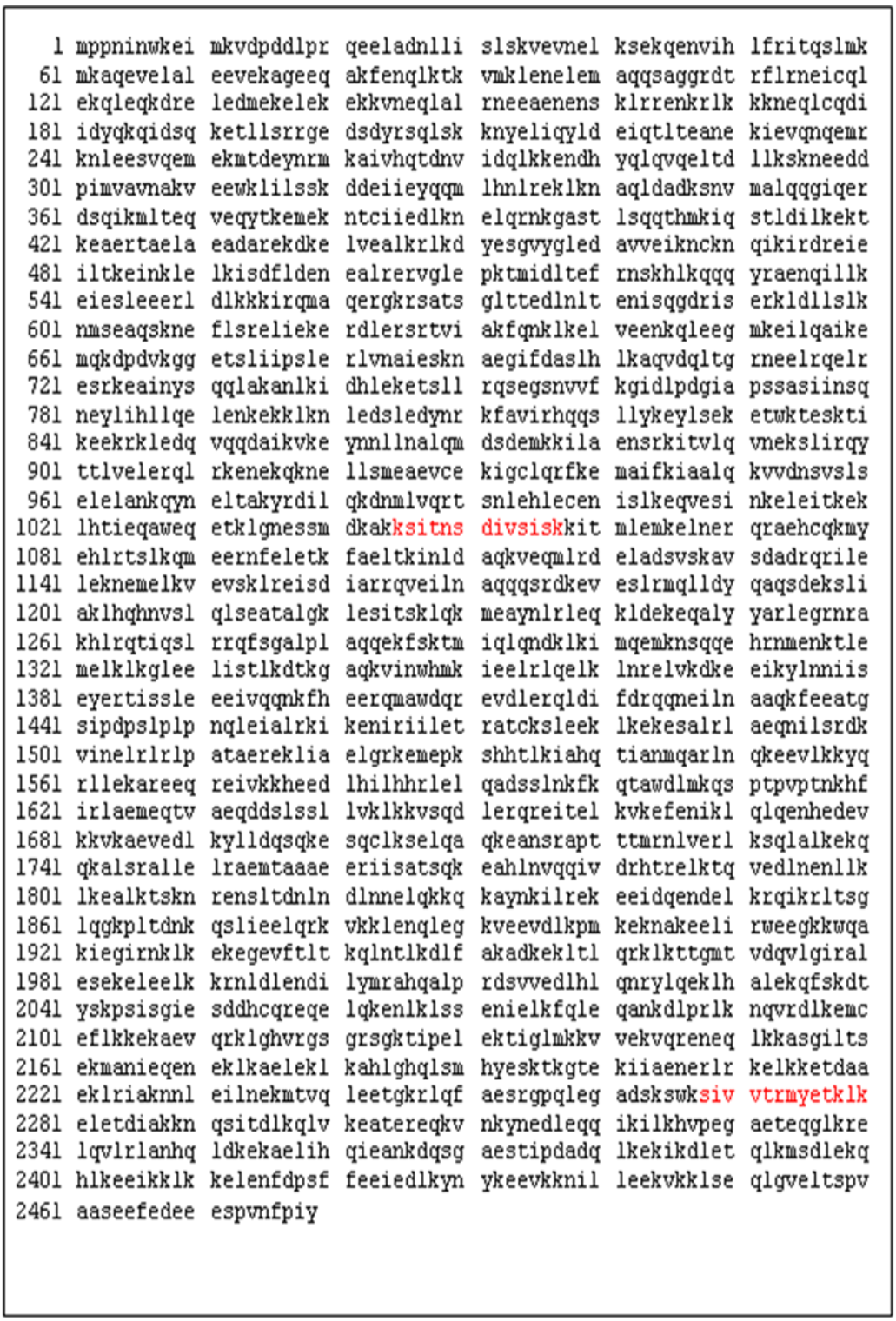
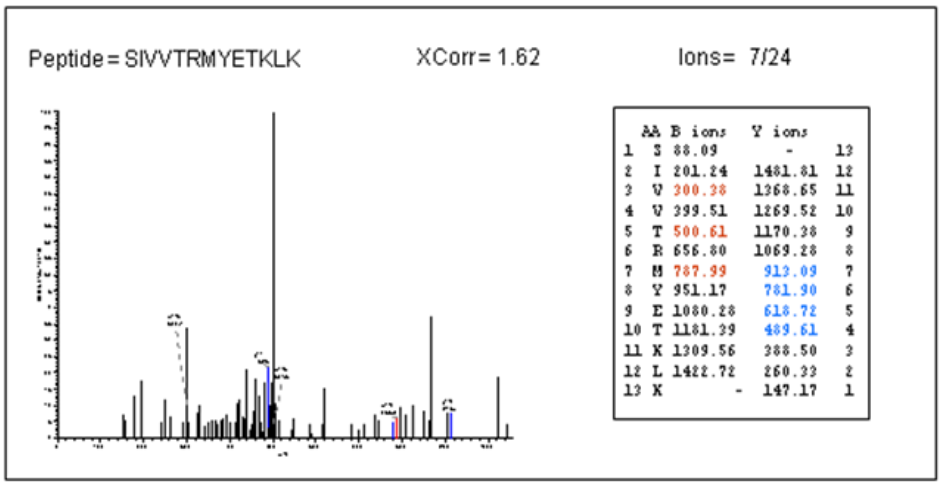


Figure 3.10A. ESI-mass spectrometric determination of CEP290 peptides isolated from APC7 immunoprecipitates

Two peptides from CEP290 were identified by mass spectrometry (highlighted in red).

Bi)



Bii)

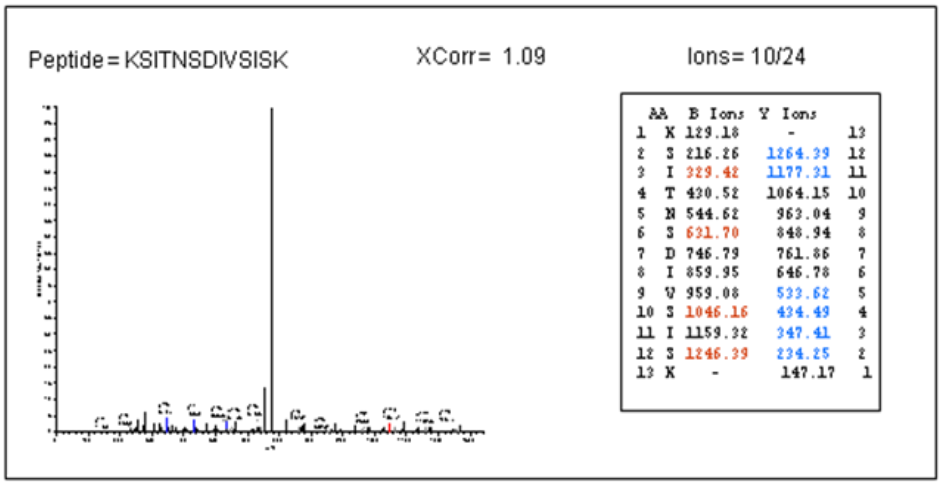


Figure 3.10Bi-ii. ESI-mass spectrometric determination of CEP290 peptides isolated from APC7 immunoprecipitates

(Bi) A mass spectrogram and table illustrating the ions generated from the CEP290 peptide SIVVTRMYETKLK by ESI-MS/MS is shown. Seven out of a possible sixteen B (red) and Y ions (blue) were identified. The peptide generated an Xcorr of 1.62. **(Bii)** A mass spectrogram and table illustrating the ions generated from the CEP290 peptide KSITNSDIVSISK by ESI-MS/MS is shown.

A)

```
  1 mghgsdysls evlwvranlf sdvqfkmsht kitlftnedn phgnesakas qartkagdlr
 61 dtgifldlmh lkkpggfdis lfyrdiisia ededlrvhfe esskledllq kvhaketrkr
121 alsrlklkln kdivisvgiy nlvqkalkpp piklyreine pvktktrtfn tstddlllps
181 dtkwsqiygs rqiilekeet eelkrfddpg lmlngfkplv llkkrhylrp slsvypeesl
241 vigsstlfsa llikclekev aalcrytprr nippyfvalv tqeevlddqk iqvtppgfql
301 vflpfaddkr kmpftekvta tpeqvdkmka iieklrftyr sdsfenpvlq qhfrnleala
361 ldlnepeqav dltlpkveai nrrlgslvde fkelvhppdy npegkvtkrk hdnegsgskr
421 pkveyseeel kthiskgtlg kftvpmlkea crayglksgl kkqellealt khfqd
```

Bi)

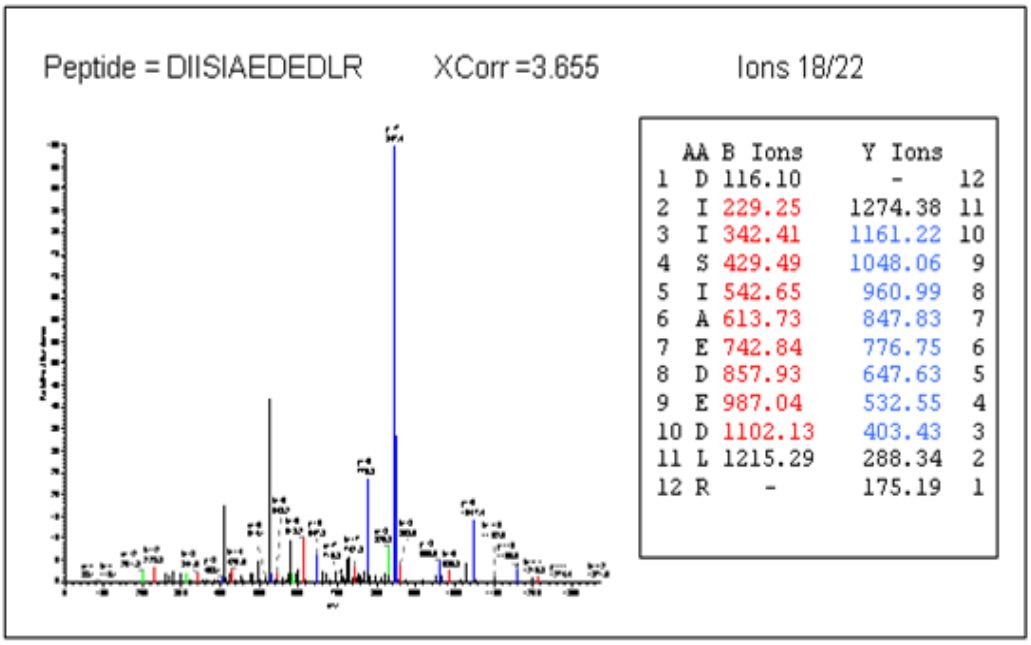
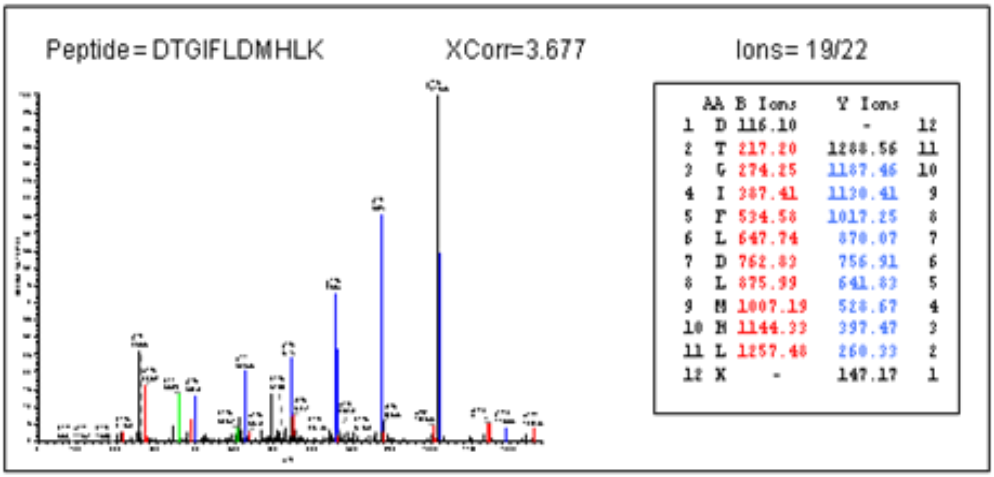


Figure 3.11A and Bi. ESI-mass spectrometric determination of Ku 70 KDa peptides isolated from APC7 immunoprecipitates.

(A) Three peptides from Ku 70 KDa were identified by mass spectrometry (highlighted in red). **(Bi)** A mass spectrogram and table illustrating the ions generated from the Ku 70 KDa peptide DIISIAEDEDLR by ESI-MS/MS is shown. Eighteen out of a possible sixteen B (red) and Y ions (blue) were identified.

Bii)



Biii)

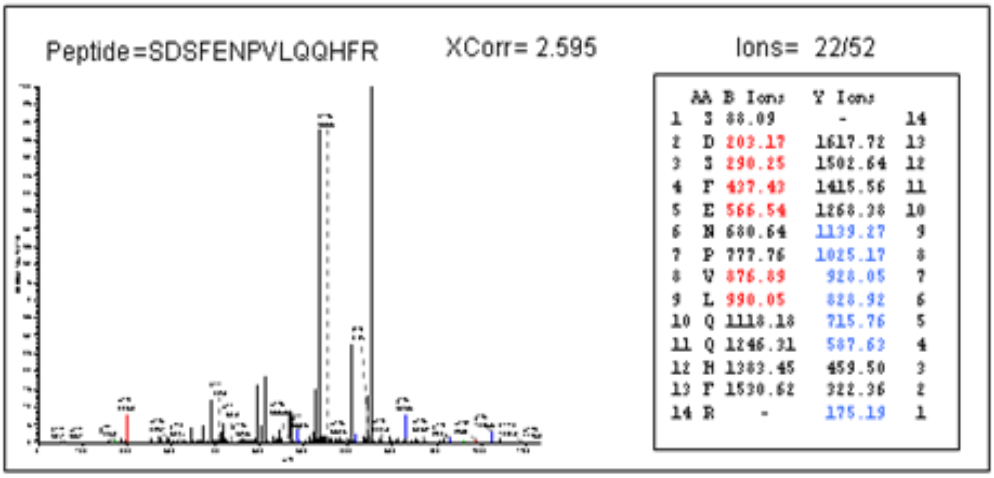


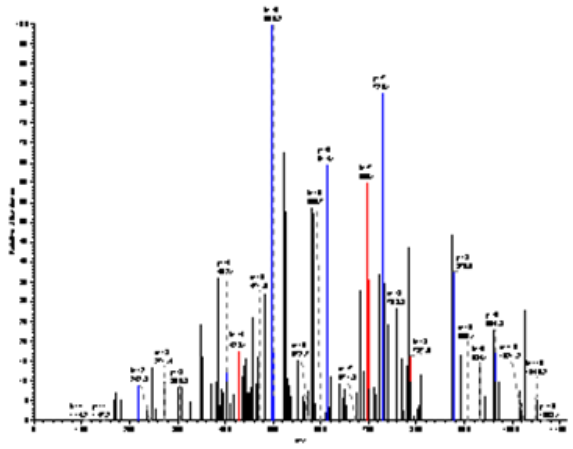
Figure 3.11Bii-iii. ESI-mass spectrometric determination of Ku 70 KDa subunit peptides isolated from APC7 immunoprecipitates.
(Bii) A mass spectrogram and table illustrating the ions generated from the Ku 70 KDa peptide DTGIFLDMHLK by ESI-MS/MS is shown. **(Biii)** A mass spectrogram and table illustrating the ions generated from the Ku70 KDa peptide SDSFENPVLQQHFR by ESI-MS/MS is shown.

A)

```
  1 masndytqqa tqsygayptq pgqgysqqss qpygqqsysg ysqstdtsgy gqssyssygq
 61 sqntgygtqs tpqgygstgg ygssqssqss ygqqssypgy gqqpapssts gsygsssqss
121 sygqpqsgsy sqqpsyggqq qsygqqqsyn ppqgygqqnq ynsssggggg gggggnygqd
181 qssmssgggs gggygnqdqs ggggsggygq qdrggrgrgg sggggggggg gynrsgggye
241 prgrgggrgg rggmggsdrg gfnkfggprd qgsrhdseqd nsdnntifvq glgenvties
301 vadyfkqigi iktnkktgqp minlytdret gklkgeatvs fddppsakaa idwfdgkefs
361 gnpikvsfat rradfnrggg ngrggrgrgg pmgrggyggg gsgggggrggf psgggggggq
421 qragdwkcpn ptcenmnfsw rnecnqckap kpdgpgggpg gshmggnygd drrggrggyd
481 rggyrgrggd rggfrggrgg gdrggfgpgk mdsrgehrqd rrerpyl
```

Bi)

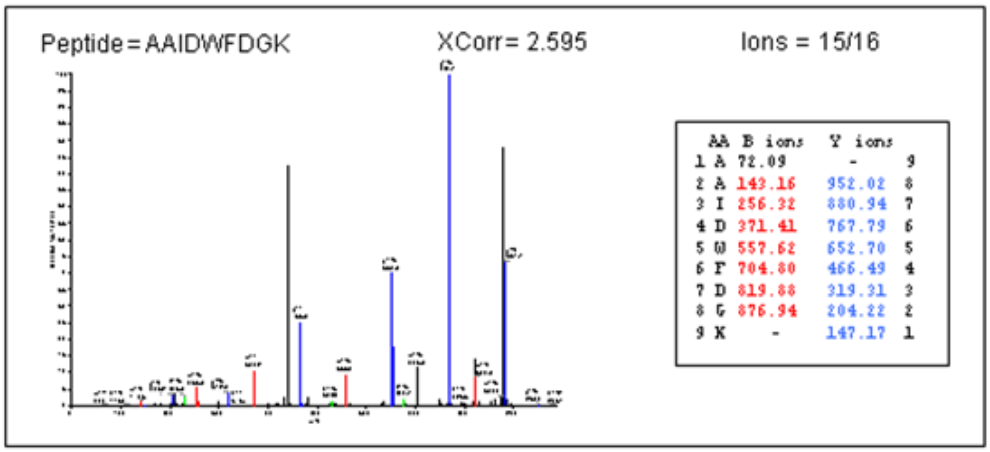
Peptide = LKGEATVSFDDPPSA XCorr = 4.047 Ions = 30/60



	AA	B Ions	Y Ions	
1	L	114.17	-	16
2	K	242.34	1549.64	15
3	G	299.39	1421.47	14
4	E	428.51	1364.42	13
5	A	499.58	1235.30	12
6	T	600.69	1164.22	11
7	V	699.82	1063.12	10
8	S	786.90	963.99	9
9	F	934.07	876.91	8
10	D	1049.16	729.74	7
11	D	1164.25	614.65	6
12	P	1261.36	499.56	5
13	P	1358.48	402.44	4
14	S	1445.56	305.33	3
15	A	1516.63	218.25	2
16	K	-	147.17	1

Figure 3.12 A and Bi. ESI-mass spectrometric determination of translocated in liposarcoma (TLS) peptides isolated from APC7 immunoprecipitates.
(A) Three peptides from TLS were identified by mass spectrometry (highlighted in red, and in blue when two peptides overlap). (B) A mass spectrogram and table illustrating the ions generated from the TLS peptide LKGEATVSFDDPPSA by ESI-MS/MS is shown. Fifteen out of a possible sixteen B (red) and Y ions (blue) were identified. The peptide generated an Xcorr of 4.047.

Bii)



Biii)

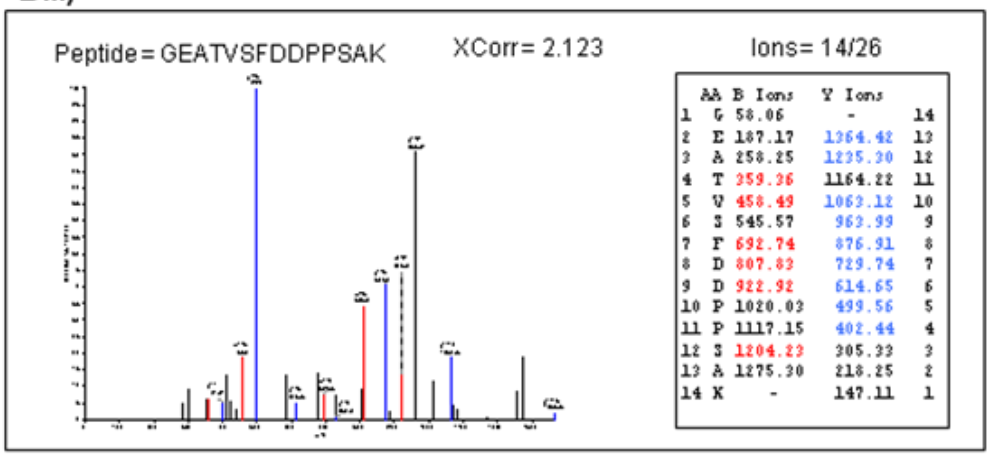


Figure 3.12Bii-iii. ESI-mass spectrometric determination of translocated in liposarcoma (TLS) peptides isolated from APC7 immunoprecipitates. **(Bii)** A mass spectrogram and table illustrating the ions generated from the TLS peptide AAIDWFDGK by ESI-MS/MS is shown. **(Biii)** A mass spectrogram and table illustrating the ions generated from the TLS peptide GEATVSFDDPPSAK by ESI-MS/MS is shown.

3.3. Discussion

Prior to this study, the only known APC7 binding proteins were other APC/C components and the transcriptional coactivators CBP and p300 (Peters 2002; Turnell, Stewart et al. 2005; Skaar and Pagano 2008). Work in this chapter provides evidence to suggest that APC7 also interacts with NF45, TIF1γ, eEF1A, CEP290, Ku70 and TLS. These observations present many implications for both APC7 and APC/C function.

As APC7 has previously been demonstrated to potentiate CBP/p300 dependent transcription (Turnell, Stewart et al. 2005), it is therefore somewhat unsurprising that five out of six of the novel APC7-interacting proteins have been demonstrated to function as transcription factors. The putative interaction between APC7 and NF45 suggests that APC7 and the APC/C holoenzmye may interact with the ARRE-2 transcription complex and hence may regulate IL-2 transcription in response to T-cell receptor activation (Corthesy and Kao 1994; Kao, Chen et al. 1994). Data presented in this chapter suggests that APC7 interacts with Ku70, which also regulates IL-2 transcription in conjunction with NF90/NF45 (Aoki, Zhao et al. 1998; Shi, Qiu et al. 2007). A role for APC7 in transcription is also implied due to interactions with TLS, TIF1γ and CEP290.

Identification of NF45, TLS, Ku70 and eEF1A as APC7 interacting proteins suggests that APC7 is involved at multiple stages of RNA processing. This is implied by the observations that NF90/NF45 regulates post-transcriptional mRNA stabilization and controls mRNA export (Shim, Lim et al. 2002; Gwizdek, Ossareh-Nazari et al. 2003; Shi, Zhao et al. 2005), while TLS is also thought to be involved in pre-mRNA splicing and mRNA export (Calvio, Neubauer et al. 1995). Moreover NF90/NF45, Ku70 and eEF1A are all involved in regulating translation (Kuhn, Stefanovsky et al. 1993; Xu, Busald et al. 2000; Hotokezaka, Tobben et al. 2002; Silvera, Koloteva-Levine et al.

2006), therefore it seems likely that APC7 or possibly the entire APC/C may also be involved in this process (Xu, Busald et al. 2000; Hotokezaka, Tobben et al. 2002; Silvera, Koloteva-Levine et al. 2006). Indeed there is already evidence to suggest that APC5 functions to regulate translation (Koloteva-Levine, Pinchasi et al. 2004), and it seems likely therefore that this may be in conjunction with APC7.

A number of the novel APC7 protein interactions identified in this chapter suggest that APC7 might be involved in the maintenance of genomic integrity. Firstly DNA-PK is crucial for DSB repair (Carter, Vancurova et al. 1990; Collis, DeWeese et al. 2005), while its regulatory component Ku is required for telomere maintenance (Bailey, Meyne et al. 1999; Samper, Goytisolo et al. 2000). Secondly TLS knockout mice show an increased sensitivity to IR that may be related to the ability of TLS to mediate binding of homologous DNA, which is an important step in homologous recombination (Kuroda, Sok et al. 2000). NF45/NF90 may also function in the maintenance of genomic integrity as suggested by interaction with DNA-PK (Ting, Kao et al. 1998). Interaction between APC7 and Ku70 also provides evidence to suggest that the APC/C may cooperate with Ku in order to initiate chromosome replication (Novac, Matheos et al. 2001).

The APC/C is localised to centrosomes where it stimulates degradation of proteins such as cyclin B and ninein-like protein (NLP), in order to facilitate centrosomal maturation (Tugendreich, Tomkiel et al. 1995; Casenghi, Meraldi et al. 2003; Wang and Zhan 2007). In accordance with this my study presents evidence to support the notion that APC7 is required for correct centrosome function. This is suggested by interaction between APC7 and the centrosomal component CEP290, and also as a result of interaction with eEF1A, which is highly homologous to a centrosomal protein in sea urchin embryos and is also capable of interacting with actin (Kuriyama, Savereide et al.

1990; Owen, DeRosier et al. 1992), and may therefore mediate mitotic spindle formation.

The interaction between APC7 and TIF1γ, suggests that APC7 and TIF1γ might cooperate to regulate transcription or that TIF1γ may be important for ubiquitylation of APC/C substrates, by functioning as substrate adaptor or due to its ability to function as an E3 ubiquitin ligase (Venturini, You et al. 1999; Dupont, Zacchigna et al. 2005).

Taken together the results in this chapter suggest a role for APC7 in isolation or as a component of the APC/C holoenzyme in transcription, post-transcriptional RNA processing, centrosome function, initiation of DNA replication and also imply that the APC/C may be involved in maintenance of genome integrity. Interestingly, a number of the novel APC7 binding proteins identified in this chapter have previously been demonstrated to bind and function together. For instance, the NF90 homologue NF110, which may bind to APC7 as a result of its interaction with NF45, has previously been shown to bind to TLS (Saunders, Perkins et al. 2001). In addition DNA-PK interacts with NF90/NF45 (Ting, Kao et al. 1998), therefore suggesting that these proteins may form a multi-component complex with APC7, and further validates the efficacy of the data obtained.

Work presented in chapters four and five focuses on validating the interactions between TIF1γ-APC7 and NF45-APC7, and also on determining the functional significance of the interactions.

CHAPTER 4

TIF1γ COOPERATES WITH THE APC/C TO REGULATE TRANSITION THROUGH MITOSIS

4.1. Introduction

TIF1γ is a member of the TRIM/RBCC family and was originally identified and characterised as a transcription factor due to its homology with TIF1α (Venturini, You et al. 1999). Subsequent work demonstrated that TIF1γ is fused to the RET receptor tyrosine-kinase domain in papillary thyroid carcinomas (PTC) (Klugbauer and Rabes 1999), suggesting that TIF1γ regulates cellular proliferation and differentiation. In accordance with this finding, TIF1γ has more recently been demonstrated as a Smad4 ubiquitin ligase, important for enhancing proliferation in postnatal cells and regulating embryogenesis after TGF-β treatment (Dupont, Zacchigna et al. 2005). Moreover TIF1γ also binds to R-SMADs in response to TGF-β signalling in order to mediate erythroid differentiation (He, Dorn et al. 2006).

In the previous chapter I presented evidence to suggest that APC7 interacts with TIF1γ, eEF1A, CEP290, Ku70, and TLS. However mass spectrometric analysis is only one method of identifying protein-protein interactions, therefore in this chapter my objective was to confirm the interaction between APC7 and TIF1γ, and go on to determine the functional significance. I decided to focus on the TIF1γ-APC7 interaction, due to TIF1γs role in human cancer (Klugbauer and Rabes 1999; Venturini, You et al. 1999).

4.2. Results

4.2.1. Generation of GST−TIF1γ fusion proteins

In order to confirm the interaction between TIF1γ and APC7 identified by mass spectrometry in the previous chapter (Fig 3.8), it was essential to perform a coimmunoprecipitation Western blot between TIF1γ and APC7. Given the lack of a commercial TIF1γ antibody, it was necessary to generate one. I therefore constructed

two fragments that encompassed the N–terminal and the central regions of TIF1γ (Fig 4.1 C), using full length TIF1γ cDNA, and inserted them into pGEX–4T1. After validating DNA sequences, BL21 RIL *E. coli* were transformed with the appropriate pGEX constructs and the fusion proteins were expressed and purified. Expression was validated by Coomassie staining following SDS–PAGE (Fig 4.2). Given that the GST–TIF1γ–F1 fusion protein showed higher levels of expression relative to GST–TIF1γ–F2 (Fig 4.2), I decided to use GST–TIF1γ–F1 in order to generate polyclonal antibodies using a commercial supplier (Eurogentec).

4.2.2. Generation of TIF1γ antibody and validation of its specificity

In order to test the efficacy and specificity of the antibodies TIF1γ98 and TIF1γ78 that were generated for this study, I performed Western blot analysis of whole cell extracts from the indicated cell lines (Fig 4.3A and 4.4) using these antibodies. Interestingly, both antibodies detected multiple bands of varying molecular weights. This may be due to the existence of multiple TIF1γ splice products (Venturini, You et al. 1999). The TIF1γ78 antibody predominantly recognises a protein band that runs below the 45K molecular weight marker and fails to recognise any bands with the same nominal size of 180K as TIF1γ (Fig 4.4), and was therefore not suitable for further evaluation. In contrast the TIF1γ98 antibody recognises a band that has a nominal size of 180k and also a number of weakly expressed sub 180K bands (Fig 4.3A). In order to determine whether these corresponded to TIF1γ, I assayed the ability of the TIF1γ98 antibody to recognise the putative TIF1γ bands, after knockdown of TIF1γ using two distinct siRNAs in HeLa cells. 72 hours after siRNA–mediated knockdown, cells were harvested and lysed in urea buffer, proteins were then analysed by Western blot.

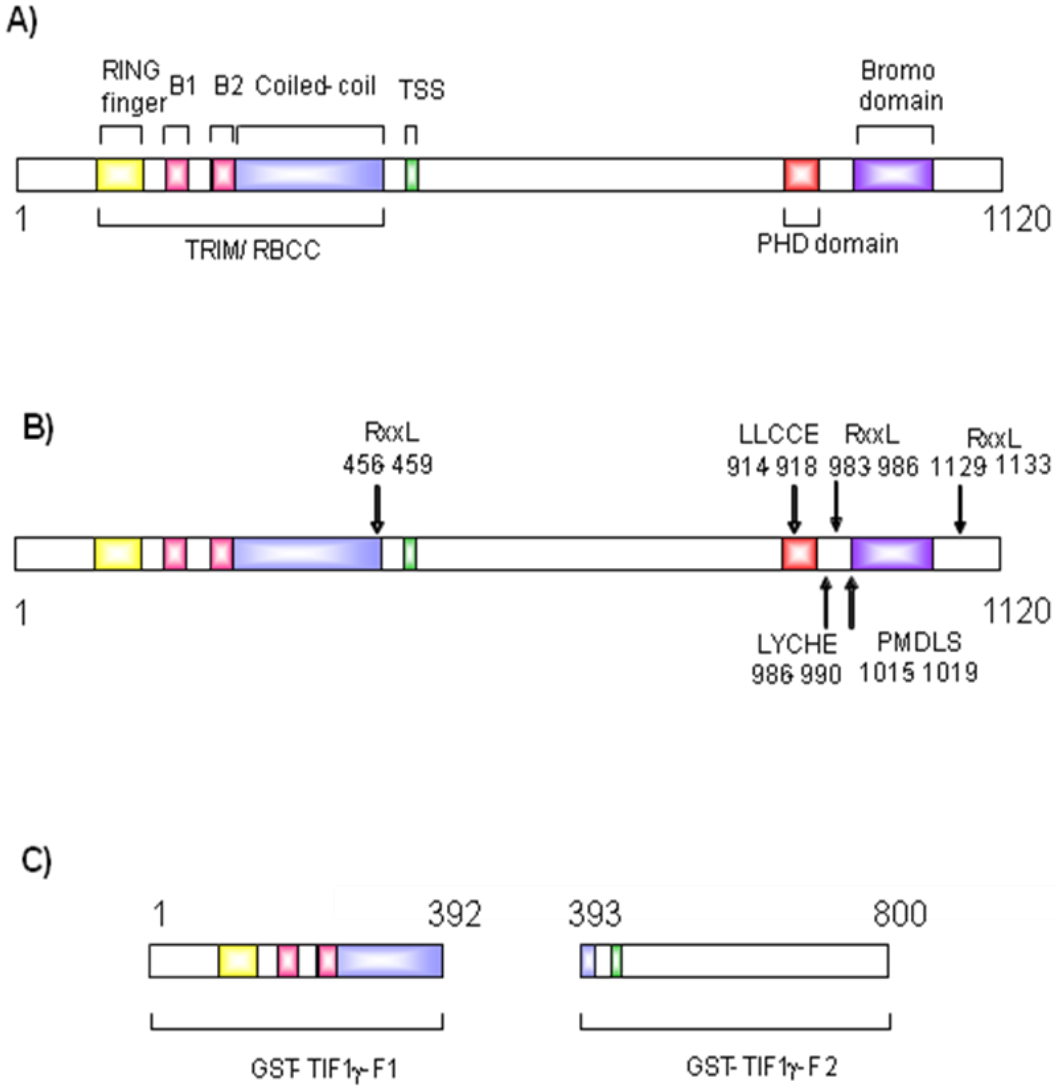


Fig 4.1A-C. Generation of a GST-TIF1γ N-Terminal fragment.
(A) A schematic representation of the full length TIF1γ protein, depicting the TRIM/RBBC motif, which consists of a RING domain, B-boxes and a coiled-coil. The TIF1 signature sequence (TSS), plant homeo domain (PHD) and bromodomains are also displayed (B) A schematic representation of full length TIF1γ detailing putative Rb family binding motifs (LXCXE), D-boxes (RXXL) and a CtBP binding motif (PXDLS). (C) A schematic representation of GST-TIF1γ fragments.

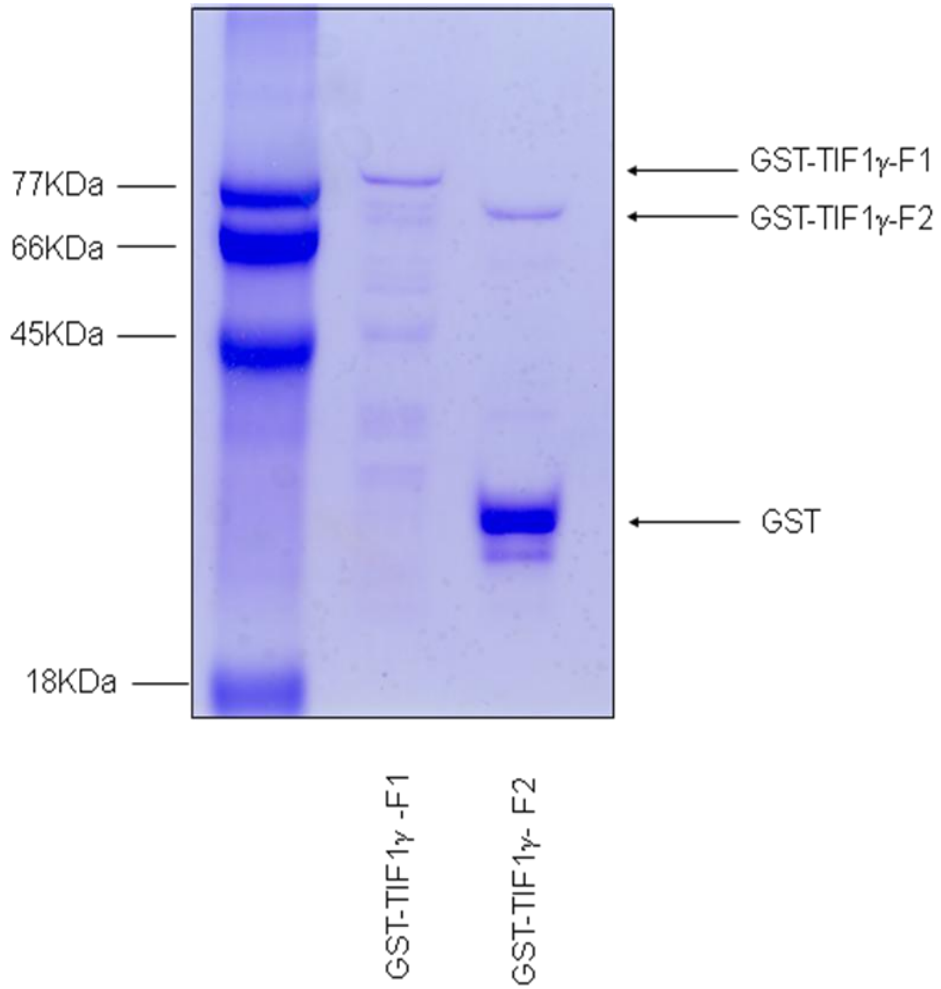


Fig 4.2. Bacterial expression of GST-TIF1γ fragments
20 µg of purified GST-TIF1γ-F1 and GST-TIF1γ-F2 were resolved by SDS-PAGE and stained with 0.1% (w/v) Coomassie brilliant blue. Molecular weight markers are indicated to the left of the image

Significantly both siRNAs knocked down expression of the 180k band to a high degree, indicating that this band corresponds to TIF1γ. All but one of the multiple sub 180k bands recognised by the TIF1γ98 antibody also displayed reduced expression after treatment with TIF1γ siRNA, indicating that they may be TIF1γ degradation products or hitherto unknown TIF1γ splice variants (Fig 4.3B). The only band recognised by the TIF1γ98 antibody that doesn't exhibit reduced expression following treatment with TIF1γ siRNA, runs midway between the 66K and 77k molecular weight markers, and may therefore represent a related protein such as another TRIM/RBCC family member. In light of this I decided to determine whether the bands recognised by the TIF1γ98 antibody resembled the expression pattern of various FLAG-TIF1γ constructs in 293 cells. Results obtained reveal the existence of a TIF1γ cDNA expression product that runs between 66k and 77k (Fig 4.5) suggesting that the band of similar size that is recognise by the TIF1γ98 antibody might correspond to a TIF1γ splice variant that is insensitive to the TIF1γ siRNAs utilised in this study

4.2.3. Binding of the APC/C to TIF1γ *in vivo*

To confirm that the APC/C and TIF1γ interact I performed immunoprecipitation from A549s using various APC/C subunit antibodies and the TIF1γ polyclonal antibody TIF1γ98. Proteins were then eluted, separated by SDS-PAGE and detected by Western blotting with the indicated antibodies (Fig 4.6A and B). As anticipated, TIF1γ forms complexes with APC/C subunits 1-8 *in vivo*.

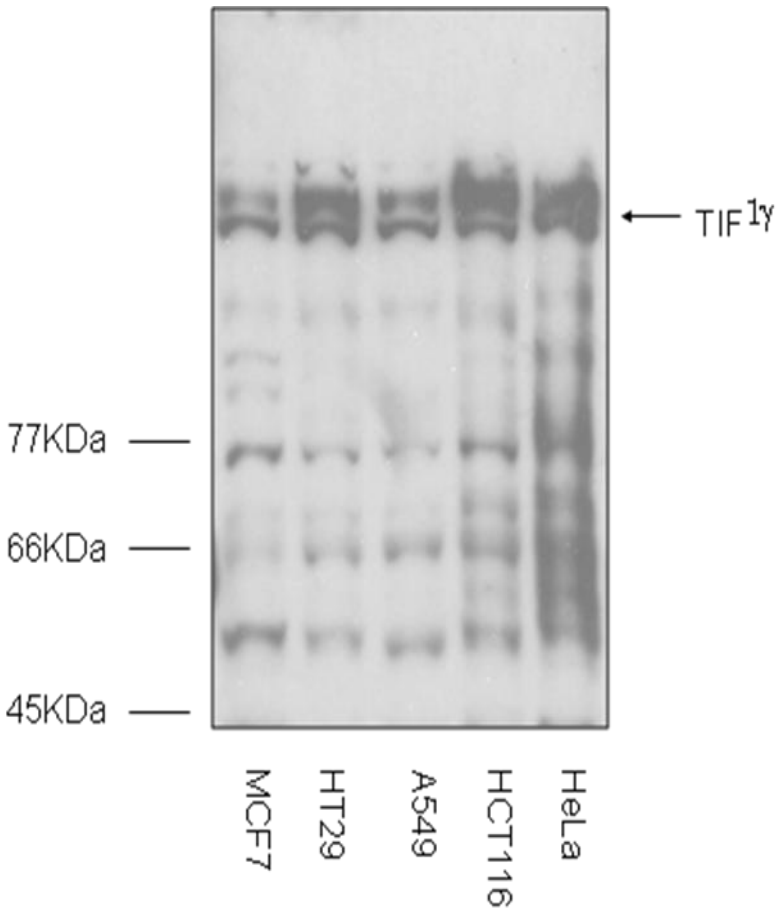


Fig 4.3A. Evaluation of the TIF1γ98 antibody

(A) 30µg of whole cell extracts from the indicated cell lines were resolved by SDS-PAGE and analyzed by Western blotting using the anti-TIF1γ polyclonal antibody TIF1γ98.

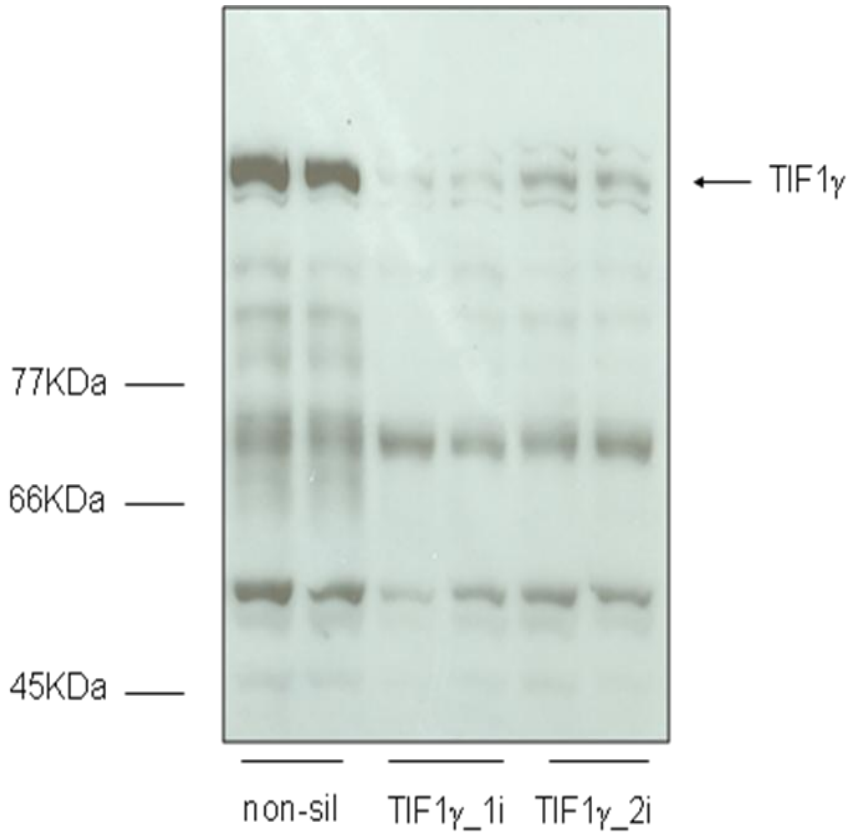


Fig 4.3B. Validation of the TIF1γ98 antibody

Following TIF1γ knockdown in HeLa cells, whole cell extracts were analyzed by Western blotting in order to determine whether the TIF1γ98 antibody is specific to TIF1γ. non-sil, non-silencing RNA oligonucleotides; TIF1γ-1i and TIF1γ-2i refer to instances where TIF1γ expression has been ablated using distinct RNAi treatments.

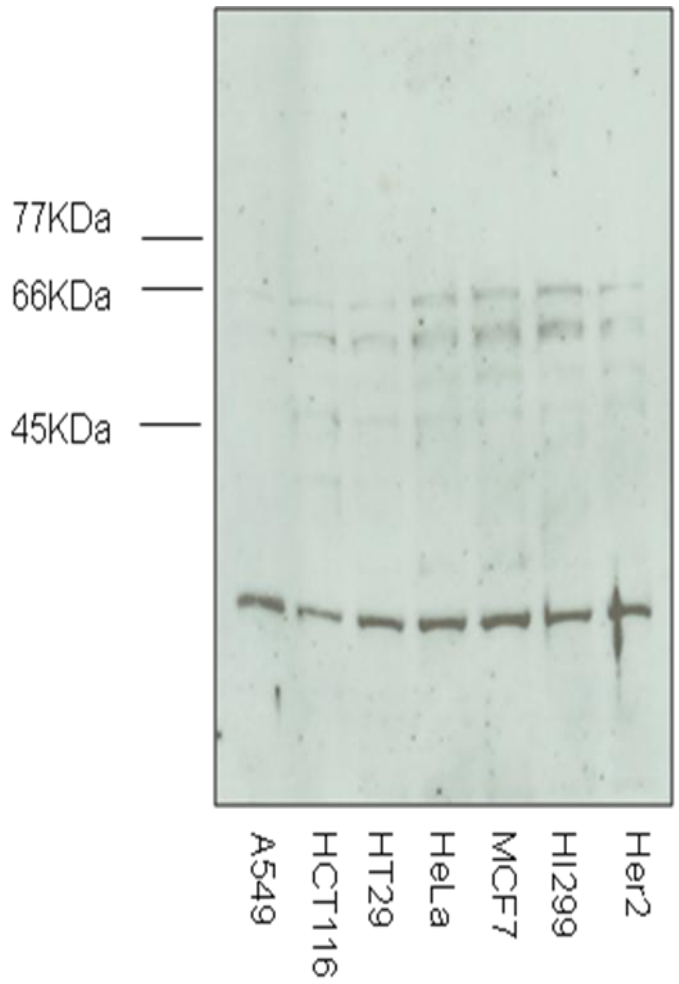


Fig 4.4. Evaluation of the TIF1γ78 antibody

30µg of whole cell extracts from the indicated cell lines were resolved by SDS-PAGE and analyzed by Western blotting using the anti-TIF1γ antibody TIF1γ78.

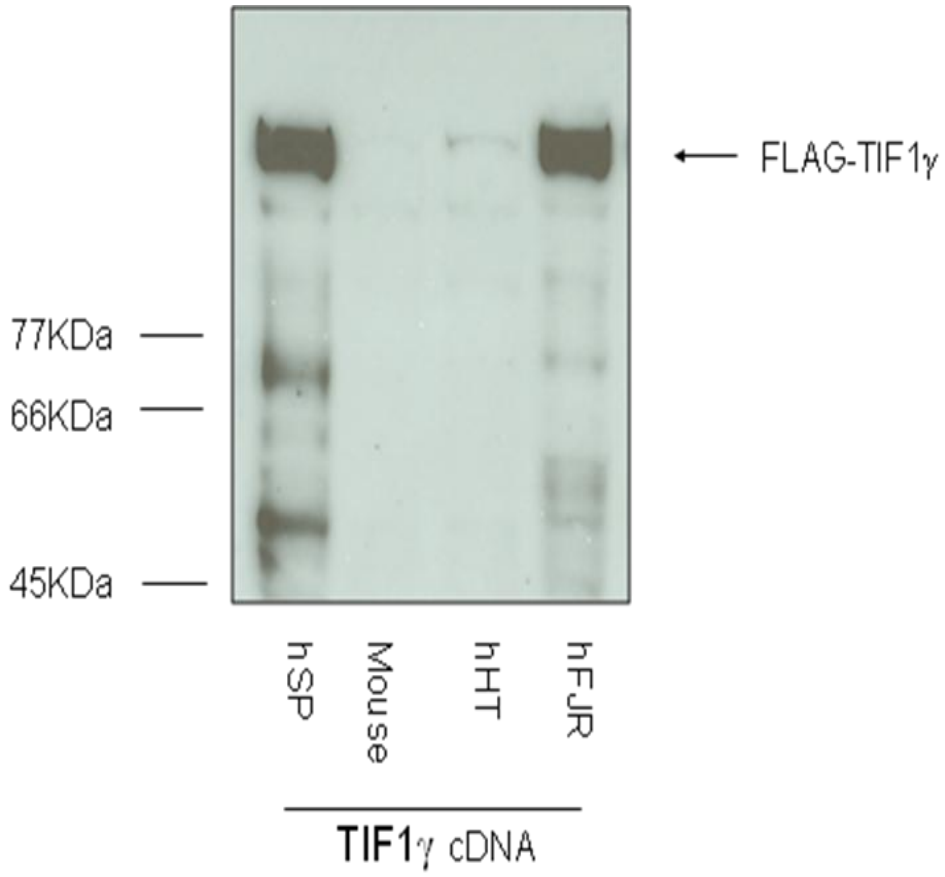


Fig 4.5. *In vivo* expression of FLAG-TIF1γ

The indicated FLAG-tagged human and mouse TIF1γ cDNAs were expressed in 293 cells for 48hrs. 50μg of whole cell extracts were then analysed by Western blotting with an anti-FLAG antibody. Under each lane the prefix h denotes a human cDNA and is followed by the initials of the person the clone was sourced from.

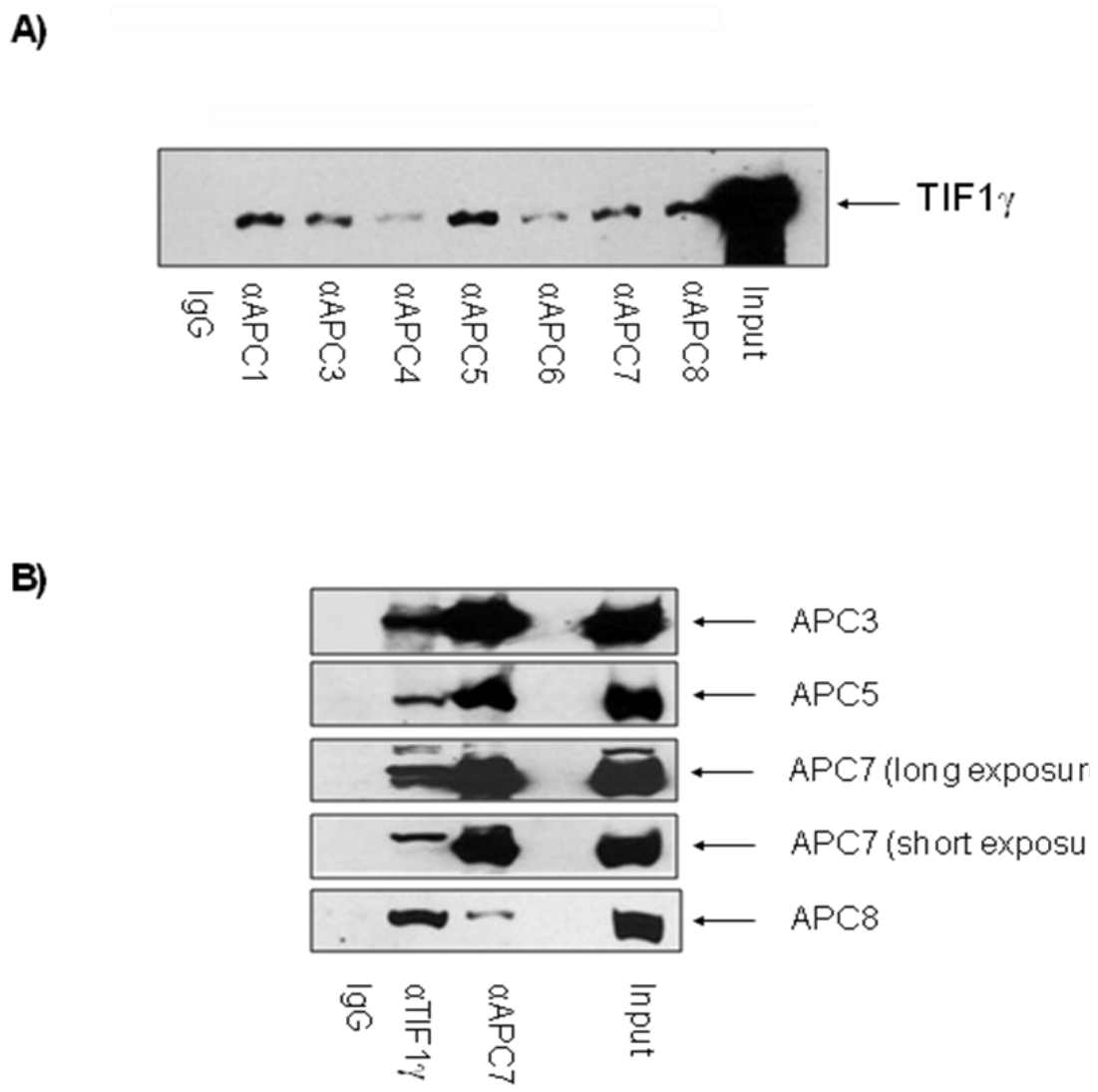


Fig 4.6 A and B. *In vivo* binding of TIF1γ and the APC/C.
(A) The indicated APC/C subunits were immunoprecipitated from 5mg of A549 lysate with the relevant antibody. After precipitation with Protein G-Sepharose, immuno-complexes were washed extensively, incubated with sample buffer and resolved by SDS-PAGE. Specific binding was analyzed by Western blotting using the TIF1γ98 antibody. (**B**) TIF1γ and also APC7 (as a positive control) were immunoprecipitated from 5mg of A549 lysate from A549 cells with the anti-TIF1γ or anti-APC7 . After precipitation with Protein G-Sepharose, immuno-complexes were washed extensively, incubated with sample buffer and resolved by SDS-PAGE. Specific APC/C subunit binding was analyzed by Western blotting using the indicated antibodies.

4.2.4. TIF1γ isn't an APC/C substrate *in vitro*

Analysis of TIF1γ's protein sequence revealed the presence of three putative destruction boxes (Fig 4.1). Taken together with TIF1γ's interaction with the APC/C this suggested that TIF1γ is ubiquitylated by the APC/C. In order to examine this possibility active APC/C complexes were immunoprecipitated with an anti-APC3 antibody, and added to a ubiquitin ligase reaction mix containing L-α-[^{35}S]-methionine-labelled cyclin B or TIF1γ. As anticipated APC3 and its associated APC/C holoenzyme was able to polyubiquitylate cyclin B (Fig 4.7A), therefore demonstrating the efficacy of this assay. However APC3 immunoprecipitates were not capable of ubiquitylating TIF1γ (Fig 4.7B).

4.2.5. CDH1 maintains TIF1γ stability *in vivo*

To further explore the possibility that TIF1γ degradation is mediated by the APC/C I transfected HeLas with an siRNA against the APC/C coactivator CDH1. 72 hours later cells were lysed in urea buffer and analysed by Western blotting to determine the effect of CDH1 knockdown on TIF1γ stability. Interestingly reduction of CDH1 levels results in reduced expression of TIF1γ (Fig 4.8), therefore suggesting that APC/C^{CDH1} may prevent TIF1γ degradation by targeting a TIF1γ ubiquitin ligase for destruction.

4.2.6. Binding of TIF1γ to APC/C substrates and coactivators *in vivo*

Given that TIF1γ interacts with the APC/C and is not itself an APC/C substrate it seemed likely that TIF1γ may therefore regulate the APC/C's function as a ubiquitin

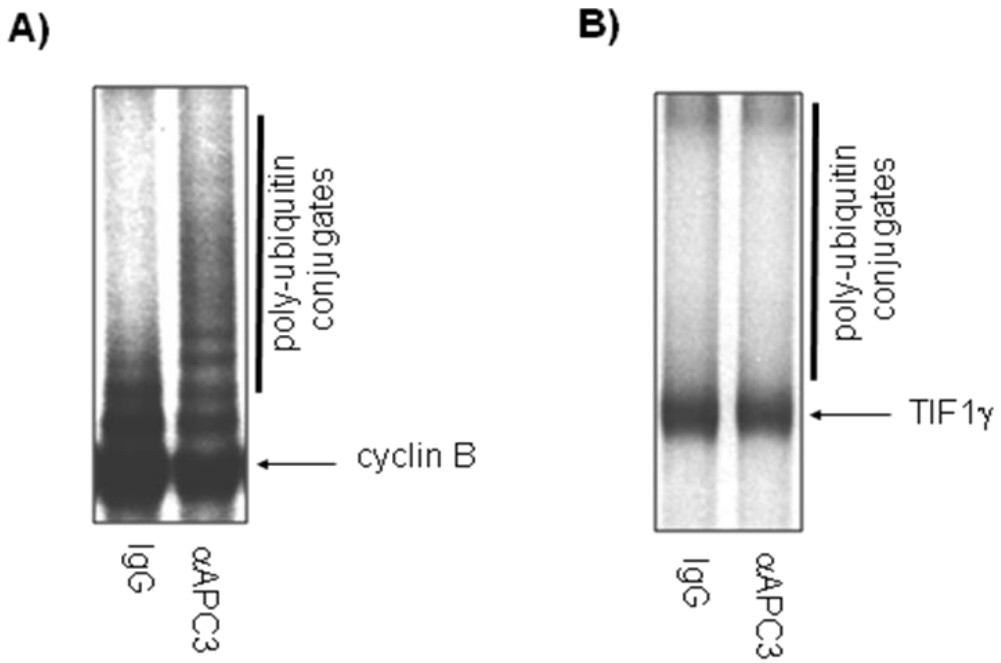


Fig 4.7. Determination of TIF1γ as a substrate for APC/C dependent ubiquitylation

20µg of the indicated antibodies were incubated with 5mg of A549 lysate. Bound proteins were then precipitated using protein G-Sepharose, washed, and incubated with with rabbit E1 and human E2 enzymes: UBCH10, UBCH4 and UBCH5, and 1µl of [35S]-labelled, *in vitro*-translated cyclin B (**A**) or TIF1γ (**B**). The reaction mixture was then incubated with sample buffer and separated by SDS-PAGE. After fluorography the gel was dried and subjected to autoradiography. 2µl of radiolabelled inputs were loaded in the first lane of the gel.

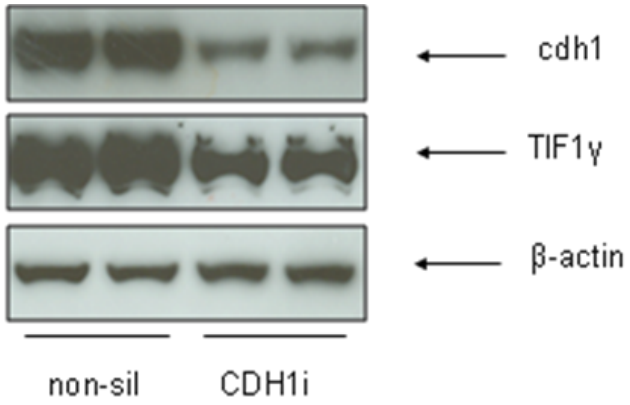


Fig 4.8. CDH1 knockdown results in destabilisation of TIF1γ
HeLa cells were treated with non-silencing siRNA or with CDH1 siRNA, and harvested 96 hours post transfection. Whole cell extracts were then examined by Western blotting with the indicated antibodies. non-sil, non-silencing RNA oligonucleotides; CDH1i refers to treatment with siRNA.

ligase during mitosis. In order to further explore this possibility, I decided to determine whether TIF1γ could interact with the APC/C's mitotic coactivator CDC20, and the APC/C's mitotic substrates cyclin A and cyclin B. To do this I carried out immunoprecipitations from HeLa cells using the anti-TIF1γ polyclonal antibody TIF1γ98. Precipitated proteins were eluted, separated by PAGE and Western blotted with the indicated antibodies (Fig 4.9A-C). These studies reveal that TIF1γ is associated with CDC20 and cyclin A, but not cyclin B *in vivo*.

4.2.7. TIF1γ displays a band shift during mitosis

Results from our lab demonstrate that CBP and mediator of DNA damage checkpoint protein 1 (MDC1), which function in concert with the APC/C during early mitosis display a band shift after treatment with the mitotic spindle poison nocodazole, suggesting that they might be phosphorylated either during mitosis or following activation of the SAC and therefore indicates that they have important functions during mitosis. Given that the results described above strongly suggest that TIF1γ is important for the function of the APC/C during early mitosis, I decided to examine whether TIF1γ also displays a similar band shift following nocodazole treatment. Interestingly TIF1γ undergoes a band shift after nocodazole treatment (Fig 4.10), indicating that TIF1γ is either phosphorylated in response to the SAC or constitutively phosphorylated during mitosis. Therefore data in this section further strengthens a role for TIF1γ during mitosis.

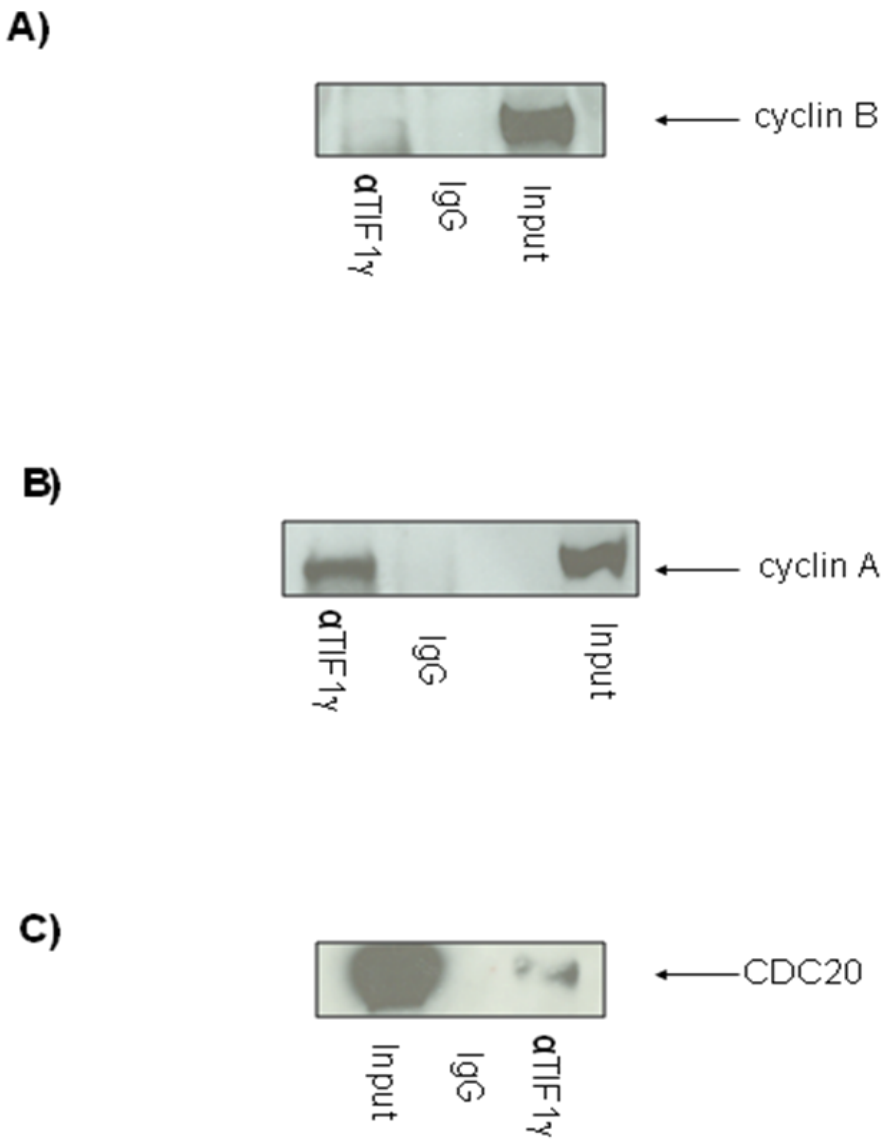


Fig 4.9A-C. TIF1γ interacts with cyclin A and CDC20, but not cyclin B *in vivo*

TIF1γ was immunoprecipitated from 5mg of HeLa lysate with the TIF1γ98 antibody. After precipitation with Protein G-Sepharose, immuno-complexes were washed extensively, incubated with sample buffer and resolved by SDS-PAGE. Specific binding was analyzed by Western blotting using anti-cyclin B (A), anti-cyclin A (B) and anti-CDC20 (C) antibodies.

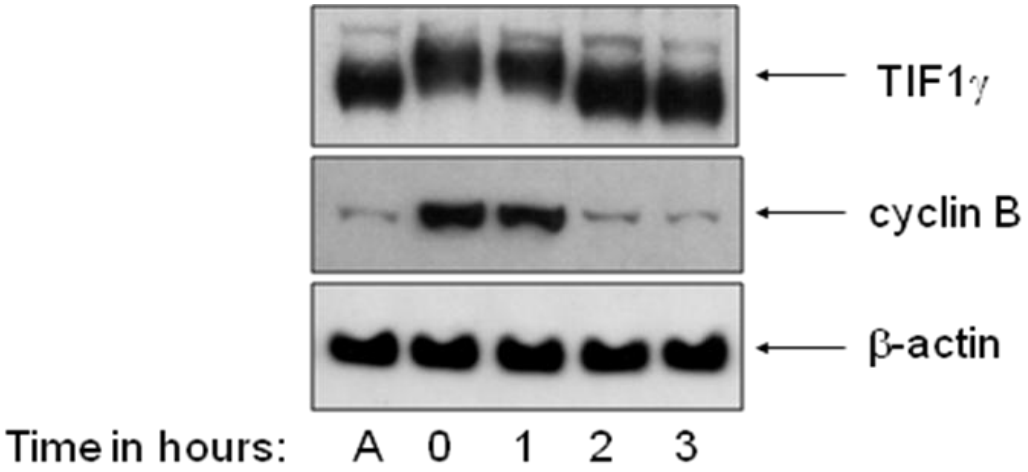


Fig 4.10. TIF1γ band shifts after treatment with nocodazole
A549s were incubated with nocodazole for 16 hours. Mitotic cells were harvested by shake off, washed to remove nocodazole and replated in medium. Cells were harvested at the indicated times, lysed and analys Western blotting with the indicated antibodies. A, refers to asynchr cells

4.2.8. TIF1γ is required for degradation of mitotic APC/C substrates

Previous work in this section details interactions between TIF1γ and the APC/C, CDC20 or cyclin A and also mitotic band shift of TIF1γ, suggesting that TIF1γ might be required for the degradation of the APC/C's mitotic substrates. To test this possibility I transfected HeLa cells with TIF1γ_1 and TIF1γ_2 siRNAs. 72 hours later the cells were harvested and lysed in urea buffer, proteins were then analysed by Western blot. Ablation of TIF1γ expression correlated with stabilisation of the APC/C substrates cyclin A, cyclin B, PLK1 and CDC20 when compared to the non-silencing control (Fig 4.11). Results in this section suggest that TIF1γ might regulate ubiquitylation and hence degradation of APC/C substrates *in vivo.*

4.2.9 TIF1γ enhances APC/C ligase activity

The ability of TIF1γ to interact with APC7, CDC20 and cyclin A *in vivo*, coupled to the observation that TIF1γ mediates degradation of the APC/C's mitotic substrates, suggests that TIF1γ might regulate the APC/C's E3 ubiquitin ligase activity. In order to investigate this possibility, I took advantage of the observation that immunoprecipitated APC/C complexes are capable of polyubiquitylating cyclin A and cyclin B in the presence of E1 and E2 enzymes *in vitro* (King, Peters et al. 1995; Geley, Kramer et al. 2001). Initially I performed TIF1γ knockdown in HeLa cells with TIF1γ_1 and TIF1γ_2 siRNAs. 72 hours later I performed immunoprecipitation with an anti-APC3 antibody. Precipitated immune-complexes were then added to a ubiquitin ligase reaction mix containing rabbit E1, human E2 enzymes (UBCH10, UBCH5 and UBCH4), and L-α-[^{35}S]-methionine-labelled cyclin A or cyclin B substrates. Samples were then separated

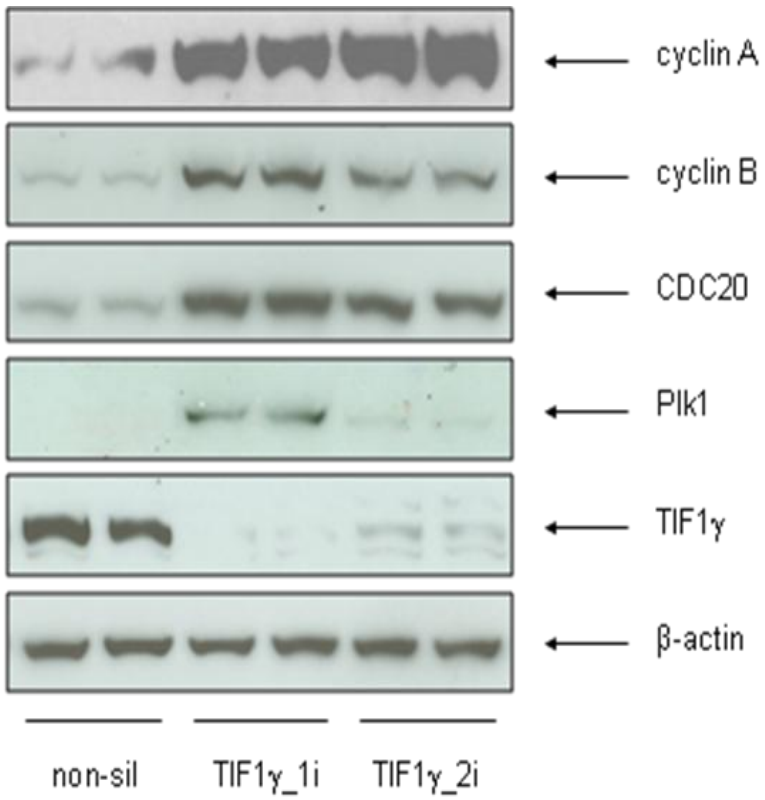


Fig 4.11. TIF1γ knockdown results in stabilisation of the APC/C's mitotic substrates

HeLa cells were treated with non-silencing siRNAs or two distinct TIF1γ siRNAs and harvested 96 hours post transfection. Whole cell extracts were then examined by Western blotting with the indicated antibodies. non-sil, non-silencing RNA oligonucleotides; TIF1γ_1i and 2i refer to instances where the expression of this protein has been ablated with two distinct siRNAs.

by SDS-PAGE and subjected to fluorography and autoradiography. Interestingly ablation of TIF1γ expression partially inhibited the ability of APC3 immunoprecipitates to polyubiquitylate cyclin A (Fig 4.12A) and cyclin B (Fig 4.12B), suggesting that TIF1γ regulates APC/C ligase activity.

4.2.10. TIF1γ is not associated with APC/C E3 ubiquitin ligase activity *in vitro*

Results above reveal that TIF1γ regulates APC/C ligase activity, therefore to confirm whether TIF1γ is directly associated with APC/C ligase activity, I performed immunoprecipitations from A549 cells using the TIF1γ98 polyclonal antibody, and an anti-APC3 antibody. Precipitated immune-complexes were then added to a ubiquitin ligase reaction mix as in section 4.2.9, again containing L-α-[^{35}S]-methionine-labelled cyclin A or cyclin B substrates. Samples were then separated by SDS-PAGE and subjected to fluorography and autoradiography. As expected, complexes that were immunoprecipitated with an anti-APC3 monoclonal antibody were capable of polyubiquitylating cyclin A (Fig 5.7A; page 161) and cyclin B (Fig 5.7B; page 161). In contrast immune complexes associated with TIF1γ were unable to catalyse mono or polyubiquitylation of cyclin A and cyclin B (Fig 5.7A and B; page 161) and hence suggests that TIF1γ might not be associated with APC/C ubiquitin ligase activity.

4.2.11. TIF1γ mediates passage through mitosis

Despite the inability of TIF1γ to precipitate APC/C ligase activity, the fact remains that TIF1γ is capable of interacting with the APC/C and mediating degradation of APC/C substrates *in vivo*, indicating that TIF1γ regulates mitotic progression. In order to test this assumption HeLa cells were harvested 72 hours after transfection with TIF1γ_1 and

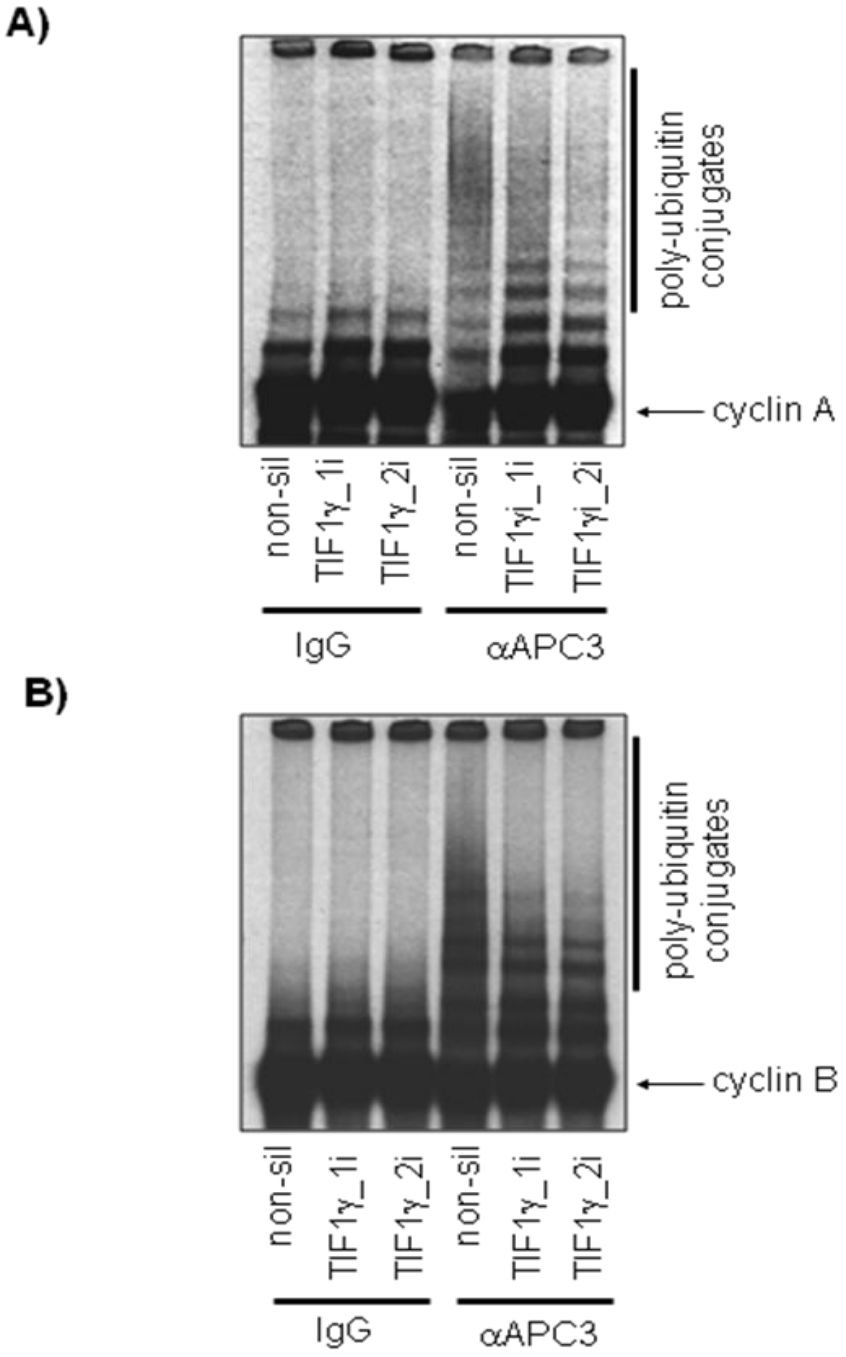


Fig 4.12A and B. TIF1γ enhances APC/C E3 ubiquitin ligase activity *in vitro*.

20µg of the indicated antibodies were incubated with 5mg of HeLa lysate, which had been pretreated with the indicated siRNAs. Bound proteins were then precipitated using protein G-Sepharose, washed, and incubated with with rabbit E1 and human E2 enzymes: UBCH10, UBCH4 and UBCH5, and 1µl of [35S]-labelled, *in vitro*-translated cyclin A (**A**) or cyclin B (**B**). The reaction mixture was then incubated with sample buffer and separated by SDS-PAGE. After fluorography the gel was dried and subjected to autoradiography. 2µl of radiolabelled inputs were loaded in the first lane of the gel. non-sil, non-silencing RNA oligonucleotides; TIF1γ_1i and 2i refer to instances where the expression of this protein has been ablated with two distinct siRNAs.

Significantly, treatment with both TIF1γ siRNAs increased the proportion of cells in mitosis when compared to the non-silencing control (Fig 4.13); TIF1γ_1 siRNA treatment caused the number of mitotic cells to increase by approximately 10 fold (Fig 4.13B); the TIF1γ_2 siRNA also enlarged the mitotic population by approximately 4 fold (Fig 4.13C). The mitotic index taken from three independent experiments is also displayed in the form of bar chart (Fig 4.13D). The extent of TIF1γ knockdown was assessed by Western blot (Fig 4.13E). In summary data in this section establishes that TIF1γ is required for mitotic progression *in vivo*.

4.2.12. TIF1γ is required for the metaphase-anaphase transition

Work above (section 4.2.11) demonstrates that TIF1γ is important for mitotic progression, therefore in order to elucidate the role of TIF1γ in mitotic progression I examined the mitotic distribution after TIF1γ knockdown. To do this I transfected HeLa cells with TIF1γ_1 and TIF1γ_2 siRNAs. Cells were then trypsinised and plated out onto poly-L-lysine treated 12 well slides 48 hours later. The cells were grown on slides for a further 24 hours and fixed in ice-cold methanol and stained with anti-α-tubulin and anti-phospho-histone H3 antibodies. The relative mitotic distribution was then quantified using the criteria set out in Fig 4.14. Interestingly, TIF1γ knockdown increased the number of metaphase-like cells, which are characterised by the presence of mitotic spindles and an inability to form a metaphase plate (Fig 4.15A), and were therefore counted as metaphase cells. After TIF1γ knockdown (Fig 4.15C) there was a large increase in the number of metaphase cells when compared to the non-silencing control, which was accompanied by a complementary reduction in anaphase and telophase cells (Fig. 4.15B). Taken together these results demonstrate that the increase

in the mitotic index of TIF1γ knockdown cells (section 4.2.11) can be attributed to an inability to progress into anaphase.

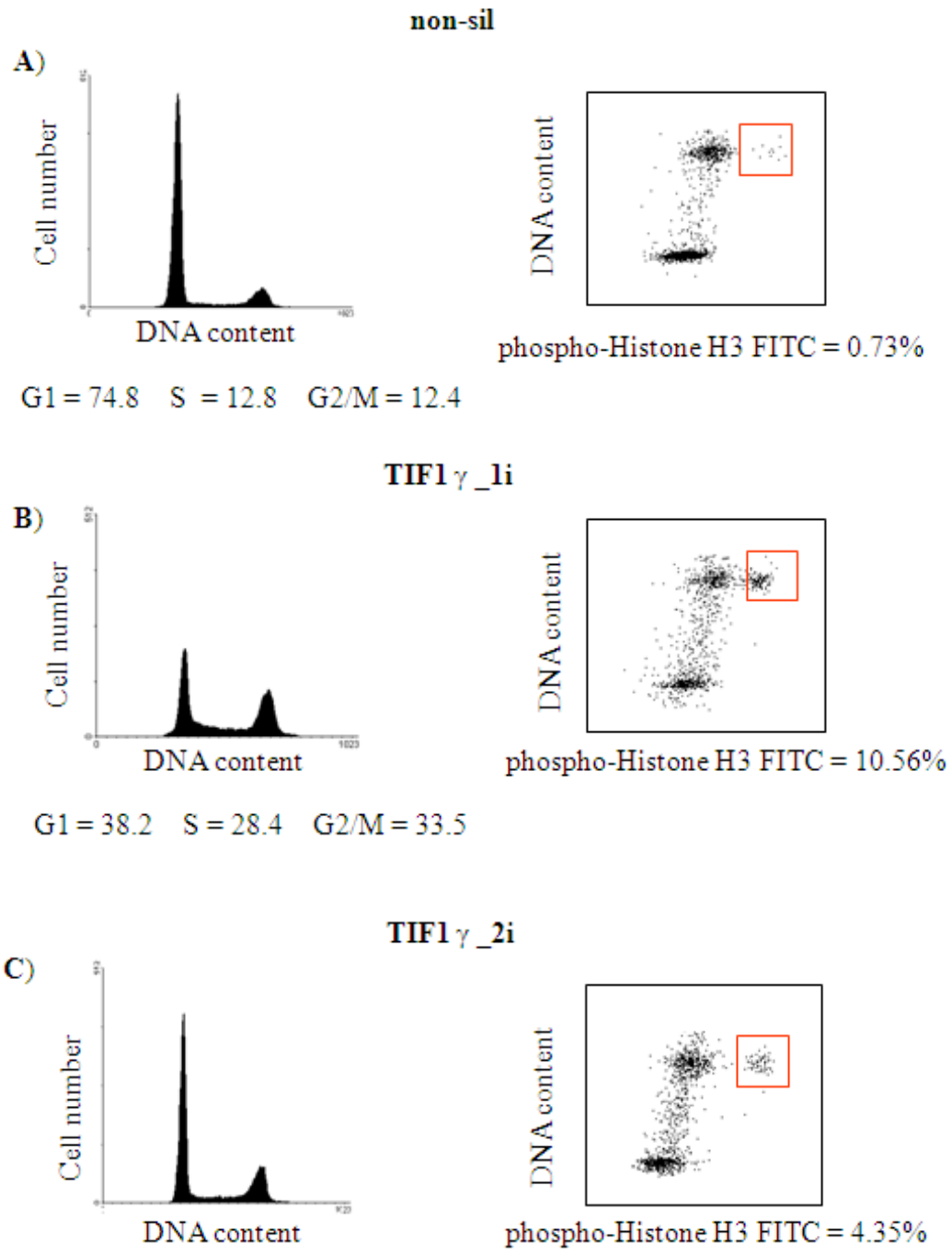
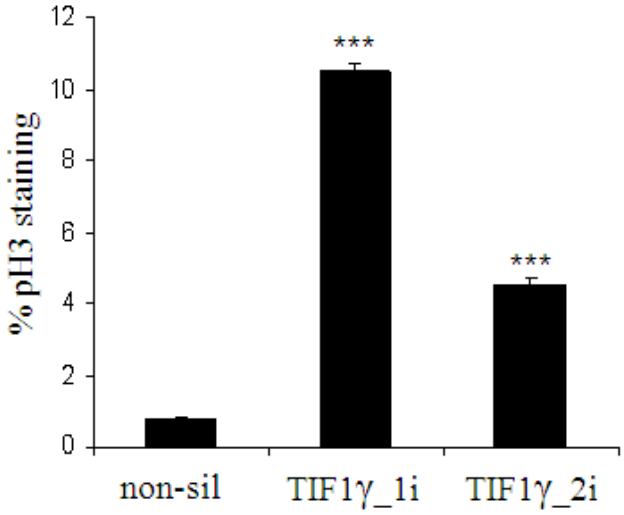


Fig 4.13 A-C. Cell cycle distribution and mitotic index of HeLas following TIF1γ RNAi treatment.

HeLa cells were harvested 72 hours after treatment with non-silencing (**A**) or TIF1 γ (**B+C**) siRNAs. Cells were then stained with propidium iodide (PI) and phospho-Histone H3 (pH3) antibody, and examined by flow cytometry. The cell cycle distribution is shown by histogram and also by dot plot; cells positive for pH3 staining are indicated by the presence of red box. non-sil, non-silencing RNA oligonucleotides; TIF1γ_1i and 2i refer to instances where the expression of this protein has been ablated with two distinct siRNAs

D)



E)

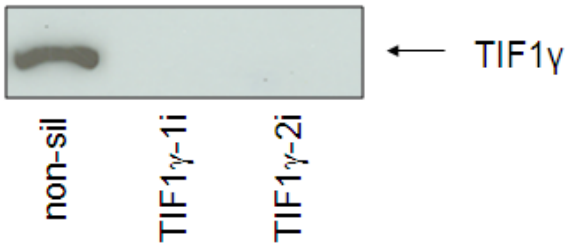


Fig 4.13 D and E. Cell cycle distribution and mitotic index of HeLas following TIF1γ RNAi treatment.

The proportion of cells positive for pH3 staining taken from three independent experiments is displayed for each siRNA treatment in a bar chart (**D**). The extent of TIF1γ knockdown was examined by Western blotting of whole cell extracts (**E**). non-sil, non-silencing RNA oligonucleotides; TIF1γ_1i and 2i refer to instances where the expression of this protein has been ablated with two distinct siRNAs. Error bars represent standard deviation. ***P<0.001.

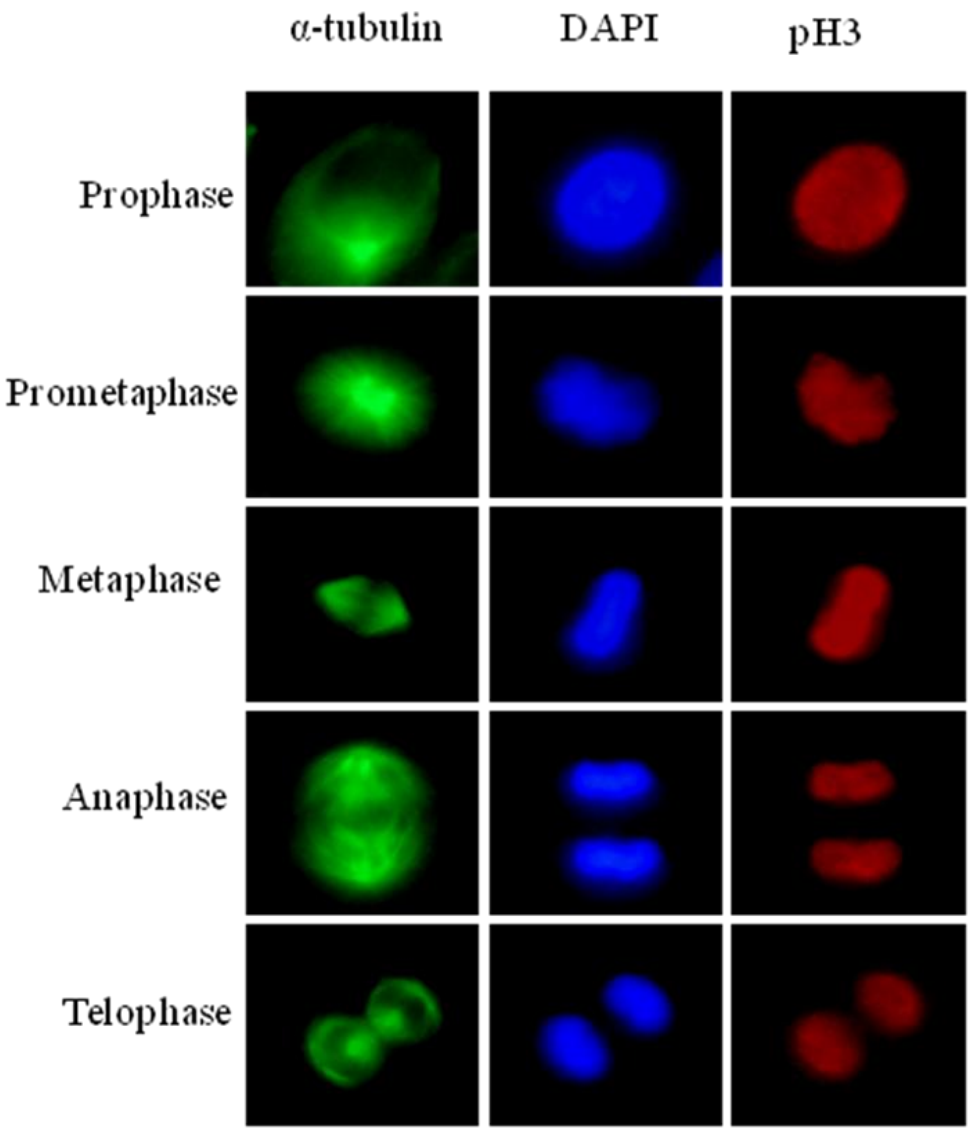


Fig 4.14. Visualisation of mitosis by immunofluorescence
HeLa cells were fixed in methanol and stained with anti-α-tubulin and anti-phospho-histone H3 antibodies in order to illustrate the criteria used to asses the mitotic distribution.

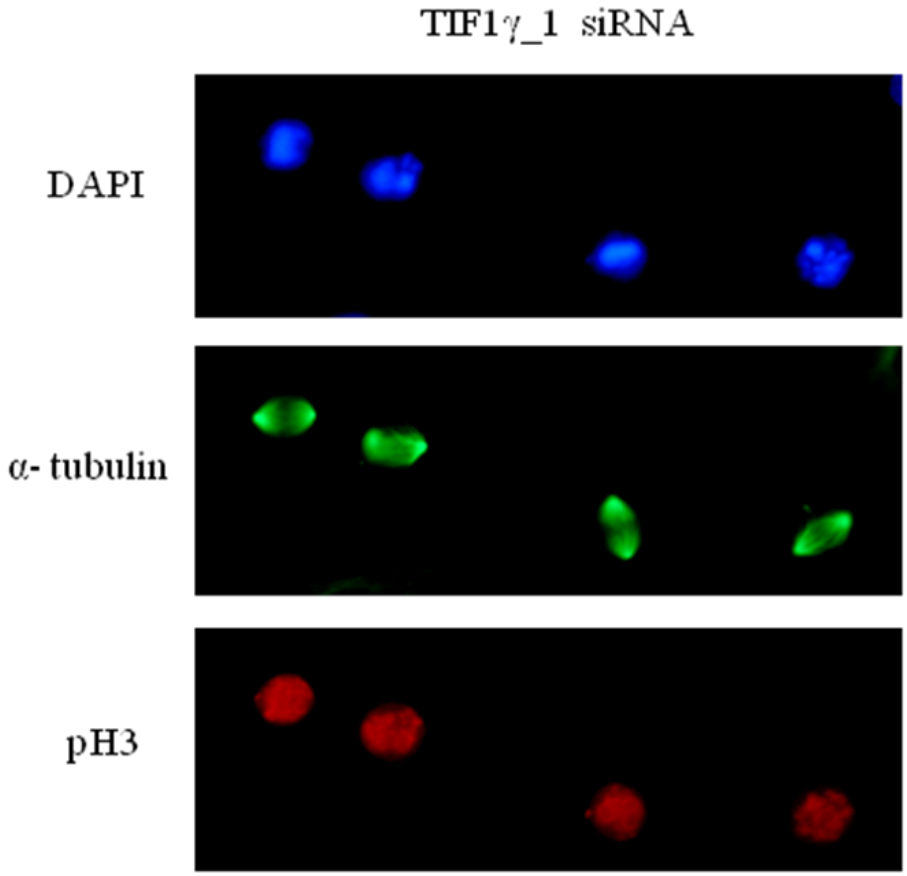
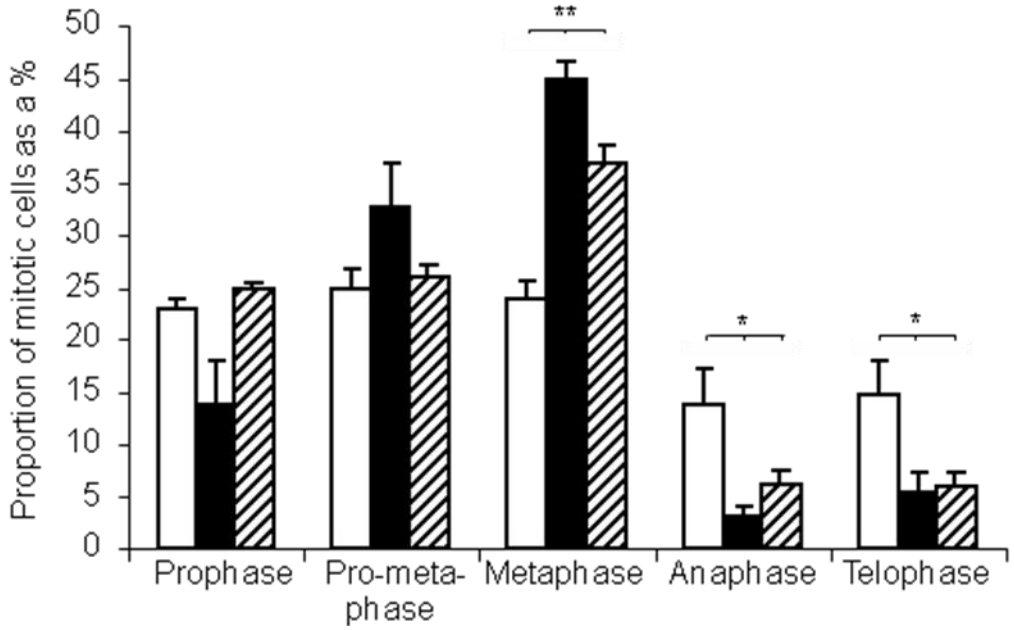


Fig 4.15A. TIF1γ knockdown causes a metaphase like arrest
HeLa cells were stained with anti-α-tubulin and anti-phospho-histone H3 (pH3) antibodies. Cells that had undergone treatment with TIF1γ_1 siRNA (TIF1γ_1i) displayed an increase in cells arrested in a metaphase like state, which is characterised by the presence of metaphase spindles and an inability to form a stable metaphase plate.

B)



C)

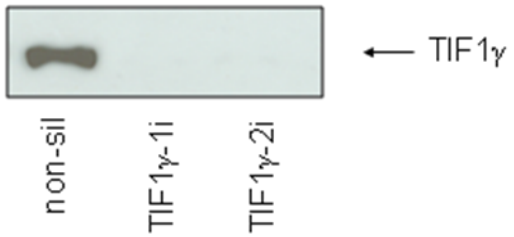


Fig. 4.15B and C. TIF1γ knockdown alters the mitotic distribution
(A) HeLa cells were stained with anti-α-tubulin and anti-phospho-histone H3 antibodies in order to determine their mitotic distribution following treatment with non-silencing siRNA (non-sil) or TIF1γ siRNA (TIF1γ-1i and 2i). (B) The levels of TIF1γ protein expression were assayed by Western blot following TIF1γ siRNA treatment. non-sil, non-silencing RNA oligonucleotides; TIF1γ-1i and 2i refers toTIF1γ siRNA treatment. The bar chart represents three independent experiments. Error bars denote standard deviation. *P<0.05; **P<0.01.

4.3. Discussion

Co-immunopricipitation experiments in this chapter demonstrate the existence of *in vivo* protein complexes comprising TIF1γ and APC/C subunits 1-8, which is consistent with mass spectrometry data from chapter 3 detailing interaction between APC7 and TIF1γ. In addition TIF1γ also interacts with the APC/C's mitotic coactivator CDC20 and substrate cyclin A, suggesting that TIF1γ is required for the activity of mitotic APC/C. Consistent with these observations I also demonstrate that TIF1γ regulates the polyubiquitylation and degradation of a number of the APC/C's mitotic substrates, and also regulates passage through mitosis by facilitating entry into anaphase.

The TIF1γ98 antibody that was used for TIF1γ immunoprecipitation and Western blotting was generated using GST–TIF1γ–F1. This decision was taken due to higher levels of expression in comparison to GST–TIF1γ–F2, which was predominantly composed of GST (Fig 4.2). The TIF1γ98 antibody is specific to TIF1γ, as it recognises a protein with the same nominal size of 180K as TIF1γ (Fig 4.3A) that is knocked down after treatment with two distinct TIF1γ siRNAs (Fig 4.3B). In addition, the TIF1γ98 antibody recognises a number of sub 180K bands (Fig 4.3A), all but one of which display reduced expression following TIF1γ knockdown (Fig4.3B), suggesting that they correspond to TIF1γ breakdown products or splice variants. It is likely that these sub 180K bands are products of alternative TIF1γ splicing, as expression of a FLAG-tagged 'intronless' cDNA in 293 cells gives rise to a similar pattern of bands (Fig 4.5), which might arise due to the presence of alternative start sites within the cDNA. The only protein band recognised by the TIF1γ98 antibody that doesn't display reduced intensity following treatment with TIF1γ siRNAs runs midway between the 66k and 77k molecular weight markers, and may therefore correspond to a TIF1γ splice variant that

doesn't contain complementary sequence to the siRNAs used. Alternatively, this band might correspond to a protein that is homologous to TIF1γ, such as another TRIM/RBCC family member, which all have a similar architecture within their TRIM/RBBC domains (Nisole, Stoye et al. 2005). In particular PML splice variants with a molecular weight of ~66Kda have been described (de The, Lavau et al. 1991; Fagioli, Alcalay et al. 1992). Although, I have been unable to successfully coimmunoprecipitate APC7 and PML from asynchronous cells (data not shown).

A number of observations from this chapter strongly imply that TIF1γ mediates mitotic progression by regulating the function of the APC/C. For instance, TIF1γ interacts with the APC/C's early mitotic coactivator CDC20 and early mitotic substrate cyclin A *in vivo* (Fig 4.9B and C). In addition, TIF1γ interacts with a higher molecular weight form of APC7 *in vivo* (Fig 4.6B), which may correspond to a phosphorylated form of APC7 that has previously been identified during mitosis (Kraft, Herzog et al. 2003). TIF1γ fails to immunoprecipitate cyclin B (Fig 4.9A) suggesting that any potential interaction between these two proteins is extremely transient, or that only a very small percentage of the total cellular pool of these proteins is bound together. Alternatively, the inability of TIF1γ to interact with cyclin B suggests that TIF1γ doesn't regulate the ability of the APC/C to target cyclin B for degradation. If this is the case, TIF1γ may only be associated with an APC/C sub-pool that mediates degradation of cyclin A, and possibly other early mitotic substrates such as NEK2A and HOXC10, which unlike cyclin B are not stabilised during early mitosis as a result of SAC activation (den Elzen and Pines 2001; Geley, Kramer et al. 2001; Gabellini, Colaluca et al. 2003; Hayes, Kimata et al. 2006).

Further evidence to support a role for TIF1γ as a mitotic APC/C regulatory protein is provided by the ability of TIF1γ to enhance polyubiquitylation of cyclins A and B *in*

vitro (Fig 4.12) and mediate degradation of the APC/C substrates cyclin A, cyclin B, CDC20 and Plk1 *in vivo* (Fig 4.11). In addition to, and consistent with, the ability of TIF1γ to associate with the APC/C and regulate the stability of APC/C mitotic substrates, it appears that TIF1γ regulates transition through mitosis, as ablation of TIF1γ expression with two distinct siRNAs (Fig 4.13E) causes an increase in the mitotic index when compared to a non-silencing control (Fig 4.13A-C).

Given that TIF1γ knockdown prevents degradation of cyclin A and cyclin B (Fig 4.11), and that TIF1γ only binds to cyclin A (Fig 4.9B) it seems likely that TIF1γ knockdown might stabilise cyclin B indirectly, and possibly also CDC20 and PLK1, by directly regulating cyclin A degradation. Significantly over-expression of *wt* cyclin A has previously been shown to delay chromosome alignment while TIF1γ knockdown increases the number of cells arrested in metaphase with unaligned chromosomes (Fig 4.15A and B) (den Elzen and Pines 2001), further suggesting that TIF1γ regulates cyclin A degradation. Chromosome alignment is a prerequisite for SAC inactivation and subsequent degradation of cyclin B (Geley, Kramer et al. 2001), and would therefore explain the ability of TIF1γ knockdown to stabilise cyclin B indirectly. A delay in the metaphase-anaphase transition due to cyclin B stabilisation would also prevent the degradation of CDC20 and PLK1, which occurs during anaphase (Acquaviva and Pines 2006), again demonstrating that TIF1γ knockdown could indirectly stabilise mitotic APC/C substrates purely as a result of a failure to degrade cyclin A.

Despite the fact that TIF1γ enhances the ability of the APC/C to polyubiquitylate its substrates *in vitro* (Fig 4.12) and regulates APC/C substrate stability *in vivo* (Fig 4.11), the TIF1γ98 antibody failed to immunoprecipitate APC/C ligase activity (Fig 5.7A and B). Notably, work from our laboratory demonstrates that a number of antibodies against various APC/C subunits fail to precipitate APC/C ligase activity (A Turnell, personal

communication), suggesting that the inability of the TIF1γ98 antibody to precipitate APC/C ligase activity could merely be due to the antibody being unsuitable for this application. Given that TIF1γ enhances APC/C ligase activity (Fig 4.12) it seems likely that the ability of TIF1γ to function as a RING dependent E3 ubiquitin ligase may be important for APC/C ligase activity (Dupont, Zacchigna et al. 2005). Indeed TIF1γ can modify SMAD4 activity through monoubiquitylation (Dupont, Mamidi et al. 2009), indicating that TIF1γ might also regulate APC/C ligase activity by monoubiquitylating APC/C subunits. Alternatively the RING domain of TIF1γ may directly enhance the polyubiquitylation of APC/C substrates.

Following CDH1 knockdown TIF1γ expression is reduced (Fig 4.8), indicating that the APC/C might indirectly regulate TIF1γ stability by controlling the accumulation of a TIF1γ E3 ubiquitin ligase. However CDH1 knockdown has previously been demonstrated to result in premature entry into S-phase due to cyclin A and SKP2 accumulation (Sudo, Ota et al. 2001; Hsu, Reimann et al. 2002; Bashir, Dorrello et al. 2004; Wei, Ayad et al. 2004; Havens, Ho et al. 2006). Therefore the observed reduction in TIF1γ expression could be due to the possibility that TIF1γ levels are reduced in S phase.

A recent report demonstrates that APC1 is targeted to PML bodies after expression of apoptin (Heilman, Teodoro et al. 2006), suggesting that the APC/C may be recruited to PML bodies in the absence of this viral protein. The observation that the TRIM/RBCC motif of TIF1α in isolation and as part of the intact protein, is required for recruitment into PML associated tracks that form after adenovirus infection (Yondola and Hearing 2007), coupled to the ability of TIF1α to hetero-oligomerise with PML (Zhong, Delva et al. 1999), indicates that TIF1α may be a component of PML-NBs in the absence of viral infection. This also suggests that other TIF1 family members could also be components

of PML-NBs, given that the TRIM/RBCC motif within members of the TIF1 family display up to 70% identity (Venturini, You et al. 1999).

If TIF1γ is a component of PML-NBs it may be important for recruitment of APC/C components into these structures, indeed, unpublished observations from our lab reveal that APC/C components are present in PML bodies in the absence of viral infection. In addition it has been suggested that SUMOylation actually occurs in PML-NBs, therefore APC/C components may undergo SUMOylation in these structures. If this were the case, TIF1γ may have the potential to function as an E3 SUMO ligase for the APC/C, which is suggested by the presence of a RING finger domain and a PHD domain in TIF1γ, both of which been have been proposed as SUMO E3 ligases (Capili, Schultz et al. 2001; Quimby, Yong-Gonzalez et al. 2006; Mascle, Germain-Desprez et al. 2007).The possibility that TIF1γ functions as a SUMO ligase suggests that TIF1γ may regulate transition through mitosis by via SUMOylation of APC/C subunits. This is given credence by the requirement for the SUMO E1 UBC9 in yeast, for degradation of APC/C mitotic substrates (Dieckhoff, Bolte et al. 2004).

Given the ability of the TIF1 family proteins (Le Douarin, Nielsen et al. 1996; Moosmann, Georgiev et al. 1996; vom Baur, Zechel et al. 1996; Venturini, You et al. 1999; Beckstead, Ortiz et al. 2001) and the APC/C to function in transcription (Turnell, Stewart et al. 2005), the interaction between the APC/C and TIF1γ (Fig 4.6A and B) might be also be important for regulation of transcription. TIF1γ may function as a cofactor in APC/C dependent transcription, recruiting repressive complexes via its TSS motif. Interestingly TIF1γ contains two putative LXCXE RB binding motifs (Fig 4.1B), and as such may facilitate binding of the APC/C to RB (Binne, Classon et al. 2007). Alternatively the presence of a bromodomain in TIF1γ and the inferred capacity for

chromatin binding, might also allow recruitment of APC/C complexes and its associated transcription factors such as CBP/p300 to DNA (Turnell, Stewart et al. 2005).

CHAPTER 5

THE APC/C COOPERATES WITH NF90/NF45 TO REGULATE CYTOKINE TRANSCRIPTION

5.1. Introduction

NF90 and its binding partner NF45 facilitate gene expression by regulating transcription (Flanagan, Corthesy et al. 1991; Corthesy and Kao 1994; Kao, Chen et al. 1994; Aoki, Zhao et al. 1998; Reichman, Parrott et al. 2003; Ranpura, Deshmukh et al. 2007; Shi, Godfrey et al. 2007; Shi, Qiu et al. 2007), mRNA processing and translation(Ting, Kao et al. 1998; Xu and Grabowski 1999; Saunders, Perkins et al. 2001; Shim, Lim et al. 2002; Zhou, Licklider et al. 2002; Leung, Andersen et al. 2003; Shi, Zhao et al. 2005). The majority of study directed towards NF90 and NF45 has been concerned with their ability to activate IL-2 transcription from the IL-2 promoter ARRE-2 (Corthesy and Kao 1994; Kao, Chen et al. 1994; Aoki, Zhao et al. 1998; Shi, Godfrey et al. 2007; Shi, Qiu et al. 2007). NF90 and NF45 are thought to be components of the ARRE-2 transcriptional regulatory complex, which functions as a constitutive repressor. The ARRE-2 regulatory complex also undergoes conversion into an IL-2 transcriptional activator following events such as PMA and ionomycin stimulation or TCR ligation, which stimulate the nuclear translocation and subsequent recruitment of NFAT1 to the ARRE-2 transcriptional regulatory complex (Shaw, Utz et al. 1988; Randak, Brabletz et al. 1990). Notably, NFAT1 has also been implicated in transcription of a number of other cytokine promoters such as TNF and GMCSF (Cockerill, Shannon et al. 1993; Oum, Han et al. 2002), suggesting that NF90/NF45 might also regulate transcription of these genes.

In chapter three I presented mass spectrometric evidence of an interaction between APC7 and a number of other proteins including the proposed ARRE-2 transcriptional regulatory complex component NF45. However, mass spectrometric analysis is only one method of identifying protein-protein interactions, therefore in this chapter my objective was to confirm the validity of the interaction between NF45 and APC7, and

also NF45's binding partner NF90, I choose to focus on the interaction between NF45 and APC7 due to NF45's well defined role in transcription, which might relate to APC7's ability to regulate transcription (Turnell, Stewart et al. 2005). I was also fortunate to obtain some excellent reagents that were sourced from collaboration with Professor Peter Kao (Stanford). Details of my findings are presented in this chapter.

5.2. Results

5.2.1. Binding of the APC/C to NF45 and NF90 *in vivo*

To confirm the interaction between APC7 and NF45, which was identified by mass spectrometry in chapter 3 (Fig 3.7), I performed coimmunoprecipitation and Western blotting analysis from A549 cells using an antibody that specifically recognises NF45. Also, given that NF45 is generally present with its binding partner NF90, I decided to perform a coimmunoprecipitation using an antibody that specifically recognises NF90. Precipitated proteins were then eluted, separated by SDS-PAGE and subsequently Western blotted for APC5 (Fig 5.1A) and APC7 (Fig 5.1B). As anticipated APC5 forms complexes with NF90 (Fig 5.1A), while APC7 associates with NF45 and NF90 (Fig 5.1B).

5.2.2. Colocalisation of NF90 and APC3 by microscopy

In order to determine the particular sub-cellular locations of NF90 and the APC/C, I performed immunofluorescence microscopy on interphase HeLa cells using anti-NF90 antibody and the anti-APC3 antibody AF3.1 (Fig 5.2). The results revealed that APC3 and NF90 partially colocalise in the nucleus, and that these complexes are particularly prevalent at the periphery of the nucleus. Furthermore APC3 and NF90 complexes were also identified in the cytoplasm (Fig 5.2).

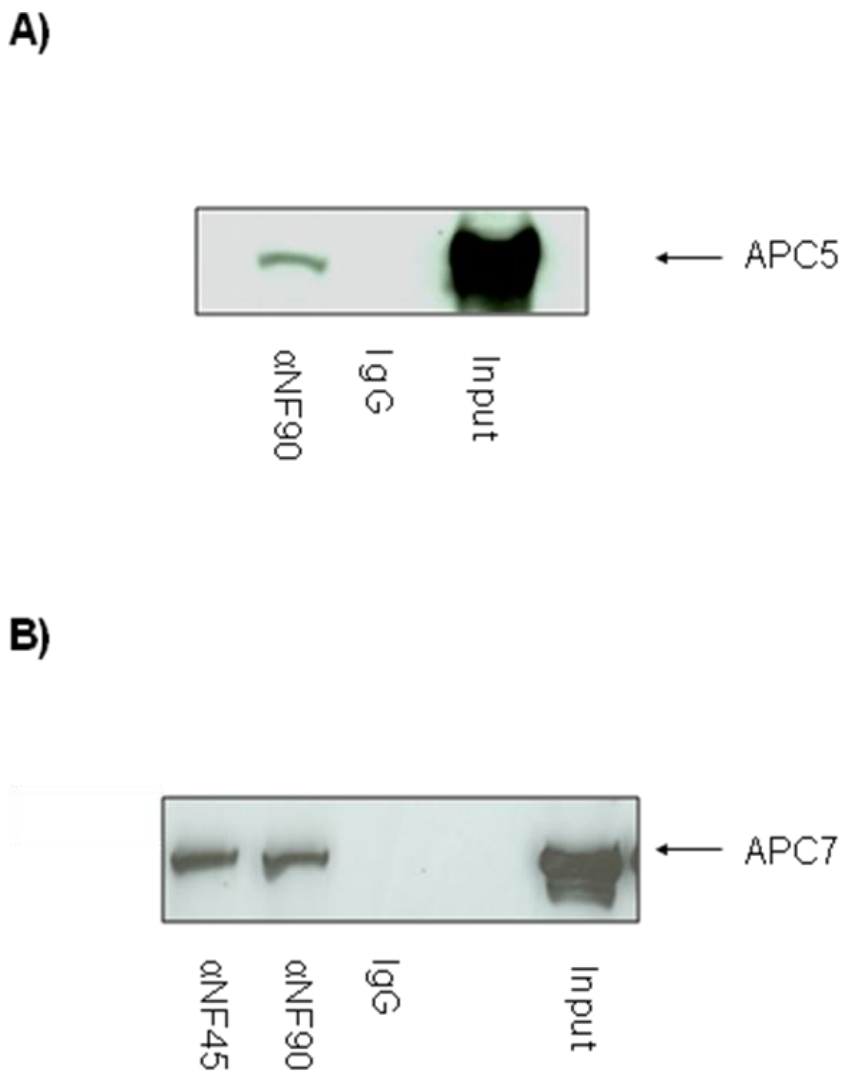


Fig 5.1A and B. *In vivo* binding of NF45 and NF90 to APC/C components.

NF45 and NF90 were immunoprecipitated from 5mg of lysate from A549 cells using anti-NF45 and anti-NF90 antibodies. After precipitation with Protein G-Sepharose, immuno-complexes were washed extensively, incubated with sample buffer and resolved by SDS-PAGE. Specific binding was analyzed by Western blotting using anti-APC5 (A) or anti-APC7 antibody (B).

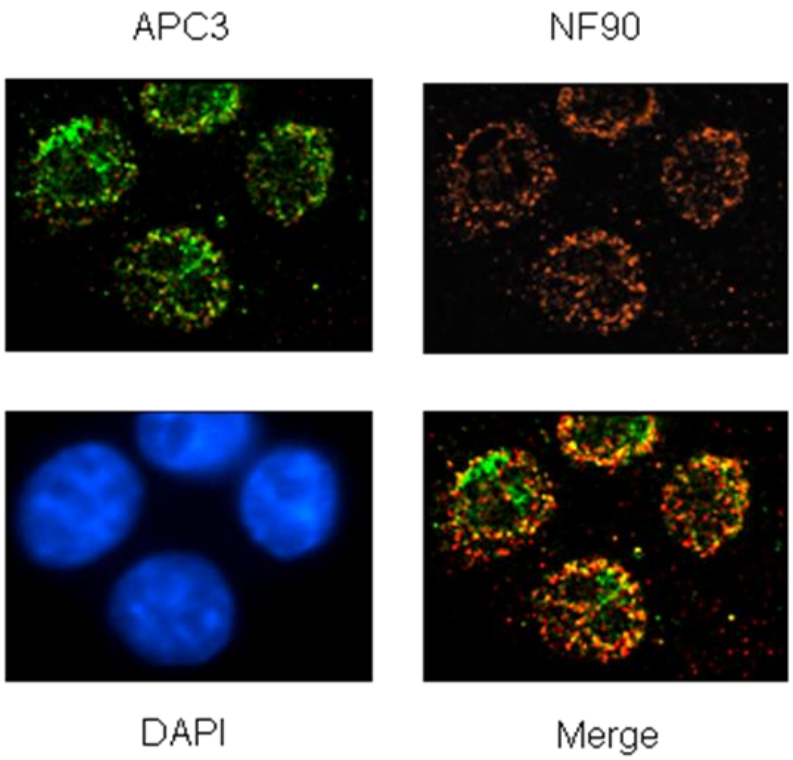


Fig 5.2. APC3 and NF90 colocalise in the nucleus and the cytoplasm
Hela cells were fixed in paraformaldehyde and extracted with acetone. T
cells were then incubated with antibodies against the indicated protei
Protein staining was visualised using a deconvaluting microscope.

5.2.3. APC5 and APC7 bind directly to NF90 *in vitro*

As co-IP studies demonstrated the existence of NF90-APC/C complexes *in vivo,* I resolved to establish whether these interactions occur directly, by performing GST-pulldowns. I therefore subcloned full-length NF90 and NF45 from pcDNA 3.1 into pGEX 4T-1. After validation of DNA sequences, BL21 RIL *E. coli* were transformed with the appropriate pGEX constructs and the fusion proteins were expressed and purified. In order to determine the binding capacity of GST-NF45 and GST-NF90 for APC5 and APC7, L-α-[^{35}S]-methionine-labelled APC5 and APC7 were incubated with GST-NF45 and GST-NF90. After GST pulldown with glutathione-agarose, binding proteins were specifically eluted with glutathione and resolved by SDS-PAGE. Following fluorography and autoradiography it was seen that NF90 binds directly to APC5 (Fig 5.3A) and to APC7 *in vitro* (Fig 5.3B). In contrast NF45 demonstrated a very weak affinity for APC5 (Fig 5.3A). These findings suggest that APC5 and APC7 bind to NF90 preferentially.

5.2.4. Generation of GST-NF90 fusion fragments, and determination of APC/C binding sites on NF90 *in vitro*

In order to delineate the regions of NF90 that are responsible for binding to APC5 and APC7, I generated GST-NF90 fusion fragments (Fig 5.4Aii), which were cloned from full length NF90 cDNA and inserted into pGEX 4T-1. Sequences were validated and the GST-NF90 fragments were expressed in BL21 RIL *E. coli*. GST-pulldown, revealed that the central (fragment 2) and C-terminal (fragment 3) fragments of NF90, which both contain double stranded RNA binding motifs (dsRBMs) interact with APC5 (Fig 5.4B) and APC7 (Fig 5.4C), whereas the N-terminal fragment of NF90 (fragment1) fails to bind to these APC/C components (Fig 5.4B and C). Given that APC5 and APC7 bind

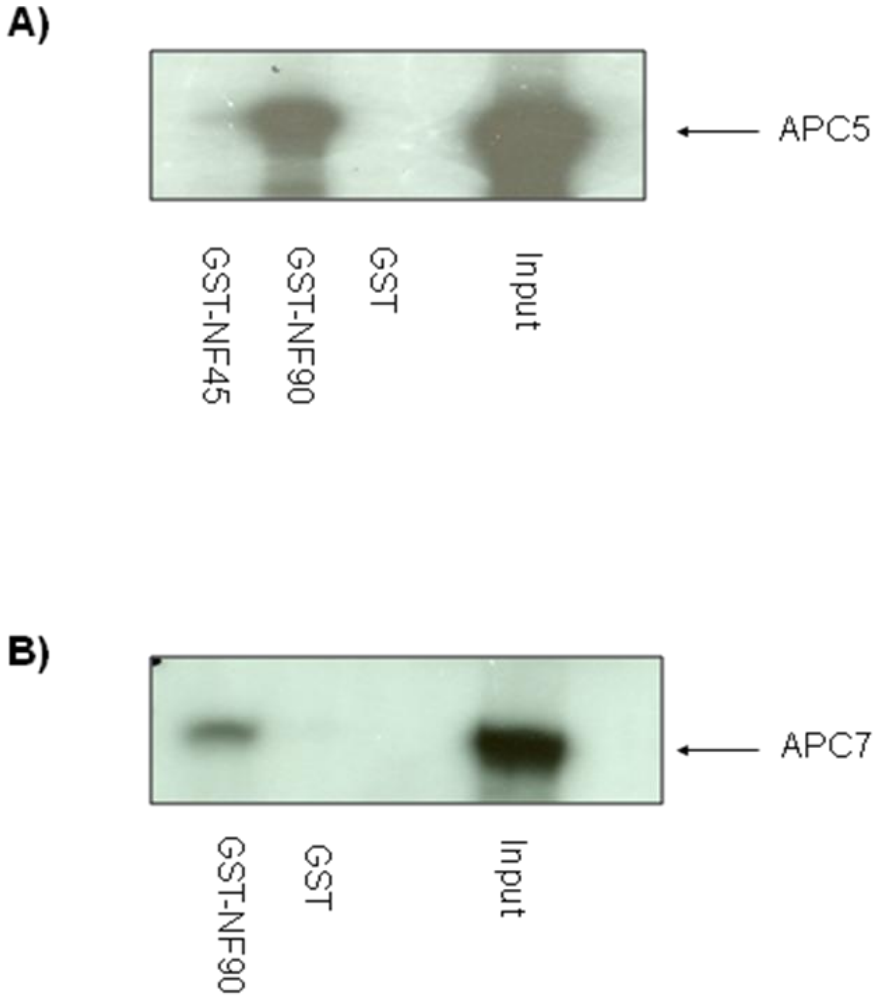


Fig 5.3A and B. *In vitro* binding of NF45 and NF90 to the APC/C.

20µg of indicated GST fusion proteins were incubated with 20µl of [^{35}S] labelled, *in vitro*-translated APC5 (A) or APC7 (B). Bound proteins were the precipitated using glutathione-Sepharose, washed and selectively eluted wit glutathione. The eluted proteins were the resolved by SDS-PAGE and the g subjected to fluorography. After fluorography the gel was dried and subjecte to autoradiography. 10% of radiolabelled inputs were loaded in the last lane the gel to asses binding capacity.

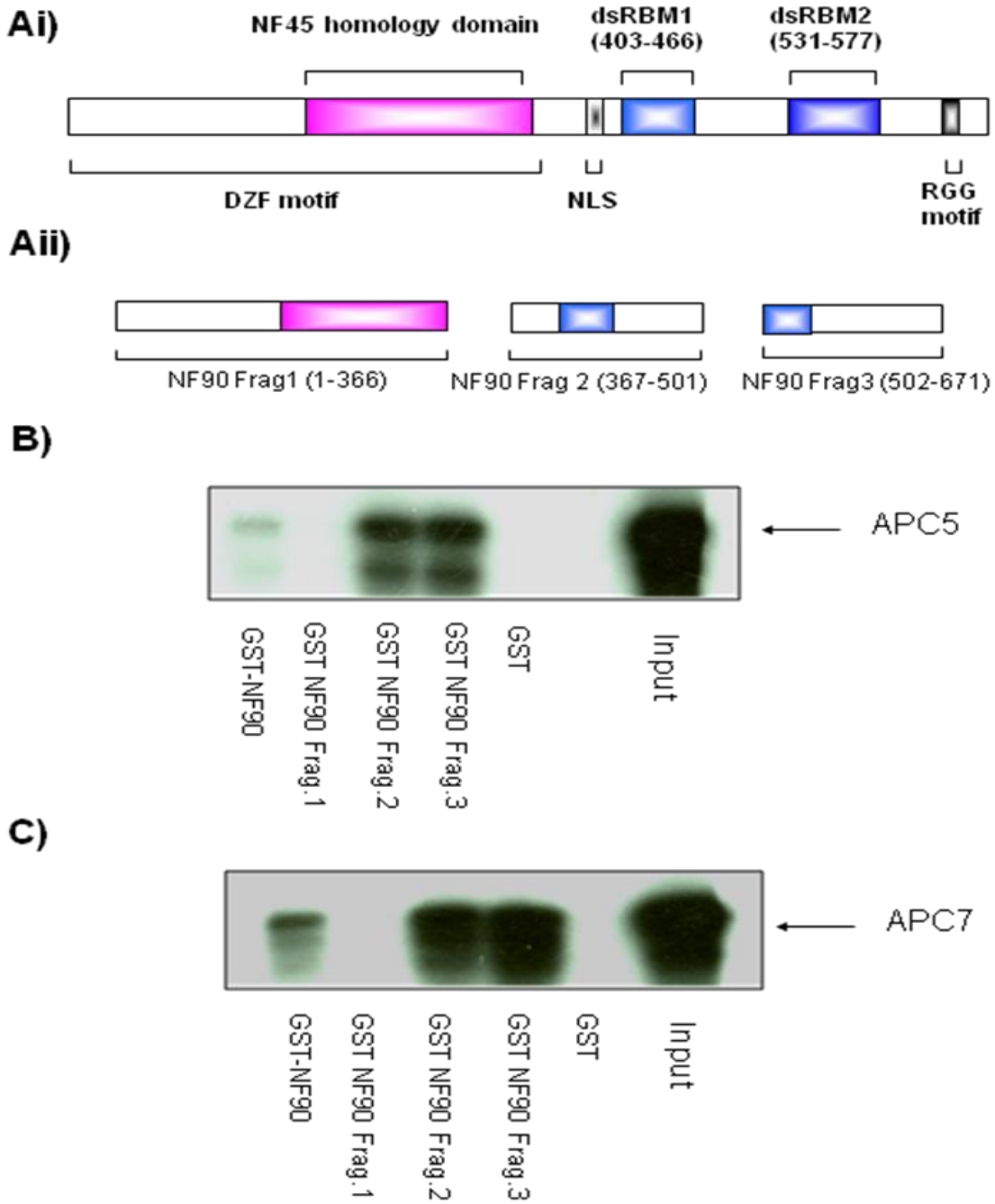


Fig 5.4A-C. *In vitro* binding of full length NF90 and NF90 fragments to the APC/C. (**Ai**) A schematic representation of the full length NF90 protein. (**Aii**) A schematic representation of GST-NF90 fragments. 20µg of indicated GST fusion proteins were incubated with 20µl of [^{35}S]-labelled *in vitro*-translated APC5 (**B**) or APC7 (**C**). Bound proteins were then precipitated using glutathione-Sepharose, washed and selectively eluted with glutathione. The eluted proteins were then resolved by SDS-PAGE and the gel was subjected to fluorography. After fluorography the gel was dried and subjected to autoradiography. 10% of radiolabelled inputs were loaded in the last lane of the gel to assess binding capacity.

to regions of NF90 that both contain dsRBMs it seemed likely that the interaction was dependent upon the dsRBMs. Therefore, in order to test this possibility I generated GST-dsRBM1 and GST-dsRBM2 fusions that exclusively expressed the dsRBMs of NF90 (Fig 5.5A). Following GST pulldown using the GST-dsRBM1 and GST-dsRBM2 of NF90 it was determined that APC5 (Fig 5.5B) and APC7 (Fig 5.5C) bind directly to NF90's dsRBM1 and dsRBM2. This observation raised the possibility that the interaction between APC/C and NF90 may be regulated by dsRNA.

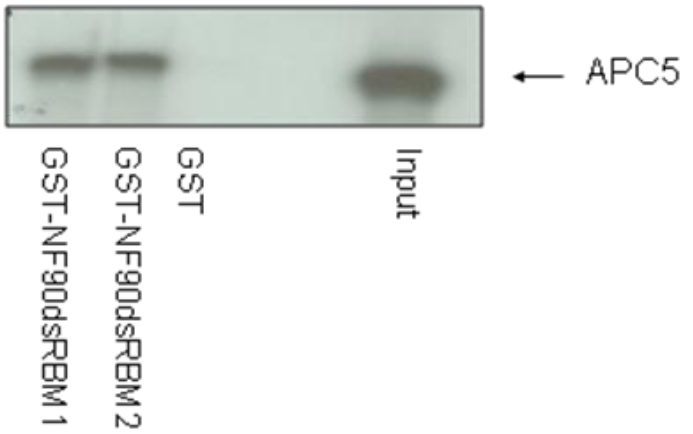
5.2.5. The *in vitro* interaction between NF90 and the APC/C is mediated by a double dsRNA intermediate

To assess whether dsRNA regulates the interaction between the dsRBMs of NF90 and APC/C components, L-α-[^{35}S]-methionine-labelled APC5 and APC7 were incubated with GST-dsRBM1 and GST-dsRBM2. After pulldown with glutathione-Sepharose beads, bound proteins were then incubated with RNase buffer alone or with RNase V1, which specifically degrades dsRNA. Any unbound proteins were then removed by extensive washing. The remaining proteins were specifically eluted with glutathione and separated by SDS-PAGE. In order to establish whether proteins had been loaded in equal amounts the gel was subjected to Coomassie blue staining (Fig 5.6A). Following fluorography and autoradiography it was seen that degradation of dsRNA ablates the interaction between the dsRBMs of NF90 and APC7 (Fig 5.6B), therefore suggesting that the interaction is bridged by a dsRNA intermediate.

A)

```
dsRBM1: 403-amnalmrlnq lkpglqyklv sqtgpvhapi ftmsvevdgn
            sfeasgpskk taklhvavkv lqdm-466
dsRBM2: 531-knpvmelnek rrglkyelis etggshdkrf vmevevdgqk
            fqgagsnkkv akayaalaal ek-592
```

B)



C)

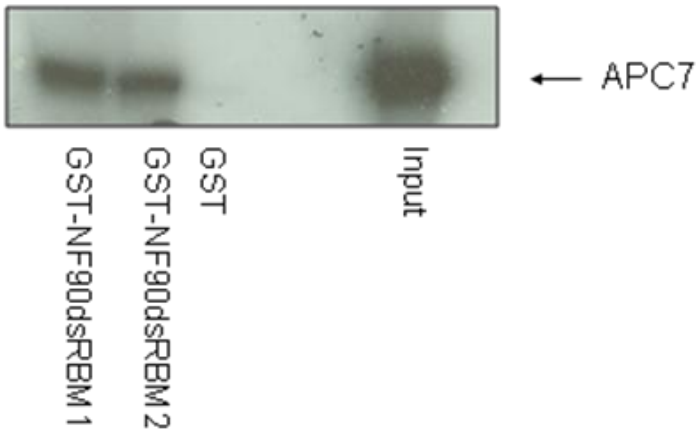


Fig 5.5A-C. *In vitro* binding of NF90-dRBM1 and NF90-dsRBM2 to APC5 and APC7

(A) The amino acid sequence of NF90 dsRBM1 and dsRBM2. 20µg of indicated GST-fusion proteins were incubated with 20µl of [35S]-labelled, *in vitro*-translated APC5 (B) or APC7 (C). Bound proteins were then precipitated using glutathione-sepharose, washed and selectively eluted with glutathione. The eluted proteins were the resolved by SDS-PAGE and the gel was subjected to fluorography. After fluorography the gel was dried and subjected to autoradiography. 10% of radiolabelled inputs were loaded in the last lane of the gel to asses binding capacity.

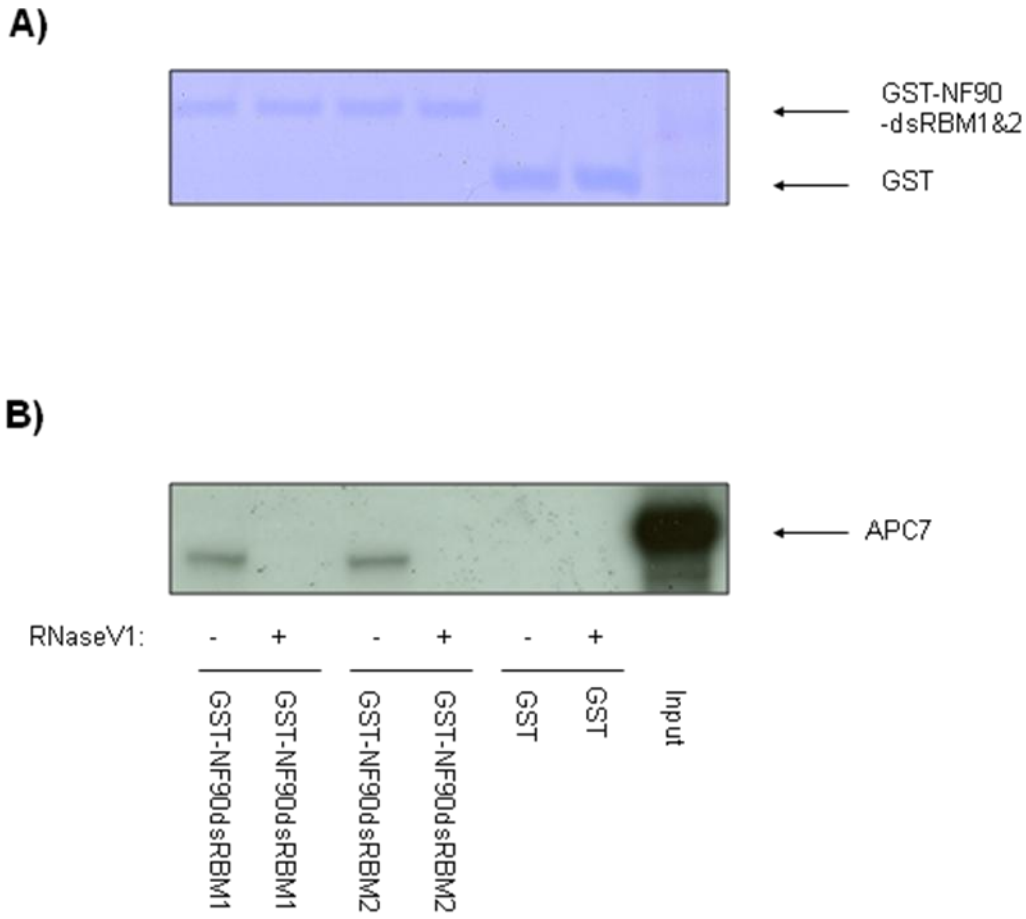


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Fig 5.6A and B. *In vitro* binding of NF90-dsRBM1 and NF90-dsRBM2 to APC7 before and after treatment with RNase V1.

20µg of indicated GST-fusion proteins were incubated with 20µl of [^{35}S]-labelled APC7. Bound proteins were then precipitated using glutathione-Sepharose, washed, selectively treated with RNaseV1 and eluted with glutathione. The eluted proteins were the resolved by SDS-PAGE and the gel was subjected to Coomassie R250 blue staining (A) to ensure equal protein loading. After fluorography the gel was dried and subjected to autoradiography (B). 10% of radiolabelled inputs were loaded in the last lane of the gel to asses binding capacity.

5.2.6. TIF1γ, NF45 and NF90 are not associated with APC/C E3 ubiquitin ligase activity

The ability of TIF1γ, NF45 and NF90 to interact with components of the APC/C *in vivo*, suggests that these proteins may be associated with APC/C E3 ubiquitin ligase activity. In order to investigate this possibility, I took advantage of the observation that immunoprecipitated APC/C complexes are capable of poly-ubiquitylating cyclin A and cyclin B in the presence of E1 and E2 enzymes *in vitro* (King, Peters et al. 1995; Geley, Kramer et al. 2001). Initially, I performed immunoprecipitations from A549 cells using anti-NF45, anti-NF90 and TIF1γ98 polyclonal antibodies, and an anti-APC3 antibody. Precipitated immune-complexes were then added to ubiquitin ligase reaction mix containing rabbit E1, human E2 enzymes (UBCH10, UBCH5 and UBCH4), and L-α-[^{35}S]-methionine-labelled cyclin A or cyclin B substrates. Samples were then separated by SDS-PAGE and subjected to fluorography and autoradiography. As expected, complexes that were immunoprecipitated with an anti-APC3 monoclonal antibody were capable of polyubiquitylating cyclin A (Fig 5.7A) and cyclin B (Fig 5.7B). In contrast immune complexes associated with NF45, NF90 or TIF1γ were unable to catalyse polyubiquitylation of cyclin A and cyclin B (Fig 5.7A and B), indicating that these specific antibodies do not co-IP active APC/C ubiquitin ligase activity.

5.2.7. Determination of NF45 and NF90 as substrates for APC/C mediated ubiquitylation

Analysis of the amino acid sequence of NF45 and NF90 resulted in the identification of putative D-boxes (RXXL) suggesting that NF45 and NF90 might be APC/C substrates. In order to authenticate these D-boxes I initially cloned NF45 and NF90 into pcDNA

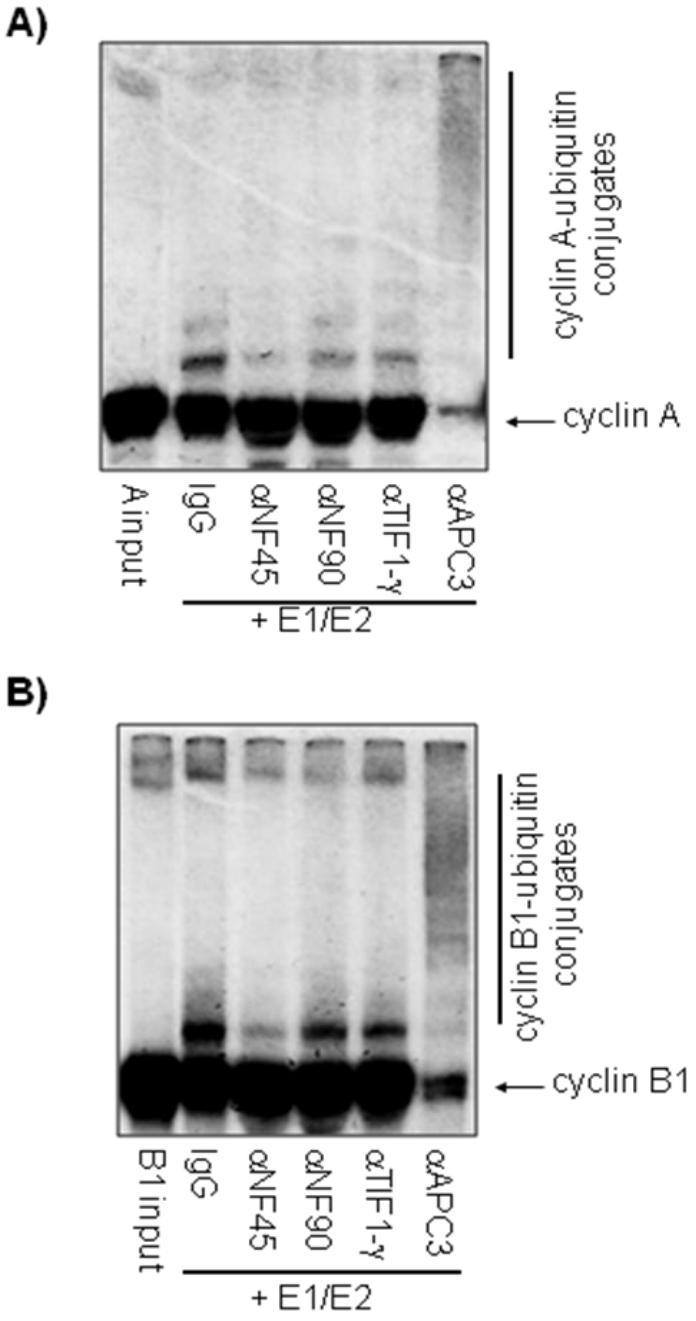

Fig 5.7A and B. NF45, NF90 and TIF1γ do not precipitate APC/C ubiquitin ligase activity

20µg of the indicated antibodies were incubated with 5mg of A549 lysate. Bound proteins were then precipitated using protein G-Sepharose, washed, and incubated with with rabbit E1 and human E2 enzymes: UbcH10, UbcH4 and UbcH5, and 1µl of [^{35}S]-labelled, *in vitro*-translated cyclin A (**A**) or cyclin B1 (**B**). The reaction mixture was then incubated with sample buffer and separated by SDS-PAGE. After fluorography the gel was dried and subjected to autoradiography. 2µl of radiolabelled inputs were loaded in the first lane of the gel.

3.1 using wild type cDNA. After validating DNA sequences, proteins were expressed using wheat germ extract. Active APC/C complexes were then immunoprecipitated with an anti-APC3 antibody, and added to a ubiquitin ligase reaction mix containing L-α-[^{35}S]-methionine-labelled cyclin A, cyclin B, NF45 or NF90. As anticipated APC3 and its associated APC/C holoenzyme was able to polyubiquitylate cyclin A and cyclin B (Fig 5.8A), therefore demonstrating the efficacy of this assay. However I was unable to detect APC/C mediated mono or poly-ubiquitylation of NF45 and NF90 (Fig 5.8B) following incubation with active APC/C ligase complexes, therefore demonstrating that these proteins are not targets for APC/C mediated ubiquitylation.

5.2.8. Exogenous NF90 and NF45 cooperate with exogenous APC/C components to potentiate transcription from the IL-2 promoter

It has previously been determined that NF90/NF45 regulates transcription from the IL-2 promoter (Corthesy and Kao 1994). In order to investigate whether APC/C components can cooperate with NF90/NF45 to regulate the IL-2 promoter, I transfected HCT116 cells with NF90/NF45 and either APC5 or APC7 in conjunction with a plasmid containing the IL-2 promoter upstream of a luciferase reporter gene. The activity of the resulting luciferase reporter protein, and hence transcription was measured 24 hours later (Fig 5.9). As expected NF90/NF45 expression stimulated luciferase activity approximately two fold. Expression of APC5 with NF90/NF45 further enhanced IL-2 reporter gene expression by approximately 90%, while expression of APC7 with NF90/NF45 increased luciferase expression by approximately 50%. Significantly, expression of APC5 or APC7 alone had little or no effect on luciferase activity (Fig 5.9).

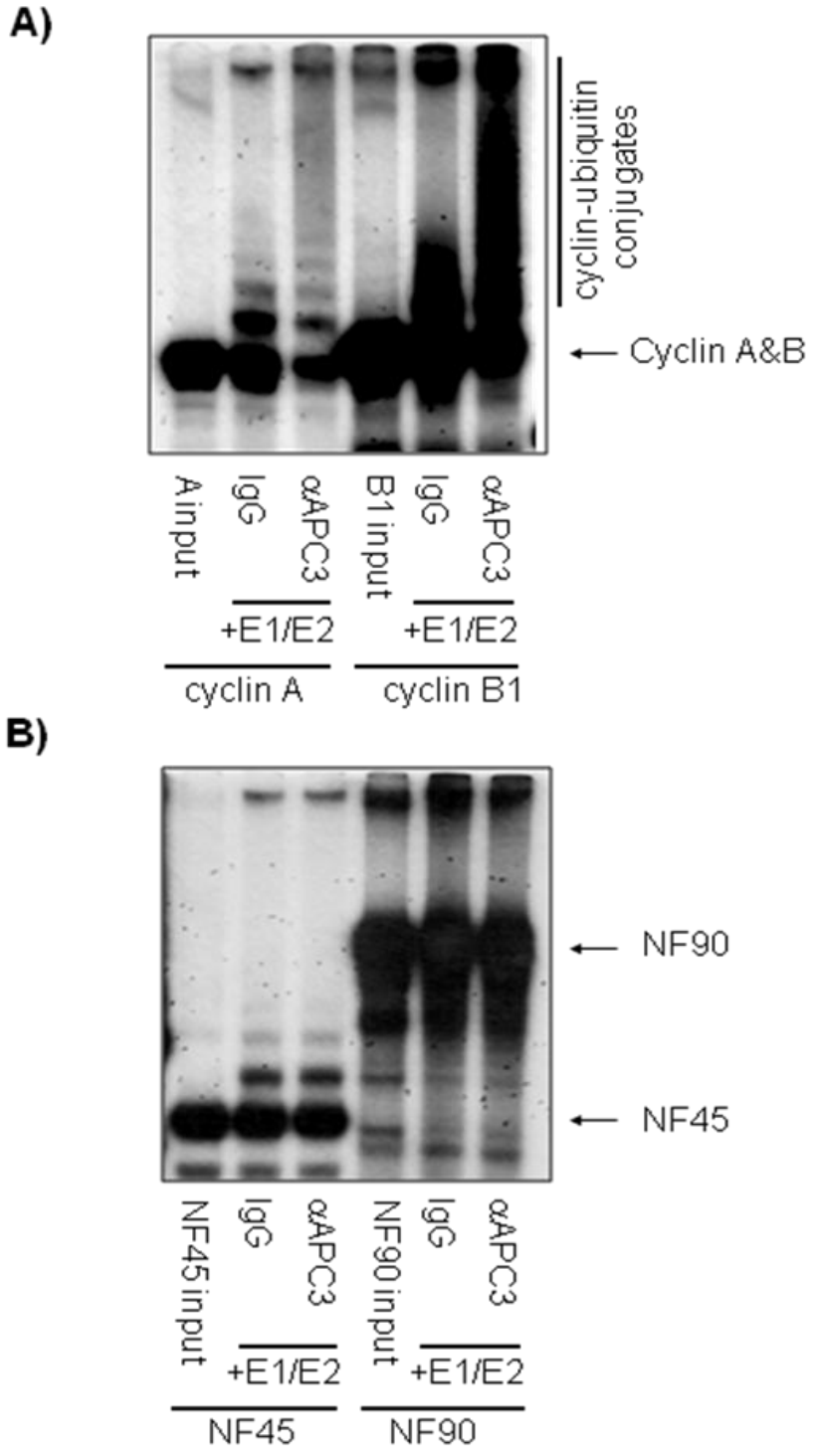

Fig 5.8A and B. Determination of NF45 and NF90 as substrates for APC/C dependent ubiquitylation

20µg of the indicated antibodies were incubated with 5mg of A549 lysate. Bound proteins were then precipitated using protein G-Sepharose, washed, and incubated with with rabbit E1 and human E2 enzymes;UbcH10, UbcH4 and UbcH5, and 1µl of [^{35}S]-labeled, *in vitro*-translated cyclin A, cyclin B1 (**A**), NF45 or NF90 (**B**). The reaction mixture was then incubated with sample buffer and separated by SDS-PAGE. After fluorography the gel was dried and subjected to autoradiography. 1µl of radiolabelled inputs were loaded in the first lane of the gel.

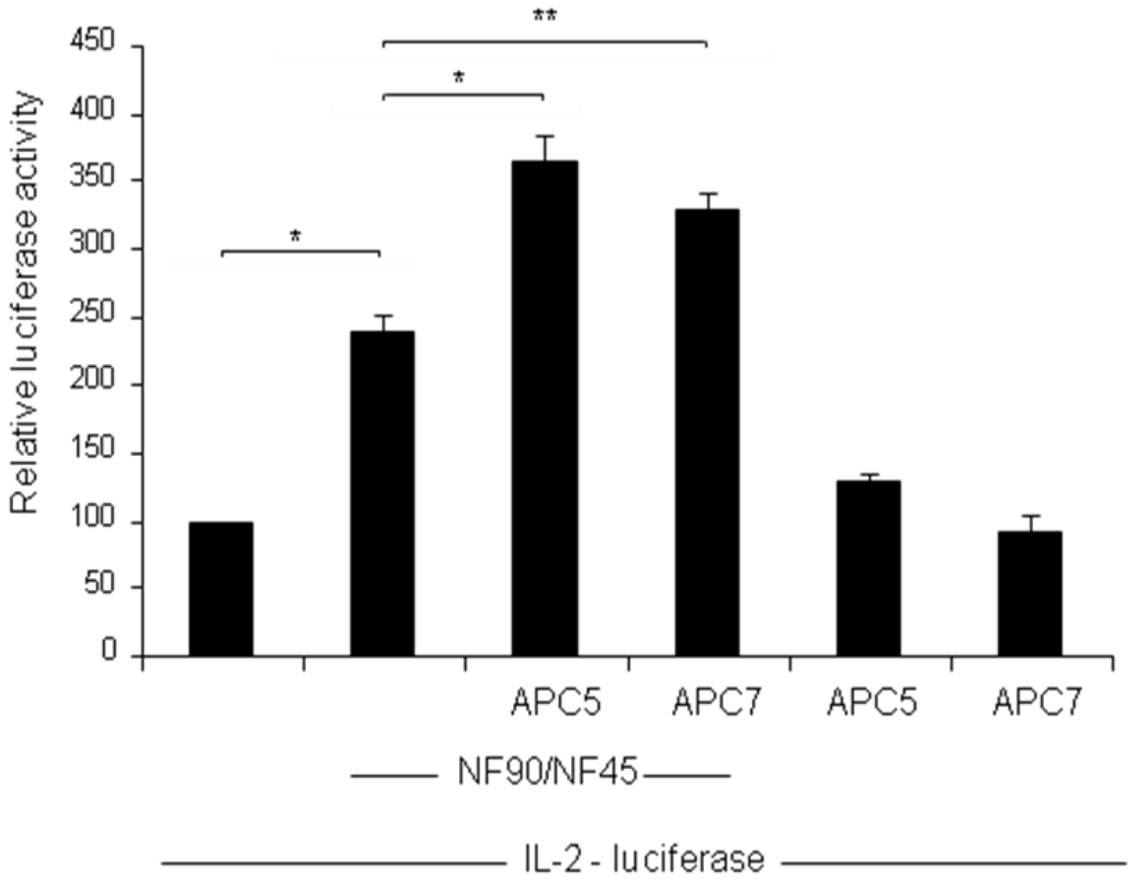

Fig 5.9. NF45 and NF90 cooperate with the APC/C to potentiate transcription from the IL-2 promoter.

HCT116 cells were transfected with a luciferase reporter construct driven by the IL-2 promoter along with the indicated protein expression constructs. Relative expression of luciferase was then quantified 24hrs post transfection. IL-2-luciferase indicates a reporter construct driven by the IL-2 promoter. The bar chart is representative of two separate experiments carried out in triplicate. Error bars represent standard deviation. *P<0.05; **P<0.01.

Results in this section demonstrate that NF90 cooperates with APC5 and APC7 to activate transcription from the IL-2 promoter *in vitro*, suggesting that the entire APC/C holoenzyme might also be important for IL-2 transcription.

5.2.9. Dynamic binding of the APC/C and NF90 to the IL-2 promoter *in vivo*

Two recent reports demonstrate that NF90 binds to the proximal IL-2 promoter in response to stimulation with PMA and ionomycin *in vivo*, which correlates with the ability of NF90 to activate IL-2 transcription in response to PMA and ionomycin stimulation *in vitro* (Shi, Godfrey et al. 2007; Shi, Qiu et al. 2007). The capacity of APC5 and APC7 to activate transcription from an IL-2 reporter construct in cooperation with NF90/NF45 *in vitro*, also suggests that the APC/C might also be associated with the IL-2 promoter *in vivo.* In order to test this I replicated a ChIP protocol that was used by Shi *et al*. (2007) to demonstrate inducible binding of NF90 to the IL-2 promoter. In short, Jurkat T-cells were either nonstimulated or stimulated with 20ng of PMA and 2uM ionomycin for 4 hours. Following treatment with 1% formaldehyde and DNA shearing, chromatin-DNA complexes were immunoprecipitated with antibodies against either NF90, APC5 or APC7. Immunoprecipitated DNA was purified and analysed by PCR using oligonucleotide primers that were specific to the IL-2 proximal promoter (Fig 5.10). As expected, PMA and ionomycin stimulation induced binding of NF90 to the IL-2 promoter. Interestingly, my analysis also revealed that APC5 and APC7 are bound to the IL-2 promoter in non-stimulated cells and that their dissociation is induced upon treatment with PMA and ionomycin. Given that in unstimulated cells IL-2 transcription is inhibited, results in this section suggest that the endogenous APC/C holoenzyme may function to repress IL-2 transcription *in vivo.*

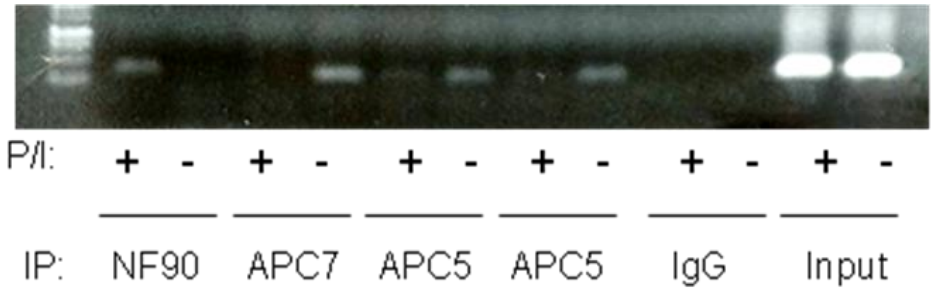

Fig 5.10. Dynamic binding of NF90, APC5 and APC7 bind to the IL-2 promoter *in vivo*

Jurkat T-cells were nonstimulated or stimulated for with PMA and ionomycin (P/I) for 4hrs. Nuclear proteins were then cross linked to chromatin with 1% formaldehyde. Sheared and restricted chromatin was then immunoprecipitated using anti-NF90, anti-APC5 and anti-APC7 antibodies, and used as a PCR template for primers specific to the IL-2 proximal promoter.

5.2.10. Exogenous NF90 and NF45 cooperate with exogenous APC/C to potentiate transcription from the TNF promoter

Given that transcription from the TNF promoter is inhibited by CsA (McCaffrey, Goldfeld et al. 1994) and that transactivation of the IL-2 promoter by NF90/NF45 is also inhibited by CsA (Kao, Chen et al. 1994), it seemed likely that the TNF promoter may also be subject to regulation by NF90/NF45 and the APC/C. To test this possibility, I transfected HCT116 cells with a plasmid containing the TNF promoter upstream of a luciferase reporter gene in combination with NF90/NF45 and either APC5 or APC7 (Fig 5.11). As expected NF90/NF45 enhances luciferase activity by approximately 130%. Addition of APC5 further increases activity by approximately 70%, whilst addition of APC7 enhances activity by 100%. Expression of APC5 or APC7 alone, had negligible effects on luciferase activity. In conclusion, the results presented in this section demonstrate that APC5 and APC7 cooperate with NF90/NF45 to stimulate transcription from the IL-2 and TNF promoters *in vitro*

5.2.11. APC7 recruits CBP/p300 to the TNF promoter in order to potentiate transcription.

Work in our laboratory has previously demonstrated an interaction between APC7 and CBP/p300, which is important for transactivation of the CDC6 and $p21^{CIP1}$ promoters (Turnell, Stewart et al. 2005); other workers have demonstrated that CBP potentiates TNF transcription (Falvo, Brinkman et al. 2000). Therefore, in order to investigate whether APC7 can regulate the TNF promoter through its interaction with CBP/p300, I made use of an APC7 deletion mutant (ΔN-APC7) previously generated in our lab, which ablates CBP/p300 binding due to removal of its E1A N-terminal homology

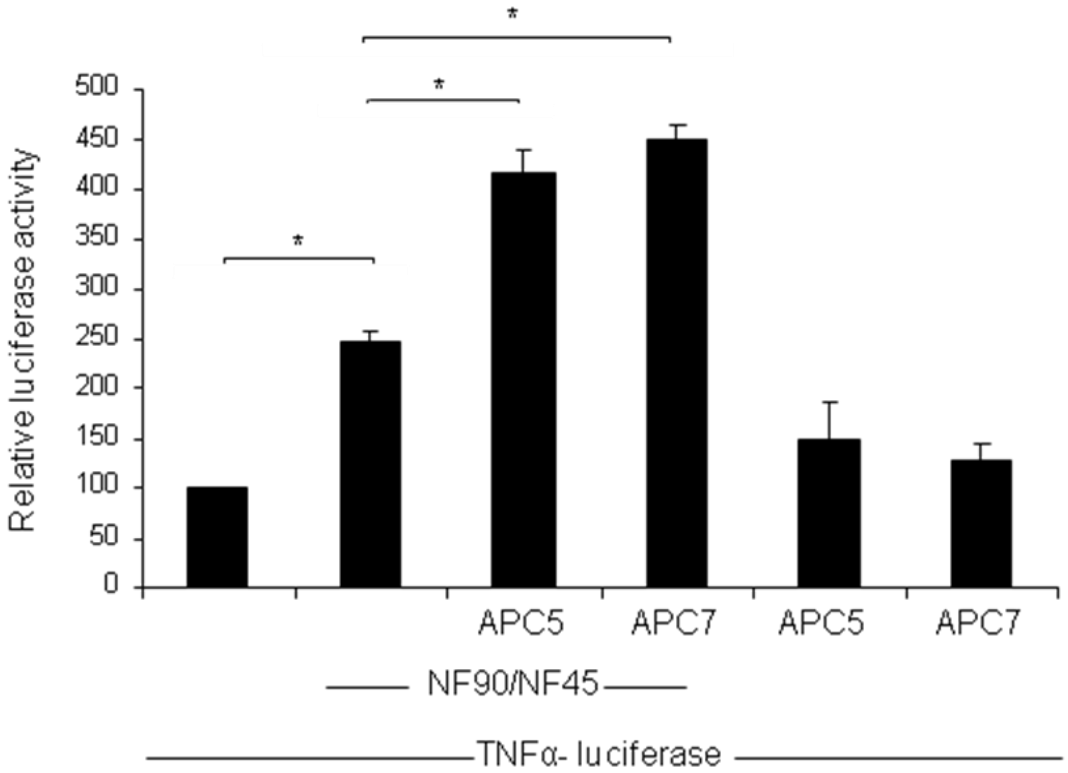

Fig 5.11. NF45 and NF90 cooperate with the APC/C to potentiate transcription from the TNF promoter.

HCT116 cells were transfected with a luciferase reporter construct driven by th TNF promoter along with the indicated protein expression constructs. Relative expression of luciferase was then quantified 24hrs post transfection. TNF-luciferase indicates a reporter construct driven by the TNF promoter. The bar chart is representative of two separate experiments carried out in triplicate. Error bars denote standard deviation. *P<0.05.

domain (Fig 5.12B). Thus HCT116 cells were transfected with a plasmid containing the TNF promoter upstream of a luciferase reporter gene in combination with NF90/NF45 and *wt* APC7 or ΔN-APC7. The activity of the luciferase reporter gene was assayed 24 hours post-transfection (Fig 5.12A). As previously demonstrated APC7 and NF90/NF45 cooperated to enhance transcription from the TNF promoter. Interestingly, ΔN-APC7 retained substantial ability to activate the TNF promoter in cooperation with NF90/NF45, although this difference is not statistically significant is does indicate that the ability of APC7 to activate TNF transcription may be partially dependent on CBP/p300.

5.2.12. Endogenous APC5 represses the TNF promoter

Over-expression of exogenous NF90/NF45 and APC/C subunits may result in the formation of non-physiological protein interactions, and therefore cause aberrant recruitment of proteins to the TNF promoter. In light of this I decided to investigate whether endogenous NF90 and APC/C are also capable of regulating TNF transcription. Thus 72hours after siRNA mediated knockdown of APC5, APC7 and NF90 (Fig 5.13B) I transfected HeLa cells with a TNF-luciferase reporter construct. The resulting luciferase activity was quantified 24 hours later (Fig 5.13A). Interestingly, following APC5 knockdown transcriptional activity of the TNF promoter was enhanced by approximately 450%, while APC7 knockdown increased the rate of transcription by approximately 200%, in comparison to the non-silencing control. In contrast there was no significant alteration in transcription from the TNF promoter after NF90 knockdown. In summary, the data presented in this section establishes that endogenous APC5 and APC7 repress transcription from the TNF promoter in the absence of PMA and

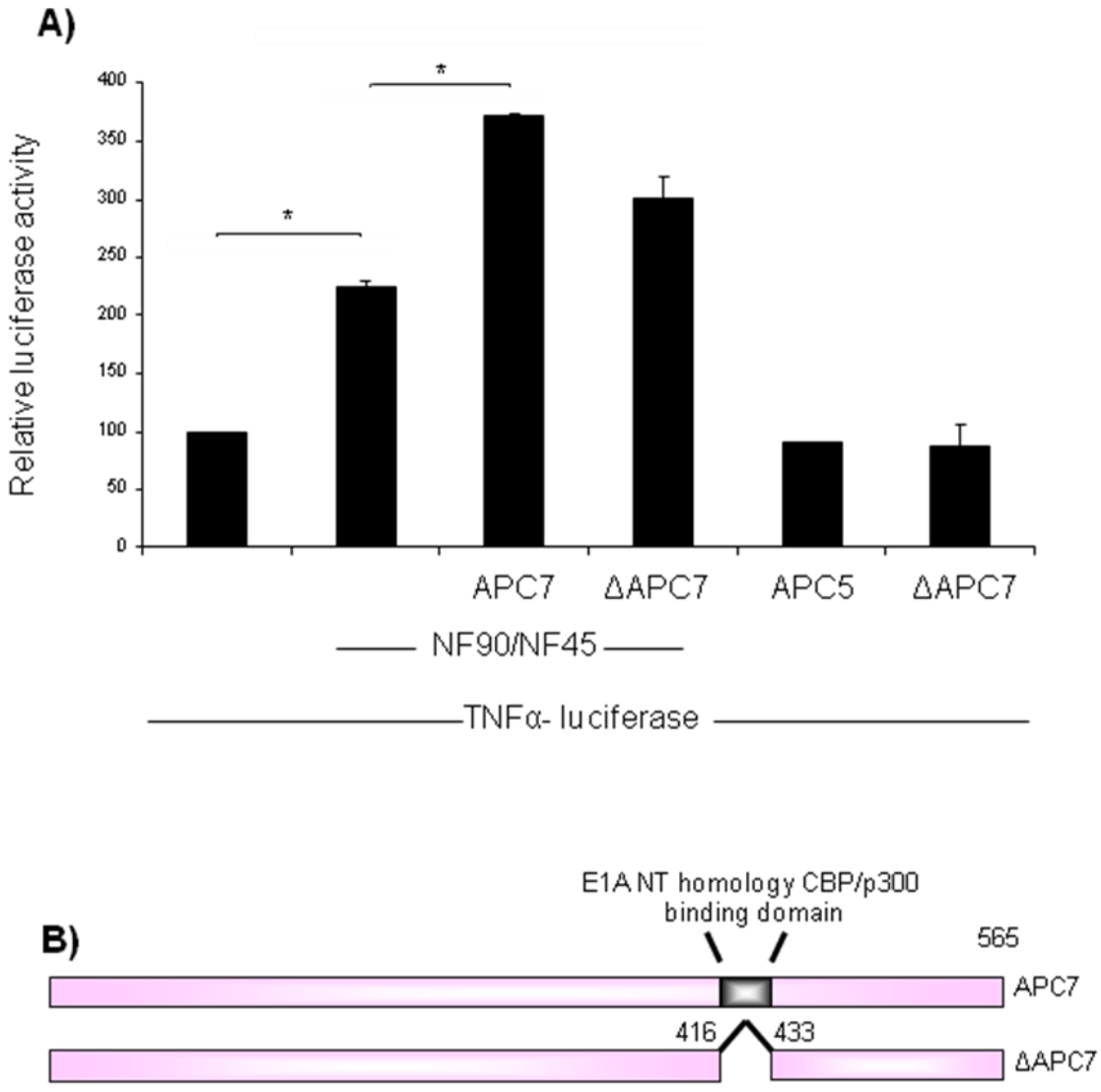

Fig 5.12A and B. CBP/p300 potentiates transcription of the TNF promoter in cooperation with NF45,NF90 and APC7.

HCT116 cells were transfected with a luciferase reporter construct driven by the TNF promoter along with the indicated protein expression constructs. (A)Relative expression of luciferase was then quantified 24hrs post transfection. TNF-luciferase indicates a reporter construct driven by the TNF promoter. (B) Schematic of APC7 WT protein and ΔAPC7, a mutant defective in CBP/p300 binding. The bar chart is representative of two separate experiments carried out in triplicate. Error bars denote standard deviation. *P<0.05.

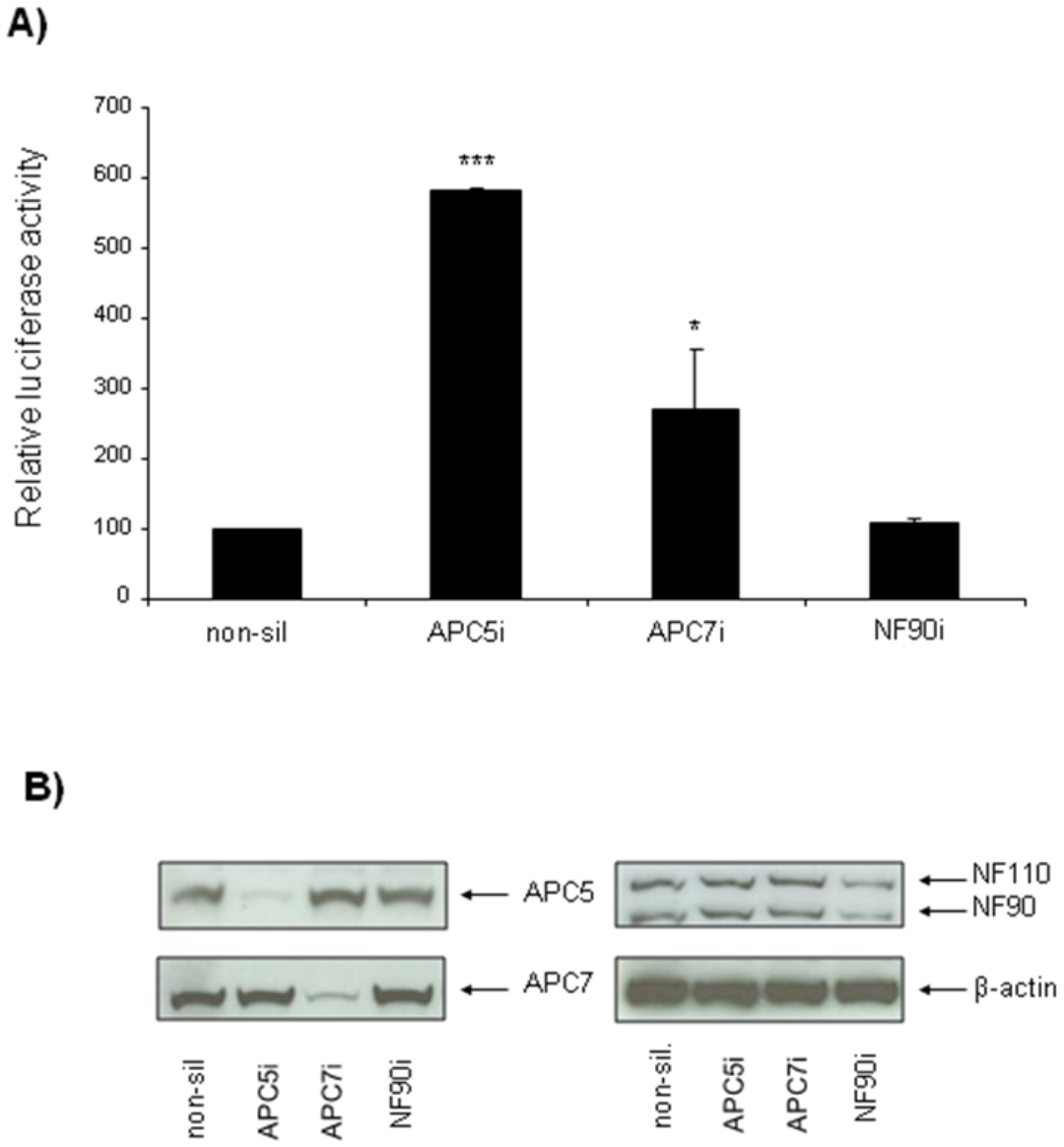

Fig 5.13A and B. Transactivation of the TNF promoter is dependent on endogenous NF90 and the APC/C.

Hela cells were transfected with siRNAs directed against NF90 and APC5. 48hrs post-transfection cells were subjected to transfection with a luciferase reporter construct driven by the TNF promoter. (A) Relative TNF promoter activity following NF90 and APC5 knockdown. (B) Whole cell extracts were analyzed by Western blotting to evaluate levels of APC5, APC7 and NF90, in order to determine knock-down efficiency. non-si., non-silencing RNA oligonucleotides; APC5i, APC7i and NF90i refer to instances where the expression of these proteins has been ablated by RNAi. The bar chart is representative of two separate experiments carried out in triplicate. Error bars denote standard deviation. *P<0.05; ***P<0.001.

ionomycin stimulation. Whereas endogenous NF90 has no significant effect on TNF transcription in the absence of PMA and ionomycin treatment.

5.2.13. The effect of NF90 and APC5 on TNF transcription *in vivo*

Work in this chapter has established that exogenous NF90/NF45 and APC/C subunits cooperate with one another to bind and enhance transcription of a synthetic TNF promoter (Fig 5.11), while endogenous APC5 and APC7 function to repress a synthetic TNF promoter (Fig13A). Taken together, these observations suggest that NF90 and the APC/C might function in concert in order to regulate the TNF promoter *in vivo*. Also, given that NF90/NF45 dependent transcription, and TNF transcription are both activated after PMA and ionomycin treatment (Hensel, Mannel et al. 1987; Richards, Dennert et al. 1989; Corthesy and Kao 1994), it seems likely that the ability of NF90 and APC/C to regulate

TNF transcription is influenced by PMA and ionomycin. To examine this possibility, I first transfected 16-Human Bronchial epithelial cells (16-HBE) with siRNA against NF90, APC5 or a non-silencing siRNA (Fig 5.14B). 5 days post transfection, I either mock treated or stimulated cells with 20ng of PMA and 2uM ionomycin for 6 hours, in order to activate NF90/NF45 dependent transcription. Cells were then harvested and whole cell RNA was extracted with a Qiagen RNeasy kit. Isolated RNA was then transcribed into cDNA by RT-PCR and utilized for quantitative realtime-PCR, in order to detect TNF gene transcripts, and hence measure levels of TNF transcription (Fig 5.14A). Prior to PMA and ionomycin stimulation levels of TNF transcription in cells treated with APC5 siRNA showed a 100% increase in relative levels of TNF transcription when compared to non-silencing control or cells treated with NF90 siRNA. As expected PMA and ionomycin stimulation of 16-HBE cells resulted in a

substantial increase in levels of TNF transcription. Following knockdown of NF90 and PMA and ionomycin stimulation there was approximately a 25% decrease in levels of TNF transcription when compared to non-silencing controls. In contrast, cells in which APC5 had been knocked down showed no significant difference in levels of TNF transcription after PMA and ionomycin stimulation when compared to non- silencing controls. Results in this section demonstrate that knockdown of APC5 has no effect on the ability of PMA and ionomycin to stimulate TNF transcription *in vivo*.

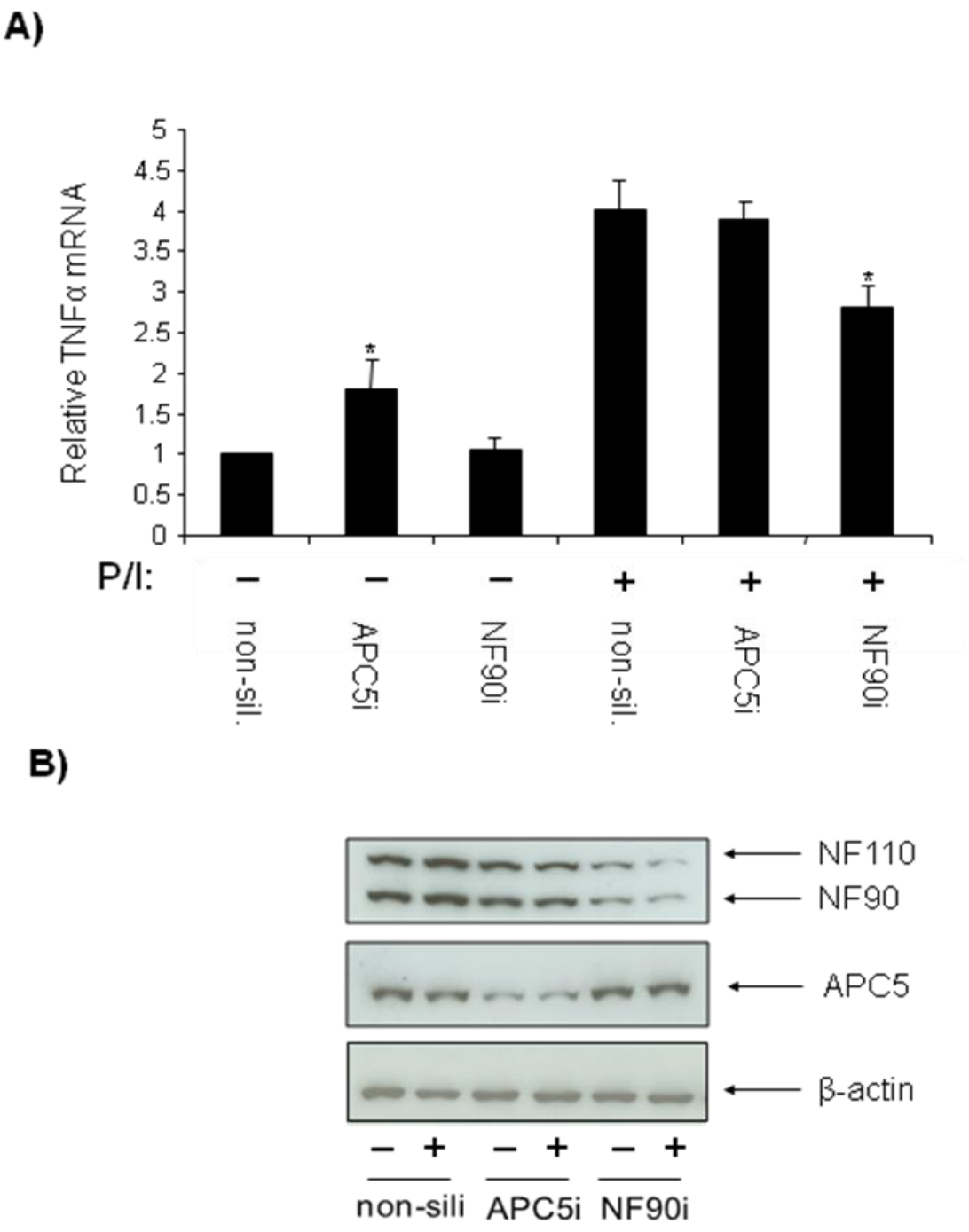

Fig 5.14A and B. NF90 and APC5 regulate TNF transcription *in vivo*.
16-HBE cells were transfected with siRNA directed against either NF90 or APC5. 96 hours post-transfection cells were stimulated with PMA and ionomycin (P/I) for 6 hours. (**A**). Relative levels of TNF mRNA were determined by realtime-PCR (**B**). Whole cell extracts were analyzed by Western blotting in order to determine the extent of NF90 and APC5 knockdown. non-sil, non-silencing RNA oligonucleotides; APC5i and NF90i refer to instances where APC5 or NF90 expression has been ablated by RNAi. The bar chart is representative of two separate experiments carried out in triplicate. Error bars denote standard deviation. *P<0.05.

5.3. Discussion

Co-IPs and GST-pulldowns in this chapter reveal the existence of protein complexes *in vivo* and *in vitro* that are composed of APC/C components and NF90/NF45 (Figs 5.1, 5.2 and 5.3), suggesting that NF90/NF45 might bind to the entire APC/C holoenzyme. These findings provide confirmation of the interaction between APC7 and NF45 that was identified by mass spectrometry in chapter 3. Interestingly, a luciferase-reporter assay and ChIP experiment strongly imply that the APC/C regulates IL-2 transcription in conjunction with NF90 *in vivo and in vitro* (Fig 5.9 and 5.10). In addition endogenous APC/C components also cooperate with NF90 to modulate transcription of the TNF promoter *in vivo* and *in vitro* (Fig 5.11-14). It should be noted that the NF90 antibody and siRNA used in this chapter also target NF110, suggesting that some the functions ascribed to NF90 in this study may actually be mediated by NF110.

A number of observations presented in this chapter and chapter 3 suggest that the APC/C holoenzyme or at least an APC/C subcomplex forms part of the ARRE-2 regulatory complex. For instance APC3 colocalises to a limited extent with NF90 in the nucleus (Fig 5.2), while APC5 and APC7 bind directly to NF90 *in vitro* (Fig 5.3). In addition APC5 and APC7 also form a complex with NF90/NF45 *in vivo* (Fig 5.1), although given that the anti-NF90 antibody used in this study also recoginses NF110, APC/C components might actually bind to NF110 and not NF90. Also, work from chapter 3 demonstrates an interaction between APC7 and the ARRE-2 regulatory component Ku70 *in vivo* (Fig 3.11) (Aoki, Zhao et al. 1998; Ting, Kao et al. 1998; Shi, Qiu et al. 2007). Moreover, APC5 and APC7 cooperate with NF90/NF45 to transactivate the IL-2 promoter *in vitro* (Fig 5.9). Lastly, data presented here demonstrates dynamic binding of NF90, APC5 and APC7 to the IL-2 promoter

following PMA and ionomycin treatment (Fig 5.10). This parallels work by Shi *et al*. (2007), which details the dissociation of Ku70 from the IL-2 promoter, and a concomitant induction of NF90 binding in response to PMA and ionomycin stimulation. Significantly, the ability of APC/C subunits to bind to the IL-2 promoter in the absence of PMA and ionomycin stimulation in a manner analogous to Ku70, correlates with reduced IL-2 transcription and therefore suggests that the endogenous APC/C holoenzyme may repress IL-2 transcription *in vivo* (see Fig 5.15 for a model of TNF and IL-2 promoter regulation by endogenous NF90/NF45, APC5 and APC7).

Analysis of TNF transcription by realtime-PCR demonstrates that NF90 functions to enhance TNF transcription in response to PMA and ionomycin treatment *in vivo* (Fig 5.14A), although given that this was shown using an NF90 siRNA that also targets NF110, TNF transcription might actually be dependent on endogenous NF110 . The ability of NF90 to transactivate a TNF promoter construct (Fig 5.11) suggests that it may regulate TNF transcription *in vivo* through binding directly to the TNF promoter. In contrast to NF90, endogenous APC5 and APC7 repress transcription from a TNF promoter construct (Fig 5.13A) while APC5 represses TNF transcription in the absence of PMA and ionomycin stimulation *in vivo* (Fig 5.14A). Indeed APC5 knockdown in 16HBE cells enhances the levels of TNF mRNA by 100% *in vivo* (Fig 5.14A). Whereas, APC5 knockdown in HeLa cells causes an approximate increase of 450% in transcription from a TNF promoter construct (Fig 5.13A). These differences may reflect disparity in the level of APC5 knockdown (Fig 5.13B and 5.14B) or differences in experimental design. Given that endogenous APC7 represses the TNF promoter *in vitro* (Fig 5.13A) it may be possible to further enhance TNF transcription *in vivo* by knocking down APC7 or additional APC/C subunits in conjunction with APC5. Interestingly, knockdown of endogenous NF90 by RNAi in the absence of PMA and ionomycin

stimulation has no effect on TNF transcription *in vitro* or *in vivo* (Fig 5.13A and 5.14A). However, one would expect a reduction in levels of transcription after NF90 knockdown given that NF90 activates the TNF promoter (Fig 5.11). This could be attributed to the inability to achieve sufficient knockdown of NF90 (Fig 5.13B and 5.14B) or suggests that endogenous NF90 is unable to bind to, and activate the TNF promoter in the absence of PMA and ionomycin stimulation, such that a reduction in NF90 expression would have no effect upon transcription from the TNF promoter in nonstimulated cells.

Direct association between NF90 and the TNF promoter could be inhibited as a result of APC5 and APC7 recruiting repressive complexes to the TNF promoter, thereby generating a closed chromatin conformation inaccessible to NF90, indeed the APC/C might be able to mediate transcriptional repression through APC8 and APC7, which contain a putative binding site for the transcriptional repressors C-terminal binding protein 1 (CtBP1) and RB respectively. Alternatively, binding of APC5 and APC7 to the TNF promoter might sterically inhibit binding of NF90 to the TNF promoter. A model for regulation of the TNF promoter by NF90 and the APC/C may mirror the dynamic binding of NF90 and APC/C subunits to the IL-2 promoter, whereby repression of the TNF promoter by APC5 in the absence of PMA and ionomycin stimulation, correlates with binding of APC5 to the TNF promoter. Upon stimulation with PMA and ionomycin, APC5 would be induced to dissociate from the TNF promoter allowing NF90 to bind and activate transcription (see Fig 5.15 for a model of IL-2 and TNF promoter regulation by endogenous NF90/NF45, APC5 and APC7). If this were the case, it would explain why knockdown of APC5 has no significant effect on TNF expression after PMA and ionomycin stimulation (Fig 5.14A) as even if APC5 were expressed to its full extent it would no longer be bound to the TNF promoter.

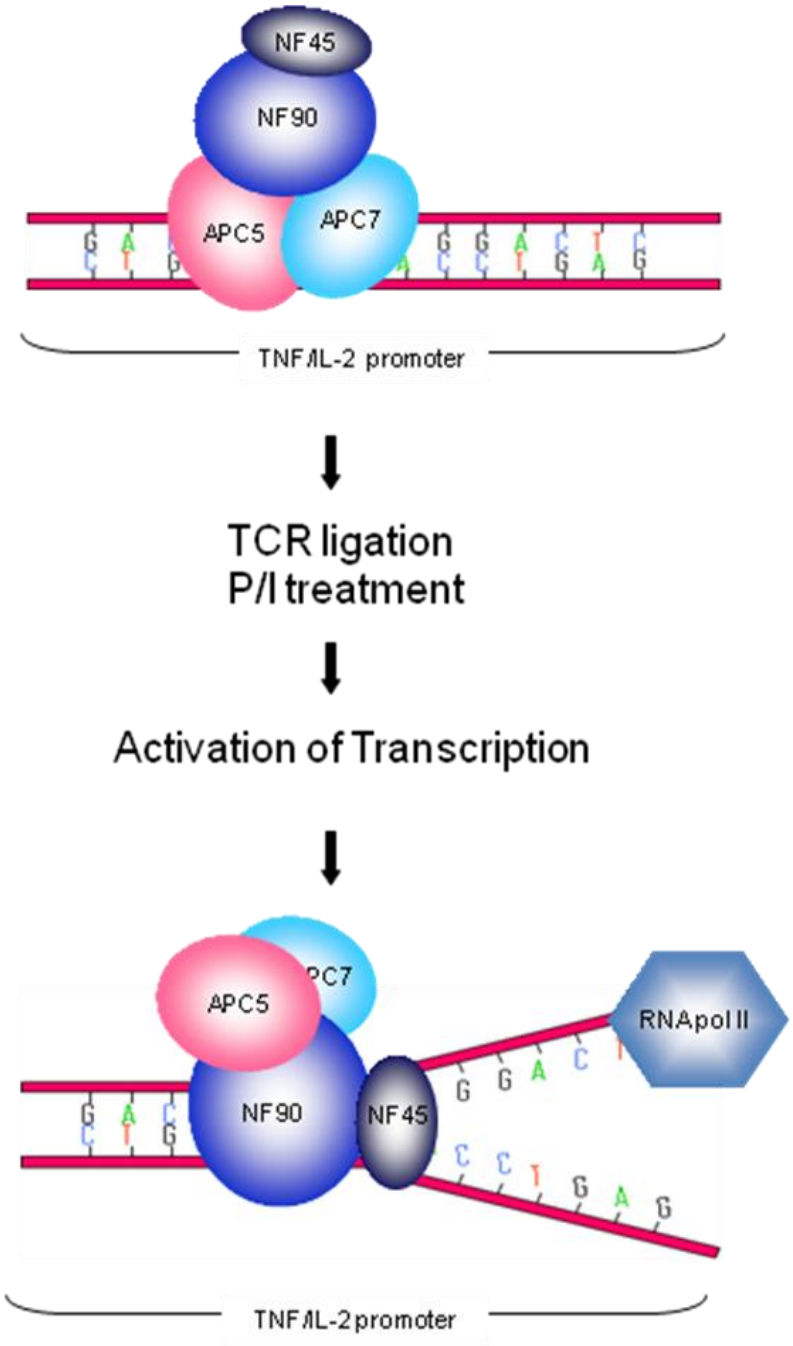

Figure 5.15. A model for IL-2 and TNF promoter regulation by endogenous NF90/NF45, APC5 and APC7.

Prior to stimulation APC5 and APC7 contact the IL-2 and TNF promoter and inhibit transcription while tethering inactive NF90/NF45 to the promoter. Upon stimulation the complex undergoes rearrangement so that APC5 and APC7 are no longer directly bound to the promoter, but remain tethered to the promoter via interaction with DNA bound NF90/NF45, which activates transcription. This model is based on the activity of the indicated endogenous proteins as defined in this study and also previous work by P. Kao *et.al.* (1994) and L. Shi *et.al.* (2007). P/I refers to stimulation with PMA and ionomycin.

Exogenous APC5 and APC7 cooperate with NF90/NF45 to transactivate the TNF and IL-2 promoters *in vitro* (Fig 5.9 and 5.11). The observation that exogenous APC5 and APC7 can't activate transcription of the IL-2 and TNF promoters in the absence of NF90/NF45 *in vitro*, suggests that exogenous APC5 and APC7 do not bind directly to these promoters and must be recruited by NF90/NF45. In contrast endogenous APC5 and APC7 inhibit TNF transcription *in vitro* and *in vivo* (Fig 5.13A and 5.14A), while immunoprecipitation of the IL-2 promoter with APC5 and APC7 (Fig 5.10) indicates that these proteins bind directly to the IL-2 promoter and repress it *in vivo*. The differences observed between the roles of exogenous APC/C subunits and endogenous APC/C subunits may be attributed to the possibility that nonphysiological over-expression of APC5 and APC7 after transfection with the corresponding expression constructs might titrate out or sequester proteins. This could result in the formation of sub-stoichiometric APC/C complexes and cause the aberrant recruitment of transcription factors to the TNF and IL-2 promoters. In addition over-expression of these subunits may deprive APC5 and APC7 of post-translational modifications that are required to induce their binding to the IL-2 and TNF promoters. High levels of APC5 or APC7 expression might titrate out factors that mediate post-translational modifications of the APC/C or could restrict their activity by altering the cell cycle distribution given the importance of the APC/C as a cell cycle regulator.

Luciferase-reporter assays in this chapter reveal that the APC/C and NF90/NF45 cooperate to regulate transcription (Figs 5.9, 5.11 and 5.12), it therefore seems likely that the interaction between the APC/C and NF90 may be important for regulation of a large panel of genes. For instance, NF90 displays a physiological association with the myc11 promoter (Shi, Qiu et al. 2007), while NF90 and its related protein NF110 regulate the PCNA promoter *in vitro* (Reichman, Parrott et al. 2003). Also, a large panel

of cytokine genes, including IL-2 and TNF, are inhibited by CsA (Hess, Tutschka et al. 1982; Pawelec and Wernet 1983; McCaffrey, Goldfeld et al. 1994), which has been demonstrated as a potent inhibitor of NF90/NF45 dependent transcription (Kao, Chen et al. 1994). Furthermore, given that PMA and ionomycin stimulation mimics ligation of the IL-2R and TNFR, the APC/C may also repress a number of other IL-2 and TNF responsive genes.

IL-2 autocrine signalling induces S phase entry, while activation of the TNFR can also stimulate cellular proliferation (Robb, Greene et al. 1984; Stern and Smith 1986). It is therefore tempting to speculate that the APC/C could exert a novel method of controlling cell cycle progression by inhibiting transcription of TNF and IL-2, therefore preventing cellular proliferation in response to autocrine signalling. During G1 the APC/C maintains cyclin A instability (Geley, Kramer et al. 2001; Rape, Reddy et al. 2006), therefore it is plausible that the APC/C may also prevent cyclin A accumulation by inhibiting IL-2 transcription, and as a result preventing an increase in levels of cyclin A, which is observed in response to IL-2R ligation (Li, Godfrey et al. 2005). If the APC/C does inhibit IL-2 transcription it would also provide a mechanism through which the APC/C could enforce G1 arrest in response to certain cellular stresses, as IL-2R ligation also increases transcription of other proteins required for cellular proliferation such as c-myc, and cyclin D (Turner 1993; Moon and Nelson 2001). Interestingly, IL-2 expression is highly elevated during mitosis in carcinoma cells (Reichert, Nagashima et al. 2000), implying that IL-2 autocrine signalling may be important for the onset of mitosis in normal cells. In addition elevation of TNF expression is associated with up regulation of cyclin A and cyclin B, which are required for entry into mitosis (Gaiotti, Chung et al. 2000; McPherson, Kubik et al. 2003).

Therefore modulation of TNF or IL-2 expression by the APC/C may be one means by which the APC/C is able to regulate the onset of mitosis.

In addition to their well characterised roles during transcription, NF90/NF45 are also known to be involved at many levels of mRNA processing such as splicing (Saunders, Perkins et al. 2001; Zhou, Licklider et al. 2002; Leung, Andersen et al. 2003), mRNA export, mRNA stabilisation (Shim, Lim et al. 2002; Shi, Zhao et al. 2005) and translation (Ting, Kao et al. 1998; Xu and Grabowski 1999), suggesting that the APC/C may also cooperate with NF90 to regulate these processes. Significantly, a role for APC7 in splicing is further supported by interaction between APC7 and the splicing factor TLS, which was identified by mass spectrometry in chapter 3 (Fig 3.12). A potential role for the APC/C in translation is strengthened by the observation that APC3 colocalises with cytoplasmic NF90 (Fig 5.2), which might occur at ribosomes, while APC5 has also previously been demonstrated to associate with ribosomes (Koloteva-Levine, Pinchasi et al. 2004). Indeed work in chapter three shows that APC7 binds the translation factor eEF1A (Fig 3.9). Lastly, the observation that the majority of nuclear APC3-NF90 complexes occur at the periphery of the nucleus (Fig 5.2), close to the nuclear membrane, coupled to the fact that NF90/NF45 are important for nuclear export of mRNA (Gwizdek, Ossareh-Nazari et al. 2003), suggests that NF90-APC/C complexes may function during mRNA export.

The inability of anti-NF45 and anti-NF90 antibodies to immunoprecipitate APC/C ligase activity (Fig 5.7) suggests that NF45/NF90 may not interact with the entire APC/C holoenzyme, but with a sub-complex that is devoid of ligase activity. Although, interaction between APC3 and NF90 contradicts this (Fig 5.2), as APC3 is crucial for activation of the APC/C's ubiquitin ligase activity (Kraft, Herzog et al. 2003; Vodermaier, Gieffers et al. 2003). Other work has suggested that NF90 and NF45 are

involved in mitotic control as their knockdown results in the appearance of multinucleated cells that could possibly arise due to deregulation of the APC/C and APC/C ubquitin ligase activity (Guan, Altan-Bonnet et al. 2008). The failure of NF90 to immunoprecipitate APC/C ligase activity could be attributed to the possibility that only a small fraction of cellular NF90/NF45 may be combined with the APC/C and therefore only a small proportion of NF90/NF45 immunoprecipitate will be associated with APC/C ligase activity. Alternatively NF90/NF45 may actually inhibit APC/C ligase function. If so, the existence of a putative consensus CDK phosphorylation site ((S/T) P X (K/R) in NF90, coupled to previously observed M phase specific phosphorylation of NF90 (Matsumoto-Taniura, Pirollet et al. 1996), suggests that during G2/M, phosphorylation of NF90 by CDK1 could potentially activate APC/C ligase function, as a result of a conformational change in NF90. Alternatively phosphorylation of NF90 could stimulate dissociation of a potentially inhibitory NF90 from the APC/C. Interestingly levels of nuclear NF110, which is virtually identical to NF90 in its first 671 amino acids (Duchange, Pidoux et al. 2000) and therefore may also bind to the APC/C, have previously been demonstrated to increase during S phase until the G2/M transition (Xu, Leonova et al. 2003), therefore suggesting that NF110 and not NF90 may be important for APC/C regulation during or prior to mitosis.

NF90/NF45 are not targets for APC/C mediated ubiquitylation *in vitro* (Fig 5.8B), despite the presence of putative D-boxes. It is possible that other members of the ARRE-2 regulatory complex may be substrates for APC/C mediated ubiquitylation, indeed Ku80 contains 1 putative D-box, while Ku70 contains 4 putative D-boxes, three of which conform to the more extensive RXXLXXXXN/D/E consensus sequence (Geley, Kramer et al. 2001).

Given the ability of APC5 and APC7 to bind to the dsRBMs of NF90 (Fig 5.5B and C) and the conserved nature of dsRBMs in all proteins from this family (Fierro-Monti and Mathews 2000), it seems likely that APC5 and APC7 may also bind to other dsRBM-containing proteins such as PKR (Green and Mathews 1992; Patel and Sen 1992) and RNA helicase A (RHA) (Zhang and Grosse 1997). Also, it seems that APC7 can interact with the dsRBMs of NF90 via a dsRNA intermediate (Fig 5.6B), indicating that APC7 might bind to RNA *in vivo*, which is consistent with a role for the APC/C in splicing, mRNA export and translational control as discussed above. Furthermore, it seems likely that the interaction between the dsRBMs of NF90 and APC7 may be a target for viral inhibition given that NF90 binds to adenovirus VA RNAII (Liao, Kobayashi et al. 1998). For example, binding of VA RNAII to NF90 could interfere with APC/C and NF90 dependent transcriptional regulation, and therefore possibly prevent activation of IFN inducible genes by NF90 (Krasnoselskaya-Riz, Spruill et al. 2002; Reichman, Parrott et al. 2003).

CHAPTER 6

FINAL DISCUSSION

6.1. Mass spectrometric analysis and future perspectives

The initial aim of this study was to identify novel APC/C interacting proteins by mass spectrometric analysis of APC7 immunoprecipitates. Using this approach I identified a number of known APC/C subunits demonstrating the efficacy of this technique and also six novel APC7-interacting proteins. The second aim of this study was to validate any novel interactions, and study their functional consequences.

I choose to examine the interaction between APC7 and NF45 due to NF45's well defined role in transcription (Corthesy and Kao 1994; Kao, Chen et al. 1994; Aoki, Zhao et al. 1998; Ting, Kao et al. 1998; Shi, Godfrey et al. 2007; Shi, Qiu et al. 2007). I also focused on the interaction between APC7 and TIF1γ as a result of *TIF1γ's* chromosomal translocation in cancer (Klugbauer and Rabes 1999), indicating that TIF1γ functions during cellular proliferation and differentiation. In the future it will be important to validate the interactions between APC7 and the additional novel APC7 interacting proteins identified in this study and go on to investigate their functional significance.

Advances in mass spectrometer technology and sample preparation protocols have recently been utilised to analyse APC7 immunoprecipitates. This work yielded 20 APC7 peptides and 9 NF90 peptides compared to 6 APC7 peptides and 0 NF90 peptides from work in chapter 3 (Dr A Turnell, unpublished observation). Given these significant improvements it is my intention to identify additional novel APC/C interacting proteins. Also, now that I am in possession of a high titre TIF1γ antibody, future work will focus on identifying novel TIF1γ interacting proteins, as reports of TIF1γ function are scarce. In particular it would interesting to identify putative substrates for TIF1γ mediated ubiquitylation, in order to complement findings by Dupont et al (2005), which suggest that TIF1γ is a ubiquitin ligase. In addition to this, it will be desirable to detect any

components of the ubiquitination machinery that TIF1γ interacts with such E2 ubiquitin transferases, DUBs or other ubiquitin ligases.

6.2. A role for TIF1γ in mitotic progression

At the beginning of this study understanding of TIF1γ was limited to a nonphysiological role in transcription and evidence of its chromosomal translocation in cancer (Klugbauer and Rabes 1999; Venturini, You et al. 1999), suggesting that TIF1γ regulates cellular proliferation and differentiation. In accordance with these findings the interaction between APC7 and TIF1γ that was identified in this study also further implicated TIF1γ in cell cycle control, as the APC/C has a well defined role in regulating proliferation (Peters 2002; Skaar and Pagano 2008). In light of this, one of the major aims of this study was to determine the function of the interaction between the APC/C and TIF1γ during cell cycle regulation.

Initial data from chapter 4 demonstrate that TIF1γ knockdown stabilises the APC/C's mitotic substrates such as cyclin A, cyclin B, and CDC20, suggesting that TIF1γ cooperates with the APC/C to regulate APC/C substrate degradation during mitosis. Additional evidence from chapter 4 further implicates TIF1γ in mitotic control, as TIF1γ band shifts after SAC activation, which is indicative of TIF1γ phosphorylation a might be required to inhibit the ability of TIF1γ to promote APC/C substrate degradation. Furthermore, TIF1γ expression increases the mitotic index and alters the mitotic distribution, by blocking cells in a metaphase-like state. It will be particularly important to supplement this TIF1γ knockdown data by attempting to complement the observed mitotic defect by expressing exogenous TIF1γ that is refractory to siRNA knockdown.

Other evidence of a role for TIF1γ during mitosis is provided by an interaction between the APC/C's early mitotic activator CDC20 and TIF1γ *in vivo*, suggesting that TIF1γ is associated with, and may regulate, the APC/C during mitosis. TIF1γ also interacts with the APC/C's mitotic substrate cyclin A *in vivo*, although given that APC/C^{CDH1} also mediates cyclin A degradation during G1 it is entirely possible that TIF1γ interacts with the G1 form of cyclin A (Hsu, Reimann et al. 2002; Havens, Ho et al. 2006). However, work from our lab has failed to demonstrate an interaction between TIF1γ and the APC/C's G1 regulator CDH1 (Fang, Yu et al. 1998; Kramer, Gieffers et al. 1998; Zachariae, Schwab et al. 1998; Jaspersen, Charles et al. 1999), indicating that TIF1γ is most likely exclusively associated with mitotic APC/C^{CDC20} and therefore with cyclin A during mitosis. Therefore, the questions of when during the cell cycle TIF1γ interacts with cyclin A, and whether ablation of TIF1γ expression results in the unexpected presence of cyclin A post-prometaphase or during early G1 should be addressed.

Despite the failure to demonstrate an interaction between TIF1γ and CDH1 in this study it is possible that TIF1γ may still regulate APC/C^{CDH1} during G1. Indeed if TIF1γ is important for APC/C^{CDH1} ligase activity during G1 then TIF1γ knockdown could result in early accumulation of cyclin A and degradation of $p27^{KIP1}$ promoting premature S phase entry that could potentially result in activation of an S phase checkpoint. This idea is given credence by evidence from chapter 4, which demonstrates that TIF1γ knockdown, like ablation of CDH1 results in an enrichment of cells in S phase (Bashir, Dorrello et al. 2004). This is notably consistent with a previous report that details inhibition of DNA synthesis following ablation of TIF1γ expression (Dupont, Zacchigna et al. 2005), suggesting an S phase block.

Given that TIF1γ interacts with the APC/C's mitotic substrate cyclin A and regulates cyclin A degradation it seems likely that TIF1γ might directly target cyclin A for

degradation during mitosis, indeed, the mitotic phenotype observed after TIF1γ RNAi treatment resembles the mitotic delay observed in cells in which *wt* cyclin A has been over-expressed (den Elzen and Pines 2001), which is akin to APC/C inhibition. Therefore, ablation of TIF1γ expression may indirectly stabilise other APC/C substrates by slowing passage through mitosis. This is consistent with the observation that TIF1γ knockdown promotes the accumulation of cyclin B while evidence from chapter 4 shows that TIF1γ is unable to interact with cyclin B *in vivo*.

Interestingly TIF1γ knockdown impairs APC/C ligase activity in vitro, suggesting that TIF1γ is important for this function. In order to further elucidate the role of TIF1γ during APC/C substrate ubiquitylation it would be of great interest to examine whether the addition of recombinant TIF1γ to an APC/C ligase assay can enhance ubiquitylation of specific APC/C substrates. Alternatively, TIF1γ might actually be capable of ubiquitylating APC/C substrates in the absence of the APC/C. To complement this future work should also focus on determining if TIF1γ is capable of direct binding to APC/C substrates and if other TIF1γ antibodies or tagged TIF1γ can precipitate APC/C ligase activity, and whether TIF1γ precipitates have a preference for specific substrates.

A large amount of the data in this study was obtained by the use of TIF1γ siRNAs, therefore it will be important to corroborate a role for TIF1γ in mitosis by other means. For instance, it will necessary to determine if TIF1γ localises to mitotic structures or undergoes posttranslational modification during mitosis. Indeed the TIF1γ band shift observed after treatment with nocodazole may indicate mitotic phosphorylation by either BUBRI in response to SAC activation or cyclin A-CDK complexes upon entry into mitosis.

Many APC/C regulatory molecules such as cyclin B and CDC20 are actually directly targeted for degradation by the APC/C (Dawson, Roth et al. 1995; Sigrist, Jacobs et al.

1995; Sudakin, Ganoth et al. 1995; Prinz, Hwang et al. 1998; Shirayama, Zachariae et al. 1998). However work in this study suggests that the APC/C doesn't directly target TIF1γ for degradation, as I was unable to ubiquitylate TIF1γ with APC3 immunoprecipitates *in vitro* while ablation of CDH1 expression causes a reduction in TIF1γ expression. Consequently it will be important to determine if APC/C^{CDH1} is capable of indirectly controlling TIF1γ stability by regulating the stability of a putative TIF1γ ubiquitin ligase, a particularly attractive candidate is SKP2, which is targeted for degradation by the APC/C^{CDH1} during G1 (Bashir, Dorrello et al. 2004; Wei, Ayad et al. 2004). Alternatively the observed reduction in TIF1γ levels after CDH1 knockdown may be due to cell cycle effects, as loss of CDH1 expression has previously been shown to induce premature S phase (Bashir, Dorrello et al. 2004). It would also be interesting to determine whether TIF1γ is capable of postranslationally modifying the APC/C, which is suggested by the presence of a RING domain in the N-terminus of TIF1γ that is thought to confer TIF1γ with ubiquitin ligase activity and may be also be capable of mediating SUMOylation (Dupont, Zacchigna et al. 2005; Quimby, Yong-Gonzalez et al. 2006).

Lastly it will be important to identify gene promoters that are regulated by TIF1γ, and whether the APC/C and TIF1γ cooperate during transcriptional control. Also given that TIF1γ and the APC/C regulate the activity of a number of transcription factors in response to TGF-β signalling it will be interesting to investigate possible cross talk between TIF1γ and the APC/C in this pathway (Wan, Liu et al. 2001; Dupont, Zacchigna et al. 2005; He, Dorn et al. 2006).

6.3. The APC/C regulates cytokine transcription

A previous report from our lab demonstrates that the APC/C regulates transcription (Turnell, Stewart et al. 2005), which taken together with the interaction between APC7 and NF90/NF45 from chapter 5, suggested that the APC/C may regulate IL-2 transcription in conjunction NF90/NF45. I therefore undertook work to elucidate the function of the APC/C during IL-2 transcription

Initial investigation concerning the function of the interaction between NF90/NF45 and the APC/C revealed that NF90/NF45 is not ubiquitylated by the APC/C or associated with active APC/C ubiquitin ligase activity. Subsequent luciferase reporter assays revealed that when over-expressed NF90/NF45 and APC/C components cooperate with one another to activate transcription from the IL-2 and the TNF promoters. In contrast it appears that endogenous APC/C proteins actually function to repress transcription of promoters that are regulated by NF90/NF45, as APC/C subunits repress a synthetic TNF promoter *in vitro* and TNF mRNA production *in vivo*. In addition APC/C subunits also associate with the IL-2 promoter in the absence of PMA and ionomycin stimulation, which suggests that APC/C subunits constitutively repress the IL-2 promoter. Protein over-expression could result in aberrant protein function through incorrect protein localisation, nonphysiological protein interactions or by titrating out interacting proteins. Therefore, my tendency is to lean towards the observation that the endogenous APC/C appears to repress cytokine transcription. Together with binding data this suggests the existence of complex consisting of the APC/C and NF90/NF45, which regulates cytokine transcription through direct dynamic binding of its constituents to the promoter (see Fig 5.15 for a model of TNF and IL-2 promoter regulation by endogenous NF90/NF45, APC5 and APC7). It may be possible to alter the function of exogenously introduced APC/C subunits by carefully titrating the proteins levels down. Also it

should be determined what effect over-expression and knockdown of APC/C subunits has on the cell cycle distribution and therefore the promoter activity of cytokine genes.

A number of studies indicate that TNF and IL-2 regulate the cell cycle both by autocrine and paracrine signalling. For instance, IL-2 stimulates progression through G1 and is elevated during mitosis in cancer cells while TNF promotes accumulation of mitotic cyclins (Gaiotti, Chung et al. 2000; Reichert, Nagashima et al. 2000; Moon and Nelson 2001; McPherson, Kubik et al. 2003). Given that the APC/C is thought to control the cell cycle by potentiating transcription of $p21^{CIP1}$ and CDC6 it is tempting to speculate that the APC/C can also regulate proliferation by modulating TNF and IL-2 transcription (Turnell, Stewart et al. 2005). Therefore it would be interesting to determine if APC/C knockdown can enhance proliferation through up regulation of IL-2 and TNF signalling. To complement this work it may be possible to demonstrate the cell cycle dependent association and repression of the IL-2 and TNF promoters by the APC/C. It is also possible that association of the APC/C with cytokine promoters may be altered during stress responses. For instance, expression of TNF is elevated following exposure to IR (Rube, Wilfert et al. 2002), therefore it is possible that the APC/C may be induced to dissociate from the TNF promoter following IR treatment enhancing TNF transcription. Alternatively, the APC/C might be recruited to IL-2 and TNF promoters following cytostatic stresses such as nutrient starvation in order to inhibit cellular proliferation.

A recent report suggests that NF90/NF45 may regulate the cell cycle, as their knockdown inhibits DNA synthesis, and results in aberrant mitosis (Guan, Altan-Bonnet et al. 2008); mitotic phosphorylation of NF90 also suggests a role for NF90 in mitotic control (Matsumoto-Taniura, Pirollet et al. 1996). These findings indicate that NF90/NF45 may cooperate with the APC/C to regulate DNA synthesis or mitosis by

regulating APC/C ligase activity. It will be important to address this notion in the future.

An isolated report details the presence of APC5 at ribosomes (Koloteva-Levine, Pinchasi et al. 2004), suggesting that the APC/C is important during translation. This notion is further supported by the ability of NF90 to regulate translation and the fact that work in chapter 5 shows colocalisation between NF90 and APC3 in the cytoplasm(Ting, Kao et al. 1998; Xu and Grabowski 1999; Vumbaca, Phoenix et al. 2008), suggesting that they cooperate in translational control. In addition it appears that the APC/C may be involved in regulating NF90's role during mRNA processing such as splicing and nuclear export(Shim, Lim et al. 2002; Zhou, Licklider et al. 2002; Leung, Andersen et al. 2003). This is suggested by work in chapter 5, which indicates that APC3 and NF90 may function together in mRNA nuclear export, due to the fact that they colocalise at the periphery of the nucleus. Interaction between the splicing factor TLS and APC7 as identified by mass spectrometry in chapter 3 also supports the notion that the APC/C functions in concert with NF90/NF45 to control splicing. In light of these factors, future work should focus on elucidating the role that APC/C-NF90/NF45 complexes play during mRNA processing.

CHAPTER 7

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